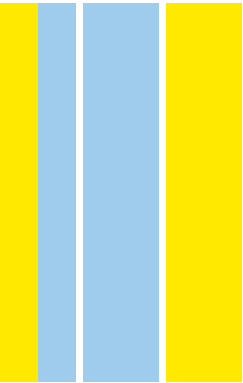


DOUTORAMENTO
PROGRAMA GRADUADO EM ÁREAS DA BIOLOGIA BÁSICA E APLICADA (GABBA)

Behavioral and endocrinological investigation of male sexual behavior

Susana Valente

D
2021

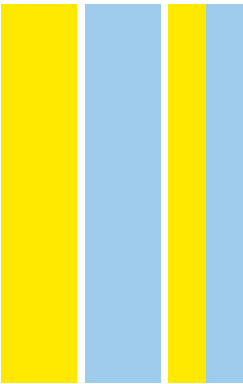


Susana Valente. Behavioral and endocrinological investigation of male sexual behavior



Behavioral and endocrinological investigation of male sexual behavior

Susana Valente



Susana Isabel Soares Valente

**Behavioral and endocrinological investigation
of male sexual behavior**

Tese de Candidatura ao grau de Doutor em
Áreas da Biologia Básica e Aplicada (GABBA),
Programa Doutoral da Universidade do Porto,
submetida ao Instituto de Ciências Biomédicas de
Abel Salazar

Orientador

Doutora Susana Lima

Investigador Principal

Champalimaud Research

Co-orientador

Doutor João Relvas

Investigador Principal

Faculdade de Medicina

Universidade do Porto



“With respect to sexuality, I have often speculated on it, and have always concluded that we are too ignorant to speculate: no physiologist can conjecture why the two elements go to form a new being, and more than that, why nature strives at uniting the two elements from two individuals.”

(Darwin, 1861)

To my person, Isa

Acknowledgments

My PhD was a ride in the biggest roller coaster. The tracks had ups and downs, stops at the highest points where I felt on the top of the world and cheerfully screamed “yeeeeahhhhhh” and vertiginous reversed falls and loops that brought tears to my eyes and made me question “what the hell am I doing here?!?” After sharp curves and sudden changes of speed the ride is over. This thesis is the end of the ride. And what a ride!

Thank you GABBA for giving me the ticket to go on this ride. Proud to be a GABBA!

This ride started a long, long, long time ago at IGC’s garden where I and Susana Lima discussed my project, complete out of the box. It was love at first sight. Thank you for the friendship, opportunity and freedom I had to explore all the questions that made this a great project.

I feel very lucky to have taken this ride along with the Limettes (past and present members). Amazing, funny and bright people always ready to give you a hand. As the first PhD student I saw and helped the lab grow, and now that I am leaving, I feel that a part of me will always stay.

I am very proud to be part of a vibrant and bright community as the Champalimaud research which I also had the joy to follow its progress. Thank you to all the platforms and people that make this a great community. At the Champalimaud I grew to be the scientist and person I am today but also made truly amazing friendships that now are part of my family.

To my family, for their support and pride on having the first PhD. To my amazing extended family that was always by my side.

A big thank you to Nuno, my soulmate, for being with me throughout this ride, for waiting for this ride to end, for your embrace and patience (put patience on it).

Last but not least, to my furry companion for the last 19 years, Tomás Afonso, for all the joy and for being my stress ball.

Resumo

O comportamento sexual dos machos roedores segue uma sequência básica de padrões específicos de comportamento apetitivo e consumatório para cada espécie. Os padrões apetitivos envolvem a aproximação e investigação olfativa da fêmea (principalmente anogenital), e o comportamento consumatório é caracterizado por sequências de tentativas de monta seguidas por múltiplas montas com intromissão peniana e finalmente ejaculação. Após a ejaculação, os machos entram numa fase de inatividade sexual, com uma redução drástica no interesse pela fêmea, denominado período refratário pós-ejaculatório (PERP).

O trabalho de Thomas E. McGill, pioneiro no estudo do comportamento sexual do macho de ratinho, persiste até ao momento como o melhor compêndio de dados sobre a variabilidade no comportamento entre diferentes linhagens de ratinho. O comportamento sexual do macho de ratinho é descrito como extremamente variável entre subespécies e dentro de linhagens da mesma subespécie. Em vez de ser considerada como um impasse, esta variabilidade fenotípica pode ser vista como uma vantagem, como uma forma alternativa para obter informação sobre os mecanismos evolutivos, genéticos e neuronais subjacentes a esse comportamento fundamental.

Um dos principais objetivos da neuroetologia evolutiva é explicar os mecanismos neuronais subjacentes ao aparecimento de comportamentos específicos de cada espécie. Esta tese explora algumas dessas diferenças comportamentais por meio de um estudo comparativo sobre o comportamento sexual de machos de ratinho de diferentes linhagens de duas subespécies irmãs: PWK / PhJ e PWD / PhJ - linhagens consanguíneas de *M.m. musculus* previamente não estudadas; e Balb / cJ e C57BL / 6J - linhagens de laboratório consanguíneas e domesticadas de *M. m. domesticus*.

Começamos com uma descrição detalhada do comportamento sexual masculino dentro das quatro linhagens. A um nível macroscópico, o comportamento apresentou-se consistente entre as quatro linhagens. Mas, quantitativamente, bastante diferente: enquanto os machos *musculus* necessitam de significativamente menos estimulação pré-ejaculatória e uma elevada percentagem reiniciou uma segunda série copulatória num curto período de tempo, os machos *domesticus* necessitaram de mais interação sexual e apenas um número residual reiniciou o acasalamento após a ejaculação. Uma vez que o comportamento sexual é o resultado de uma interação dinâmica entre macho e fêmea, para garantir que os parâmetros medidos eram de

fato relativos ao macho, numa experiência subsequente avaliamos o impacto da linhagem da fêmea nos padrões de acasalamento. Observamos um impacto nas fases consumatória e inibitória, no entanto, o tamanho do efeito demonstrou-se dependente da linhagem do macho. Em particular, a notável discrepância na latência para recuperar o interesse sexual após a ejaculação entre os machos PWK e BL6 não foi afetada pela linhagem da fêmea.

Os mecanismos subjacentes à variação observada na latência do PERP ou à sua função têm recebido pouca atenção. Como tal, permanecem até hoje como um dos maiores mistérios do comportamento sexual masculino. No entanto, o facto de ser altamente conservado entre as espécies implica um papel evolucionário e /ou ecologicamente importante. Embora sem ter sido formalmente testada, no campo da endocrinologia persiste a hipótese de que o pico de prolactina concomitante com a ejaculação está envolvido na subsequente diminuição da actividade sexual, a observação que caracteriza o PERP.

Neste contexto, na segunda parte desta tese, tiramos partido das diferentes durações de PERP dos machos PWK e BL6 para investigar o suposto papel da hormona prolactina no período refratário. Deste modo, começamos por monitorizar a dinâmica de libertação de prolactina durante o comportamento sexual nos machos de ambas as linhagens. Em seguida, através de uma abordagem farmacológica, testamos se manipulações agudas dos níveis de prolactina, seja mimetizando a libertação natural durante o comportamento sexual ou inibindo a sua ocorrência, têm impacto na actividade sexual ou alteram a duração do período refratário, respectivamente. Os nossos resultados mostram que, embora a prolactina seja libertada durante o comportamento sexual nos machos de ratinho, esta não é suficiente nem necessária para induzir o PERP. Portanto, o nosso trabalho refuta a ideia de que a prolactina libertada durante a cópula está envolvida no estabelecimento do PERP, uma hipótese vigente no campo da endocrinologia comportamental.

Sumarizando, além de introduzir as linhagens de ratinho PWK/PWD como um novo sistema modelo importante para estudar o comportamento sexual de ratinho, esta tese contribuiu para o enriquecimento do conhecimento sobre o PERP.

Abstract

Sexual behavior in male rodents follows a basic sequence of species-typical patterns of appetitive and consummatory behavior. Appetitive patterns involve approach and olfactory investigation of the female (mostly anogenital sniffing), and consummatory behavior is characterized by bouts of mount attempts followed by multiple mounts with penile intromission and finally ejaculation. Following ejaculation, males enter a phase of sexual inactivity, with a drastic reduction on sexual interest in the female, named the post ejaculatory refractory period (PERP).

Thomas E. McGill pioneered the study of mouse sexual behavior, persisting to date as the best collective body of data on the issue of behavioral variability across different strains of mice. The male mouse sexual behavior is described as extremely variable between subspecies and within strains of the same subspecies. These phenotypic differences, rather than being a nuisance, can be taken to our advantage and used to gather insights into the evolutionary, genetic and neuronal mechanisms underlying this fundamental behavior.

A major goal of evolutionary neuroethology is to explain the neuronal mechanisms underlying the appearance of species-specific behaviors. This thesis explores some of these behavioral differences through a comparative study on male mouse sexual behavior of different strains from two closely related subspecies: PWK/PhJ and PWD/PhJ – previously unstudied inbred wild derived strains of *M.m. musculus*; and Balb/cJ and C57BL/6J - domesticated inbred laboratory strains of *M. m. domesticus*.

We started with a detailed description of male sexual behavior within the four strains. The behavior was consistent within, but vastly different across subspecies: while *musculus* males needed significantly less pre-ejaculatory stimulation and a high percentage of the males restarted a second copulatory series in a short period of time, *domesticus* males needed more sexual interaction and only a residual number re-mated after ejaculation. Since sexual behavior is the result of a dynamic interaction between male and female, to ensure that the parameters measured are indeed a function of the male, in a subsequent inter-strain mating assay, we assessed the impact of the female on the mating patterns. The female strain had an effect on both consummatory and inhibitory phases. However, the effect size was dependent on the male strain. In particular, the remarkable latency discrepancy to recover sexual interest after ejaculation in PWK and BL6 strains was not affected by the female.

The mechanisms underlying the observed variation in the latency of the PERP or its concrete function are scarcely studied and hence, until now remain as one of the biggest mysteries of male sexual behavior. However, the fact that it is highly conserved across species implies an evolutionary and or ecologically important role. Albeit without formal testing, it has been hypothesized that the prolactin surge observed around the time of ejaculation is involved in the immediate subsequent decrease of sexual activity, the hallmark of the PERP.

Hence, in the second part of this thesis, we took advantage of the different PERP durations for PWK and BL6 males to investigate the putative role of the hormone prolactin in the refractory period. For that, we started by monitoring the dynamics of PRL release during sexual behavior in the male mice. After that, using a pharmacological approach, we tested if acute manipulations of prolactin levels, either mimicking the natural release during sexual behavior or inhibiting its occurrence, have an impact in sexual activity or alters the refractory period length, respectively. Our results show that, although prolactin is released during sexual behavior in male mice, it is not sufficient nor necessary to induce the PERP. Therefore, our work refutes the idea that prolactin released during copulation is involved in the establishment of the PERP, a long-standing hypothesis in the field of behavioral endocrinology.

Overall, our work not only revealed the PWK/PWD mouse strains as an important model system to study mouse sexual behavior but also shed light into the previously little understanding of sexual satiety.

Author Contributions

Susana Valente and Susana Q. Lima designed the experiments.

Chapter 2: Susana Valente and the master student Gonalo Andr  performed the experiments and behavior annotation. Tiago Marques wrote the MATLAB code. Susana Valente analyzed the data.

Chapter 3: Susana Valente performed the experiments, prolactin quantification, annotation of the behavior, IdTracker data acquisition and analyzed the data. Tiago Marques wrote the MATLAB code.

Susana Valente wrote this thesis.

Financial Support

The research leading to these results was funded by the Champalimaud Foundation, UIDB/04443/2020, LISBOA-01-0145-FEDER-022170, LISBOA-01-0145-FEDER-022231, ERC Consolidator Grant (772827).

Susana Valente was supported by Funda o para a Ci ncia e Tecnologia grant number SFRH / BD / 51011 / 2010.

Publications

Valente, S., Marques, T. & Lima, S.Q. No evidence for prolactin’s involvement in the post-ejaculatory refractory period. *Commun Biol* 4, 10 (2021). <https://doi.org/10.1038/s42003-020-01570-4>

Motivation and structure of this thesis

Although male mouse sexual behavior is highly stereotyped, there is a vast variability regarding latencies and repetitions of the mating elements. This variability can be taken into our advantage to gain insight into the neural mechanisms underlying this fundamental behavior.

Recent studies including new strains of mice from the *M.m.musculus* subspecies report that, when compared to most domesticated inbred strains from the *M.m.domesticus* subspecies, inbred wild derived mice display a plethora of differences in a wide range of phenotypes, including their small size, vitality and high exploration (Gregorová and Forejt 2000; Fernandes et al. 2004; Tordoff 2008). Although wild derived strains were included in many of the large-scale genomic projects (Wade et al. 2002; Frazer et al. 2007; Yang et al. 2007; Chang et al. 2017) and as new models for disease (Lee et al. 2011; Onos et al. 2019) and assortative mate choice (Zinck and Lima 2013) studies, their potential as models for sexual behavior has not been assessed. Insofar, the majority of the research in male mouse sexual behavior were conducted in strains of domesticated inbred laboratory mice of the *M.m.domesticus* subspecies.

The present Thesis is composed by four Chapters. Chapter 1 consists of a general introduction where I present a literature review of relevance for the topics discussed in the following chapters. The following two Chapters correspond to the body of original research produced during my doctoral studies. In Chapter 2 I investigate male mouse sexual behavior exploring the probability of the differences reported between the subspecies being also extended to sexual behavior. I conducted a comparative study on male mouse sexual behavior of different strains from the two closely relates subspecies: PWK/PhJ and PWD/PhJ – previously unstudied inbred wild derived strains of *M.m. musculus*; and Balb/cJ and C57BL/6J - domesticated inbred laboratory strains of *M. m. domesticus*. A major finding of this study was that, contrary to the BL6 males that can take days to resume copulatory behavior after ejaculation, a high percentage of PWK males restarted a second copulatory series within a short period of time (< 120min). This remarkable latency disparity to recover sexual interest after ejaculation between PWK and BL6 strains led me to the Chapter 3 of this thesis where we investigated a possible mechanism responsible for this temporal difference. Several lines of evidence have led to the hypothesis that the prolactin surge around the time of ejaculation plays a role in the immediate subsequent decrease of sexual activity. Therefore, we

investigated the potential role of the hormone prolactin in the establishment of the post ejaculatory refractory period. I conclude the thesis with Chapter 4 that consists of a general discussion where I summarize and contextualize our findings. I discuss the generality of our results and bring up some of the questions and research avenues that arise from our work.

List of figures

Figure 1.1. Phases of the sexual cycle.....	3
Figure 2.1. Within-strain mouse sexual behavior ethogram.....	13
Figure 2.2. <i>Musculus</i> males require less mount duration to reach ejaculation.	14
Figure 2.3. <i>Musculus</i> males have a short refractory period.	15
Figure 2.4. Between-strain mouse sexual behavior ethogram.....	17
Figure 2.5. Male mouse sexual behavior is relative and adaptable to the female.	19
Figure 2.6. Sexual dynamics across the behavioral session.	20
Figure 2.7. The refractory period is an absolute trait, intrinsic to PWK and BL6 males.....	21
Figure 2.8. Occurrence of s-PERP cannot be predicted from individual consummatory parameters.	22
Figure 2.9. s-PERP duration is on average weakly correlated with individual copulatory parameters.	23
Figure 3.1. Prolactin is released during sexual behavior in male mice.	37
Figure 3. 2. Domperidone induces a sharp rise in the levels of circulating PRL of similar magnitude to the release during copulation.....	39
Figure 3.3. Mouse sexual behavior ethogram for Domperidone manipulation.	40
Figure 3.4. Acute prolactin release does not induce a refractory period-like state.	41
Figure 3.5. Bromocriptine blocks prolactin release during consummatory behavior.....	42
Figure 3.6. Mouse sexual behavior ethogram for Bromocriptine manipulation.....	43
Figure 3.7. Blocking prolactin release during copulation does not decrease the duration of the refractory period.....	45

List of tables

Table 2.1. Pairing combinations between <i>Mus musculus domesticus</i> and <i>Mus musculus musculus</i> subspecies.....	30
Table 2.2. Definitions of the measures of copulatory behavior used in this study. Time in minutes.....	31

Content

Acknowledgments	iv
Resumo	v
Abstract	vii
Author Contributions	ix
Financial Support	ix
Publications	ix
Motivation and Structure of the Thesis.....	x
List of Figures	xii
List of Tables	xiii
1. Introduction.....	1
1.1 Rodent sexual behavior.....	2
1.2 Phases of rodent sexual behavior	3
1.2.1 Appetitive phase.....	4
1.2.2 Consummatory phase	4
1.2.3 inhibitory phase	7
1.3 Outlook	8
2. Male sexual behavior	9
2.1 Summary	9
2.2 Introduction	10
2.3 Results.....	11
2.3.1 Within-strain behavioral assays: subspecies characteristics.....	11

2.3.2	Between-strain behavioral assays: the role of the female	16
2.3.3	The post ejaculatory refractory period	21
2.4	Discussion.....	24
2.4.1	Male mating strategy	24
2.4.2	Female role in mating behavior	26
2.4.3	Predicting the post ejaculatory refractory period	27
2.5	Conclusion	28
2.6	Methods	29
3.	The post ejaculatory refractory period	33
3.1	Summary	33
3.2	Introduction	33
3.3	Results.....	36
3.3.1	Prolactin is released during sexual behavior in male mice	36
3.3.2	Acute prolactin release does not induce a refractory period-like state	38
3.3.3	Blocking prolactin release during copulation does not decrease the duration of the refractory period	42
3.4	Discussion.....	46
3.5	Methods	49
4.	General discussion	54
4.1	Male mouse mating behavior	55
4.2	The inhibitory phase and the role of prolactin	58
4.3	Closing Remarks.....	59
	References	60
	Supplementary data.....	71
	Supplementary data 1	73
	Supplementary data 2	74
	Supplementary data 3	75
	Supplementary data 4	77
	Supplementary data 5	79
	Appendix A	80

Chapter 1

1. Introduction

Unlike many behaviors, such as feeding or drinking, which are crucial for the survival of the individual, sex is not required for the viability of the individual, but rather for the continuity of the species (Crawford et al. 1993). Evolution came up with thousands of solutions when it comes to sexual behavior, from the splendorous appearance of attraction signals, to the amazing variety of courtship rituals, the enormous diversity of copulating organs or the fact that it is a highly rewarding behavior in many species. But sex is also dangerous, as it exposes animals to predation, for example. Therefore, sexual behavior must be tightly controlled, to maximize fertilization and minimize risk (Ågmo 1999). Consequently, to obtain a wholistic view of this behavior and its underlying neuroendocrine mechanisms, it is fundamental to consider all its components, from initiation to consummation, but also its termination.

While the first organisms reproduced simply by cloning themselves, producing progeny identical to the parents, evolution led to the appearance of sexual reproduction, the most commonplace reproductive strategy amongst eukaryotes (Otto 2009; Dimijian 2017). Several hypotheses have been put forward to explain the appearance and maintenance of sexual reproduction, from the production of variable offspring that can keep up with changing environments to the elimination of deleterious mutations (Otto 2009; Dimijian 2017), even though heated controversy still marks this field. During the evolution of sexual reproduction, several steps occurred leading to the existence of gametes of very different size (egg and sperm), each containing only one half of the genetic material, which can produce a new individual upon fusion (fertilization) (Baker and Parker 1973). Together with the appearance of the egg/sperm, complementary behavioral phenotypes, female- and male-like, whose dimorphic actions lead to the execution of sexual behavior also emerged, ensuring that sperm is delivered to the egg and fertilization is achieved (Breed and Moore 2016; Dixson 2018; Anholt et al. 2020).

This thesis pertains to sexual behavior, to the general quest of understanding the mechanisms underlying the coordinated action of two individuals, how it leads to the expulsion of sperm from the male reproductive organ inside the female for egg fertilization. Because

sexual behavior and its underlying mechanisms differ widely across taxa, this thesis will focus on rodents, in particular the house mouse, the object of study during my PhD.

1.1 Rodent sexual behavior

Rodents (especially the house mouse, *Mus musculus*, and the rat, *Rattus norvegicus*) are, for many decades, one of the most used animal models (Ellenbroek and Youn 2016). Rodents are used in many research fields, from immunology to development, or cancer biology to endocrinology. Rats and mice are important subjects of study in behavioral neuroscience, especially when the goal is to investigate neuroendocrine mechanisms underlying behavior (Georgiadis et al. 2012; Bialy et al. 2019). But rodents are also fascinating to study even if no application to humans is considered. First, they are the largest existent group of mammals, representing half of the species in this clade, and can inhabit almost all places on earth, withstanding extreme environments from deserts to the artic. They exhibit a myriad of behavioral adaptations: some can swim, while others fly or burrow complex underground tunnels. Their size varies tremendously, from some of the smallest mammals on earth, to the dog sized capibara (Nowak 1983).

Rodents belong to the few mammalian groups in which details of male copulatory behavior have been studied extensively (Dewsbury 1972; Burns-Cusato et al. 2004; Hull and Rodríguez-Manzo 2017). Among the most complete descriptive papers on rodent sexual behavior, are the publications from Frank Beach and colleagues showing “normative data on sexual behavior in male rats”, introducing a systematic classification of behavior patterns, that pushed the field forward (Beach 1956; Beach and Jordan 1956; Larson 1956). Beach was followed by Thomas E. McGill with pioneering studies of male mouse sexual behavior (McGill 1962; McGill 1963; McGill and Blight 1963a; McGill 1969). His body of work currently persists as the best collective body of data on strain differences in mating behavior in male mice (Burns-Cusato et al. 2004)

In broad strokes, after the perception of cues arising from a potential mate, the male/female will pursue each other until reaching their target. Once met, both will thoroughly investigate each other, increasing sexual arousal. Usually the female turns into the male’s direction and then runs away, forcing the male to chase her. When the male approaches the female from the rear, she will display hopping and darting sequences of locomotion before allowing the male to mount her. Following the mount, the female runs away in order to pace the rate of copulation. The male typically chases her until she stops again and holds a lordosis crouch, allowing him

to mount. If the male stops chasing, then she will have to solicit to initiate another mount. When mounting results in intromissions, the male will perform pelvic thrusts corresponding to the penile insertion. The chasing and mounting will continue until ejaculation occurs, ending the sexual series (McClintock 1984; Erskine 1989; Hull and Dominguez 2007; Ågmo and Snoeren 2017).

1.2 Phases of rodent sexual behavior

Unlike humans, non-human animals almost always show highly stereotyped motor patterns when copulating. In rodents the copulatory behavior consists of series of mount attempts (without intromission), mounts (with intromission, no sperm transfer) ending with a final mount leading to ejaculation (sperm transfer) (Mosig and Dewsbury 1976). The sequence of events, beginning with the introduction of a receptive female and ending with genital disengagement following ejaculation can be used to divide the sexual cycle into three phases: appetitive, consummatory and inhibitory (Frank A. Beach 1976; Lenschow and Lima 2020) (Fig. 1.1).

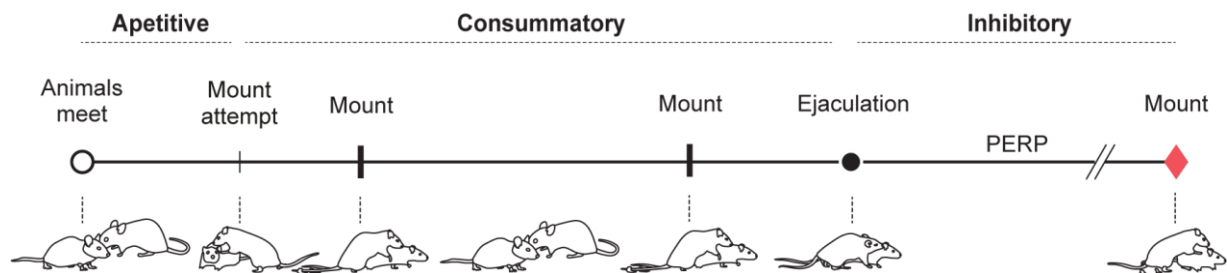


Figure 1.1. Phases of the sexual cycle.

The sexual cycle comprises 3 phases: Appetitive – from the moment the animals meet to the first mount attempt or mount; Consummatory – from the first mount attempt or mount to ejaculation, and Inhibitory – from ejaculation to the first consummatory event of the next copulatory series - called post-ejaculatory refractory period (PERP).

1.2.1 Appetitive phase

By its nature, sexual intercourse requires a close and cooperative association between male and female that is not present in any other social behavior. The appetitive phase is loosely defined as containing a series of behaviors that increase the probability of gaining access to consummation. The appetitive phase consists of bouts of intense social touch and anogenital and facial sniffing, interleaved with male chasing and female teasing and running away. Appetitive behaviors can also include ultrasonic vocalizations, urine marking and rooting (male putting his head under the female and raising the head to sniff) (Frank A Beach 1976; McClintock 1984; Knobil and Neill 2016; Ågmo and Snoeren 2017).

It is believed that this initial phase serves two main purposes. First, to gain information about prospective mates, such as sex, reproductive state and identity. For example, major urinary proteins were shown to allow individual recognition in mice (Hurst 2009). Second, cues emitted by both sexes are known to increase sexual arousal. Several molecules that can promote attraction and sexual receptivity have been identified. For example, darcin is a male major urinary protein that is innately attractive to females, that can also drive learning of neutral cues (Roberts et al. 2010) while ESP1, a protein present in male tears, was shown to induce female sexual receptivity (Haga et al. 2010). Other pheromones present in other tissues, such as in anal glands, have been identified in a variety of rodent species (Tirindelli et al. 2009).

How all these cues are integrated to initiate and maintain investigatory behavior, to the point where the male attempts to copulate and the female stops rejecting the male's attempts is still understudied.

1.2.2 Consummatory phase

When sexual excitement is increased to a point that a copulatory threshold is reached, the consummatory phase is initiated. The goal of the consummatory phase is to bring sperm to the egg. In rodents, like many vertebrates, this is achieved by inserting the male reproductive organ inside the female one, and then triggering ejaculation (Hull et al. 2006; Nelson and Kriegsfeld 2017). Also, similarly to many other species, ejaculation depends on the sensory stimulation of the male organ, which is mostly achieved through the repeated insertion of the penis inside the female. The beginning of the consummatory phase is usually controlled by the male, once he tries to mount the female.

1.2.2.1 Mounting

In an attempt to mount, the male will approach the female from the back and will lift his fore paws, touching her on the flanks (Dewsbury 1972). If the female is in the non-fertile phase of the cycle, she will unequivocally reject his attempts of copulation, by walking away, or even kicking and punching if the male is too insisting (McGill 1962; Zinck and Lima 2013; Nelson and Kriegsfeld 2017; Lenschow and Lima 2020). When fertile and receptive, the male's attempts of copulation can end up in failed mount attempts, either because he does not grab the female properly, or because she can initially reject him (personal observation). Eventually, most ovulating females will accept the male and will adopt the lordosis posture, arching of the back that aids penetration. Despite the fact that lordosis behavior is dependent on the concentration of sex hormones, its trigger can differ quite drastically across different species of rodents (Dewsbury 1972; Frank A Beach 1976).

If successful, male mounting allows penile insertion in the female vagina and the execution of pelvic thrusting, an oscillating behavior where powerful muscles in the pelvic floor of the male sustain "pendulum-like" behavior that can lead to several rounds of penile insertion. Although there is a high degree of conservation in consummatory behavior, different rodent species vary quantitatively in their copulatory behavior. Dewsbury (Dewsbury 1972) developed a classification system of the rodent copulatory behavior based on a binary choice:

a) Single or multiple-mount pattern

For golden mice ejaculation can occur in the first mount. However, this single-mount pattern is mostly considered an individual trait observed in several species, rather than a species characteristic (Young and Grunt 1951; Dewsbury 1972). For males with multiple-mounts, a number of mounts with no sperm transfer is a prerequisite to the occurrence of ejaculation. This number varies across and within species, from 1-4 mounts in guinea pigs (Young and Grunt 1951) to 10 mounts in rats (Beach and Jordan 1956), contrasting with the dozens of mounts preceding ejaculation in mice (McGill 1962), gerbils, hamsters and a great number of *Peromyscus* species (Dewsbury 1983).

b) Single or multiple-intromission pattern

In many species a series of shallow pelvic thrusts precedes intromission and serves to locate the vagina (Bermant 1965). However, once penetration is achieved, the males of many species, such as laboratory rats (Beach and Jordan 1956), hamsters (Beach and Rabedeau

1959; Boland et al. 1977) and gerbils (Dewsbury 1972) cease pelvic thrusting and dismount. By contrast, other species such as the house mouse, *Mus musculus*, (McGill 1962), california mice (Dewsbury 1975) and guinea pigs (Young and Grunt, 1951) can perform a large number of pelvic thrusts after the first intromission is attained. The frequency of intromissions is specific for each species and interestingly, for species with multiple-intromissions per mount, the frequency can sometimes decrease across the mount (Moralí et al. 2003).

The mount duration is variable (and therefore is composed of variable number of intromissions in species with multiple-intromission pattern) across species, across individuals of the same species and even across mounts from the same individual (Dewsbury 1972; Burns-Cusato et al. 2004). How the mount duration and the number of intromissions per mount is controlled is unknown, but in the mouse for example, mount duration seems to be mostly controlled by the female (when using naturally cycling females, personal observation). While in the beginning of the mount the female is expressing lordosis, at some point she will start trying to escape. Following each mount males will engage in genital grooming, and several seconds or minutes may pass before the next mount. During this inter-mount interval, the female may also approach the male and execute proceptive behaviors (McClintock and Adler 1978; Erskine 1989). In fact, the inter-mount interval seems to be an important parameter for the female. For both female mice and rat pacing of the sexual interaction, which can be achieved when in environments where the female can escape the male after each mount, seems to be rewarding (Mermelstein and Becker 1995; Pfaus et al. 1995; Martínez and Paredes 2001; Johansen et al. 2008). In males, it has been shown that mounts and intromissions are rewarding as they will establish place preference for contexts where these behaviors were executed (Kudwa et al. 2005).

In both patterns, the sexual episode ends with a final mount leading to ejaculation.

1.2.2.2 Ejaculation

Through mechanisms that are not fully understood, the sensory stimulation obtained with penile insertion is needed for ejaculation to occur. Independently of the species-specific mounting pattern, ejaculation can be divided in two main phases: emission, where seminal fluid and sperm are mixed and the necessary coordination between the different components of the autonomic system is achieved to open and close different pathways (Hull and Rodríguez-Manzo 2017). Once emission is achieved, the rhythmic contraction of muscles in the

reproductive organ will lead to the second phase, expulsion of sperm into the female (Hull and Rodríguez-Manzo 2017). In many species, such as the mouse, ejaculation is accompanied by characteristic behaviors, including shivering and collapsing to the side of the female, where the male can stay immobile for several seconds (McGill 1962). After, the male will detach from the female and will engage in genital grooming.

Besides sperm and seminal fluid, many rodent species leave behind a plug, composed of secretions of the seminal and coagulating glands which coagulates within the vagina, blocking the access to the female's vagina and therefore further copulation by other males (Hartung and Dewsbury 1978; Komisaruk and González-Mariscal 2017).

1.2.3 inhibitory phase

Following ejaculation, sexual motivation drops and the male enters an inhibitory period called the post-ejaculatory refractory period (PERP). This recovery period is comprised of two phases: an absolute period where the male no longer responds to any sexual stimulus, and a relative period, which can be terminated when in the presence of a new female, which can instigate sexual motivation (Beach and Holz-Tucker 1949).

The PERP interval varies significantly from less than 60 seconds in hamsters (Boland et al. 1977), a few minutes in rats (Beach and Jordan 1956), to days in mice (McGill 1962; McGill and Blight 1963b). Following Dewsbury's (Dewsbury 1972) classification system, based on the PERP duration, males can have a single or multiple-ejaculation pattern. In some species, such as vervets (Gartlan 1969), guinea pigs (Young and Grunt 1951) and inbred strains of mice (McGill, 1962; Mosig & Dewsbury, 1976) copulatory activity is considered effectively terminated with the first ejaculation. Conversely, species like rats (Beach and Jordan 1956), several *Peromyscus*, golden mice (Dewsbury 1972; Dewsbury 1983) and hamsters (Boland et al. 1977) are capable of several ejaculations before the point of sexual exhaustion/satiety is reached. Male rats can ejaculate five to seven times before becoming sexually sated with only 6% ejaculating in the next day (Beach and Jordan 1956; Sachs and Barfield 1976). Deer mice may ejaculate four to five times prior to satiation and 80% of the males can have a new ejaculation just after one day of recovery (Dewsbury 1983) while hamsters can ejaculate around 9 times, needing only 8h to reach the same proportion (Beach and Rabedeau 1959). During the successive ejaculatory series leading to exhaustion, the refractory period increases

progressively between each ejaculation (Boland et al. 1977; Komisaruk and González-Mariscal 2017)

Although there is a large body of literature describing rodent sexual behavior, there is still paucity of knowledge over the mechanisms underlying the refractory period and the behavioral variability.

1.3 Outlook

As already mentioned, the large behavioral diversity observed within the rodent order has for long fascinated evolutionary biologists interested in understanding biodiversity, since one can compare multiple species that arise from a common ancestor. But this diversity of closely related species can also be taken advantage by researchers interested in understanding the neuronal mechanisms underlying behavior. Nervous systems have been shaped by evolution and comparative approaches take advantage of the resulting behavioral diversity across related species to gain insight into the neural mechanisms underlying behavior, since the brains are very similar.

One successful example is the observation of the relationship between neurotransmitter receptor density and reproductive strategy. The prairie voles and the meadow voles are two different species of the *Microtus* genus that are similar in appearance and in many aspects of nonsocial behavior, but display marked differences in social organization: prairie voles are monogamous and the meadow are polygamous. A comparative neurotransmitter screen revealed that, in males, the pattern of the vasopressin receptor was as different as their social behavior (Insel et al. 1994). Altering this pattern by simply infusing a vasopressin antagonist, prairie voles decreased the amount of time they spent with their partner (Lim and Young 2004). Along these same lines, by genetically increasing the expression of this receptor, meadow voles showed a behavior similar to the monogamous voles (Young et al. 1999).

Inbred animals provide a well-defined and stable genotype for analysis, a valuable tool to relate central and behavioral functioning. In the case of mice, a wealth of laboratory strains is available making them a great model system for comparative studies. But in order to delve into the mechanisms, first we must perform behavioral studies, characterize in detail the behavioral differences between the subjects and, when possible, take advantage of behavioral manipulations to gain insights into the possible sources of the behavioral variations.

2. Male sexual behavior

2.1 Summary

Naturally occurring variations in behavior between closely related species are many times the outcome from small differences in otherwise very similar brains, and therefore can provide unique access into the mechanisms underlying behavior. The house mouse, *Mus musculus*, one of the most commonly used mammalian laboratory animals, is in a unique position for such comparative studies, due to the existence of closely related subspecies and the availability of hundreds of strains.

Here we describe the sexual behavior of two previously unstudied strains of house mouse from the *Mus musculus musculus* subspecies, the inbred wild derived PWD and PWK, and compare it to the sexual behavior of two commonly used strains from the *Mus musculus domesticus* subspecies, the laboratory strains BalbC and BL6. Our results show that, in the context of within-strain copulation (male and female from the same strain) although the overall macroscopic organization of sexual behavior is maintained, *musculus* and *domesticus* differ significantly in quantitative measures of copulatory behavior. In particular, *musculus* males seem to require overall less mounting behavior in order to achieve an ejaculation, as they perform less mounts and mounts of shorter duration to ejaculate. However, the overall mounting required to achieve ejaculation is not an intrinsic property of the male, as it changes when males copulate with females from other strains. This is, the consummatory aspects of sexual behavior of both subspecies are flexibly modulated by the sexual interaction. Finally, the influence of the female on the percentage of males that restart mounting after ejaculation varies across strains: while for the BL6 and PWK males, the PERP seems to be an absolute trait, not affected by the female strain/the dynamics of the sexual interaction, for PWD males its occurrence seems to be dependent on the female.

In summary, this type of behavioral manipulations revealed that consummatory and inhibitory aspects of sexual behavior are complex traits, which depend on external factors and intrinsic male factors, respectively, and may offer new insights into the mechanisms controlling ejaculation and the refractory period.

2.2 Introduction

Based on genetic profiles, the house mice entail three *Mus musculus* subspecies, *castaneus*, *musculus*, and *domesticus*, that diverged from a common ancestor about one million years ago (Frazer et al. 2007). The distinct subspecies have evolved to dominate largely nonoverlapping segments of the world, with *domesticus* in the Mediterranean region and Western Europe; *musculus* in Eastern Europe and Northern Asia; and *castaneus* in southeast Asia. However, although speciation is kept, *musculus* and *domesticus* cohabit in a narrow region from Denmark to Bulgaria, called hybrid zone (Nachman and Payseur 2012).

Beginning in the 1960s, Thomas E. McGill conducted pioneering studies of male mouse sexual behavior. His body of work remains the best collective body of data on strain differences in mating behavior in male mice. Insofar, the majority of the studies on mouse sexual behavior were performed with domesticated strains of *Mus musculus domesticus* (Burns-Cusato et al. 2004; Park 2011; Knobil and Neill 2016). Classical laboratory strains, like the commonly used C57BL/6J, were developed over 100 years ago from crosses between domesticated European and East Asian fancy mice (Wade et al. 2002). More recently, novel inbred mouse strains that are genetically distinct from the commonly used models have been developed from wild-caught *Mus musculus musculus* mice (Gregorová and Forejt 2000). Studies including new strains of inbred wild derived *musculus* subspecies reported that, when compared to most domesticated inbred strains from the *domesticus* subspecies, inbred wild derived animals display a plethora of differences in a wide range of phenotypes, including their small size, vitality and high exploration (Gregorová and Forejt 2000; Fernandes et al. 2004; Tordoff 2008). These wild-derived inbred strains have been included in many of the large-scale genomic projects (Wade et al. 2002; Frazer et al. 2007; Yang et al. 2007; Chang et al. 2017) and as new models for disease (Lee et al. 2011; Onos et al. 2019) and assortative mate choice (Zinck and Lima 2013) studies. Interestingly, in this study it was shown that, when no choice is available, wild derived *musculus* females will mate with *domesticus* males, providing evidence that the assortative mate preference of *musculus* females is not caused by an inability to mate with more domesticated males. However, the potential to use wild derived animals as models for sexual behavior has not been assessed.

In this study, we present the first characterization of male sexual behavior for the PWD and PWK strains of the *musculus* subspecies. We also describe inter-subspecies behavioral differences between *musculus* and *domesticus* through a comparative study on male mouse

sexual behavior. We observed that the behavior was consistent within, but vastly different between subspecies: while *musculus* males needed significantly less pre-ejaculatory stimulation and a high percentage of the males restarted a second copulatory series in a short period of time, *domesticus* males needed more sexual interaction and only a residual number re-mated after ejaculation. As sexual behavior is dependent on the sexual interaction with a female, in a subsequent inter-strain mating assay, we assessed the impact of the female strain on the mating pattern. The female strain had an effect on both consummatory and inhibitory phases. However, the effect size was dependent on the male strain.

2.3 Results

2.3.1 Within-strain behavioral assays: subspecies characteristics

To characterize the sexual behavior dynamics of the *domesticus* and *musculus* subspecies, we performed a within-strain behavioral assay. For that we used two representative strains of each subspecies: C57BL/6J and Balb/cj as *Mus musculus domesticus* (from here on *domesticus*: BL6 and BalbC, respectively), and the inbred wild-derived mice PWK/PhJ and PWD/PhJ as *Mus musculus musculus* (from here on *musculus*: PWK and PWD, respectively). Sexually experienced males were paired with sexually experienced hormonally-primed females from the same strain. Animals were allowed to interact until the male performed the first attempt of copulation after ejaculation (red diamonds) or until 120 minutes if no attempt was made (Fig. 2.1a; please see Methods for details).

2.3.1.1 Musculus males require less mount duration to reach ejaculation

All strains showed the stereotypic pattern of rodent sexual behavior comprised of mounts, mounts with intromissions and ejaculation as described by McGill (1962). Interestingly, just by observing the raster plots depicting the behavior (Fig. 2.1b), we can immediately observe differences in the dynamics of sexual behavior between the two subspecies, in particular the difference in the amount of total mounting behavior.

The 4 inbred strains were compared on 14 different parameters (Table 2.2 in Methods). Strain differences were noted for the majority. Quantification of the individual behavioral

parameters showed that, in comparison to *domesticus*, *musculus* engage in sexual behavior with a shorter latency (Fig. 2.2a) and require less total mount duration in order to reach ejaculation, seen by the decreased number (Fig. 2.2b) and duration (Fig. 2.2c, d) of mounts. Particularly, BL6 males which exhibit a mount rate significantly higher comparatively to the other 3 strains (Fig. 2.2e), which is accompanied by a decreased latency between mounts, Inter-mount-interval (IMI, Fig. 2.2f). Nevertheless, the mount success index is the same for all 4 strains (Fig. 2.2g) as well as the pattern of mount distribution throughout the behavioral session (Fig. 2.2i).

Altogether these results show that although qualitatively similar, *musculus* and *domesticus* males seem to have quantitatively different matting needs as *musculus* need less mounting duration to reach ejaculation.

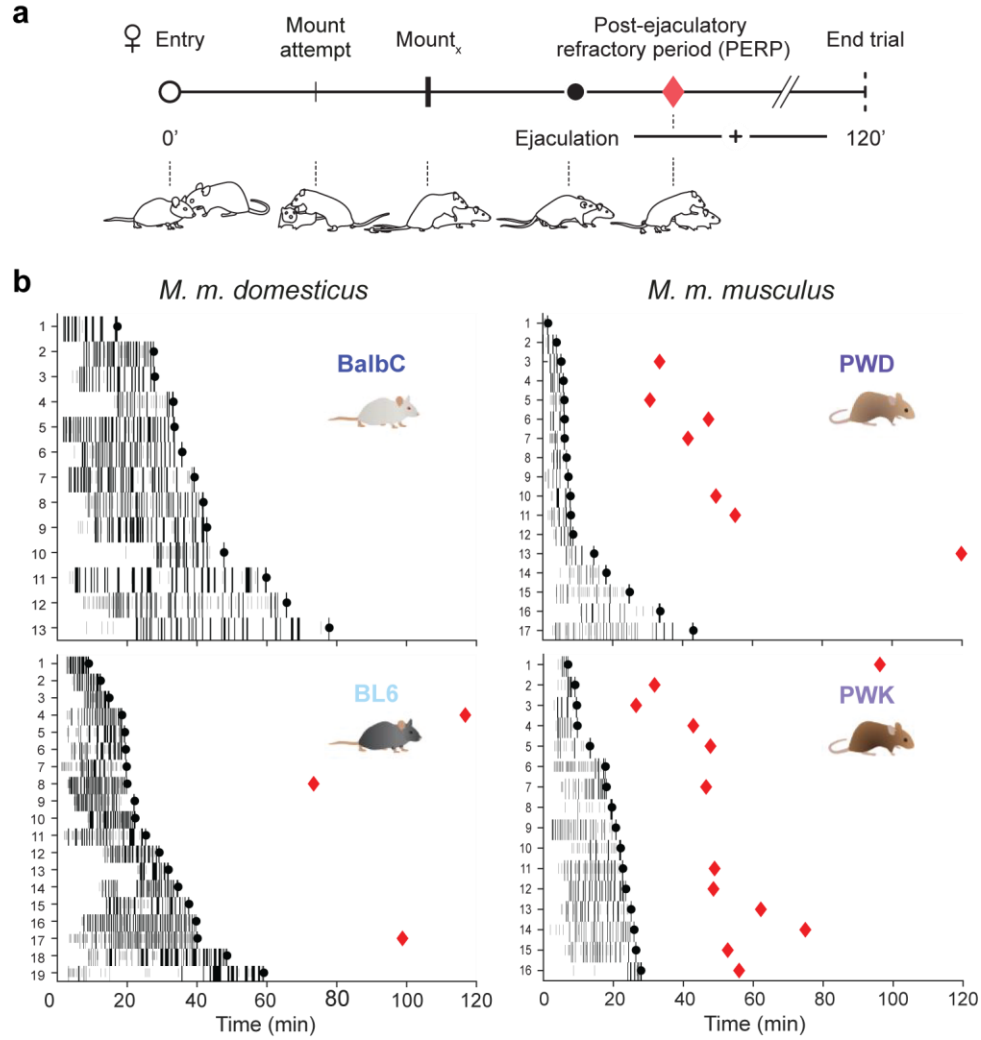


Figure 2.1. Within-strain mouse sexual behavior ethogram.

a Timeline for sexual behavior assay using sexually trained *domesticus* (BalbC, BL6) and *musculus* (PWD, PWK) animals. Each male was tested once and the behavioral trial corresponded to the second ejaculatory event. **b** Raster plot aligned to female entry ($t = 0'$), representing the sexual behavior performed by the male, with mount attempts represented in small grey bars, mounts in long black bars (width correlated with mount duration), ejaculation with a black circle and PERP (latency to the first consummatory event (mount attempt or mount) after ejaculation) represented with a red diamond. Plots sorted by latency to ejaculation. $n_{\text{BalbC}} = 13$; $n_{\text{BL6}} = 19$; $n_{\text{PWD}} = 17$; $n_{\text{PWK}} = 16$.

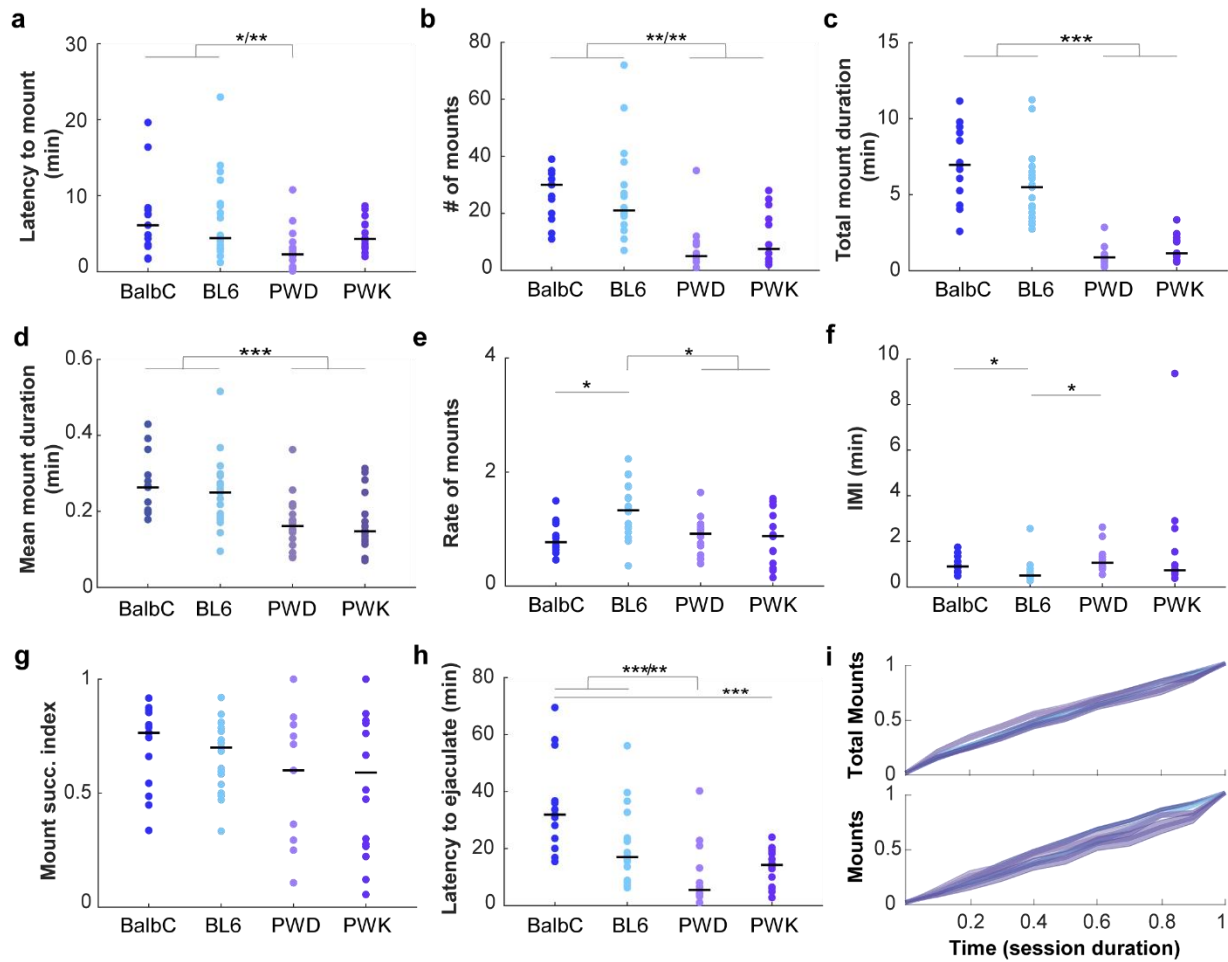


Figure 2.2. *Musculus* males require less mount duration to reach ejaculation.

Quantification of **a** latency to mount (first mount attempt or mount), **b** number of mounts, **c** total mount duration, **d** mean mount duration (total mount duration/#mounts), **e** rate of mounts (#mounts/lat. ejaculate), **f** Inter-mount interval (IMI), **g** mount success index [#mounts/(#mount attempt + #mounts)], **h** latency to ejaculate (from **a**). **i** Cumulative distributions of total mounts (mount attempts + mounts) and mounts along the behavioral assay. Histogram aligned to the first consummatory event with 0,1min bins. Time normalized for the duration of the session from first consummatory event to ejaculation. $n_{\text{BalbC}} = 13$; $n_{\text{BL6}} = 19$; $n_{\text{PWD}} = 17$; $n_{\text{PWK}} = 16$. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) by Kruskal Wallis followed by Dunn-Sidak. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

2.3.1.2 *Musculus* males have a shorter refractory period

After ejaculation males enter an inhibitory phase characterized by decreased sexual activity named the post-ejaculatory refractory period (PERP). We compared the occurrence (within the 120 minutes after ejaculation) and latency of PERP between the 2 subspecies. We characterized the latency as short-PERP (s-PERP) if the male resumed sexual activity within the 120 minutes period and long-PERP (l-PERP) if no attempt was made.

We found that *musculus* and *domesticus* also diverge in these parameters: none of the tested BalbC re-initiated the behavior after ejaculation and only 15,8% (3 out of 19) of the BL6 had a s-PERP. Conversely, *musculus* strains contain a high percentage of animals with s-PERP, specially PWK (Fig. 2.3a; PWD: 41,2% vs PWK: 75%).

Although with only 3 animals with s-PERP, BL6 have a significantly higher PERP duration comparatively to PWK males (Fig. 2.3b, BL6: 58.7 ± 7.9 vs PWK: 28.3 ± 7.74 , $P = 0.034$ by Kruskal-Wallis test with Dunn-Sidak correction).

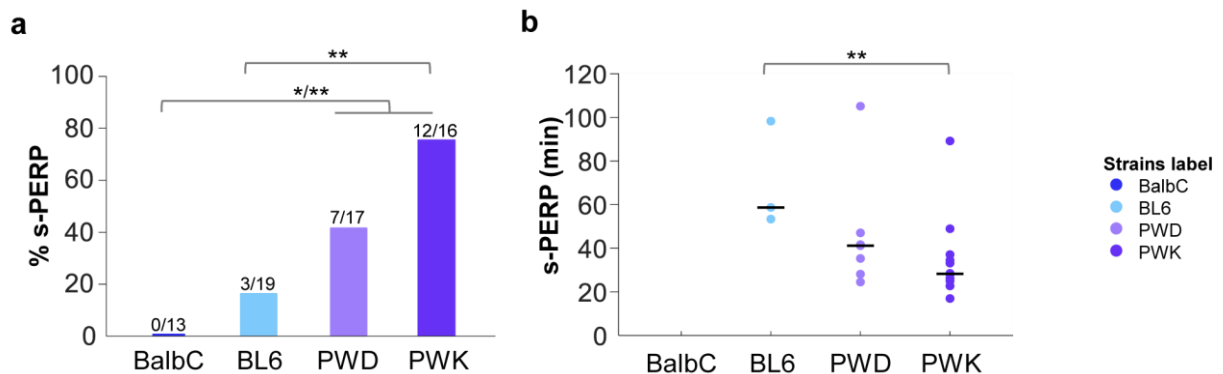


Figure 2.3. *Musculus* males have a short refractory period.

a Quantification of the percentage of males that re-initiated the consummatory behavior after ejaculating (s-PERP) and **b** duration of s-PERP. $n_{\text{BalbC}} = 13$; $n_{\text{BL6}} = 19$; $n_{\text{PWD}} = 17$; $n_{\text{PWK}} = 16$. Significance was calculated with **a** permutation and **b** median $\pm$ M.A.D. (median absolute deviation with standard scale factor) by Kruskal-Wallis test with Dunn-Sidak correction * $P < 0.05$, ** $P < 0.01$.

Together with the previous results, our data suggests that *musculus* and *domesticus* differ significantly in the amount of total mount duration needed to reach ejaculation and also in the time interval taken until the first mount of a new copulatory series, the PERP.

2.3.2 Between-strain behavioral assays: the role of the female

As presented above, besides presenting similar organization of sexual behavior, *musculus* and *domesticus* males show a very different number of mounts, as well as latencies to conduct the sexual interaction. Although not quantified, we can subjectively report that the female behavior is also different between subspecies. *Musculus*' females fight back and reject the male before mounting is allowed, exhibiting more proceptive behaviors, teasing the male with darts in his direction and then running away, soliciting his attention. Conversely, *domesticus*' females appear more submissive and the complete solicitation behavior is very rare.

2.3.2.1 Male mouse consummatory behavior is relative and adaptable to the female

As females display such different behaviors towards the male, we hypothesized that the difference observed between *musculus* and *domesticus* could rely on different female cues influencing the arousal elicited in the male and/or differences during the copulatory sequence that could affect the mount duration or the course to reach ejaculation. Therefore, in a second set of experiments we tested the impact of the female strain in the mating behavior, by systematically comparing the behavior when allowing the male to copulate with females from the different strains (Fig. 2.4; Methods table 2.1).

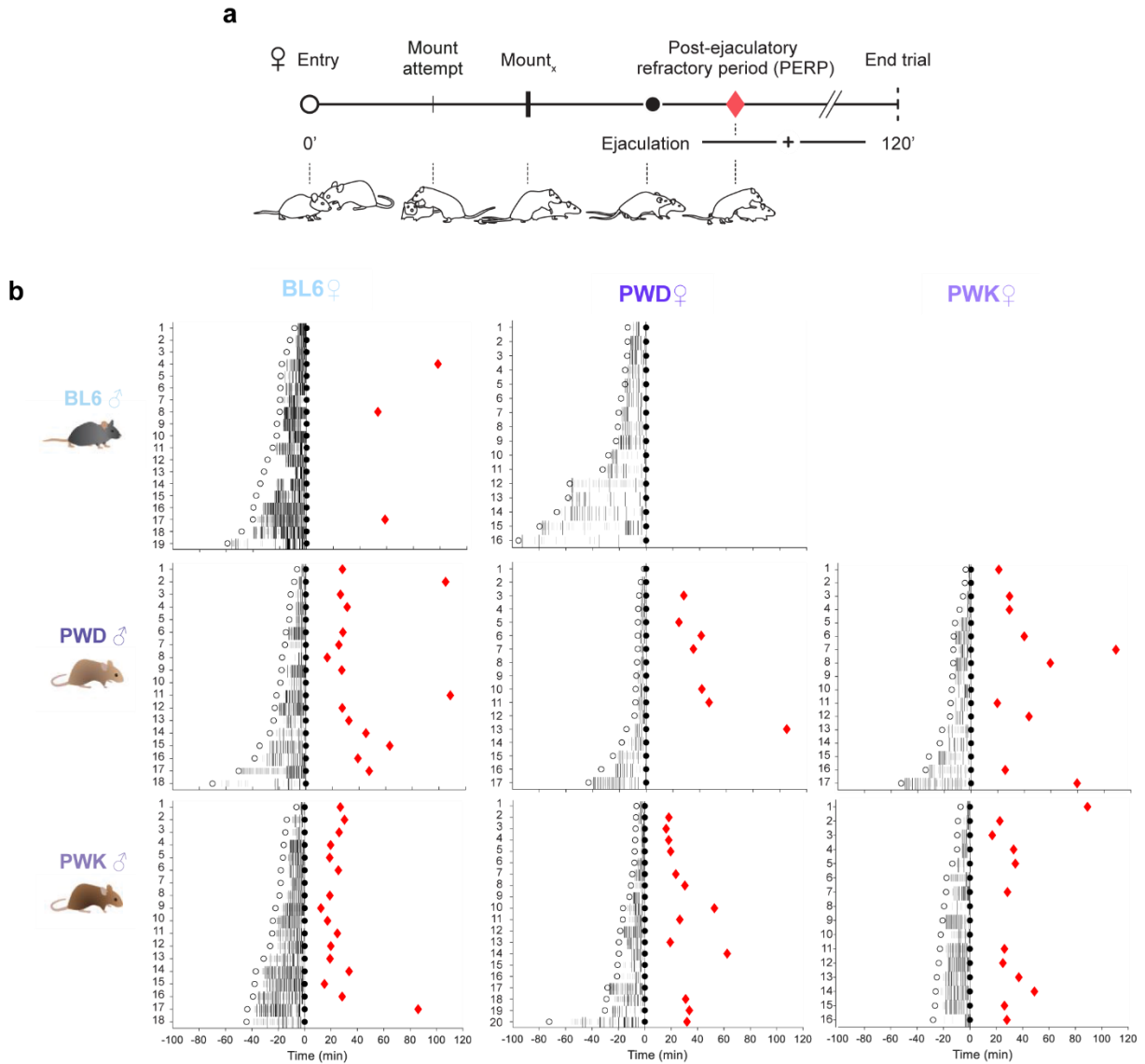


Figure 2.4. Between-strain mouse sexual behavior ethogram.

a Timeline for sexual behavior assay using sexually trained *domesticus* (BL6) and *musculus* (PWD, PWK) animals. Each male was tested once and the behavioral trial corresponded to the second ejaculatory event. **b** Raster plot aligned to ejaculation ($t = 0'$), representing the sexual behavior performed by the male, with mount attempts represented in small grey bars, mounts in long black bars (width correlated with mount duration), ejaculation with a black circle and PERP (latency to the first consummatory event (mount attempt or mount) after ejaculation) represented with a red diamond. The plots sorted by latency to ejaculation. $n_{BL6 \times BL6} = 19$; $n_{BL6 \times PWD} = 16$; $n_{PWD \times PWD} = 17$; $n_{PWD \times PWK} = 17$; $n_{PWD \times BL6} = 18$; $n_{PWK \times PWK} = 16$; $n_{PWK \times PWD} = 20$; $n_{PWK \times BL6} = 18$.

Because of technical difficulties (BalbC males did not manage to mate with *musculus* females), we did not continue with the BalbC strain.

Female subspecies have a major impact on BL6 male sexual behavior as all behavioral parameters, except the latency to ejaculate, are affected (Fig. 2.5). Comparatively to when copulating with females from their own strain, BL6 males mating with PWD females exhibit shorter latency to start mounting (Fig. 2.5a) and require less total mount duration, as the number, duration and rate of mounts significantly decrease (Fig. 2.5b-e). Although requiring less mounting behavior, the latency to ejaculate is the same (Fig. 2.5h), in part as a result of an increased inter-mount interval (Fig. 2.5f) and a lower mount success rate (Fig. 2.5g). Moreover, the dynamics of the sexual interaction across the session is altered, as mounts with PWD female are more concentrated in the beginning to middle of the session compared to BL6 female, where the mounts are more evenly distributed along the whole session (Fig. 2.6a), suggesting a higher motivation or sexual arousal with the PWD female.

Behavioral studies using wild-derived strains observed notable strain-related differences (Koide et al. 2000). Surprisingly, BL6 males were not able to mate with PWK females. The females actively rejected the BL6 males which only managed to perform few mounts due to its size leverage, but never managed to reach ejaculation. Several attempts were made with different pairs (n=20), always with the same outcome. Therefore, we excluded the combination BL6 male – PWK female.

Musculus males showed a very consistent behavior when interacting with *musculus* females (Fig. 2.5). Conversely, interaction with BL6 females induced some behavior alterations, especially for PWD males. For PWD males, both latency and number of repetitions are affected. They take longer to start mounting and to ejaculate (Fig. 2.5a and h, respectively) needing more mounts and an overall longer total mount duration to reach ejaculation (Fig. 2.5b and c, respectively). The mating dynamics is also slightly altered with more mounts towards the end of the session with BL6 females (Fig. 2.6b), suggesting a reduced motivation or sexual arousal with the BL6 female. Similarly, for PWKs an increase in the number of mounts and an increase in the overall period of mounting duration was observed (Fig. 2.5b and c, respectively). However, the overall structure of the mating pattern (Fig. 2.6c) were maintained for the three female's subspecies. Despite the described female effects, for both *musculus* the duration and rate of the mounts execution (Fig. 2.5d and e, respectively) as well as the male efficiency (Fig. 2.5g) were not affected.

These results show that the execution of the consummatory behavior is not an intrinsic property of the male but rather flexible and dependent on the particular female the male is interacting with, with BL6 and PWK males at the opposite ends of the spectrum.

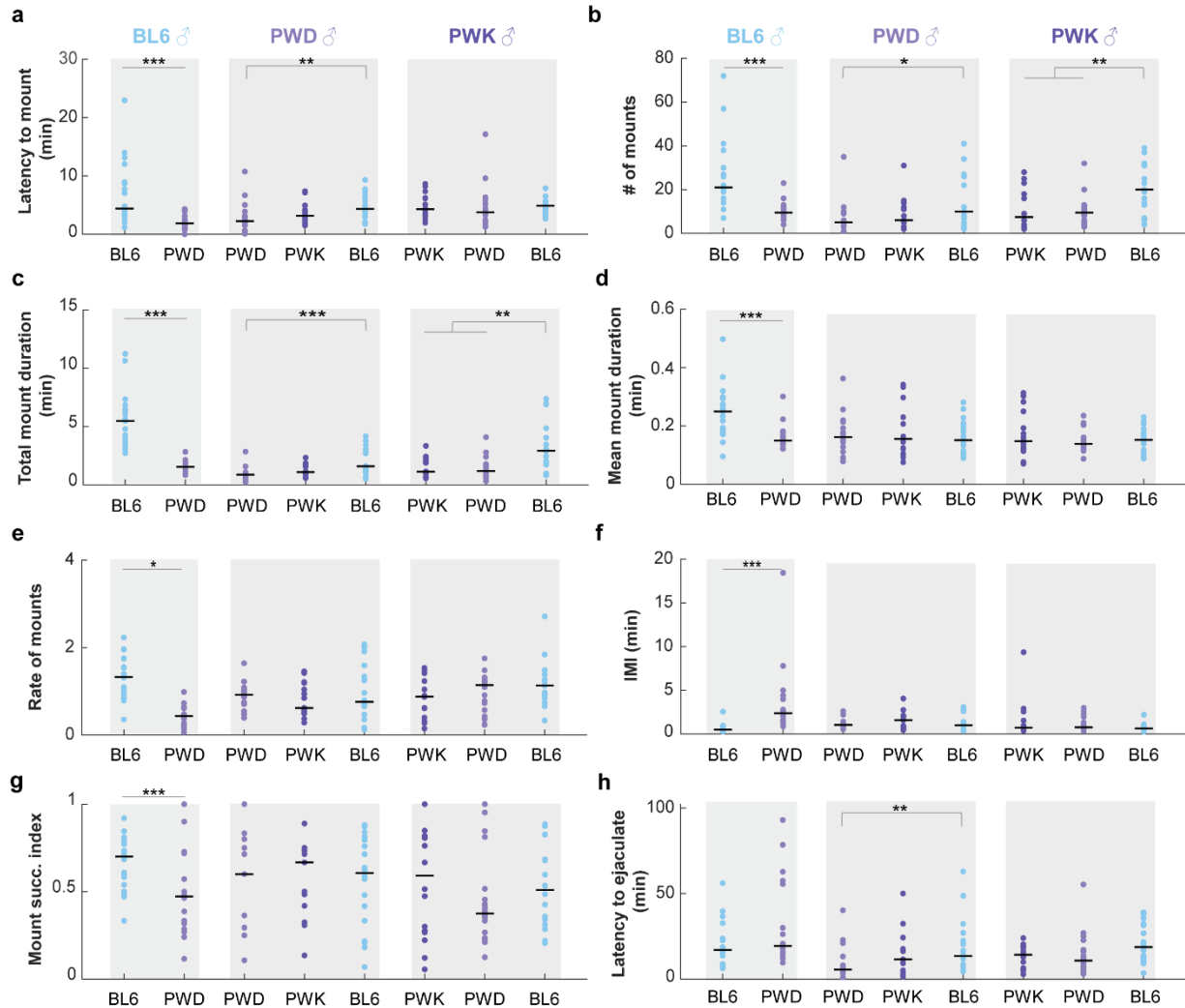


Figure 2.5. Male mouse sexual behavior is relative and adaptable to the female.

Quantification of **a** latency to mount (first mount attempt or mount), **b** number of mounts, **c** total mount duration, **d** mean mount duration (total mount duration/#mounts), **e** rate of mounts (#mounts/lat. ejaculate), **f** Inter-mount interval (IMI), **g** mount success index [#mounts/(#mount attempt + #mounts)], **h** latency to ejaculate (from **a**). $n_{BL6 \times BL6} = 19$; $n_{BL6 \times PWD} = 16$; $n_{PWD \times PWD} = 17$; $n_{PWD \times PWK} = 17$; $n_{PWD \times BL6} = 18$; $n_{PWK \times PWK} = 16$; $n_{PWK \times PWD} = 20$; $n_{PWK \times BL6} = 18$. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) by Kruskal Wallis followed by Dunn-Sidak. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

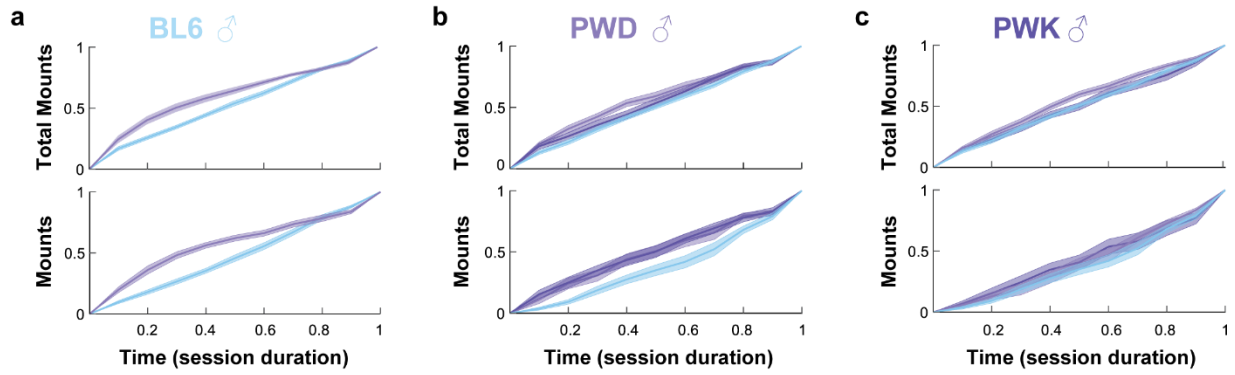


Figure 2.6. Sexual dynamics across the behavioral session.

Cumulative distributions of total mounts (mount attempts + mounts) and mounts along the behavioral assay for **a** BL6 ($n_{BL6 \times BL6} = 19$; $n_{BL6 \times PWD} = 16$), **b** PWD ($n_{PWD \times PWD} = 17$; $n_{PWD \times PWK} = 17$; $n_{PWD \times BL6} = 18$) **c** PWK ($n_{PWK \times PWK} = 16$; $n_{PWK \times PWD} = 20$; $n_{PWK \times BL6} = 18$) males. Histogram aligned to the first consummatory event with 0,1min bins. Time normalized for the duration of the session from the first consummatory event to ejaculation.

2.3.2.2 The refractory period is an absolute trait, intrinsic to BL6 and PWK males

The previous results show that the female strain can influence differently several aspects of the consummatory behavior. This section evaluates if this influence is extended to the PERP.

The results follow the trend observed in section 2.3.1.2. The low percentage of BL6 resuming the behavior after ejaculation dropped to zero when paired with PWD females (Fig. 2.7a). As observed for the consummatory behavior, *musculus* subspecies are differently influenced by the female strain: for PWD, although the latency was not affected, the proportion of animals with s-PERP increased significantly with BL6 females (Fig 2.7b and a, respectively; PWD: 42.18% vs BL6: 83.33, $P = 0.0152$ by permutation). Conversely, PWK's percentage of s-PERP or its duration was not affected by female strain (Fig. 2.7a and b, respectively).

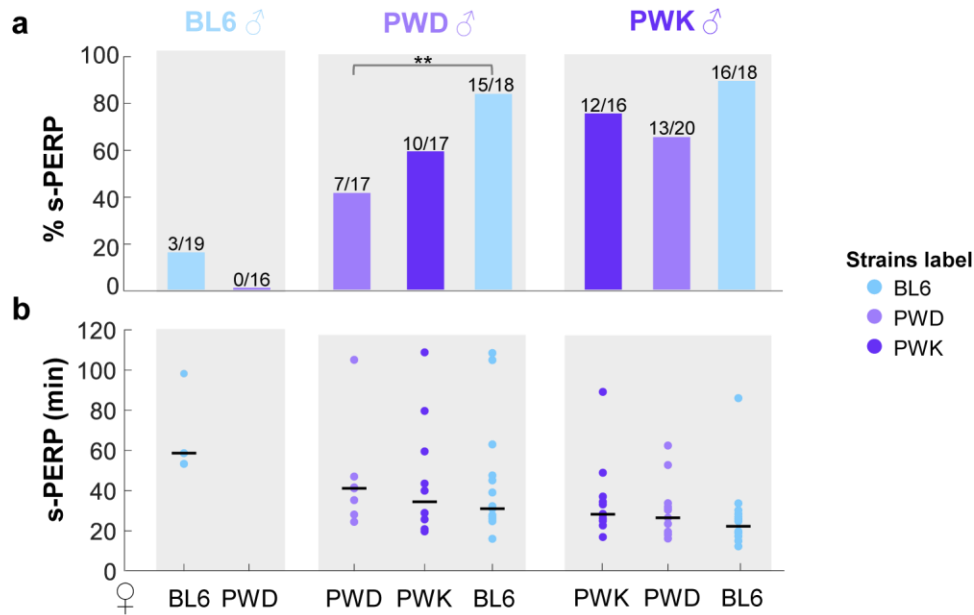


Figure 2.7. The refractory period is an absolute trait, intrinsic to PWK and BL6 males.

Quantification of the percentage of males that **a** re-initiated the consummatory behavior after ejaculating (s-PERP) and **b** duration of s-PERP. $n_{BL6 \times BL6} = 19$; $n_{BL6 \times PWD} = 16$; $n_{PWD \times PWD} = 17$; $n_{PWD \times PWK} = 17$; $n_{PWD \times BL6} = 18$; $n_{PWK \times PWK} = 16$; $n_{PWK \times PWD} = 20$; $n_{PWK \times BL6} = 18$. Significance was calculated with **a** permutation and **b** median $\pm$ M.A.D. (median absolute deviation with standard scale factor) by Kruskal-Wallis test with Dunn-Sidak correction $**P < 0.01$.

2.3.3 The post ejaculatory refractory period

2.3.3.1 Animals with s-PERP and I-PERP display similar consummatory needs

Following the previous analysis, we questioned if there is any signature of the consummatory behavior that differs between animals with s-PERP (individuals that restarted mounting within the 120min after ejaculation) and I-PERP (individuals that did not restarted mounting within the 120min after ejaculation). To do so we combined the data for each male strain (all the female strains combinations) and sorted the individuals in s-PERP and I-PERP and repeated the analysis for all the behavioral parameters as previously done.

From the 35 BL6 males only 3 animals restarted mounting within the 120 min after ejaculation. Although no statistical analysis was applied because of the low number of s-PERP individuals, we decided to include this data to investigate if these animals would fall within the

I-PERP distribution or if they would lay in a different cluster. It was not the case, as BL6 s-PERP and I-PERP fall within the same distribution (Fig. 2.8).

Overall, the occurrence of PERP cannot be predicted from individual consummatory behaviors as no differences were found between s-PERP and I-PERP animals for both musculus strains (Fig. 2.8).

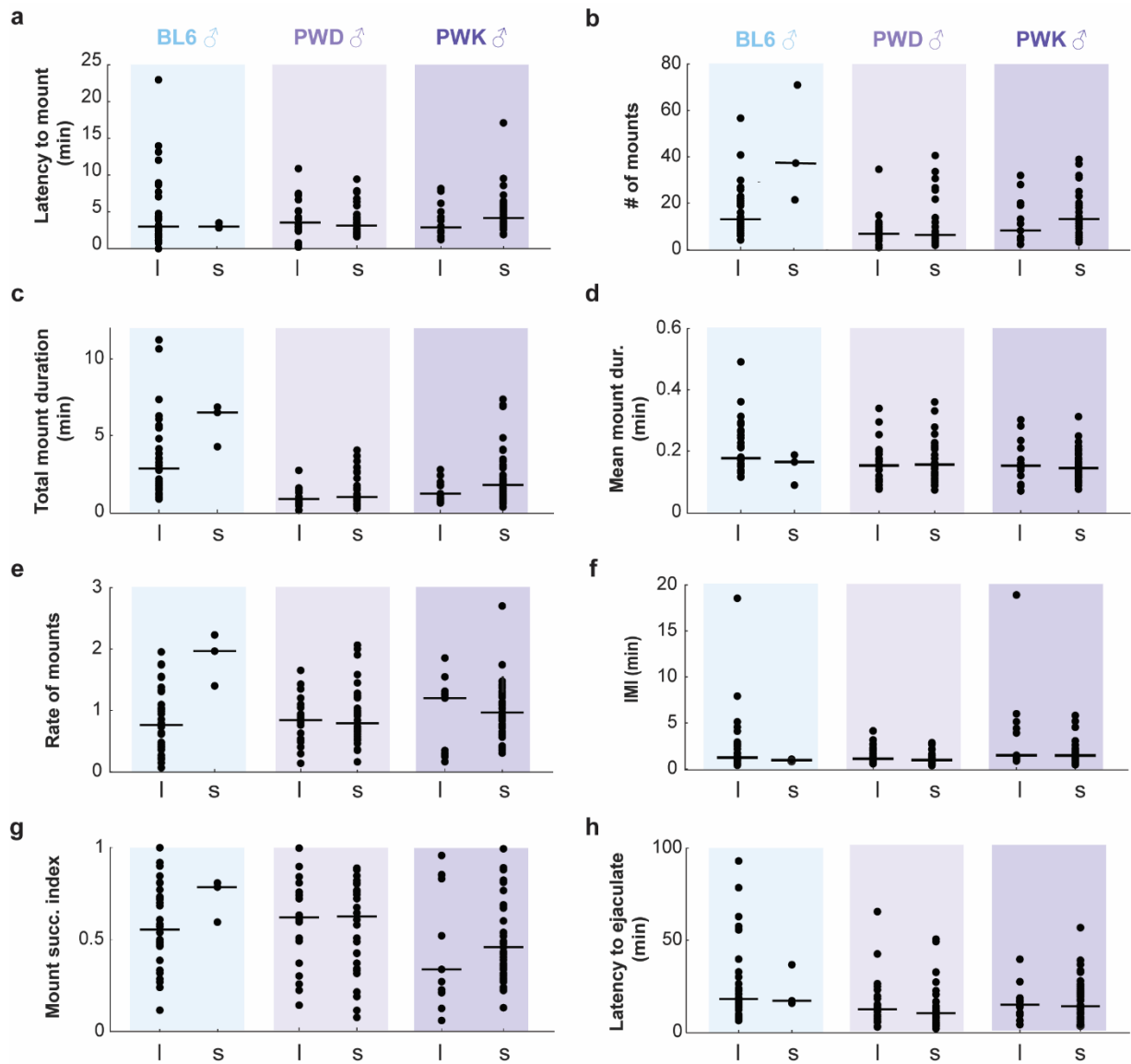


Figure 2.8. Occurrence of s-PERP cannot be predicted from individual consummatory parameters.

Quantification of **a** latency to mount (first mount attempt or mount), **b** number of mounts, **c** total mount duration, **d** mean mount duration (total mount duration/#mounts), **e** rate of mounts (#mounts/lat. ejaculate), **f** Inter-mount interval (IMI), **g** mount success index [#mounts/(#mount attempt + #mounts)],

h latency to ejaculate (from **a**) for individuals that did (**s**: s-PERP) or did not (**l**: l-PERP) restarted mounting within the 120min after ejaculation. $n_{BL6 \times BL6} = 19$; $n_{BL6 \times PWD} = 16$; $n_{PWD \times PWD} = 17$; $n_{PWD \times PWK} = 17$; $n_{PWD \times BL6} = 18$; $n_{PWK \times PWK} = 16$; $n_{PWK \times PWD} = 20$; $n_{PWK \times BL6} = 18$. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) by Kruskal Wallis followed by Dunn-Sidak.

2.3.3.2 s-PERP duration

On a final observation, we wanted to investigate if the s-PERP duration is correlated with any of the measured copulatory parameters. To do so we combined the data from all the female crosses for each *musculus* male strain and performed a correlation analysis between s-PERP duration and the measured copulatory parameters (Fig. 2.9 and Supplementary data 1). Overall similarity scores were weak and no correlation was found between s-PERP duration and independent consummatory measures.

Taken together, both percentage of animals with s-PERP or its duration cannot be predicted from individual consummatory parameters.

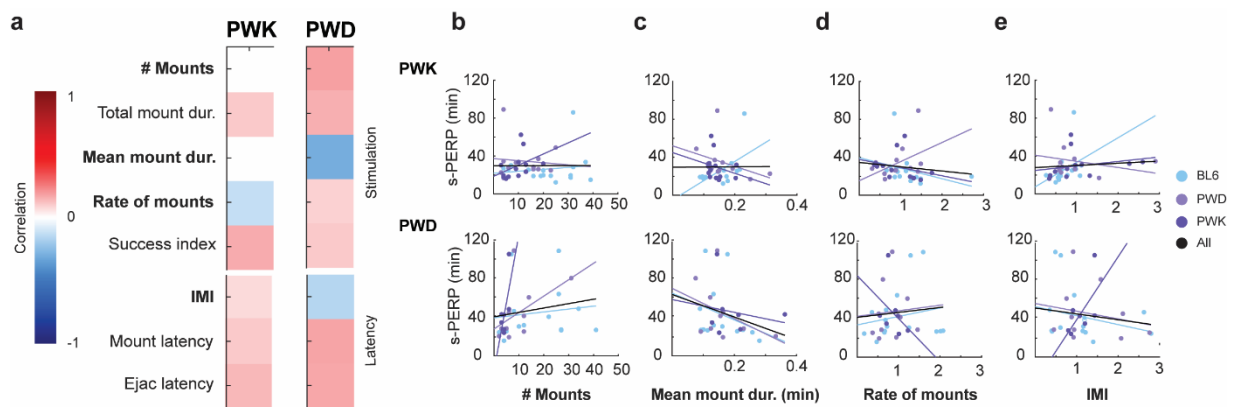


Figure 2.9. s-PERP duration is on average weakly correlated with individual copulatory parameters.

a Pair-wise correlation for PWK (left) and PWD (right) strains between s-PERP duration and 8 individual copulatory parameters grouped in 2 groups related to stimulation (number of mounts, total mount duration, mean mount duration, rate of mounts and mount success index) and latency (inter-mount interval, latency to mount and latency to ejaculate). Correlation between s-PERP and **b** number of mounts, **c** mean mount duration, **d** rate of mounts and **e** IMI for PWK (top) and PWD (bottom) male strain.

2.4 Discussion

The majority of the studies on mouse sexual behavior were performed taking advantage of domesticated strains of *Mus musculus domesticus*. We extended these findings through a comparative study of different strains from two closely related subspecies: PWK/PhJ and PWD/PhJ – previously unstudied inbred wild derived strains of *M.m. musculus*; and Balb/cJ and C57BL/6J - domesticated inbred laboratory strains of *M. m. domesticus*. Subspecies differ in the amount of sexual stimulation needed to reach ejaculation and, on the latency to restart a second copulatory series. Also, female strain had an effect on both consummatory and inhibitory phases, however, the effect size was dependent on the male strain.

2.4.1 Male mating strategy

Similarly to other common laboratory rodents, the copulatory behavior of *Mus musculus* consists of series of mount attempts (without intromission), mounts (with intromission, no sperm transfer) ending with a final mount leading to ejaculation (sperm transfer). Many of these common measures of male copulatory behavior vary markedly within the small number of inbred strains in which copulation has been compared (McGill 1962; McGill 1963; McGill and Blight 1963b; Levine et al. 1966; McGill and Ransom 1968; Mosig and Dewsbury 1976).

Our results show that although displaying identical qualitative patterns of behavior, there are pronounced quantitative differences that segregate the two subspecies: *domesticus* have a long mating pattern while *musculus* a short one; regarding length and repetition of the parameters.

A common observation in *domesticus* strains is the large number of mounts, which seems to be a characteristic of the subspecies. Mosing and Dewsbury (1976) reported a median of 132 mounts for C3H/HeJ strain. Similarly, in the present study the *domesticus* strains performed a significantly larger number of mounts. These values are higher than the ones reported previously for the same strains (McGill 1962; McGill 1963; Batty 1978). This observation may be related to methodological differences with the previous studies and the high variability observed for the annotation of the common measures of the copulatory behavior.

Another common observation is the fact that for most strains a single ejaculation typically ends the male's interest in sexual behavior for a long period of time (McGill 1962; McGill 1963;

McGill and Blight 1963b). A relationship between long copulations and long PERP latencies have been proposed (Mosig and Dewsbury 1976; Preston and Stockley 2006). Accordingly, in the present study, BL6 and BalbC needed a median of 30 mounts before reaching ejaculation, ending the male sexual behavior (only 15% of BL6 restarted a second copulatory series and none of the BalbC did it). On the other hand, PWK and PWD needed 4 to 6-fold lower number of mounts before ejaculating, with a high percentage of animals restarting a second copulatory series under 40 minutes. However, some exceptions were reported by (Mosig and Dewsbury 1976) for C3H/HeJ that restarted a second mating episode closer to 40 minutes after 132 mounts and AKR/J with a PERP of 23 minutes after a mean of 51 mounts.

Although opposing strategies, both *musculus* and *domesticus* mating strategies are common in nature. In the wild multiple paternity occurs frequently, which means that females will mate with different males during their receptive window (Bronson 1979; Thonhauser et al. 2014). Under these circumstances males tend to adopt a strategy with a smaller number of mounts, decreased latency to ejaculate and shorter PERP in order to increase paternity chances and avoiding takeover by another male or female rejection (Parker 1998a; Thonhauser et al. 2014). Or, on the other hand, reducing the probability of the female to mate again by adopting a strategy with a prolonged copulation (Oglesby et al. 1981; Huck and Lisk 1986; Stockley and Preston 2004), although reducing the ability to inseminate a second ejaculate, potentially through physical exhaustion, reduced female receptivity or sperm depletion (Toner and Adler 1986).

One possible explanation for PERPs with long latencies, like the BL6 that can take days to re-initiate sexual interaction, may rely on the volume of sperm allocated in each ejaculation. Typically, multiple ejaculators deliver smaller volumes of sperm allowing a next ejaculation, rather than in one large single dose as in single ejaculators (Dewsbury 1982; Parker 1998a; Williams et al. 2005). In this context, further experiments are needed in order to evaluate if the mating strategies observed are correlated with the volume of sperm allocated in each ejaculation between BL6 and PWK/PWD; and if the total volume of ejaculate of PWK/PWD mating series until sexual satiation resembles the one of BL6.

2.4.2 Female role in mating behavior

Females display a complex pattern of species-typical sexual behavior, varying in the amount of solicitation displays, interaction during copulation and the duration of the receptive posture, among others (Dewsbury 1972; Doty 1974). Taking this into consideration, to ensure that the parameters measured are indeed a function of the male, in a subsequent inter-strain mating assay, we assessed the impact of the female strain on the mating pattern.

Female effects on male behavior were observed for inter-subspecies mating. Overall, the male consummatory behavior became more similar to the behavior of the female subspecies. This is, when BL6 males mated with PWD females, their behavior was similar to that of *musculus* males, as the number and duration of mounts to reach ejaculation was reduced. Conversely, both *musculus* males behaved more like *domesticus* males, showing an increase in the number and duration of mounts executed with BL6 females. A similar result was observed in a study with rats in a seminatural environment, where wild males were allowed to copulate with domestic females, displaying behavior similar to that of domesticated males. Conversely, if domesticated males were allowed to copulate with wild females, they behaved more like wild males (McClintock and Adler 1978).

In a study in rats, where females were treated with the dopamine receptor antagonist flupentixol (which has the effect of prolonging the time spent in lordosis and eliminating proceptive solicitation and pacing), males took significantly longer to initiate copulation (Everitt 1990). In agreement with this observation i) PWD males took longer to initiate copulation with BL6 females which shows reduced solicitation behaviors or pacing of the mounts; while ii) BL6 males reduced significantly the latency to copulate with PWD females, a strain that exhibit proceptive behaviors and actively terminates the mounting event. Therefore, the differences in the latencies to initiate the behavior and in the inter-mount interval that were observed when males copulate with females from other strains could be derived from differences in social interaction with the female, in particular the execution of proceptive behaviors.

For BL6 males, all consummatory events were altered when mated with a PWD female except for the latency to ejaculate. Although we could speculate that i) each mount with the PWD female were more rewarding increasing the latency for the next stimulation and therefore keeping the same latency to ejaculate; ii) and/or that the males have a “sensory integrator” that is not only dependent on the quality of the interaction but also depends on its duration, where

a specific amount of time is needed to induce ejaculation; the most probable explanation is the decrease on male mounting efficiency due to pacing of the mounting from the female.

For PWD males, both latencies to start mounting and to ejaculate and the overall copulatory stimulation increased when mating with BL6 females. PWK males had a more conserved behavioral pattern as only the number of mounts and an overall period of sexual stimulation was significantly increased. Contrary to BL6 males, for which the mount duration was dependent on the female the male interacted with, the mount duration for *musculus* was unaffected, suggesting that this behavioral trait is male dependent (the male is the one controlling the beginning and end of the mounting behavior).

Relatively to the PERP, only the percentage of PWD males with s-PERP was affected, increasing significantly with BL6 females. This observation may be related to the level of sexual satiety with the previous copulatory series that can be lower with BL6 females.

Altogether these observations indicate a major role of the female in mediating some of the aspects of the sexual encounter.

2.4.3 Predicting the post ejaculatory refractory period

While most parameters annotated during consummatory behavior are dependent of one another, the PERP occurrence and duration is independent of the remaining measures. Frank Beach (1956) reported a reliable correlation ($r = +0.50$) between the mean interval between mounts (IMI) and the PERP duration of laboratory rats. Subsequent studies performed by Sachs (1978) demonstrated the reliability of this relationship among different individuals, genotypes and species of rodents ($r = +0.82$; $df=16$, $P < 0.0001$). The paucity of studies on mouse sexual behavior and the fact that for some strains PERP can last for days, limited the extension of this analysis for mice. However, although the aforementioned correlation was kept, as most studies with *Mus musculus* showed significantly higher IMI and PERP latencies compared with the remaining species, *Mus musculus* were skewed from the general correlation, laying along a different regression line of their own ($+1.00$; $df = 1$, $P < 0.01$).

Frank Beach (1956) considered the IMI and PERP as two fundamental measures of the temporal patterning of copulation, reflecting pauses between mounts. Proposing that the IMI could be a mini-PERP, a temporarily reduced sexual excitation, so that the copulatory threshold has to be regained before another sexual act occur. This implies that both measures are

influenced by the male motivation. The generalization that the male ceases to copulate because sexual motivation is diminished (Ågmo 1999) is supported by the Coolidge effect - restoration of mating behavior with a different female from the one with which the male reached ejaculation (Wilson et al. 1963). Sexually exhausted male rats do not engage in sexual behavior for a period of 30–90 minutes after they have copulated ad lib with the same female. However, if the female is replaced by a different sexually receptive female, the male will resume copulation (Phillips-Farfán and Fernández-Guasti 2009; Tlachi-López et al. 2012).

Recently, some reviews covering the neurohormonal regulation of the PERP provide compelling evidence that the PERP may be peripherally regulated rather than centrally (Hull and Rodríguez-Manzo 2017; Seizert 2018). Therefore, the motivational explanation for the reduced sexual activity after ejaculation requires further investigation. In agreement, in our results, no correlation was found between IMI and PERP latency. Moreover, no differences were found between s-PERP and I-PERP subpopulations nor correlations between the consummatory parameters and s-PERP latency. Implying that there is no signature in the behavioral pattern that can foresee the occurrence of s-PERP or its latency.

2.5 Conclusion

To our knowledge no study has investigated the sexual behavior of the inbred wild derived *musculus* strains PWK and PWD.

Male sexual behavior was consistent within males from the same subspecies, but vastly different between subspecies: while *musculus* males needed significantly less pre-ejaculatory stimulation and a high percentage of the males restarted a second copulatory series; *domesticus* males needed more sexual interaction and only a residual number re-mated after ejaculation. Female strain had an effect on both consummatory and inhibitory phases, with effect size dependent on the male strain. However, the remarkable disproportioned latency to recover sexual interest after ejaculation in PWK and BL6 strains was not affected by the female. This strain-related difference is a valuable starting point to pin down possible causative mechanisms underlying the different behavioral phenotypes, providing new insight into an old and still open question on the field: how is the PERP established and maintained.

2.6 Methods

Animals. *Mus musculus domesticus* BL6 and BalbC (C57BL/6J and Balb/cJ) and *Mus musculus musculus* PWD and PWK (PWD/PhJ and PWK/PhJ) mice were ordered from The Jackson Laboratories and maintained in our animal facility. Animals were weaned at 21 days and housed in same-sex groups in stand-alone cages (1284L, Techniplast, 365 x 207 x 140 mm) with access to food and water ad libitum. Mice were maintained on a 12:12 light/dark cycle and experiments were performed during the dark phase of the cycle, under red dim light. All experiments were approved by the Animal Care and Users Committee of the Champalimaud Neuroscience Program and the Portuguese National Authority for Animal Health (Direcção Geral de Veterinária). Females were kept house grouped and males were isolated before the sexual training. Both males and females were sexually experienced and habituated to be handled and to the assay routine. The data from each male's first mating test were discarded; the behavioral assay was the second ejaculatory event of each male. Males interacted with different females in each sexual encounter.

Ovariectomy and hormonal priming. All females underwent bilateral ovariectomy under isoflurane anesthesia (1-2% at 1L/min). After exposing the muscle with one small dorsal incision (1 cm) a small incision was made in the muscle wall, at the ovary level, on each side. The ovarian arteries were cauterized and both ovaries were removed. The skin was sutured, and the suture topped with iodine and wound powder. The animals received an ip injection of carprofen before being housed individually with food supplemented with analgesic (MediGel, 1mg carprofen /2 oz cup) for 2 days recovery and then re-grouped in their home cages. Female mice were primed subcutaneously 48 hours before the assay with 0,1ml estrogen (1mg/ml, Sigma E815 in sesame oil) and 4 hours before the assay with 0,1ml progesterone (5mg/ml, Sigma 088K0671 in sesame oil).

Behavioral assays. Each behavioral assay was performed in a different group of males and, within each trial, experiments were conducted in parallel for both *domesticus* and *musculus* strains. To characterize the male sexual behavior of each strain we runed a within-strain assay were *domesticus-domesticus* and *musculus-musculus* pairings were done for the same strain. Subsequently, to evaluate the effect of the female subspecies in the behavior, we did a between-strain assay where males of each strain were combinatorically paired with females of each strain. BalbC was only included in the within-stray assay as this strain revealed to have low copulatory interest. BL6 male – PWK female combination was also excluded as

the females would not allow copulation. Table 2.1 shows the different combinations that were performed in this study and their corresponding number.

Trials were conducted in the male home cage (1145T, Techniplast, 369 x 156 x 132 mm) stripped from nesting, food and water; covered with a transparent acrylic lid. The trial started with the entry of the female in the setup (t = 0 min) and ended once the male performed the first attempt of copulation after ejaculation (red diamonds) or after two hours if no attempt was made (Fig. 1a and 2a). Trials were aborted after 1 hour in the case the male did not display sexual behavior.

Table 2.1. Pairing combinations between *Mus musculus domesticus* and *Mus musculus musculus* subspecies

♀ \ ♂	<i>M. m. domesticus</i>		<i>M. m. musculus</i>	
	BalbC	BL6	PWD	PWK
BalbC	13	--	--	--
BL6	--	19	18	18
PWD	--	16	17	20
PWK	--	--	17	16

Behavior analysis. The behavior was recorded from the side with pointgrey cameras (FL3- U3-13S2C-CS) connected to a computer running a custom Bonsai software (Lopes et al. 2015). Behavior was manually annotated using the open-source program Python Video Annotator (<https://pythonvideoannotator.readthedocs.io>) and analyzed using Matlab. The copulatory behavior of house mice is composed of mount attempts (MA, without intromission), mounts (mounts with intromission but no sperm transfer), and ejaculations (final part of the last mount, with semen transfer). These are organized into "series", with each series beginning with a mount attempt/mount and ending with an ejaculation. The annotated measures are summarized in table 2.2.

Table 2.2. Definitions of the measures of copulatory behavior used in this study. Time in minutes

Events	Description
MA	Mount attempt. Characterized by the male mounting the female in any angle of her body and failing to gain vaginal penetration.
Mount	Mount. Begins with male clasping the female until he dismounts. Characterized by the male mounting the female rear, clasping her, and displaying pelvic thrusting with vaginal penetration; no sperm is transferred to the female.
Ejaculation	Ejaculation. End of the final mount of a copulatory series. Characterized by a male shudder grasping the female and falling to the side taking the female with him and remaining completely still before genital disengagement.
PERP	Post ejaculatory refractory period. Latency between ejaculation and the next MA or mount.
Annotations	Description
Latency to mount	Latency to mount. Time from the introduction of the female to the first MA or mount.
MA latency	Mount attempt latency. Time from the introduction of the female to the first MA.
Mount latency	Mount latency. Time from the introduction of the female to the first mount.
IMI	Inter-mount interval. Time from the end of one mount to the beginning of the next.
Latency to ejaculate	Latency to ejaculate. Time from latency to mount to ejaculation.
s-PERP	Short PERP. Initiation of a second copulatory series within 120min after ejaculation.
I-PERP	Long PERP. No initiation of a second copulatory series within 120min after ejaculation.
PERP latency	PERP duration. Time from the ejaculation of one series to the first MA or mount of the next copulatory series.
# Ma	Total number of MA.
# Mounts	Total number of mounts.

# Total mounts	Total mounts. Sum of MA + mounts.
Rate of mounts	Rate of mounts. #mounts / latency to ejaculate.
Total mount duration	Total mount duration. Sum of the duration of all mounts.
Mean mount duration	Mean mount duration. Total mount duration / # mounts.
Success index	Mount success index. Percentage of mounts from total mounts done by the male [$\#mounts / (\#MA + \#mounts)$] * 100%

Statistical analysis. The statistical details of each experiment, including the statistical tests used and exact value of n are detailed in each figure legend. Data was analyzed with MATLAB R2019b and presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor). Behavioral comparisons were done with Kruskal Wallis followed by Dunn-Sidak multiple comparison test (Fig. 2.2a-h, 2.3b, 2.5a-h, 2.7b and 2.8a-h). Percentage of animals with s-PERP were compared by permutation (Fig. 2.3a, 2.7a). The measures of strength and direction of a linear relationship between the two variables were measures with Pearson correlation coefficient r (Fig. 2.10 and Supplementary Fig. 1). Significance was accepted at $P < 0.05$ for all tests with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

3. The post ejaculatory refractory period

3.1 Summary

In many species, ejaculation is followed by a state of decreased sexual activity, the post-ejaculatory refractory period. Several lines of evidence have suggested prolactin, a pituitary hormone released around the time of ejaculation in humans and other animals, to be a decisive player in the establishment of the refractory period. However, data supporting this hypothesis is controversial.

We took advantage of two different strains of house mouse, a wild derived and a classical laboratory strain that differ substantially in their sexual performance, to investigate prolactin's involvement in sexual activity and the refractory period. First, we show that there is prolactin release during sexual behavior in male mice. Second, using a pharmacological approach, we show that acute manipulations of prolactin levels, either mimicking the natural release during sexual behavior or inhibiting its occurrence, do not affect sexual activity or shorten the refractory period, respectively. Therefore, we show compelling evidence refuting the idea that prolactin released during copulation is involved in the establishment of the refractory period, a long-standing hypothesis in the field of behavioral endocrinology.

3.2 Introduction

Sexual behavior follows the classical sequence of motivated behaviors, terminating with an inhibitory phase after ejaculation: the post-ejaculatory refractory period (PERP) (Masters and Johnson 1966). The PERP is highly conserved across species and includes a general decrease in sexual activity and also inhibition of erectile function in humans and other primates (Seizert 2018). This period of time is variable across and within individuals and is affected by many factors, such as age (McGill 1963; Levin 2009) or the presentation of a new sexual partner (Wilson et al. 1963; Rodriguez-Manzo 1999). The PERP is thought to allow replacement of sperm and seminal fluid, functioning as a negative feedback system where by inhibiting too-

frequent ejaculations an adequate sperm count needed for fertilization is maintained (Hernandez et al. 2006; Rojas-Durán et al. 2015).

Several lines of evidence have suggested the hormone prolactin (PRL) to be a key player in the establishment of the PERP (Drago 1984; Kruger et al. 2002). PRL is a pleiotropic hormone, first characterized in the context of milk production in females, but for which we currently know several hundred physiological effects in both sexes (Freeman et al. 2000a; Grattan and Kokay 2008). The association of PRL to the establishment of the PERP in males is based on several observations. First, it was shown that PRL is released around the time of ejaculation in humans and rats (Oaknin et al. 1989; Krüger et al. 1998; Exton et al. 1999; Exton et al. 2000; Exton et al. 2001; Kruger, Haake, Chereath, et al. 2003; Brody and Krüger 2006; Krüger et al. 2006; Egli et al. 2010). Anecdotally, no PRL release has been observed in a subject with multiple orgasms (Haake et al. 2002). Second, chronically abnormal high levels of circulating PRL are associated with decreased sexual drive, anorgasmia and ejaculatory dysfunctions (Svare et al. 1979; Buvat 2003). Finally, removal of PRL-producing pituitary tumors or treatment with drugs that inhibit PRL release reverse sexual dysfunctions (Sato et al. 1997; Melmed et al. 2011). Taking these observations into consideration, it has been hypothesized that the PRL surge around the time of ejaculation plays a role in the immediate subsequent decrease of sexual activity, the hallmark of the PERP. In fact, this idea is widespread in behavioral endocrinology textbooks (Grattan and Bridges 2009) and the popular press(https://en.wikipedia.org/wiki/Refractory_period;<https://www.humanitas.net/treatments/prolactin>).

PRL is primarily produced and released into the bloodstream from the anterior pituitary (Riddle et al. 1933; Freeman et al. 2000a) and consistent with its functional diversity, PRL receptors are found in most tissues and cell types of the body (Nagano and Kelly 1994; Aoki et al. 2019). Therefore, PRL may depress sexual activity directly, via PRL receptors present in the male reproductive tract. In fact, PRL has been shown to impact the function of accessory sex glands and to contribute to penile detumescence (Aoki et al. 1995). PRL can also affect central processing, as it can reach the central nervous system either via circumventricular regions lacking a blood-brain barrier (Ganong 2000) or via receptor-mediated mechanisms (Grattan et al. 2001), binding its receptors which have widespread distribution, including in the social brain network (Brown et al. 2010). Hence, circulating PRL can impact the activity of neuronal circuits involved in the processing of socio-sexual relevant cues and thus sexual performance. Circulating PRL reaches the central nervous system on a timescale that supports the rapid

behavioral alterations that are observed immediately after ejaculation (in less than 2 minutes)(Kirk et al. 2019) . Through mechanisms that are not yet well established, PRL is known to elicit fast neuronal responses (Georgescu et al. 2020) besides its classical genomic effects (Bole-Feyssot et al. 1998). In summary, circulating PRL can impact several systems involved in sexual behavior on a time scale compatible with the establishment of the PERP.

However, despite data supporting the involvement of the ejaculatory PRL-surge in the establishment of the PERP, this hypothesis has received numerous critics (Levin 2003; Levin 2009; Corona et al. 2012; Turley and Rowland 2013; Seizert 2018). While in humans it is well established that chronically high levels of PRL reduces libido (Buvat 2003), some authors suggest that those results were erroneously extended to the acute release of PRL around ejaculation (Levin 2003; Levin 2009; Corona et al. 2012; Turley and Rowland 2013; Seizert 2018). Furthermore, there is controversy in relation to PRL dynamics during sexual behavior, since in most studies PRL levels were quantified during fixed intervals of time, and not upon the occurrence of particular events, such as ejaculation. In fact, some reports in rats suggest that PRL levels are elevated through the entire sexual interaction (Kamel et al. 1977; Kamel and Frankel 1978). Finally, formal testing of the impact of acute PRL manipulations on sexual activity and performance is still missing (but see (Kruger, Haake, Haverkamp, et al. 2003) for an acute manipulation in humans).

In the present study, we tested the role of PRL in sexual activity and in the establishment of the PERP in the mouse. The sequence of sexual behavior in the mouse is very similar to the one observed in humans (Lenschow and Lima 2020), making it an ideal system to test this hypothesis. Also, we took advantage of two strains of inbred mice that are representative of two different mouse subspecies (C57BL/6J: laboratory mouse, predominantly *Mus musculus domesticus* and PWK/PhJ: inbred wild-derived, *Mus musculus musculus* (Gregorová and Forejt 2000) and exhibit different sexual performance. Through routine work in our laboratory, we observed that while most BL6 males take several days to recover sexual activity after ejaculation, a large proportion of PWK males will re-initiate copulation with the same female within a relatively short period of time. This difference in PERP duration can be taken to our advantage, widening the dynamic range of this behavioral parameter and increasing the probability of detecting an effect with pharmacological manipulations.

First, we show that there is PRL release during sexual behavior in male mice. Second, using a pharmacological approach, we show that acute manipulations of prolactin levels, either

mimicking the natural release during sexual behavior or inhibiting its occurrence, do not affect sexual activity or shorten the refractory period, respectively.

3.3 Results

3.3.1 Prolactin is released during sexual behavior in male mice

We first asked if PRL is released during copulation in the two strains of male mice. To monitor PRL dynamics during sexual behavior we took advantage of a recently developed ultrasensitive ELISA assay that can detect circulating levels of PRL in very small volumes of whole blood (5-10 microliters), allowing the assessment of longitudinal PRL levels in freely behaving mice (Guillou et al. 2015). Sexually trained laboratory mice (C57BL/6J, from here on BL6) and inbred wild-derived mice (PWK/PhJ, from here on PWK) were paired with a receptive female and allowed to mate (see Methods for details). During the sexual interaction males were momentarily removed from the cage to collect tail blood after which they returned to the behavioral cage, resuming the sexual interaction with the female. We collected blood samples upon the execution of pre-determined, easily identifiable, behavioral events that correspond to different internal states of the male: before sexual arousal (baseline, before the female was introduced in the cage), at the transition from appetitive to consummatory behavior (MA, mount attempt, immediately after the male attempted to mount the female), during consummatory behavior (mount, after a pre-determined number of mounts with intromissions, BL6=5 and PWK=3) and after ejaculation (ejaculation, after the male exhibited the stereotypical shivering, falling to the side and separating from the female) (Fig.3.1a, see Methods for details).

Baseline levels of circulating PRL in male mice were low for both strains (BL6 0.86 ± 0.46 ; PWK: 2.31 ± 1.37 ng/ml; please see (Guillou et al. 2015) for BL6), but are significantly increased during sexual interaction (BL6: $F_{3,7} = 21.26$; $P < 0.0001$; PWK $F_{3,8} = 17.18$; $P < 0.0001$, RM One-way ANOVA) (Fig. 3.1a). While in the case of BL6 males PRL levels only increased during the consummatory phase, PRL levels in PWK males are significantly increased already at the transition from appetitive to consummatory behavior (Baseline vs MA 16.30 ± 6.67 ng/ml, $P = 0.001$, Tukey's multiple comparisons test) (Fig. 3.1a). In both strains, PRL levels after ejaculation are similar to the levels reached during consummatory behavior (BL6 $P = 0.71$ vs PWK $P = 0.95$, Tukey's multiple comparisons test), in marked contrast to humans, where PRL seems to be released only around the time of ejaculation (Exton et al. 2001).

Contrary to PWK males, which in the presence of a receptive female always engaged in sexual behavior and ejaculated, a large percentage of BL6 males never attempted to mount the female (15 out of 23, Supplementary Data 2). In such case, the session was aborted after 30 minutes of social interaction and a blood sample was collected (Fig. 3.1b). In this case, PRL levels of BL6 males did not differ from baseline (baseline 0.99 ± 0.67 vs Social 1.25 ± 0.63 ; $P = 0.282$, Paired t test), further suggesting that PRL is only released in the context of a sexual interaction.

Because PRL is known to be released under stress (Gala 1990) and to ensure that the changes observed in circulation are not a result from the blood collection procedure itself, all animals were initially habituated to the collection protocol in another cage, alone. To ensure that the habituation protocol was effective, in a separate experiment we measured PRL levels in the absence of any behavior. Four blood samples were collected 20 minutes apart from BL6 and PWK males in their home cage (Fig. 3.1c). In both cases, circulating PRL levels were not altered, ensuring that the observed increases were not caused by the manipulation (BL6: $F_{3,7} = 2.08$; $P = 0.18$; PWK $F_{3,7} = 2.94$; $P = 0.11$, RM One-way ANOVA).

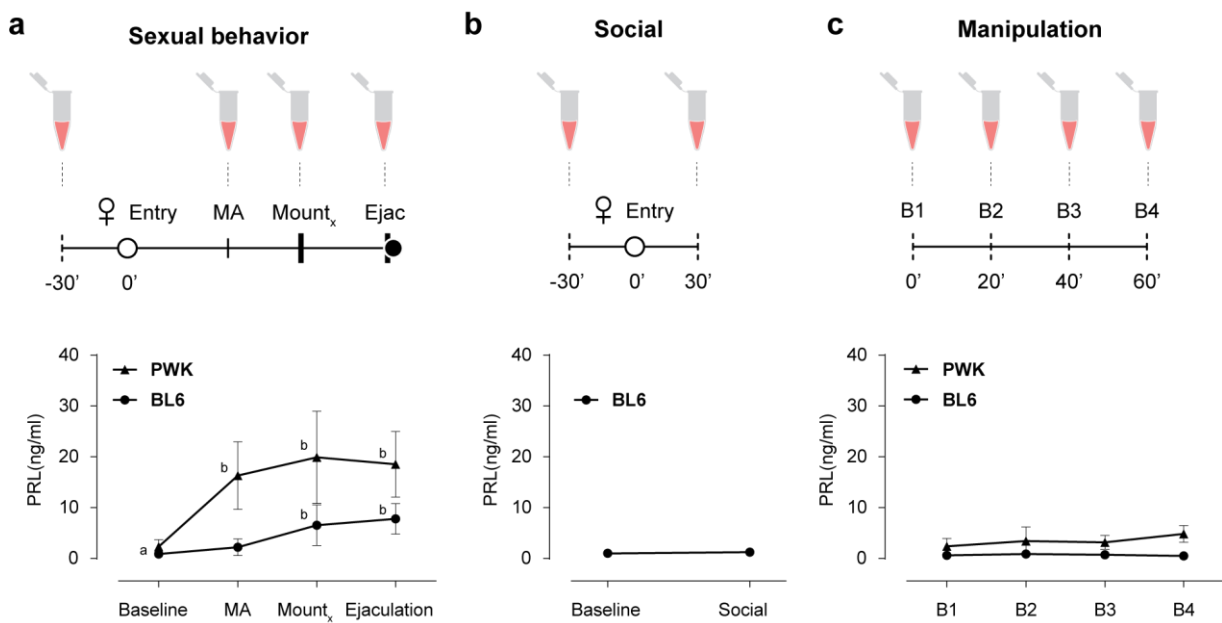


Figure 3.1. Prolactin is released during sexual behavior in male mice.

a Timeline for blood collection and $[PRL]_{\text{blood}}$ during sexual behavior (MA- mount attempt; BL6_xmounts = 5; PWK_xmounts = 3). RM One-way Anova for BL6 ($n = 8$) $F_{3,7}=21.26$, $P < 0.0001$ and PWK ($n = 9$) $F_{3,8}=17.18$, $P < 0.0001$, followed by Tukey's multiple comparison test $_{ab} P \leq 0.01$. **b** Timeline for blood

collection and [PRL]_{blood} during social behavior in BL6 males (n = 15) $P = 0.282$, two-tailed Paired t test. **c** Timeline for blood collection and [PRL]_{blood} during repeated sampling in resting condition. RM One-way Anova for BL6 (n = 8) $F_{3,7} = 2.08$; $P = 0.18$ and PWK (n = 8) $PWK F_{3,7} = 2.94$; $P = 0.11$. Data represented as mean $\pm$ SD.

Together, these results demonstrate that PRL is released during sexual behavior in male mice, but not during a social interaction or due to the blood collection protocol, prompting us to examine the role of PRL release during sexual behavior.

3.3.2 Acute prolactin release does not induce a refractory period-like state

To investigate if the increase in circulating levels of PRL that occurs during mating is sufficient to decrease sexual activity, a hallmark of the PERP, we employed a pharmacological approach to acutely elevate PRL levels before the animals became sexually aroused and assess if the male mice behave as if in a PERP-like state. PRL is produced in specialized cells of the anterior pituitary, the lactotrophs, and its release is primarily controlled by dopamine originating from the hypothalamus (Freeman et al. 2000b). Dopamine binds D2 receptors at the membrane of the lactotrophs, inhibiting PRL release. Suppression of dopamine discharge leads to disinhibition of lactotrophs, which quickly release PRL into circulation (Lyons and Broberger 2014; Grattan 2015). To acutely elevate PRL levels, we performed an intraperitoneal injection of the D2 dopamine receptor antagonist domperidone, which does not cross the blood brain barrier (Costall et al. 1979; Cocchi et al. 1980), and measured PRL levels 15 minutes after the procedure (Fig. 3.2a). As expected, domperidone administration led to a sharp rise in the levels of circulating PRL (BL6: baseline 0.4957 ± 0.4789 vs domp 12.54 ± 2.032 , $P < 0.0001$; PWK: baseline 4.82 ± 1.645 vs domp 25.87 ± 7.154 ; $P < 0.0001$, Paired t test) (Fig. 3.2b) of similar magnitude to what is observed during copulation (ejaculation time point from Fig. 3.1a).

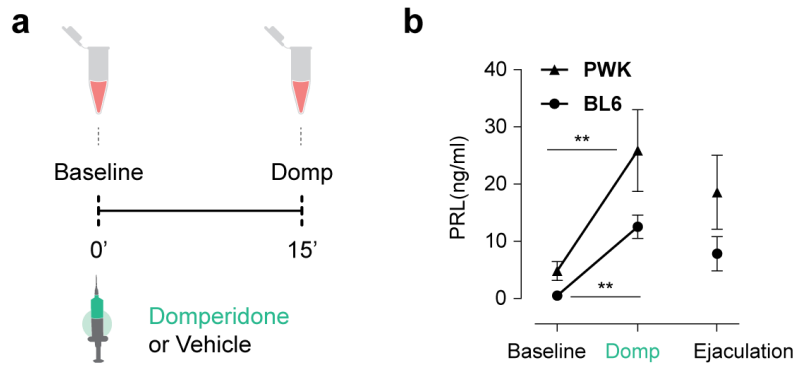


Figure 3. 2. Domperidone induces a sharp rise in the levels of circulating PRL of similar magnitude to the release during copulation.

a Timeline for blood collection and [PRL]_{blood} after Vehicle (Veh - black) or Domperidone (Domp - green) administration. Values from the ejaculation time point (Fig. 1a) were included as a reference. $n_{BL6} = 8$, $n_{PWK} = 9$.

Therefore, on a separate experiment we investigated how domperidone-treated male mice behave with a receptive female. If PRL is sufficient to induce a PERP like state, treated males should exhibit decreased sexual activity, which could be manifested in distinct manners, such as on the latency to initiate consummatory behavior or on the vigor of copulation. Each male from the two strains was tested twice, once with vehicle and another time with domperidone, in a counter-balanced manner and all the annotated behaviors are depicted over time on Fig. 3.3a and b (see Methods for details). Some BL6 males did not reach ejaculation in one of the sessions. A similar frequency was observed among unmanipulated animals (Supplementary Data 2). Despite differences in the dynamics of sexual behavior between BL6 and PWK males, administration of domperidone does not seem to affect sexual performance, as we could not detect any significant difference in the latency to start mounting the female, frequency of attempts to mount the female, time taken to ejaculate or proportion of animals that reached ejaculation (Fig. 3.4a-d). Domperidone administration also does not seem to affect the dynamics of the sexual interaction across the session or within each mount (Fig. 3.4e and f, respectively) or other measures of sexual behavioral performance (Supplementary Data 3). Moreover, the majority of males reached ejaculation (latency to ejaculate since female entry: BL6: 16.2 ± 7 with a maximum of 62 min; PWK: 12.3 ± 6.7 with a maximum of 59 min, Supplementary Data 3) within the time window where PRL levels remain elevated after domperidone administration (Guillou et al. 2015).

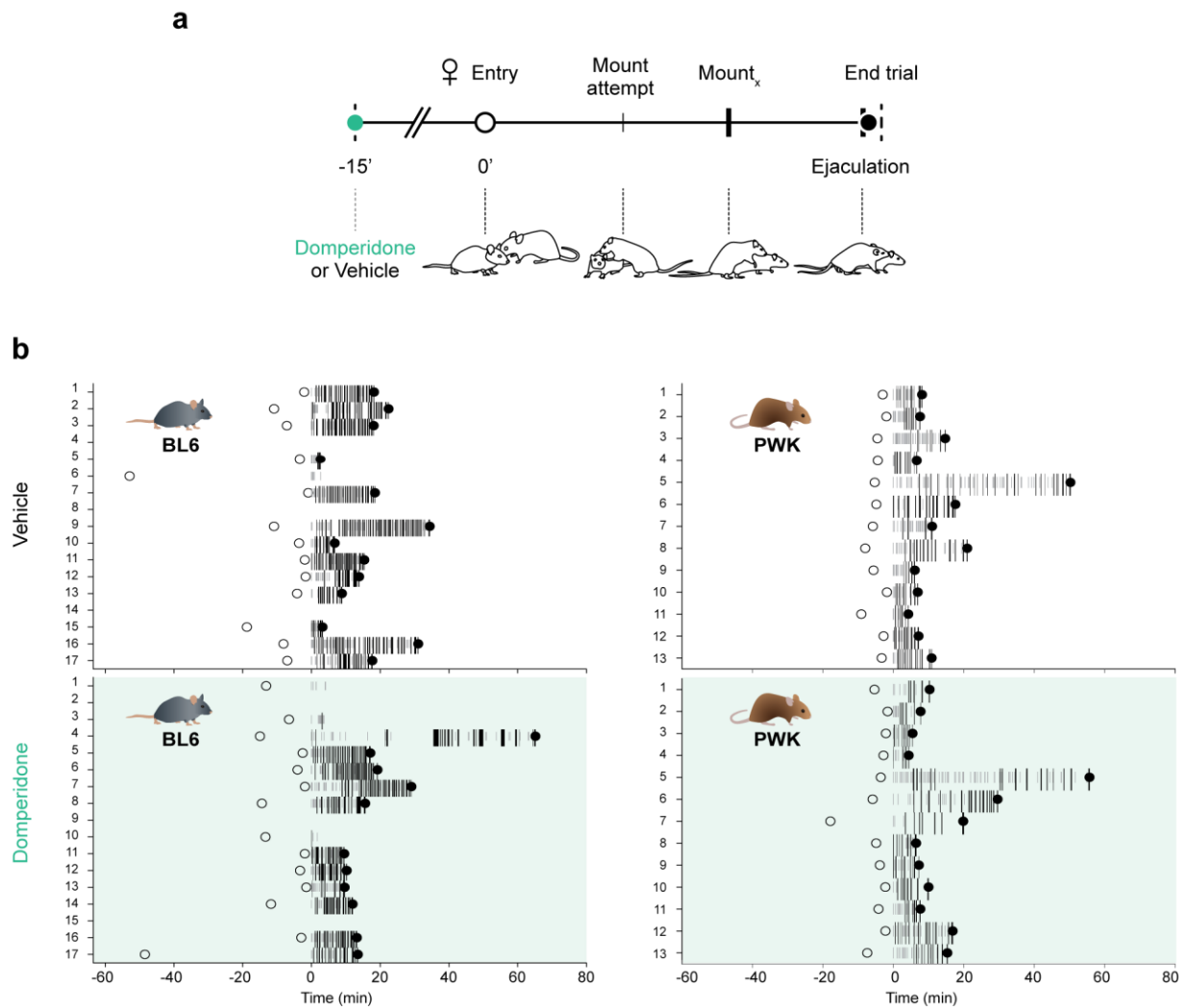


Figure 3.3. Mouse sexual behavior ethogram for Domperidone manipulation.

a Timeline for sexual behavior assay using sexually trained BL6 and PWK males pre-treated ($t = -15$ min) with Veh (black) or Domp (green). Each animal was tested twice, in a counterbalanced manner: once with Veh and once with Domp. **b** Raster plot aligned to the first consummatory event (first mount attempt or mount), representing the sexual behavior executed by the male, with mount attempts represented in small grey bars, mounts in long black bars (width correlated with mount duration) and ejaculation with a black circle. Time of female entry in the apparatus represented with an open circle. $n_{BL6} = 17$; $n_{PWK} = 13$.

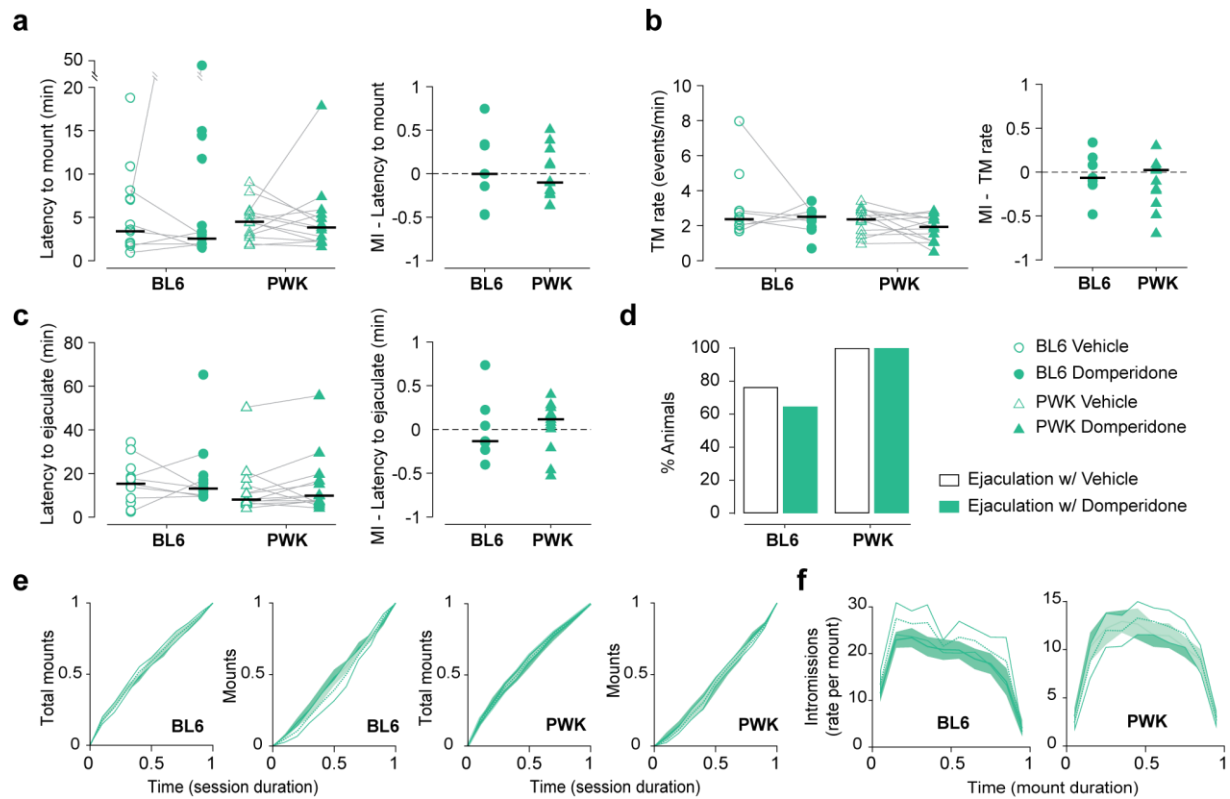


Figure 3.4. Acute prolactin release does not induce a refractory period-like state.

Quantification of **a** latency to mount (first mount attempt or mount, BL6 $P = 1.0$, PWK $P = 0.6355$), **b** rate of total mounts (TM, mount attempts + mounts, BL6 $P = 0.9375$, PWK $P = 0.3054$) and **c** latency to ejaculate (from a, BL6 $P = 0.8125$, PWK $P = 0.21631$). With BL6 represented by circles and PWK by triangles (Veh - open, Domp - solid). Each line represents data of an individual. Only animals that ejaculated in both sessions were considered in the statistics ($n_{BL6} = 7$, $n_{PWK} = 13$). Individuals that did not ejaculate in one of the trials are represented as unconnected dots (not used in statistics). MI [modulation index (Domp - Veh)/(Domp + Veh)] between the two conditions for both strains. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test. **d** Percentage of animals that reached ejaculation in the Veh (open) and Domp (solid) condition. **e** Cumulative distributions of total mounts and mounts along the behavioral assay. Histogram aligned to the first consummatory event with 0,1min bins. Time normalized for the duration of the session from the first consummatory event to ejaculation. **f** Rate of intromissions executed during the mount. Histogram for all mounts of each session, aligned to beginning of the mount with 0,1min bins. Time normalized for the duration of the mount.

In summary, domperidone administration, which causes an acute elevation of circulating PRL levels similar to what is observed at the end of copulation, does not have an inhibitory effect on any behavioral parameter related to sexual activity on the two strains of mice tested, that is, it does not induce a PERP-like state.

3.3.3 Blocking prolactin release during copulation does not decrease the duration of the refractory period

The release of PRL which is observed during sexual behavior has been proposed to be crucial in the establishment of the PERP (Kruger et al. 2002). To test this hypothesis, we acutely inhibited PRL release during sexual behavior by taking advantage of bromocriptine, a D2 receptor agonist. Bromocriptine's activation of D2 receptors on the lactotrophs' membrane blocks PRL release, a well-established procedure to inhibit the discharge of this hormone from the pituitary (Brown et al. 2010; Molik and Błasiak 2015). If PRL is indeed necessary for the establishment of the PERP, we expected that after ejaculation, drug-treated males would regain sexual activity faster than controls.

To test bromocriptine's efficiency in blocking PRL release during sexual behavior, we first injected a group of males with bromocriptine (or vehicle) and measured PRL levels at three time points: i) before the drug or vehicle injection, ii) before the female was inserted in the cage and then iii) after ejaculation (Fig. 3.5a). As shown in Fig. 3.5b, bromocriptine administration efficiently blocked PRL release in both subspecies of mice, since PRL levels after ejaculation are not different from baseline (BL6: B1 vs ejac, $P = 0.3$, B2 vs ejac $P = 0.99$; PWK: B1 vs ejac, $P = 0.97$, B2 vs ejac $P = 0.99$; Tukey's multiple comparisons test after RM Two-way Anova).

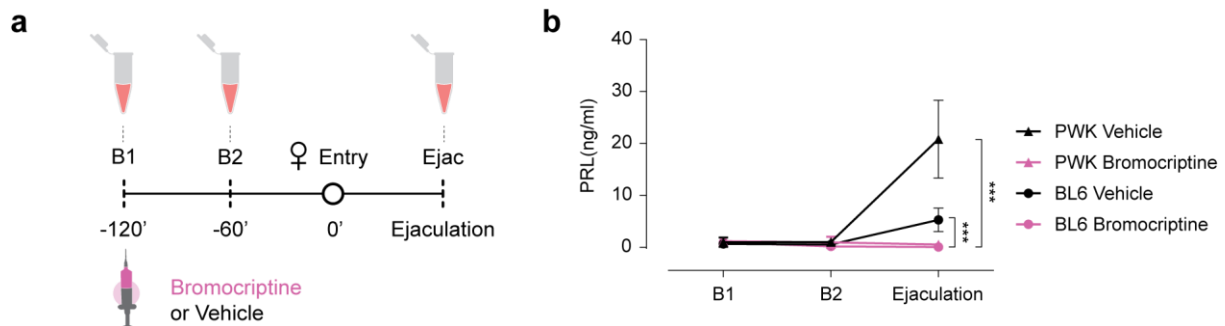


Figure 3.5. Bromocriptine blocks prolactin release during consummatory behavior.

a Timeline for blood collection and **b** [PRL]_{blood} after Vehicle (Veh - black) or Bromocriptine (Bromo - pink) administration. RM two-way Anova with treatment (Veh or Bromo) as between subject's factor and time (B1, B2 and Ejac) as the within subject's factor, followed by Tukey's multiple comparison test: BL6 ($n = 5$ each) Treatment $F_{1,8}=21.08$, $P = 0.0018$ Ejac_{veh} vs. Ejac_{bromo} $P < 0.0001$; Time $F_{2,16}=18.41$, $P < 0.0001$, B1_{bromo} vs. Ejac_{bromo} $P = 0.3$; and PWK ($n_{veh} = 6$, $n_{bromo} = 4$) Treatment $F_{1,8}=28.43$, $P = 0.0007$ Ejac_{veh} vs. Ejac_{bromo} $P < 0.0001$; Time $F_{2,16}=23.97$, $P < 0.0001$, B1_{bromo} vs. Ejac_{bromo} $P = 0.97$.

In a separate experiment we then tested the effect of the pharmacological manipulation on the establishment of the PERP and its duration. In this case, the male and female were allowed to remain in the cage undisturbed for a period of up to 2 hours after ejaculation. Each male from the two strains was tested twice, once with vehicle and a second time with bromocriptine, in a counter-balanced manner. Each session ended once the male performed the first attempt of copulation after ejaculation (red diamonds) or after two hours if no attempt was made (Fig. 3.6a, see Methods for details). All the annotated behaviors are depicted over time on Fig. 3.6b (see Methods for details).

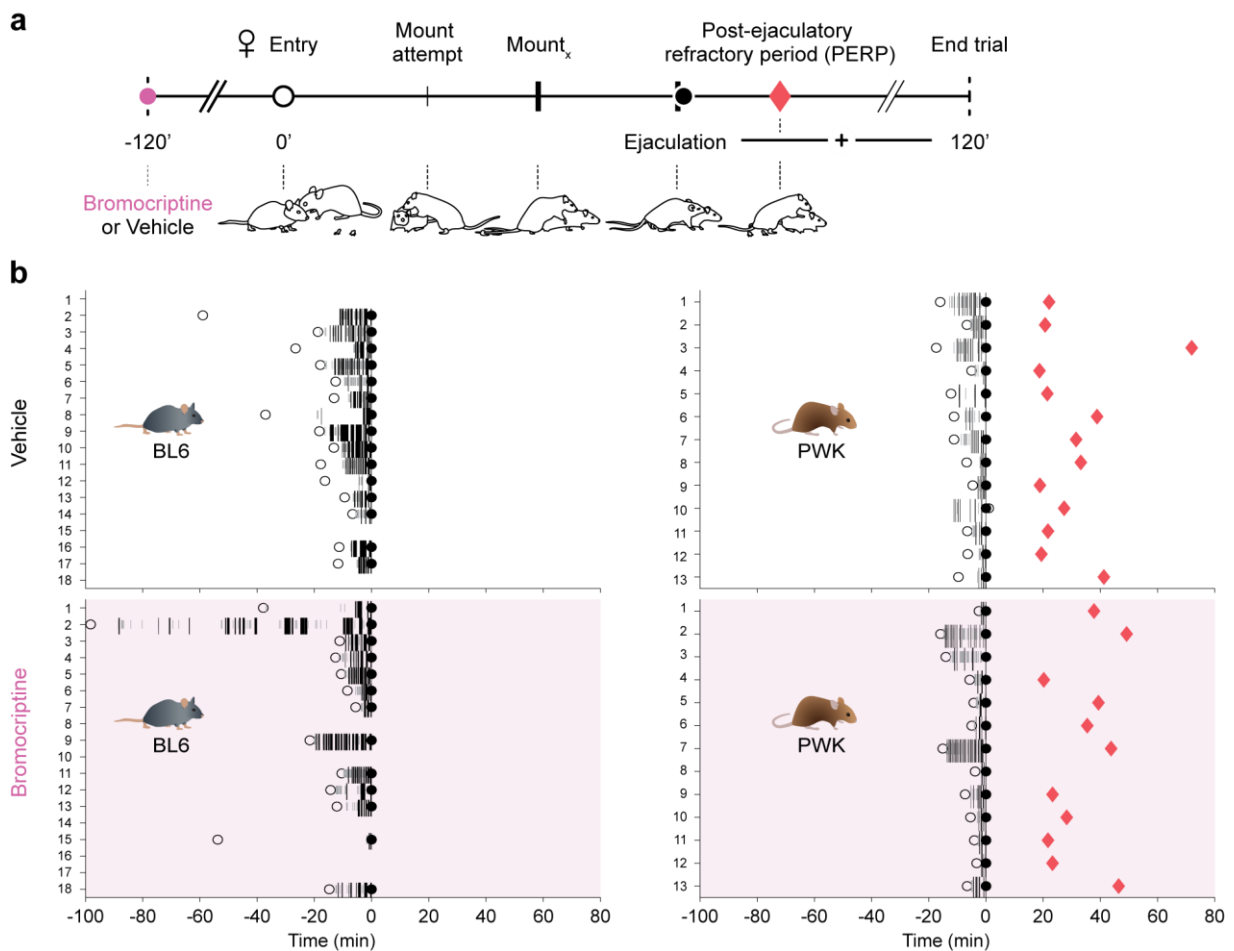


Figure 3.6. Mouse sexual behavior ethogram for Bromocriptine manipulation.

a Timeline for sexual behavior assay using sexually trained BL6 and PWK males pre-treated ($t=-120$ min) with Veh (black) or Bromo (pink). Each animal was tested twice in a counterbalanced manner. **b** Raster plot aligned to ejaculation, representing the sexual behavior performed by the male,

with mount attempts represented in small grey bars, mounts in long black bars (width correlated with mount duration), ejaculation with a black circle and PERP (latency to the first consummatory event (mount attempt or mount) after ejaculation represented with a red diamond. Time of female entry in the apparatus represented with an open circle. $n_{BL6} = 18$; $n_{PWK} = 13$.

As shown in Fig. 3.7a, inhibiting PRL release during sexual behavior did not change the proportion of male mice of the two strains that reached ejaculation (Supplementary Data 2) or regained sexual activity in the 2 hours after ejaculation (corresponding to the proportion of red diamonds in Fig 3.6b). In addition, and contrary to what was expected, we observed a significant increase in the PERP of PWK males (Fig. 3.7b, veh: 21.7 ± 4.18 vs bromo: 35.4 ± 16.3 , $P = 0.007$ by Wilcoxon signed rank test). Administration of bromocriptine also seems to affect the initial sexual arousal, as we could detect a decrease in the latency to start mounting the female (Fig. 3.7c, trend for B6 males and significant for PWK, veh: 4.06 ± 4.35 vs bromo: 1.93 ± 1.13 , $P = 0.06$; and veh: 2.78 ± 1.7 vs bromo: 1.48 ± 0.55 , $P = 0.01$, respectively, by Wilcoxon signed rank test). This observation was not due to an increase in activity/locomotion of the bromocriptine treated males, as the average male speed before and after the female entry was not affected by the manipulation, nor the distance between the pair (Supplementary Data 4). Despite the locomotor activity being the same, the average male speed projected towards the female increased significantly for PWK males treated with bromocriptine as they moved in a goal directed way, directionally towards the female (Supplementary Data 4).

Once consummatory behavior was initiated, control and bromocriptine-treated males exhibited similar levels of sexual performance, as we could not detect any difference in the frequency of attempts to mount the female or time taken to reach ejaculation (Fig 3.7d and e). Other aspects of the sexual interaction were also not altered (Supplementary Data 5). Furthermore, bromocriptine administration does not seem to affect the dynamics of the sexual interaction across the session or within each mount (Fig. 3.7f and g respectively).

In summary, blocking PRL release during copulation does not affect the proportion of animals that regain sexual activity within 2 hours after ejaculation and contrary to what was expected, bromocriptine leads to an increase in the duration of the PERP of PWK males. Except for a decrease in the latency to start mounting, maintaining circulating PRL low, at levels similar to what is observed prior to the sexual interaction, does not affect any of the parameters of sexual performance analyzed in both strains of mice.

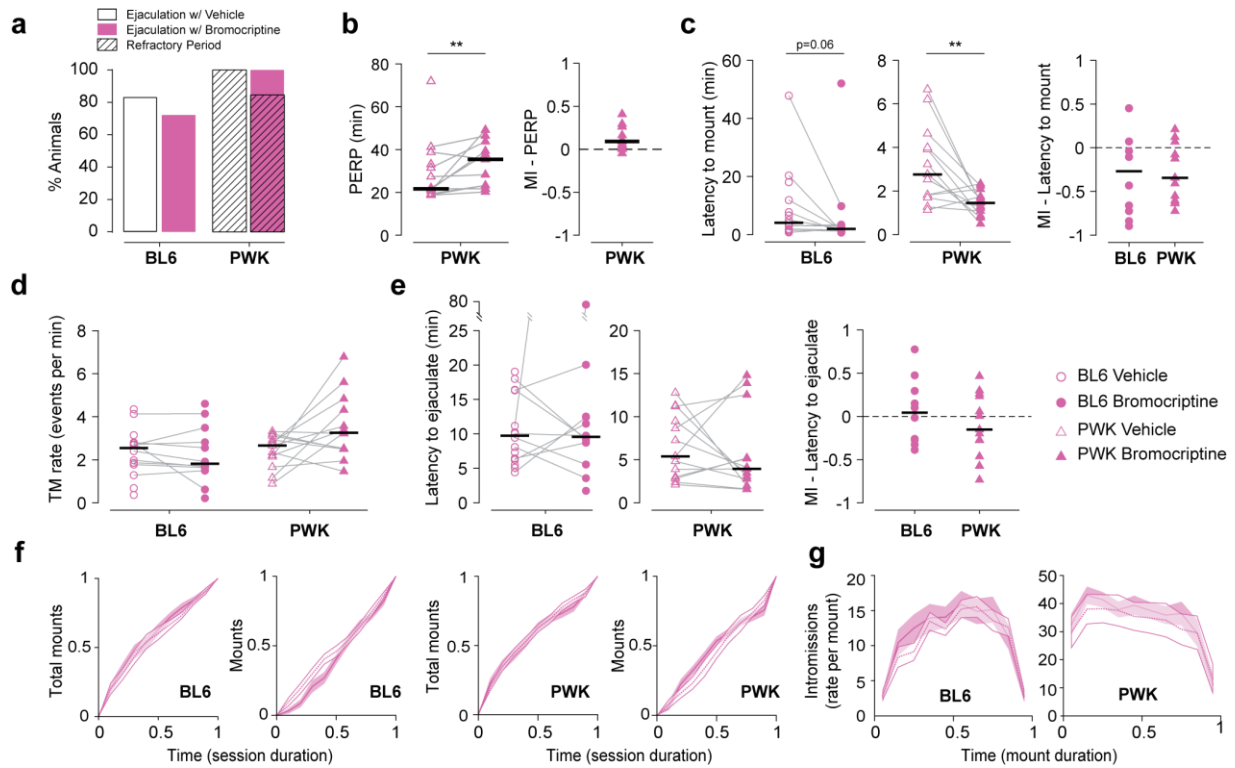


Figure 3.7. Blocking prolactin release during copulation does not decrease the duration of the refractory period.

a Percentage of animals that reached ejaculation (solid color) and re-initiated the consummatory behavior after ejaculating (PERP, dashed color) in Veh (white) and Bromo (pink) conditions. Quantification of **b** PERP duration ($P = 0.007$), **c** latency to mount (first MA or mount) (BL6 $P = 0.0644$, PWK $P = 0.0132$), **d** rate of total mounts (TM, MA+mounts) (BL6 $P = 0.32$, PWK $P = 0.0681$) and **e** latency to ejaculate (from **f**, BL6 $P = 0.92$, PWK $P = 0.68$). With BL6 represented by circles and PWK by triangles (Veh – open, Bromo - solid). Each line represents data from an individual; only animals that ejaculated in both sessions were considered in the statistics ($n_{BL6} = 10$, $n_{PWK} = 13$). Individuals that did not ejaculate in one of the trials are represented as unconnected dots (not used in statistics). Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test. MI [modulation index (Bromo - Veh)/(Bromo + Veh)] between the two conditions for both strains. ** $P < 0.01$. **f** Cumulative distributions of total mounts and mounts along the behavioral assay. Histogram aligned to the first consummatory event with 0,1min bins. Time normalized for the duration of the session from the first consummatory event to ejaculation. **g** Rate of intromissions executed during the mount. Histogram for all mounts of each session, aligned to beginning of the mount with 0,1min bins. Time normalized for the duration of the mount.

3.4 Discussion

The post ejaculatory refractory period or PERP is highly conserved across species and is characterized by a general decrease in sexual activity after ejaculation (Seizert 2018). The pituitary hormone PRL is released during copulation and has been put forward as the main player in the establishment of the PERP (Kruger et al. 2002). However, the involvement of PRL in the establishment and duration of the PERP is controversial and has not been formally tested (Seizert 2018). Here we show that despite being released during copulation as previously shown in other taxa, PRL is neither sufficient nor necessary for the establishment of the PERP.

We first showed that PRL is released during copulation in male mice. The proportion of BL6 males that engaged in sexual behavior when tail blood is collected is lower than the proportion of animals that copulated in the rest of the experiments, suggesting that the procedure affects their sexual activity. However, once they start mating, all animals reached ejaculation. Importantly, once habituated, the procedure itself does not lead to PRL release. This opens up the possibility to perform such type of experiments using an “within-animal” design, a very important point particularly when there is a large inter-individual variability, while decreasing the number of animals used.

Despite being released during sexual behavior in mice, PRL dynamics are quite different from what has been observed in humans. In men, PRL seems to only be released around the time of ejaculation (Exton et al. 2000; Exton et al. 2001), and only when ejaculation is achieved (Exton et al. 2000). Indeed, the fact that PRL surge was only observed when ejaculation was achieved was one of the main results that lead to the idea that PRL may play a role in the acute regulation of sexual activity after orgasm in humans (Krüger et al. 2005). In contrast, in mice we observed an increase in circulating levels of PRL in sexually aroused PWK males and in BL6 males during the consummatory phase. The discrepancy between our results and the results published by others might be a result of the sampling procedure. Despite the fact that in human studies blood was continuously collected, PRL detection was performed at fixed time intervals and not upon the occurrence of particular events, such as ejaculation. Therefore, when averaging PRL levels across individuals, each participant might be in a slightly different internal state. Also, because PRL concentration is determined over fixed intervals of time, it is difficult to pinpoint the PRL surge to the time of ejaculation (even though the human studies show that sexual arousal per se is not accompanied by an increase in PRL levels) (Kruger et al. 2002). To our knowledge, a single study assessed PRL levels during sexual behavior in male mice, stating that PRL is released after ejaculation (Bronson and Desjardins 1982). In this case, blood

was also continuously sampled at fixed intervals of time. In contrast, in our study the blood was collected upon the execution of particular events, such as the first mount attempt, a pre-defined number of mounts and ejaculation. Thus, even though the intervals between PRL measurements are different for each mouse, we ensure that PRL levels are measured for all individuals in a similar internal state. Independently of the differences in the dynamics of circulating PRL levels, the increase we observe seems to be specific to a sexual encounter, since PRL levels in BL6 males that never attempt copulation remain unaltered from baseline.

In order to test if PRL by itself is sufficient to decrease sexual activity, we injected domperidone to induce an artificial PRL-surge. In this case, the male mouse should behave like a male that just ejaculated: for example, exhibit longer latency to initiate the sexual interaction, which in the case of BL6 mice should take days. Even though domperidone administration leads to circulating levels of PRL that are similar to the ones observed at the end of a full sexual interaction, this manipulation did not cause any alteration in terms of sexual performance, as all behavioral parameters remained unaltered for both strains of mice. Although most likely other neuromodulatory systems were affected by our manipulation (via PRL), the fact that the domperidone manipulation did not cause a PERP-like state might still be due to the fact that the full repertoire of neuromodulators and hormones accompanying an ejaculation was not present (Pfaus 2009). Further experiments could test this idea by examining if combinations of different neuromodulators and hormones administered together with PRL can induce a PERP-like state.

Last, we asked if the elevation in PRL levels during sexual behavior is necessary for the establishment and duration of the PERP. For that we took a complementary pharmacological approach, where we injected bromocriptine, a D2 receptor agonist that temporarily inhibits the release of PRL. PRL levels after ejaculation in bromocriptine-treated males are similar to pre-copulatory levels. If PRL is indeed necessary to establish the PERP, we would expect a decrease in its duration that should easily be observed in the PWK males (since they regain sexual activity on average 30 minutes after ejaculation) or even in the BL6 (which take days). We observed a decrease in the latency to start mounting the female and, contrary to our expectation, a significant increase in the PERP duration of PWK males. We believe these effects may be mediated by the direct effect of bromocriptine, rather than an effect of PRL itself. First, baseline PRL levels are already very low in male mice and therefore the manipulation most likely did not affect them. Second, systemic administration of dopamine agonists has shown that anticipatory measures of sexual behavior are more sensitive to disruption than are

consummatory measures of copulation (Pfaus and Phillips 1991; Hull et al. 2004). This agrees with our results, where we observed a significant decrease in the latency to initiate mounting with bromocriptine, while no other parameter of sexual performance was affected. Interestingly, bromocriptine-treated PWK males seem more ballistic in their approach to the female, suggesting a more goal-directed behavior towards the female. Bromocriptine (and domperidone) might also have an effect outside the central nervous system as D2 receptors are expressed in the human and rat seminal vesicles (Hyun et al. 2002). It is not known if direct manipulation of these receptors in the seminal vesicles has an impact on the PERP, which could explain our results.

In this study we investigated the role of PRL in the PERP of two different strains of mice that belong to the two main subspecies of house mouse, *Mus musculus musculus* (PWK) and *Mus musculus domesticus* (BL6), for three main reasons. As already presented, the two strains have very different PERP duration, widening the dynamic range of this behavioral parameter and increasing the probability of detecting an effect of the manipulations. Second, in addition to differences in PERP duration, BL6 and PWK males have very different sexual performance, reflected in several behavioral parameters as for example the number of mounts needed to reach ejaculation. Despite the differences (which were not explored as they are outside the scope of the present study), the effects of the pharmacological manipulations were similar across the two strains, causing either no effect or changing the behavior in the same direction (shorter latency to start mounting the female in bromocriptine treated males). This is an important point that strengthens our conclusions. Finally, although fundamental for many present-day discoveries, the usage of the common inbred strains of mice comes at a cost, due to the limitations in their genetic background that sometimes leads to results that are specific to the strain of mouse used (Welker and Loos 1986; puglisi-Allegra and Cabib 1997; Cabib et al. 2002). Wild derived strains of mice are valuable tools that can complement the genetic deficiencies of classical laboratories strains of mice (Gregorová and Forejt 2000; Fernandes et al. 2004). Despite the fact that larger numbers of animals are used (because experiments are repeated on each mouse strain), this approach is already routinely used in other fields, such as in immunological studies (Deschepper et al. 2004) providing greater confidence to the results obtained from the effect of pharmacological manipulations on behavior, for example.

As initially discussed, PRL can affect peripheral and central pathways that have been implicated in sexual performance and motivation. Even though our manipulations indiscriminately affect both, our results refute the idea that PRL decreases sexual activity, the

hallmark of the PERP. What could be the role of copulatory PRL? PRL release may be the “side-effect” of the neuromodulatory changes that occur during sexual behavior, this is, merely the result of reduction in DA levels (DA inhibits PRL release) and/or the increase in oxytocin and serotonin (known stimulating factors of PRL release) instead of having the principal role in the establishment of PERP (McIntosh and Barfield 1984a; McIntosh and Barfield 1984b; Carmichael et al. 1987). The fact that PRL levels are already elevated during sexual interaction in BL6 and PWK males, further suggests that PRL cannot promote by itself reduced sexual activity, at least in male mice. Other studies point towards a role of PRL in the establishment of parental behavior (Brown 1985; Schradin and Anzenberger 1999; Stagkourakis et al. 2020) New behavioral paradigms will be fundamental in unravelling this mystery.

3.5 Methods

Animals. BL6 (*Mus musculus domesticus*, C57BL/6J) and Wild (*Mus musculus musculus*, PWD/PhJ and PWK/PhJ) mice were ordered from The Jackson Laboratories and maintained in our animal facility. Animals were weaned at 21 days and housed in same-sex groups in stand-alone cages (1284L, Techniplast, 365 x 207 x 140 mm) with access to food and water ad libitum. Mice were maintained on a 12:12 light/dark cycle and experiments were performed during the dark phase of the cycle, under red dim light. All experiments were approved by the Animal Care and Users Committee of the Champalimaud Neuroscience Program and the Portuguese National Authority for Animal Health (Direcção Geral de Veterinária).

Females were kept house grouped and males were isolated before the sexual training. Both males and females were sexually experienced and habituated to be handled and to the assay routine. To enter the study, a male had to ejaculate 3 times (in 4 sessions). Males interacted with different females in each sexual encounter. Each manipulation was performed in a different group of males and, within each manipulation, experiments were conducted in parallel for both BL6 and PWK. Trials were conducted in the male home cage (1145T, Techniplast, 369 x 156 x 132 mm) stripped from nesting, food and water; covered with a transparent acrylic lid. The trial started with the entry of the female in the setup (t=0min).

Ovariectomy and hormonal priming. All females underwent bilateral ovariectomy under isoflurane anesthesia (1-2% at 1L/min). After exposing the muscle with one small dorsal incision (1 cm) a small incision was made in the muscle wall, at the ovary level, on each side.

The ovarian arteries were cauterized and both ovaries were removed. The skin was sutured, and the suture topped with iodine and wound powder. The animals received an ip injection of carprofen before being housed individually with food supplemented with analgesic (MediGel, 1mg carprofen /2 oz cup) for 2 days recovery and then re-grouped in their home cages.

Female mice were primed subcutaneously 48 hours before the assay with 0,1ml estrogen (1mg/ml, Sigma E815 in sesame oil) and 4 hours before the assay with 0,1ml progesterone (5mg/ml, Sigma 088K0671 in sesame oil).

Blood collection. Tail-tip whole blood samples were collected from the male tail, immediately diluted in PBS-T (PBS, 0.05% Tween20) and frozen at -20°C where it was stored until use (Guillou et al. 2015).

To profile $[\text{PRL}]_{\text{blood}}$ during sexual behavior (Fig. 1a), baseline blood was collected 30 minutes before ($t=-30\text{min}$) the female entry ($t=0\text{min}$). From this point on, blood collection was locked to the onset of specific behaviors: once the male did the first mount attempt (MA), after executing a fixed number of mounts (Mx) and after ejaculation (after the male exhibited the stereotypical shivering, falling to the side and decoupling from the female). We choose $\text{Mx}=5$ for BL6 and $\text{Mx}=3$ for PWK to ensure that the males would have sufficient sexual interaction without reaching ejaculation. Contrarily to PWK males that, in the presence of a receptive female, the majority engages in sexual behavior, BL6 do not. Thus, after 30 minutes interacting with the female without displaying sexual interest, we collected a blood sample and terminated the trial (Fig. 1b, social). Because blood collection is an invasive procedure and PRL is also released under stress we evaluated if the manipulation itself could induce PRL release. For that we collected blood every 20 minutes for 1 hour from males resting in their home cage (Fig. 1c).

Domperidone is a D2 dopamine receptor antagonist that was previously used to study the inhibitory tone of dopamine on PRL release from the pituitary, inducing a PRL peak 15 minutes after ip injection (Guillou et al. 2015). To test the magnitude of the PRL release of the two mouse strains under domperidone (Fig 2a), we conducted a pilot study where we collected a blood sample before (baseline) and 15 minutes after domp injection (20mg/kg, abcam Biochemicals). We opted to manipulate $[\text{PRL}]_{\text{blood}}$ through domperidone instead of injecting PRL directly to induce a PRL release similarly to a natural occurring instead of adding a recombinant form.

Bromocriptine is a D2 dopamine receptor agonist known to inhibit endogenous prolactin release (Brown et al. 2010). To test its efficacy on blocking PRL release during sexual behavior (Fig. 3a) we conducted a second pilot study where the males were injected (100 µg bromo or vehicle) 2 hours before the trial started. Blood samples were collected just before injection, 1 hour after injection and after ejaculation.

Prolactin quantification. [PRL]_{blood} quantification was done as previously described (Guillou et al. 2015). Briefly, a 96-well plate (Sigma-Aldrich cls 9018–100EA) was coated with 50 µl capture antibody antirat PRL (anti-rPRL-IC) (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), AFP65191 (Guinea Pig), NIDDK-National Hormone and Pituitary Program (NHPP, TORRANCE, CA) at a final dilution of 1:1000 in PBS of the antibody stock solution, reconstituted in PBS as described in the datasheet (Na₂HPO₄ 7.6 mM; NaH₂PO₄ 2.7 mM and NaCl 0.15M; pH 7.4). The plate was protected with Parafilm® and incubated at 4°C overnight in a humidified chamber. The coating antibody was decanted and 200 µl of blocking buffer (5% skimmed milk powder in PBS-T) was added to each well to block nonspecific binding. The plate was left for 2 hours at room temperature on a microplate shaker. In parallel, a standard curve was prepared using a 2-fold serial dilution of Recombinant mouse Prolactin (mPRL; AFP-405C, NIDDK-NHPP) in PBS-T with BSA 0.2 mg/mL (bovine serum albumin; Millipore 82–045–1). After the blocking step, the plate was washed (3 times for 3 minutes at room temperature with PBS-T), 50 µl of quality control (QC), standards or samples were loaded in duplicate into the wells and incubated for 2 hours at room temperature on the microplate shaker. The plate was washed, and the complex was incubated for another 90 minutes with 50 µl detection antibody (rabbit alpha mouse PRL; a gift from Patrice Mollard Lab) at a final dilution of 1:50 000 in blocking buffer solution. Following a final wash, this complex was incubated for 90 minutes with 50 µl horseradish peroxidase-conjugated antibody (anti rabbit, IgG, Fisher Scientific; NA934) diluted in 50% PBS, 50% blocking buffer. One tablet of O-phenylenediamine (Life technologies SAS 00–2003) was diluted into 12 ml Citrate-phosphate buffer pH 5, containing 0.03% hydrogen peroxide. 100 µl of this substrate solution was added to each well (protected from light), and the reaction was stopped after 30 minutes with 50 µl of 3M HCl. The optical density from each well was determined at 490nm using a microplate reader (SPECTROstar^{Nano}, BMG LABTECH). An absorbance at 650nm was used for background correction.

A linear regression was used to fit the optical densities of the standard curve vs their concentration using samples ranging from 0.1172ng/ml to 1.875ng/ml, using the former as the lower limit of detection. Appropriate sample dilutions were carried out in order to maintain detection in the linear part of the standard curve, PRL concentrations were extrapolated from the OD of each sample. To control for reproducibility of the assay, trunk blood of males injected with domperidone was immediately diluted in PBS-T and pulled to be used as quality control (QC). Loading of the wells was done vertically left to right and QC was always loaded on the top row. The formula $OD (Co,t) = OD (Ob) + \alpha (QC).t$ was used to correct the ODs for loading dwell time (OD: optical density, Co: corrected, t: well number, Ob: observed, α : QC linear regression' α). Coefficient of variability was kept to a maximum of 10%.

Behavioral assays. Each male underwent two trials: one with vehicle and one with drug (domperidone or bromocriptine). Administrations were counter balanced between animals and spaced seven days. In the first assay, for pharmacological induction of acute PRL release (Fig. 2b), the male was injected ip with domperidone or vehicle 15 minutes before the trial started ($t=-15min$). Animals were allowed to interact until the male reached ejaculation or 1 hour in the case the male did not display sexual behavior. Conversely, for pharmacological blockage of PRL release (Fig. 3b), a second group of males were pre-treated with bromocriptine or vehicle with a subcutaneous injection 2 hours before the beginning of the trial ($t=-120min$). Animals were allowed to interact until a maximum of 2 hours after the male reached ejaculation or 1h in the case the male did not display sexual behavior. For the BL6 unmanipulated group, the female was added to the male home cage without disturbing the male (Supplementary Data 2). Animals were allowed to interact until the male reached ejaculation or 1 hour in the case the male did not display sexual behavior.

Behavior analysis. The behavior was recorded from the top and side with pointgrey cameras (FL3- U3-13S2C-CS) connected to a computer running a custom Bonsai software (Lopes et al. 2015). Behavior was manually annotated using the open-source program Python Video Annotator (<https://pythonvideoannotator.readthedocs.io>) and analyzed using Matlab. The number of mount attempts (MA, mount without intromission), mounts (mounts with intromission), latency to mount (first MA or mount), latency to ejaculation and PERP (latency between ejaculation and the next mount) was calculated. Total number of mounts (TM) was calculated as the sum of MA and mounts and TM rate was calculated as TM/latency to

ejaculate. The percentage of animals that reached ejaculation and regain sexual activity under 2 hours (Refractory period) were also calculated. The modulation index (MI) was calculated as $(X_{\text{drug}} - X_{\text{vehicle}}) / (X_{\text{drug}} + X_{\text{vehicle}})$. The centroid position and individual identity of each pair was followed off-line using the open source program idtracker.ai (Romero-Ferrero et al. 2019) and used to calculate male velocity and inter individual distance with Matlab (Supplementary Data 4).

Statistical analysis. The statistical details of each experiment, including the statistical tests used and exact value of *n* are detailed in each figure legend. Comparisons were always performed within the same strain and not across strains. Data related to prolactin quantification was analyzed using GraphPad Prism 7 software and presented as mean $\pm$ S.D. For comparison within strain (Fig. 3.1a and c) a RM One-way Anova followed by a Tukey's multiple comparison test was used. Comparison of paired samples comparing two groups, statistical analysis was performed by using a paired-sample two-tailed *t* test (Fig. 3.1b, 3.2a baseline-Domp). For the effect of treatment (veh or bromo) and time of sampling (B1, B2 and Ejac) (Fig. 3.5) a RM two-way Anova followed by Tukey's multiple comparison test was used. Data related to animal behavior was analyzed with MATLAB R2019b and presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor). Animals were randomized between treatments and comparison between the two conditions were done with Wilcoxon rank sum test (Fig. 3.4a-c, 3.7b-e and Supplementary Data 3-5). Only animals that ejaculated in both sessions were included in the statistical analysis (Domperidone: $n_{\text{BL6}} = 7$, $n_{\text{PWK}} = 13$; Bromocriptine: $n_{\text{BL6}} = 10$, $n_{\text{PWK}} = 13$). Significance was accepted at $P < 0.05$ for all tests.

4. General discussion

Rodents belong to the few mammalian groups in which details of male copulatory behavior have been extensively studied (Dewsbury 1972; Burns-Cusato et al. 2004; Hull and Rodríguez-Manzo 2017). Even though a lot of effort has been made in the examination of rodent sexual behavior during the past century, our understanding about the underlying brain circuitry and mechanisms, is to date still scarce. Moreover, the field is substantially lacking comparative studies that can not only shed light into evolutionary distinct patterns of sexual behaviors but also help to unravel the underlying principles of brain function and neuromodulation of sexual behavior.

Mouse sexual behavior, as for virtually almost any research topic, is prominently studied in domesticated inbred strains of *M.m. domesticus*. Although the behavior is highly stereotypical in the house mouse, there is large inter-strain variability in respect to the latency for the occurrence of certain behaviors and in the number of iterations of consummatory actions, as the number of intromissions or the number of mounts. Moreover, it has become apparent that in some instances the results obtained using classical laboratory strains of mice were the direct product of their genetic background, meaning that sometimes findings are specific to the strain of mouse used (Welker and Loos 1986; puglisi-Allegra and Cabib 1997; Cabib et al. 2002). Awareness that the observed results may be only representative for the strain used in that particular study led to the inclusion of new strains with different genotypic backgrounds. Hence, *M.m. musculus* wild derived strains recently gained popularity and have been included in several research topics including assortative mate choice (Zinck and Lima 2013), however their potential as models for sexual behavior has not been assessed.

Therefore, the first part of this thesis aimed at i) characterizing the inter-subspecies behavioral difference between *M.m.musculus* and *M.m.domesticus* and ii) investigating the impact of the female strain on the copulatory pattern. The second part combined biochemical and pharmacological methods in order to investigate the role of the hormone prolactin in the post-ejaculatory inhibitory phase of sexual behavior.

4.1 Male mouse mating behavior

As mice are polyandrous, different mating strategies often are under a sperm competition scenario, in which sperm of different males “compete” to fertilize the ova (Parker 1998b). Under these circumstances males tend to adopt a strategy with a smaller number of mounts, decreased latency to ejaculate and shorter PERP in order to increase paternity chances and avoiding takeover by another male or female rejection (Parker 1998a; Thonhauser et al. 2014). However, alternative mating strategies also occur with prolonged copulation in order to reduce female re-mating probability (Oglesby et al. 1981; Huck and Lisk 1986; Stockley and Preston 2004), reducing the ability to inseminate a second ejaculate, potentially through physical exhaustion, reduced female receptivity or sperm depletion (Toner and Adler 1986). Surprisingly, our comparative study revealed that each subspecies characteristically showed one of these mating strategies: *musculus* males perform few mounts before ejaculation with a high percentage of the males restarting a second copulatory series under the given time (120min) while *domesticus* had a longer pre-ejaculatory stimulation (4 to 6 folds comparatively to *musculus*) and only a residual number attempted to re-mate after ejaculation. Although both mating strategies exist in nature, it remains to be tested whether the observed difference between *musculus* and *domesticus* is due to a genetic divergence between the subspecies or/and a differential capacity of males with different selection histories. Independently of the source of variation, it would be interesting to investigate if this difference in mating strategy is related to sperm allocation. In a follow up experiment, a comparison of sperm counts by ejaculate would allow to evaluate if the ejaculate investment by *domesticus* is bigger than for *musculus*, as expected. The comparison of the total sperm ejaculated in the several ejaculations until *musculus* entered a period of sexual satiety, with the sperm counts of the *domesticus* ejaculate could indicate if the total sperm available is similar and thus if *musculus* actively fractionate it. As *musculus* present smaller number of mounts, decreased latency to ejaculate and shorter PERP, we can speculate that *musculus* are still under a sperm competition scenario, while *domesticus* seem to no longer be under this reproductive pressure. Additional information could be derived from morphological data on body and testes mass as species under a higher degree of sperm competition typically have bigger testis (Parker et al. 1997; Birkhead and Møller 1998; Gage and Freckleton 2003; Stockley and Preston 2004). Also, testis size was positively correlated with the size of the genital cortex (Lauer et al. 2017), suggesting a bigger sensitivity hence, a smaller amount of stimulation to reach ejaculation. In agreement, although the size of the testis was not measured, by visual inspection *musculus*

seem to possess a higher testis/body ratio suggesting a bigger sensitivity and reduced stimulation to reach ejaculation.

Mating is rewarding in most taxa, but it is also expensive and dangerous. This could lead us to predict that there should be a strong evolutionary pressure to reinforce consummatory acts that are as efficient as possible, this is, to quickly reach ejaculation. Evolutionary, it would be expected an increased success in a mating pattern involving fewer mounts with more intromissions per mount. This would minimize the number of contacts necessary between potential mates in order to achieve ejaculation. For example, every time the male dismount and the couple splits, one of them could be caught by a predator, ending the session without ejaculation (Dewsbury 1981). The rather unfitting *domesticus* mating pattern could result from a difference on the neural control structures that process genital information. It would be interesting to investigate if these subspecies differ in their genital cortex size or neural response, suggesting a difference in sensitivity to penile stimulation. This hypothesis could be further investigated by using c-fos labelling in brain areas related to sexual behavior in males, like the medial preoptic area and medial amygdala, as a readout of a potentially differential neuronal activation between the two subspecies (Coolen et al. 1996; Coolen et al. 1998; Parfitt and Newman 1998; Hull and Dominguez 2007; Hull and Rodríguez-Manzo 2017). Moreover, imaging of the potential targeted areas could provide information on how the behavior is modulating the neuronal response.

It remains to be tested if the first sexual experience of the male is an important point. The males used in this study were all previously sexually trained with the female strain that they were subsequently tested with. It will be interesting to train them with a particular strain and test them with another one, to understand how flexible the behavior really is.

An alternative hypothesis is that the amount of mounting behavior and intromissions is controlled by the female, which could serve two purposes: mate assessment (as in mate choice) (Thonhauser et al. 2013) and/or a necessity of a certain amount of stimulation for the induction of the neuroendocrine response that must happen in order for pregnancy to be initiated (Erskine et al. 2004). Even though there are contradictory reports regarding the possibility of inducing pseudo-pregnancy in female mice (Yang et al 2009 for example), the vaginal lavage procedure required for the determination of the reproductive state of the female, which involves the gentle insertion of a pipette tip in the female's vagina, and is routinely used in our lab, can lead females to look pseudo-pregnant like, as they stop cycling. In fact, when *musculus* and *domesticus* males interact with females from the other subspecies, what we

observed is that the mounting behavior becomes more similar to the behavior of the female strain, this is: *domesticus* males mating with *musculus* females need less total mount duration and the contrary is for *musculus* males. In a pure speculative point of view, ejaculation might be triggered by the female, for example, if once the female reaches the necessary level of vaginal stimulation to induce pregnancy, the inner walls of the cervix contract, providing a signal to the male. In this case, the number of mounts and intromissions are dictated by the female. Furthermore, it is well established that female mice will pace the amount of sexual interaction when possible, this is, they will run away from the male and increase the duration of the inter-mount-interval when possible (Johansen et al. 2008). Therefore, the number of mounts might be dictated by the female. Further experiments with electromyogram recordings in the female reproductive organs could be used to test this hypothesis.

Sexual behavior is highly interactive and, from studies in the field of sperm competition, we know that males have the capacity to adapt their copulatory behavior (Ramm and Stockley 2007; Firman et al. 2013). Most notably, the distinct and repeatable difference in the type of PERP exhibited by BL6 (l-PERP) and PWK (s-PERP) was not affected by female strain. Inspired by studies about the modality of inheritance of burrow building in *Peromyscus* (Dawson et al. 1988; Weber et al. 2013) we conducted a preliminary experiment where we explored the possibility of a genetic dominance or sex-linked feature for the PERP pattern. We did reciprocal crosses between BL6 and PWK and tested the F1 with BL6 females. s-PERP pattern was observed in 30% (6/20) of the F1 sired by PWK males and 59% (10/17) of F1 sired by BL6 males. Overall, 43% of F1s (16/37) showed a s-PERP. With no obvious answer to our question, a large back-cross experiment would be needed to provide an extended genetic analysis of this behavior.

Taken together, our findings are of great importance for the field of mouse sexual behavior and neuroethology in general as this study presents:

- 1) The first characterization of mating behavior of the *musculus* PWK and PWD strains;
- 2) Introduces new models that allow the study of particular characteristics of the copulatory behavior;
- 3) Describes the latency to recover sexual interest after ejaculation for PWK and BL6 strains in detail, laying the basis for the investigation of the mechanisms underlying the variation of the inhibitory phase.

4.2 The inhibitory phase and the role of prolactin

The post-ejaculatory refractory period (PERP) remains as one of the biggest mysteries of male sexual behavior. As mentioned before, after ejaculation, males enter a sexual inhibitory phase with decreased sexual motivation. As for the consummatory phase, the PERP is also highly variable (Dewsbury 1972). The mechanisms underlying the observed variation in the PERP duration or its concrete function are scarcely studied. However, the fact that it is highly conserved across species implies an evolutionary and/or an ecologically important role (Seizert 2018).

Although our comparative study represented only a small sample of the vast number of strains available, it provided a model to explore the mechanisms underlying this behavior. It has been hypothesized that the prolactin surge around the time of ejaculation plays a role in the immediate subsequent decrease of sexual activity, the hallmark of the PERP (Kruger et al. 2002). This theory was founded in limited evidence-based data and never formally tested. Our parallel study in PWK and BL6 showed that, although released during consummatory behavior for both strains, prolactin is not sufficient nor necessary to induce the refractory period. If not prolactin then what?

Several studies have speculated about the neurobiology of the PERP, mostly by brain lesions or drug infusion, however we are still far away from a holistic understanding of this process. As discussed previously, the possibility of a single hormone/neuromodulator inducing the PERP is very unlikely. Most probably the PERP is the result of several neuromodulatory systems and the interaction between different brain areas. One possible way to investigate which neuromodulators are involved in the establishment of the PERP could be ventricular micro-dialysis (Mas et al. 1995), with sampling at specific behavioral events, followed by a quantitative measure of the major neuromodulators known to be involved in sexual behavior. As a starting point to gain insight into putative brain areas controlling this behavior, a cFos study could be pursued, comparing neuronal activation after the male executed a fixed number of mounts with the neuronal activation after ejaculation. And subsequently, monitor the activity of the candidate areas, with imaging methods for example, in freely moving animals while mating.

The results from the second part of this thesis refute a long-standing hypothesis in the field of sexual behavior endocrinology, which will hopefully force new avenues of research for

understanding the underlying mechanisms of the PERP. A growing body of knowledge suggests a peripheral rather than central regulation of the PERP. Seizert (Seizert 2018) speculates that the chain of information may start in the spinal ejaculation generator (SEG). More specifically, in the galanin-expressing ascending projections from ejaculation activated lumbar spinothalamic (LSt) cells of the SEG to the medial subparafascicular thalamus (mSPFp). The implication of these cells in the transmission of ejaculation-related information to the brain is based on the observation that i) galanin LSt cells become cFos positive only following ejaculation (and not other sexual behavior) and ii) galanin infusion into the mSPFp induces a quiescence in rats similar to the PERP. One possible way to test this hypothesis could be by inhibition of the mSPFp using DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) (Pei et al. 2010; Roth 2016) in sexual behaving animals in an attempt to block the ejaculation-related flow of information and consequently, prevent the PERP. If the hypothesis is correct, the male should continue to mount the female just after ejaculation.

4.3 Closing Remarks

This thesis investigated the mating strategies of two closely related *Mus musculus* subspecies (Chapter 2) and formally tested the role of the hormone prolactin in the establishment of the post ejaculatory refractory period (Chapter 3).

Taken together, the results obtained in this thesis emphasize the value of comparative studies in investigating mechanisms underlying complex behaviors. Importantly, although these subspecies have evolved in different contexts, laboratory colonies allow us to perform behavioral experiments under carefully controlled conditions using animals that share a common environment. Moreover, it contributes to increase the knowledge on sexual behavior research in animals by providing new research models and refuting a long-standing hypothesis that had not been formally tested, encouraging further investigation regarding the mechanisms underlying the establishment of the PERP.

References

- Ågmo A. 1999. Sexual motivation—an inquiry into events determining the occurrence of sexual behavior. *Behav Brain Res.* 105(1):129–150. doi:10.1016/s0166-4328(99)00088-1.
- Ågmo A, Snoeren EMS. 2017. A cooperative function for multisensory stimuli in the induction of approach behavior of a potential mate. *Plos One.* 12(3):e0174339. doi:10.1371/journal.pone.0174339.
- puglisi-Allegra S, Cabib S. 1997. Psychopharmacology of dopamine: the contribution of comparative studies in inbred strains of mice. *Prog Neurobiol.* 51(6):637---661. doi:10.1016/s0301-0082(97)00008-7.
- Anholt RRH, O’Grady P, Wolfner MF, Harbison ST. 2020. Evolution of Reproductive Behavior. *Genetics.* 214(1):49–73. doi:10.1534/genetics.119.302263.
- Aoki H, Fujioka T, Matsuzaka J, Kubo T, Nakamura K, Yasuda N. 1995. Suppression by Prolactin of the Electrically Induced Erectile Response through its Direct Effect on the Corpus Cavernosum Penis in the Dog. *J Urology.* 154(2):595–600. doi:10.1016/s0022-5347(01)67118-0.
- Aoki M, Wartenberg P, Grünwald R, Phillipps HR, Wyatt A, Grattan DR, Boehm U. 2019. Widespread cell-specific prolactin receptor expression in multiple murine organs. *Endocrinology.* 160(11):2587–2599. doi:10.1210/en.2019-00234.
- Baker RR, Parker GA. 1973. The origin and evolution of sexual reproduction up to the evolution of the male-female phenomenon. *Acta Biotheoretica.*(22):22: 49–77. doi:10.1007/bf01601983.
- Batty J. 1978. Acute changes in plasma testosterone levels and their relation to measures of sexual behaviour in the male house mouse (*Mus musculus*). *Anim Behav.* 26:349–357. doi:10.1016/0003-3472(78)90053-2.
- Beach FA. 1956. Characteristics of masculine “sex drive”. In: Jones MR, editor. Vol. Vol. 4. Lincoln: University of Nebraska Press. p. 1–32.
- Beach Frank A. 1976. Cross-species comparisons and the human heritage. *Arch Sex Behav.* 5(5):469–485. doi:10.1007/bf01541337.
- Beach Frank A. 1976. Sexual attractivity, proceptivity, and receptivity in female mammals. *Horm Behav.* 7(1):105–138. doi:10.1016/0018-506x(76)90008-8.
- Beach FA, Holz-Tucker AM. 1949. Effects of different concentrations of androgen upon sexual behavior in castrated male rats. *J Comp Physiol Psych.* 42(6):433–453. doi:10.1037/h0059086.
- Beach FA, Jordan L. 1956. Sexual Exhaustion and Recovery in the Male Rat*. *Q J Exp Psychol.* 8(3):121--133. doi:10.1080/17470215608416811.
- Beach FA, Rabedeau RG. 1959. Sexual exhaustion and recovery in the male hamster. *J Comp Physiol Psych.* 52(1):56–61. doi:10.1037/h0041896.
- Bermant G. 1965. Rat sexual behavior: Photographic analysis of the intromission response. *Psychon Sci.* 2(1–12):65–66. doi:10.3758/bf03343332.

Bialy M, Bogacki-Rychlik W, Przybylski J, Zera T. 2019. The Sexual Motivation of Male Rats as a Tool in Animal Models of Human Health Disorders. *Front Behav Neurosci.* 13:257. doi:10.3389/fnbeh.2019.00257.

Birkhead TR, Møller AP. 1998. *Sperm Competition and Sexual Selection*. Academic Press.

Boland BD, Bunnell BN, Dewsbury DA. 1977. Copulatory Behavior of Golden Hamsters (*Mesocricet Us Auratus*). *Behaviour.* 61(3–4):180–205. doi:10.1163/156853977x00342.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. 1998. Prolactin (PRL) and Its Receptor: Actions, Signal Transduction Pathways and Phenotypes Observed in PRL Receptor Knockout Mice. *Endocr Rev.* 19(3):225–268. doi:10.1210/edrv.19.3.0334.

Breed MD, Moore J. 2016. *Animal Behavior* (Second Edition). :357–393. doi:10.1016/b978-0-12-801532-2.00011-8.

Brody S, Krüger THC. 2006. The post-orgasmic prolactin increase following intercourse is greater than following masturbation and suggests greater satiety. *Biol Psychol.* 71(3):312–315. doi:10.1016/j.biopsycho.2005.06.008.

Bronson FH. 1979. The Reproductive Ecology of the House Mouse. *Q Rev Biology.* 54(3):265–299. doi:10.1086/411295.

Bronson FH, Desjardins C. 1982. Endocrine Responses to Sexual Arousal in Male Mice*. *Endocrinology.* 111(4):1286–1291. doi:10.1210/endo-111-4-1286.

Brown RE. 1985. Hormones and Paternal Behavior in Vertebrates. *Am Zool.* 25(3):895–910. doi:10.1093/icb/25.3.895.

Brown RSE, Kokay IC, Herbison AE, Grattan DR. 2010. Distribution of prolactin-responsive neurons in the mouse forebrain. *J Comp Neurology.* 518(1):92–102. doi:10.1002/cne.22208.

Burns-Cusato M, Scordalakes EM, Rissman EF. 2004. Of mice and missing data: what we know (and need to learn) about male sexual behavior. *Physiol Behav.* 83(2):217–232. doi:10.1016/j.physbeh.2004.08.015.

Buvat J. 2003. Hyperprolactinemia and sexual function in men: a short review. *Int J Impot Res.* 15(5):3901043. doi:10.1038/sj.ijir.3901043.

Cabib S, Puglisi-Allegra S, Ventura R. 2002. The contribution of comparative studies in inbred strains of mice to the understanding of the hyperactive phenotype. *Behav Brain Res.* 130(1–2):103–109. doi:10.1016/s0166-4328(01)00422-3.

Carmichael MS, Humbert R, Dixen J, Palmisano G, Greenleaf W, Davidson JM. 1987. Plasma Oxytocin Increases in the Human Sexual Response*. *J Clin Endocrinol Metabolism.* 64(1):27–31. doi:10.1210/jcem-64-1-27.

Chang PL, Kopania E, Keeble S, Sarver BAJ, Larson E, Orth A, Belkhir K, Boursot P, Bonhomme F, Good JM, et al. 2017. Whole exome sequencing of wild-derived inbred strains of mice improves power to link phenotype and genotype. *Mamm Genome.* 28(9–10):416–425. doi:10.1007/s00335-017-9704-9.

Cocchi D, Gil-Ad I, Parenti M, Stefanini E, Locatelli V, Müller EE. 1980. Prolactin-Releasing Effect of a Novel Anti-Dopaminergic Drug, Domperidone, in the Rat. *Neuroendocrinology.* 30(2):65–69. doi:10.1159/000122977.

Coolen LM, Peters HJPW, Veening JG. 1996. Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison. *Brain Res.* 738(1):67–82. doi:10.1016/0006-8993(96)00763-9.

- Coolen LM, Peters HJPW, Veening JG. 1998. Anatomical interrelationships of the medial preoptic area and other brain regions activated following male sexual behavior: A combined Fos and tract-tracing study. *J Comp Neurol*. 397(3):421–435. doi:10.1002/(sici)1096-9861(19980803)397:3<421::aid-cne8>3.0.co;2-4.
- Corona G, Jannini EA, Vignozzi L, Rastrelli G, Maggi M. 2012. The hormonal control of ejaculation. *Nat Rev Urology*. 9(9):508. doi:10.1038/nrurol.2012.147.
- Costall B, Fortune DH, Naylor RJ. 1979. Neuropharmacological studies on the neuroleptic potential of domperidone (R33812). *J Pharm Pharmacol*. 31(1):344--347. doi:10.1111/j.2042-7158.1979.tb13518.x.
- Crawford LL, Holloway KS, Domjan M. 1993. The nature of sexual reinforcement. *J Exp Anal Behav*. 60(1):55–66. doi:10.1901/jeab.1993.60-55.
- Dawson WD, Lake CE, Schumpert SS. 1988. Inheritance of burrow building in *Peromyscus*. *Behav Genet*. 18(3):371–382. doi:10.1007/bf01260937.
- Deschepper CF, Olson JL, Otis M, Gallo-Payet N. 2004. Characterization of blood pressure and morphological traits in cardiovascular-related organs in 13 different inbred mouse strains. *J Appl Physiol*. 97(1):369---376. doi:10.1152/jappphysiol.00073.2004.
- Dewsbury D. 1975. Diversity and adaptation in rodent copulatory behavior. *Science*. 190(4218):947–954. doi:10.1126/science.1188377.
- Dewsbury DA. 1972. Patterns of Copulatory Behavior in Male Mammals. *Q Rev Biology*. 47(1):1–33. doi:10.1086/407097.
- Dewsbury DA. 1981. On the function of the multiple-intromission, multiple-ejaculation copulatory patterns of rodents. *B Psychonomic Soc*. 18(4):221–223. doi:10.3758/bf03333609.
- Dewsbury DA. 1982. Ejaculate Cost and Male Choice. *Am Nat*. 119(5):601–610. doi:10.1086/283938.
- Dewsbury DA. 1983. Recovery from sexual satiety in deer mice (*Peromyscus maniculatus bairdi*). *J Comp Psychol*. 97(1):34–42. doi:10.1037/0735-7036.97.1.34.
- Dimijian GG. 2017. Evolution of Sexuality: Biology and Behavior. *Bayl Univ Medical Cent Proc*. 18(3):244–258. doi:10.1080/08998280.2005.11928075.
- Dixon AF. 2018. Copulatory and Postcopulatory Sexual Selection in Primates. *Folia Primatol*. 89(3–4):258–286. doi:10.1159/000488105.
- Doty RL. 1974. A cry for the liberation of the female rodent: Courtship and copulation in rodentia. *Psychol Bull*. 81(3):159–172. doi:10.1037/h0035971.
- Drago F. 1984. Prolactin and sexual behavior: A review. *Neurosci Biobehav Rev*. 8(4):433--439. doi:10.1016/0149-7634(84)90002-2.
- Egli M, Leeners B, Kruger THC. 2010. Prolactin secretion patterns: basic mechanisms and clinical implications for reproduction. *Reproduction Camb Engl*. 140(5):643--654. doi:10.1530/rep-10-0033.
- Ellenbroek B, Youn J. 2016. Rodent models in neuroscience research: is it a rat race? *Dis Model Mech*. 9(10):1079–1087. doi:10.1242/dmm.026120.

Erskine MS. 1989. Solicitation behavior in the estrous female rat: A review. *Horm Behav.* 23(4):473–502. doi:10.1016/0018-506x(89)90037-8.

Erskine MS, Lehmann ML, Cameron NM, Polston EK. 2004. Co-regulation of female sexual behavior and pregnancy induction: an exploratory synthesis. *Behav Brain Res.* 153(2):295–315. doi:10.1016/j.bbr.2004.01.026.

Everitt BJ. 1990. Sexual motivation: A neural and behavioural analysis of the mechanisms underlying appetitive and copulatory responses of male rats. *Neurosci Biobehav Rev.* 14(2):217–232. doi:10.1016/s0149-7634(05)80222-2.

Exton MS, Bindert A, Kruger T, Scheller F, Hartmann U, Schedlowski M. 1999. Cardiovascular and Endocrine Alterations After Masturbation-Induced Orgasm in Women. *Psychosom Med.* 61(3):280–289. doi:10.1097/00006842-199905000-00005.

Exton MS, Krüger THC, Koch M, Paulson E, Knapp W, Hartmann U, Schedlowski M. 2001. Coitus-induced orgasm stimulates prolactin secretion in healthy subjects. *Psychoneuroendocrino.* 26(3):287–294. doi:10.1016/s0306-4530(00)00053-6.

Exton NG, Truong TC, Exton MS, Wingenfeld SA, Leygraf N, Saller B, Hartmann U, Schedlowski M. 2000. Neuroendocrine response to film-induced sexual arousal in men and women. *Psychoneuroendocrino.* 25(2):187–199. doi:10.1016/s0306-4530(99)00049-9.

Fernandes C, Liu L, Paya-Cano JL, Gregorová S, Forejt J, Schalkwyk LC. 2004. Behavioral Characterization of Wild Derived Male Mice (*Mus musculus musculus*) of the PWD/Ph Inbred Strain: High Exploration Compared to C57BL/6J. *Behav Genet.* 34(6):621–630. doi:10.1007/s10519-004-5589-y.

Firman RC, Klemme I, Simmons LW. 2013. Strategic adjustments in sperm production within and between two island populations of house mice. *Evolution.* 67(10):3061–3070. doi:10.1111/evo.12164.

Frazer KA, Eskin E, Kang HM, Bogue MA, Hinds DA, Beilharz EJ, Gupta RV, Montgomery J, Morenzoni MM, Nilsen GB, et al. 2007. A sequence-based variation map of 8.27 million SNPs in inbred mouse strains. *Nature.* 448(7157):1050–1053. doi:10.1038/nature06067.

Freeman ME, Kanyicska B, Lerant A, Nagy G. 2000a. Prolactin: Structure, Function, and Regulation of Secretion. *Physiol Rev.* 80(4):1523–1631. doi:10.1152/physrev.2000.80.4.1523.

Freeman ME, Kanyicska B, Lerant A, Nagy G. 2000b. Prolactin: Structure, Function, and Regulation of Secretion. *Physiol Rev.* 80(4):1523–1631. doi:10.1152/physrev.2000.80.4.1523.

Gage MJG, Freckleton RP. 2003. Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. *Proc Royal Soc Lond Ser B Biological Sci.* 270(1515):625–632. doi:10.1098/rspb.2002.2258.

Gala RR. 1990. The physiology and mechanisms of the stress-induced changes in prolactin secretion in the rat. *Life Sci.* 46(20):1407–1420. doi:10.1016/0024-3205(90)90456-2.

Ganong WF. 2000. Circumventricular Organs: Definition And Role In The Regulation Of Endocrine And Autonomic Function. *Clin Exp Pharmacol P.* 27(5–6):422–427. doi:10.1046/j.1440-1681.2000.03259.x.

Gartlan JS. 1969. Sexual and maternal behaviour of the vervet monkey, *Ceropithecus aethiops*. *J Reprod Fertil.(Suppl., 6):*137–150.

Georgescu T, Ladyman SR, Brown RSE, Grattan DR. 2020. Acute effects of prolactin on hypothalamic prolactin receptor expressing neurones in the mouse. *J Neuroendocrinol.* 32(11):e12908. doi:10.1111/jne.12908.

Georgiadis JR, Kringelbach ML, Pfaus JG. 2012. Sex for fun: a synthesis of human and animal neurobiology. *Nat Rev Urol.* 9(9):486–498. doi:10.1038/nrurol.2012.151.

Grattan DR. 2015. 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-prolactin axis. *J Endocrinol.* 226(2):T101--T122. doi:10.1530/joe-15-0213.

Grattan DR, Bridges RS. 2009. *Hormones, Brain and Behavior. (A: Endocrine Systems Interacting with Brain and Behavior).* p. 2471--2504.

Grattan DR, Kokay IC. 2008. Prolactin: A Pleiotropic Neuroendocrine Hormone. *J Neuroendocrinol.* 20(6):752--763. doi:10.1111/j.1365-2826.2008.01736.x.

Grattan DR, Xu J, McLachlan MJ, Kokay IC, Bunn SJ, Hovey RC, Davey HW. 2001. Feedback Regulation of PRL Secretion Is Mediated by the Transcription Factor, Signal Transducer, and Activator of Transcription 5b. *Endocrinology.* 142(9):3935--3940. doi:10.1210/endo.142.9.8385.

Gregorová S, Forejt J. 2000. PWD/Ph and PWK/Ph inbred mouse strains of *Mus m. musculus* subspecies--a valuable resource of phenotypic variations and genomic polymorphisms. *Folia Biol-prague.* 46(1):31---41.

Guillou A, Romanò N, Steyn F, Abitbol K, Tissier PL, Bonnefont X, Chen C, Mollard P, Martin AO. 2015. Assessment of Lactotroph Axis Functionality in Mice: Longitudinal Monitoring of PRL Secretion by Ultrasensitive-ELISA. *Endocrinology.* 156(5):1924---1930. doi:10.1210/en.2014-1571.

Haake P, Exton MS, Haverkamp J, Krämer M, Leygraf N, Hartmann U, Schedlowski M, Krueger THC. 2002. Absence of orgasm-induced prolactin secretion in a healthy multi-orgasmic male subject. *Int J Impot Res.* 14(2):3900823. doi:10.1038/sj.ijir.3900823.

Haga S, Hattori T, Sato T, Sato K, Matsuda S, Kobayakawa R, Sakano H, Yoshihara Y, Kikusui T, Touhara K. 2010. The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature.* 466(7302):118–122. doi:10.1038/nature09142.

Hartung TG, Dewsbury DA. 1978. A Comparative Analysis of Copulatory Plugs in Muroid Rodents and Their Relationship to Copulatory Behavior. *J Mammal.* 59(4):717–723. doi:10.2307/1380136.

Hernandez ME, Soto-Cid A, Rojas F, Pascual LI, Aranda-Abreu GE, Toledo R, Garcia LI, Quintanar-Stephano A, Manzo J. 2006. Prostate response to prolactin in sexually active male rats. *Reprod Biol Endocrin.* 4(1):28. doi:10.1186/1477-7827-4-28.

Huck UW, Lisk RD. 1986. Mating-induced inhibition of receptivity in the female golden hamster I. Short-term and long-term effects. *Behav Neural Biol.* 45(1):107–119. doi:10.1016/s0163-1047(86)80010-3.

Hull EM, Dominguez JM. 2007. Sexual behavior in male rodents. *Horm Behav.* 52(1):45–55. doi:10.1016/j.yhbeh.2007.03.030.

Hull EM, Muschamp JW, Sato S. 2004. Dopamine and serotonin: influences on male sexual behavior. *Physiol Behav.* 83(2):291--307. doi:10.1016/j.physbeh.2004.08.018.

Hull EM, Rodríguez-Manzo G. 2017. Male sexual behavior. In: Pfaff DW, Joëls M, editors. *Hormones, brain and behavior.* Vol. 1. third. United Kingdom: Academic Press. (González-Mariscal G, editor.). p. 1–57.

Hull EM, Wood RI, McKenna KE. 2006. Neurobiology of male sexual behaviour. In: Neill JD, editor. Knobil and Neill's Physiology of Reproduction. 3^o. Elsevier. p. 1730–1795.

Hurst JL. 2009. Female recognition and assessment of males through scent. *Behav Brain Res.* 200(2):295–303. doi:10.1016/j.bbr.2008.12.020.

Hyun J-S, Baig MR, Yang D-Y, Leungwattanakji S, Kim K-D, Abdel-Mageed AB, Bivalacqua TJ, Hellstrom WJG. 2002. Localization of Peripheral Dopamine D1 and D2 Receptors in Rat and Human Seminal Vesicles. *J Androl.* 23(1):114–120. doi:10.1002/j.1939-4640.2002.tb02604.x.

Insel T, Wang Z, Ferris C. 1994. Patterns of brain vasopressin receptor distribution associated with social organization in microtine rodents. *J Neurosci.* 14(9):5381–5392. doi:10.1523/jneurosci.14-09-05381.1994.

Johansen JA, Clemens LG, Nunez AA. 2008. Characterization of copulatory behavior in female mice: Evidence for paced mating. *Physiol Behav.* 95(3):425–429. doi:10.1016/j.physbeh.2008.07.004.

Kamel F, Frankel AJ. 1978. Hormone Release during Mating in the Male Rat: Time Course, Relation to Sexual Behavior, and Interaction with Handling Procedures*. *Endocrinology.* 103(6):2172–2179. doi:10.1210/endo-103-6-2172.

Kamel F, Right WW, Mock EJ, Frankel AI. 1977. The Influence of Mating and Related Stimuli on Plasma Levels of Luteinizing Hormone, Follicle Stimulating Hormone, Prolactin, and Testosterone in the Male Rat 1. *Endocrinology.* 101(2):421–429. doi:10.1210/endo-101-2-421.

Kirk SE, Grattan DR, Bunn SJ. 2019. The median eminence detects and responds to circulating prolactin in the male mouse. *J Neuroendocrinol.* 31(6):e12733. doi:10.1111/jne.12733.

Knobil, Neill. 2016. Physiology of reproduction_Vol2. Fourth Edition.

Koide T, Moriwaki K, Ikeda K, Niki H, Shiroishi T. 2000. Multi-phenotype behavioral characterization of inbred strains derived from wild stocks of *Mus musculus*. *Mamm Genome.* 11(8):664–670. doi:10.1007/s003350010129.

Komisaruk BR, González-Mariscal G. 2017. Behavioral Neuroendocrinology. 1st ed. Boca Raton: CRC Press.

Krüger T, Exton MS, Pawlak C, Mühlen A von zur, Hartmann U, Schedlowski M. 1998. Neuroendocrine and cardiovascular response to sexual arousal and orgasm in men. *Psychoneuroendocrino.* 23(4):401–411. doi:10.1016/s0306-4530(98)00007-9.

Kruger T, Haake P, Chereath D, Knapp W, Janssen O, Exton M, Schedlowski M, Hartmann U. 2003. Specificity of the neuroendocrine response to orgasm during sexual arousal in men. *J Endocrinol.* 177(1):57–64. doi:10.1677/joe.0.1770057.

Kruger T, Haake P, Haverkamp J, Kramer M, Exton M, Saller B, Leygraf N, Hartmann U, Schedlowski M. 2003. Effects of acute prolactin manipulation on sexual drive and function in males. *J Endocrinol.* 179(3):357–365. doi:10.1677/joe.0.1790357.

Kruger THC, Haake P, Hartmann U, Schedlowski M, Exton MS. 2002. Orgasm-induced prolactin secretion: feedback control of sexual drive? *Neurosci Biobehav Rev.* 26(1):31–44. doi:10.1016/s0149-7634(01)00036-7.

Krüger THC, Hartmann U, Schedlowski M. 2005. Prolactinergic and dopaminergic mechanisms underlying sexual arousal and orgasm in humans. *World J Urol.* 23(2):130–138. doi:10.1007/s00345-004-0496-7.

- Krüger THC, Schiffer B, Eikermann M, Haake P, Gizewski E, Schedlowski M. 2006. Serial neurochemical measurement of cerebrospinal fluid during the human sexual response cycle. *Eur J Neurosci.* 24(12):3445--3452. doi:10.1111/j.1460-9568.2006.05215.x.
- Kudwa AE, Dominguez-Salazar E, Cabrera DM, Sibley DR, Rissman EF. 2005. Dopamine D5 receptor modulates male and female sexual behavior in mice. *Psychopharmacology.* 180(2):206–214. doi:10.1007/s00213-005-2150-5.
- Larson k. 1956. Conditioning and sexual behavior in the male albino rat. *Almqvist & Wiksell.*
- Lauer SM, Lenschow C, Brecht M. 2017. Sexually selected size differences and conserved sexual monomorphism of genital cortex. *J Comp Neurol.* 525(12):2706–2718. doi:10.1002/cne.24237.
- Lee KTY, Karunakaran S, Ho MM, Clee SM. 2011. PWD/PhJ and WSB/EiJ Mice Are Resistant to Diet-Induced Obesity But Have Abnormal Insulin Secretion. *Endocrinology.* 152(8):3005–3017. doi:10.1210/en.2011-0060.
- Lenschow C, Lima SQ. 2020. In the mood for sex: neural circuits for reproduction. *Curr Opin Neurobiol.* 60:155–168. doi:10.1016/j.conb.2019.12.001.
- Levin R. 2003. Is prolactin the biological “off switch” for human sexual arousal? *Sex Relatsh Ther.* 18(2):237--243. doi:10.1080/1468199031000099488.
- Levin RJ. 2009. Revisiting Post-Ejaculation Refractory Time—What We Know and What We Do Not Know in Males and in Females. *J Sex Medicine.* 6(9):2376--2389. doi:10.1111/j.1743-6109.2009.01350.x.
- Levine L, Barsel GE, Diakow CA. 1966. Mating behaviour of two inbred strains of mice. *Anim Behav.* 14(1):1–6. doi:10.1016/s0003-3472(66)80002-7.
- Lim MM, Young LJ. 2004. Vasopressin-dependent neural circuits underlying pair bond formation in the monogamous prairie vole. *Neuroscience.* 125(1):35–45. doi:10.1016/j.neuroscience.2003.12.008.
- Lopes G, Bonacchi N, Frazão J, Neto JP, Atallah BV, Soares S, Moreira L, Matias S, Itskov PM, Correia PA, et al. 2015. Bonsai: an event-based framework for processing and controlling data streams. *Front Neuroinform.* 9:7. doi:10.3389/fninf.2015.00007.
- Lyons DJ, Broberger C. 2014. TIDAL WAVES: Network mechanisms in the neuroendocrine control of prolactin release. *Front Neuroendocrin.* 35(4):420--438. doi:10.1016/j.yfrne.2014.02.001.
- Martínez I, Paredes RG. 2001. Only Self-Paced Mating Is Rewarding in Rats of Both Sexes. *Horm Behav.* 40(4):510–517. doi:10.1006/hbeh.2001.1712.
- Mas M, Fumero B, Fernandez-Vera JR, Gonzalez-Mora JL. 1995. Neurochemical correlates of sexual exhaustion and recovery as assessed by in vivo microdialysis. *Brain Res.* 675(1–2):13–19. doi:10.1016/0006-8993(95)00029-p.
- Masters WH, Johnson VE. 1966. Human sexual response. New York: Bantam Books.
- McClintock MK. 1984. Group Mating in the Domestic Rat as a Context for Sexual Selection: Consequences for the Analysis of Sexual Behavior and Neuroendocrine Responses. *Adv Stud Behav.* 14:1–50. doi:10.1016/s0065-3454(08)60298-3.

- McClintock MK, Adler NT. 1978. The Role of the Female During Copulation in Wild and Domestic Norway Rats (*Rattus Norvegicus*). *Behaviour*. 67(1–2):67–95. doi:10.1163/156853978x00260.
- McGill TE. 1962. Sexual Behavior in Three Inbred Strains of Mice. *Behaviour*. 19(4):341–350. doi:10.1163/156853962x00087.
- McGill TE. 1963. Sexual Behavior of the Mouse after Long-Term and Short-Term Postejaculatory Recovery Periods. *J Genetic Psychology*. 103(1):53–57. doi:10.1080/00221325.1963.10532496.
- McGill TE. 1969. An enlarged study of genotype and recovery of sex drive in male mice. *Psychon Sci*. 15(5):250–251. doi:10.3758/bf03337405.
- McGill TE, Blight WC. 1963a. The sexual behaviour of hybrid male mice compared with the sexual behaviour of males of the inbred parent strains. *Anim Behav*. 11(4):480–483. doi:10.1016/0003-3472(63)90265-3.
- McGill TE, Blight WC. 1963b. Effects of genotype on the recovery of sex drive in the male mouse. *J Comp Physiol Psych*. 56(5):887. doi:10.1037/h0044719.
- McGill TE, Ransom TW. 1968. Genotypic change affecting conclusions regarding the mode of inheritance of elements of behaviour. *Anim Behav*. 16(1):88–91. doi:10.1016/0003-3472(68)90115-2.
- McIntosh TK, Barfield RJ. 1984a. Brain monoaminergic control of male reproductive behavior. I. Serotonin and the post-ejaculatory refractory period. *Behav Brain Res*. 12(3):255–265. doi:10.1016/0166-4328(84)90151-7.
- McIntosh TK, Barfield RJ. 1984b. Brain monoaminergic control of male reproductive behavior. II. Dopamine and the post-ejaculatory refractory period. *Behav Brain Res*. 12(3):267–273. doi:10.1016/0166-4328(84)90152-9.
- Melmed S, Casanueva FF, Hoffman AR, Kleinberg DL, Montori VM, Schlechte JA, Wass JAH. 2011. Diagnosis and Treatment of Hyperprolactinemia: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metabolism*. 96(2):273–288. doi:10.1210/jc.2010-1692.
- Mermelstein PG, Becker JB. 1995. Increased Extracellular Dopamine in the Nucleus Accumbens and Striatum of the Female Rat During Paced Copulatory Behavior. *Behav Neurosci*. 109(2):354–365. doi:10.1037/0735-7044.109.2.354.
- Molik E, Błasiak M. 2015. The Role of Melatonin and Bromocriptine in the Regulation of Prolactin Secretion in Animals – A Review. *Ann Anim Sci*. 15(4):849–860. doi:10.1515/aoas-2015-0042.
- Moralí G, Soto MAP, Contreras JL, Arteaga M, González-Vidal MD, Beyer C. 2003. Detailed analysis of the male copulatory motor pattern in mammals: Hormonal bases. *Scand J Psychol*. 44(3):279–288. doi:10.1111/1467-9450.00346.
- Mosig DW, Dewsbury DA. 1976. Studies of the copulatory behavior of house mice (*Mus musculus*). *Behav Biol*. 16(4):463–473. doi:10.1016/s0091-6773(76)91635-7.
- Nachman MW, Payseur BA. 2012. Recombination rate variation and speciation: theoretical predictions and empirical results from rabbits and mice. *Philosophical Transactions Royal Soc B Biological Sci*. 367(1587):409–421. doi:10.1098/rstb.2011.0249.
- Nagano M, Kelly PA. 1994. Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. *J Biological Chem*. 269(18):13337–45.

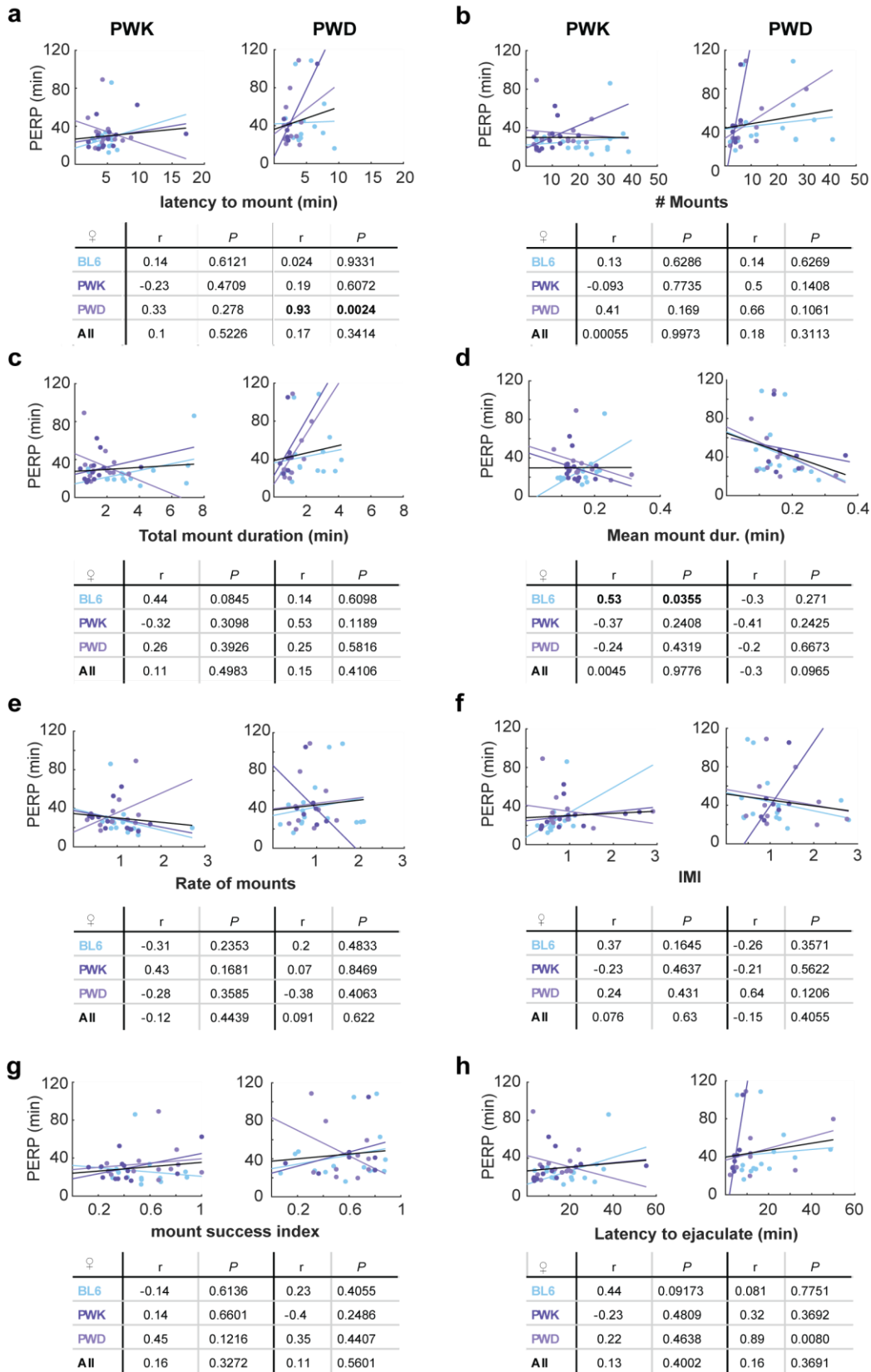
- Nelson RJ, Kriegsfeld LJ. 2017. *An Introduction to Behavioral Endocrinology*. Fifth Edition. Sinauer Associates, Inc.
- Nowak RM. 1983. *Walker's Mammals of the World*. fourth edition. Baltimore: The Johns Hopkins University Press.
- Oaknin S, Castillo ARD, Guerra M, Battaner E, Mas M. 1989. Changes in forebrain Na,K-ATPase activity and serum hormone levels during sexual behavior in male rats. *Physiol Behav*. 45(2):407--410. doi:10.1016/0031-9384(89)90148-0.
- Oglesby JM, Lanier DL, Dewsbury DA. 1981. The role of prolonged copulatory behavior in facilitating reproductive success in male Syrian golden hamsters (*Mesocricetus auratus*) in a competitive mating situation. *Behav Ecol Sociobiol*. 8(1):47--54. doi:10.1007/bf00302843.
- Onos KD, Uyar A, Keezer KJ, Jackson HM, Preuss C, Acklin CJ, O'Rourke R, Buchanan R, Cossette TL, Rizzo SJS, et al. 2019. Enhancing face validity of mouse models of Alzheimer's disease with natural genetic variation. *Plos Genet*. 15(5):e1008155. doi:10.1371/journal.pgen.1008155.
- Otto SP. 2009. The Evolutionary Enigma of Sex. *Am Nat*. 174(S1):S1--S14. doi:10.1086/599084.
- Parfitt DB, Newman SW. 1998. Fos-Immunoreactivity within the Extended Amygdala Is Correlated with the Onset of Sexual Satiety. *Horm Behav*. 34(1):17--29. doi:10.1006/hbeh.1998.1459.
- Park JH. 2011. Assessment of Male Sexual Behavior in Mice. In: Gould TD, editor. *Mood and Anxiety Related Phenotypes in Mice, Characterization Using Behavioral Tests*. Vol. 2. Springer Science+Business Media. p. 357--373.
- Parker GA. 1998a. Sperm Competition and the Evolution of Ejaculates: Towards a Theory Base. Academic Press. (Part One: General Themes). p. 3--54.
- Parker GA. 1998b. Sperm Competition and Sexual Selection. Part One Gen Themes. (*Anim. Behav.* 37(1989):3--54. doi:10.1016/b978-012100543-6/50026-x.
- Parker GA, Ball MA, Stockley P, Gage MJG. 1997. Sperm competition games: a prospective analysis of risk assessment. *Proc Royal Soc Lond Ser B Biological Sci*. 264(1389):1793--1802. doi:10.1098/rspb.1997.0249.
- Pei Y, Dong S, Roth BL. 2010. Generation of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) Using Directed Molecular Evolution. *Curr Protoc Neurosci*. 50(1):4.33.1-4.33.25. doi:10.1002/0471142301.ns0433s50.
- Pfaus JG. 2009. Pathways of Sexual Desire. *J Sex Medicine*. 6(6):1506--1533. doi:10.1111/j.1743-6109.2009.01309.x.
- Pfaus JG, Damsma G, Wenkstern D, Fibiger HC. 1995. Sexual activity increases dopamine transmission in the nucleus accumbens and striatum of female rats. *Brain Res*. 693(1-2):21--30. doi:10.1016/0006-8993(95)00679-k.
- Pfaus JG, Phillips AG. 1991. Role of Dopamine in Anticipatory and Consummatory Aspects of Sexual Behavior in the Male Rat. *Behav Neurosci*. 105(5):727--743. doi:10.1037/0735-7044.105.5.727.
- Phillips-Farfán BV, Fernández-Guasti A. 2009. Endocrine, neural and pharmacological aspects of sexual satiety in male rats. *Neurosci Biobehav Rev*. 33(3):442--455. doi:10.1016/j.neubiorev.2008.11.003.

- Preston BT, Stockley P. 2006. The prospect of sexual competition stimulates premature and repeated ejaculation in a mammal. *Curr Biol.* 16(7):R239–R241. doi:10.1016/j.cub.2006.03.018.
- Ramm SA, Stockley P. 2007. Ejaculate allocation under varying sperm competition risk in the house mouse, *Mus musculus domesticus*. *Behav Ecol.* 18(2):491–495. doi:10.1093/beheco/arm003.
- Riddle O, Bates RW, Dykshorn SW. 1933. The preparation, identification and assay of prolactin- a hormone of the anterior pituitary. *Am J Physiology-legacy Content.* 105(1):191--216. doi:10.1152/ajplegacy.1933.105.1.191.
- Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, McLean L, Beynon RJ, Hurst JL. 2010. Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *Bmc Biol.* 8(1):75. doi:10.1186/1741-7007-8-75.
- Rodriguez-Manzo G. 1999. Blockade of the establishment of the sexual inhibition resulting from sexual exhaustion by the Coolidge effect. *Behav Brain Res.* 100(1–2):245--254. doi:10.1016/s0166-4328(98)00137-5.
- Rojas-Durán F, Pascual-Mathey LI, Serrano K, Aranda-Abreu GE, Manzo J, Soto-Cid AH, Hernandez MaE. 2015. Correlation of prolactin levels and PRL-receptor expression with Stat and Mapk cell signaling in the prostate of long-term sexually active rats. *Physiol Behav.* 138:188---192. doi:10.1016/j.physbeh.2014.10.036.
- Romero-Ferrero F, Bergomi MG, Hinz RC, Heras FJH, Polavieja GG de. 2019. idtracker.ai: tracking all individuals in small or large collectives of unmarked animals. *Nat Methods.* 16(2):179–182. doi:10.1038/s41592-018-0295-5.
- Roth BL. 2016. DREADDs for Neuroscientists. *Neuron.* 89(4):683–694. doi:10.1016/j.neuron.2016.01.040.
- Sachs BD, Barfield RJ. 1976. Functional Analysis of Masculine Copulatory Behavior in the Rat. *Adv Stud Behav.* 7:91–154. doi:10.1016/s0065-3454(08)60166-7.
- Sato F, Aoki H, Nakamura K, Taguchi M, Aoki T, Yasuda N. 1997. Suppressive Effects of Chronic Hyperprolactinemia on Penile Erection and Yawning Following Administration of Apomorphine to Pituitary-Transplanted Rats. *J Androl.* 18(1):21--25. doi:10.1002/j.1939-4640.1997.tb01872.x.
- Schradin C, Anzenberger G. 1999. Prolactin, the Hormone of Paternity. *Physiology.* 14(6):223--231. doi:10.1152/physiologyonline.1999.14.6.223.
- Seizert CA. 2018. The Neurobiology of the Male Sexual Refractory Period. *Neurosci Biobehav Rev.* 92(Pharmacol. Biochem. Behav. 38 1991):350---377. doi:10.1016/j.neubiorev.2018.06.011.
- Stagkourakis S, Smiley KO, Williams P, Kakadellis S, Ziegler K, Bakker J, Brown RSE, Harkany T, Grattan DR, Broberger C. 2020. A Neuro-hormonal Circuit for Paternal Behavior Controlled by a Hypothalamic Network Oscillation. *Cell.* 182(4):960-975.e15. doi:10.1016/j.cell.2020.07.007.
- Stockley P, Preston BT. 2004. Sperm competition and diversity in rodent copulatory behaviour. *J Evolution Biol.* 17(5):1048–1057. doi:10.1111/j.1420-9101.2004.00742.x.
- Svare B, Bartke A, Doherty P, Mason I, Michael SD, Smith MS. 1979. Hyperprolactinemia Suppresses Copulatory Behavior in Male Rats and Mice. *Biol Reprod.* 21(2):529---535. doi:10.1095/biolreprod21.2.529.
- Thonhauser KE, Raveh S, Hettyey A, Beissmann H, Penn DJ. 2013. Why do female mice mate with multiple males? *Behav Ecol Sociobiol.* 67(12):1961–1970. doi:10.1007/s00265-013-1604-8.

- Thonhauser KE, Thoß M, Musolf K, Klaus T, Penn DJ. 2014. Multiple paternity in wild house mice (*Mus musculus musculus*): effects on offspring genetic diversity and body mass. *Ecol Evol.* 4(2):200–209. doi:10.1002/ece3.920.
- Tirindelli R, Dibattista M, Pifferi S, Menini A. 2009. From Pheromones to Behavior. *Physiol Rev.* 89(3):921–956. doi:10.1152/physrev.00037.2008.
- Tlachi-López JL, Eguibar JR, Fernández-Guasti A, Lucio RA. 2012. Copulation and ejaculation in male rats under sexual satiety and the Coolidge effect. *Physiol Behav.* 106(5):626–630. doi:10.1016/j.physbeh.2012.04.020.
- Toner JP, Adler NT. 1986. The pre-ejaculatory behavior of male and female rats affects the number of sperm in the vagina and uterus. *Physiol Behav.* 36(2):363–367. doi:10.1016/0031-9384(86)90030-2.
- Tordoff MG. 2008. Gene discovery and the genetic basis of calcium consumption. *Physiol Behav.* 94(5):649–659. doi:10.1016/j.physbeh.2008.04.004.
- Turley KR, Rowland DL. 2013. Evolving ideas about the male refractory period. *Bju Int.* 112(4):442–452. doi:10.1111/bju.12011.
- Wade CM, Kulbokas EJ, Kirby AW, Zody MC, Mullikin JC, Lander ES, Lindblad-Toh K, Daly MJ. 2002. The mosaic structure of variation in the laboratory mouse genome. *Nature.* 420(6915):574–578. doi:10.1038/nature01252.
- Weber JN, Peterson BK, Hoekstra HE. 2013. Discrete genetic modules are responsible for complex burrow evolution in *Peromyscus* mice. *Nature.* 493(7432):402–405. doi:10.1038/nature11816.
- Welker E, Loos HV der. 1986. Quantitative correlation between barrel-field size and the sensory innervation of the whiskerpad: a comparative study in six strains of mice bred for different patterns of mystacial vibrissae. *J Neurosci.* 6(11):3355–3373. doi:10.1523/jneurosci.06-11-03355.1986.
- Williams PD, Day T, Cameron E. 2005. The evolution of sperm-allocation strategies and the degree of sperm competition.
- Wilson JR, Kuehn RE, Beach FA. 1963. Modification in the sexual behavior of male rats produced by changing the stimulus female. *J Comp Physiol Psych.* 56(3):636. doi:10.1037/h0042469.
- Yang H, Bell TA, Churchill GA, Villena FP-M de. 2007. On the subspecific origin of the laboratory mouse. *Nat Genet.* 39(9):1100–1107. doi:10.1038/ng2087.
- Young LJ, Nilsen R, Waymire KG, MacGregor GR, Insel TR. 1999. Increased affiliative response to vasopressin in mice expressing the V1a receptor from a monogamous vole. *Nature.* 400(6746):766–768. doi:10.1038/23475.
- Young WC, Grunt JA. 1951. The pattern and measurement of sexual behavior in the male guinea pig. *J Comp Physiol Psych.* 44(5):492–500. doi:10.1037/h0055063.
- Zinck L, Lima SQ. 2013. Mate Choice in *Mus musculus* Is Relative and Dependent on the Estrous State. *Plos One.* 8(6):e66064. doi:10.1371/journal.pone.0066064.

Supplementary data

Supplementary data 1



Supplementary data 1. PERP duration is not correlated with individual copulatory parameters.

Pair-wise correlation for PWK (left) and PWD (right) strains between PERP duration and 8 individual copulatory parameters. a - h Scatter plots and similarity scores for PERP and a latency to mount, b number of mounts, c total mount duration, d mean mount duration, e rate of mounts f inter-mount interval (IMI), g mount success index and h latency to ejaculate.

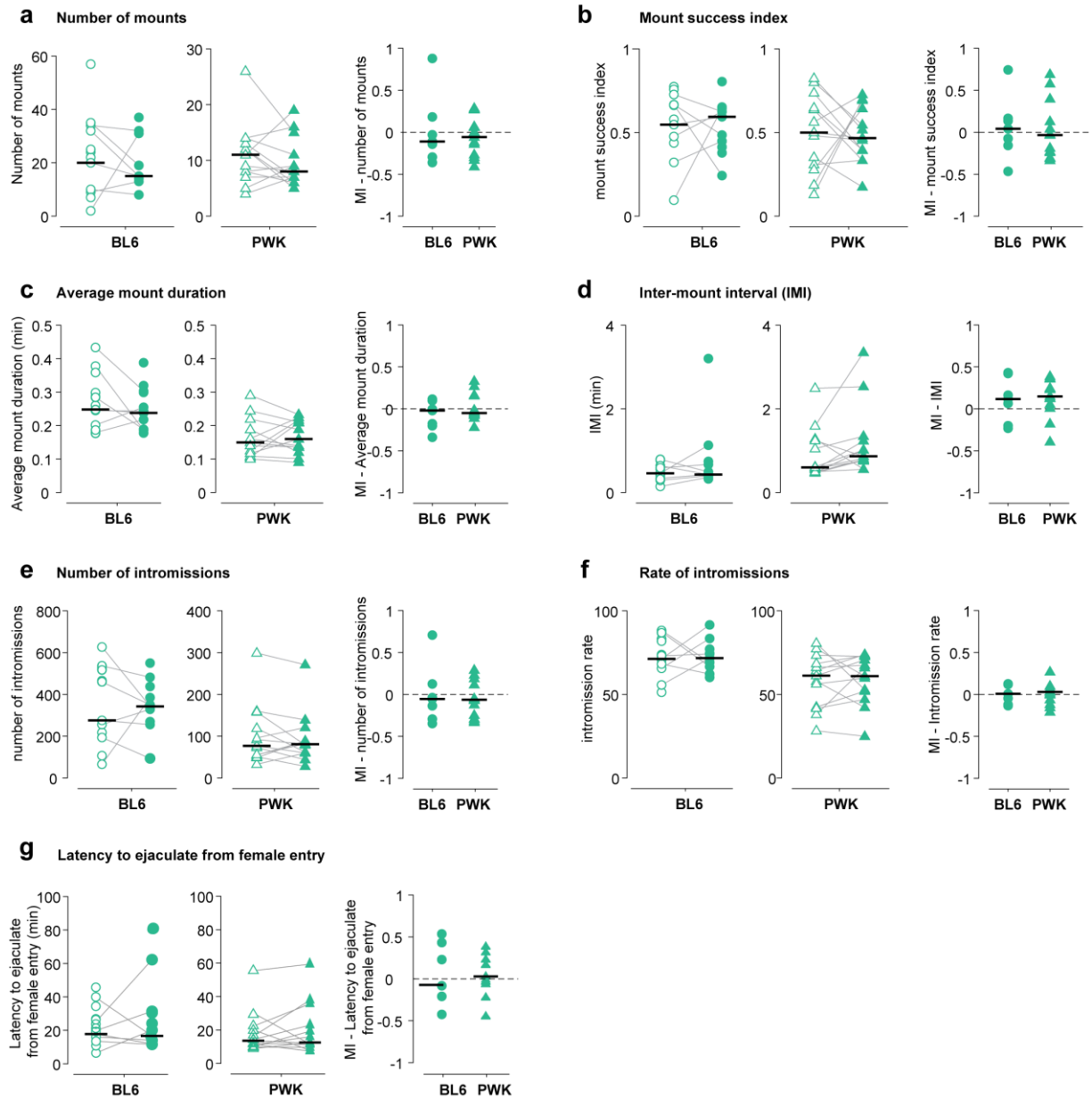
Supplementary data 2

Supplementary Data 2. Percentage of BL6 males that reached ejaculation.

Comparison of the percentage of BL6 males that ejaculated in the different type of experiments: a) PRL monitoring: tail tip blood collection during the trial; b) PRL manipulation: substance injection prior beginning of the trial; c) un-manipulated males.

Experiment type		# males that ejaculated	Total number males	% males that ejaculated
a) PRL monitoring	Fig. 1a-b	8	23	34.8
b) PRL manipulation – Veh	Fig. 2c	13	17	76.5
b) PRL manipulation – Domp	Fig. 2c	11	17	64.7
b) PRL manipulation – Veh	Fig. 3c	15	18	83.3
b) PRL manipulation – Bromo	Fig. 3c	13	18	72.2
c) Un-manipulated	---	6	9	66.7

Supplementary data 3



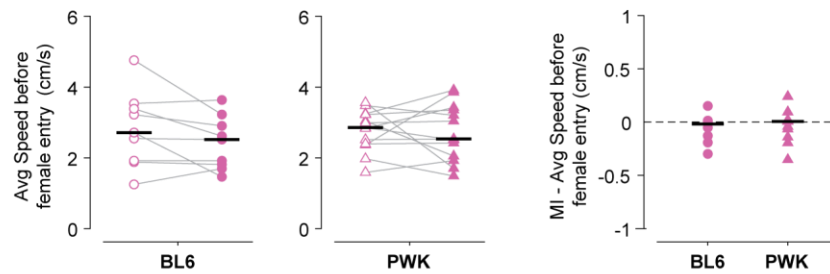
Supplementary Data 3. Additional analysis of the sexual behavior assay.

Adult sexually trained BL6 and PWK males were pre-treated with vehicle or Domperidone (total number of subjects used: BL6 $n = 17$; PWK, $n = 13$; with ejaculation in both sessions BL6 $n = 7$; PWK, $n = 13$). Quantification of **a** number of mounts, **b** mount success index [mounts/(mount attempt + mounts)], **c** mount mean duration (total mount duration/#mounts), **d** Inter-mount interval (IMI), **e** number of intromissions and **f** rate of intromissions (#intromissions/total mount duration). Each animal underwent two behavioral assays: one with vehicle and one with domperidone. Administration was counterbalanced between animals. Each line represents data of an individual; Unconnected dots represent data of an individual that did not ejaculate in the matching trial (data not used for statistics). MI [modulation index

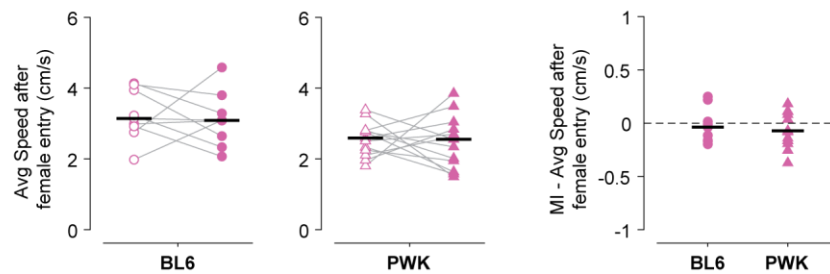
(domp-veh)/(dom+veh)]. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test.

Supplementary data 4

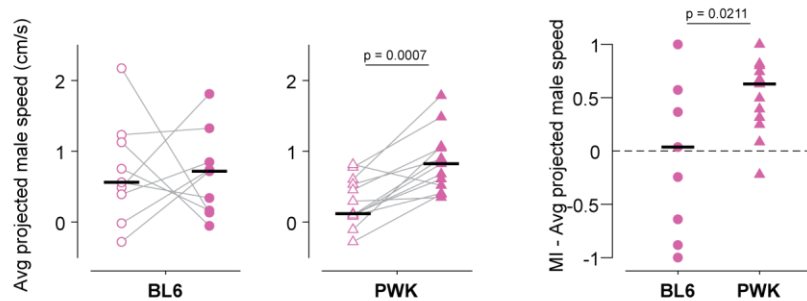
a Average speed before female entry



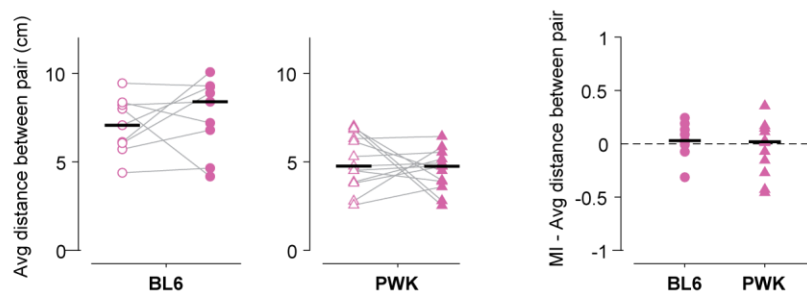
b Average speed after female entry



c Average male speed projected towards the female



d Average distance between the pair

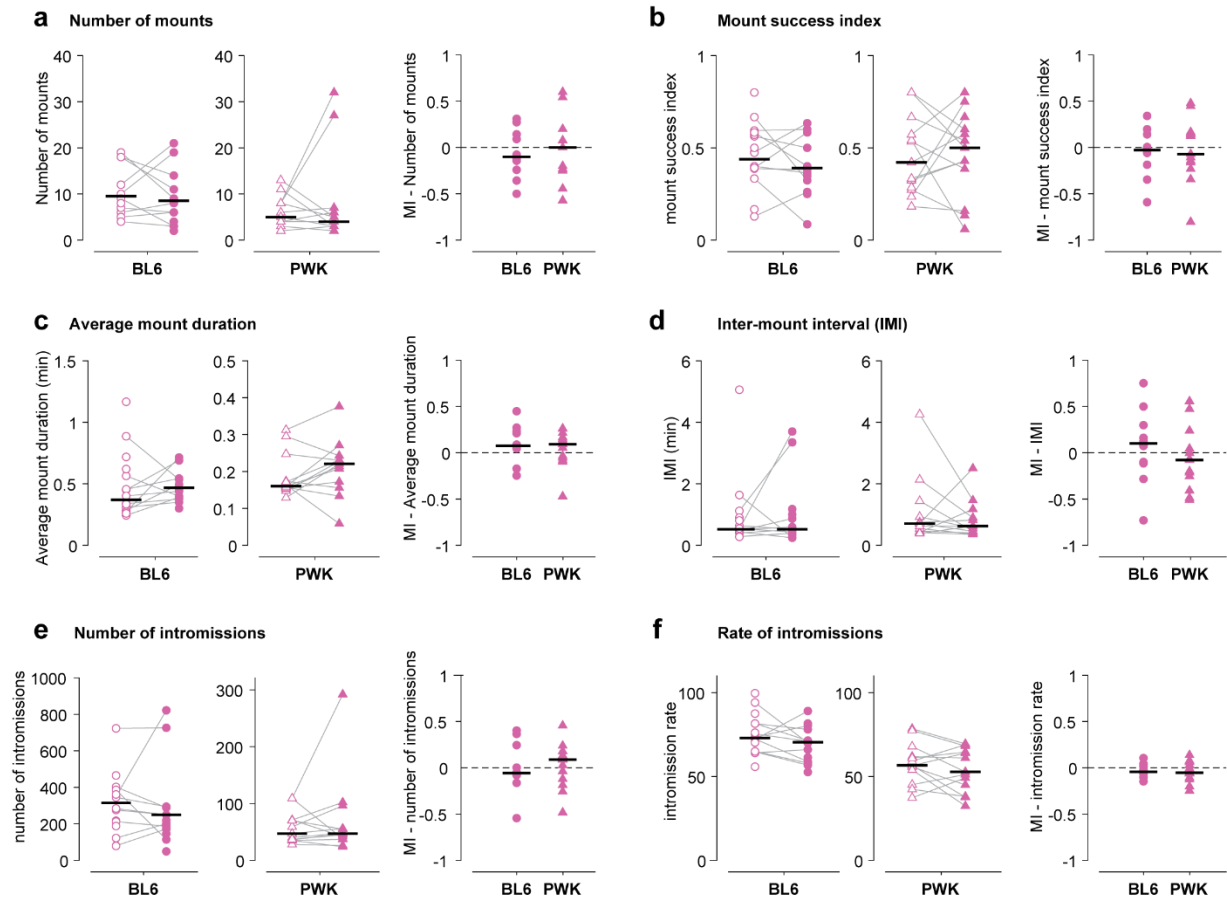


Supplementary data 4. Bromocriptine treated animals no not show increased locomotion activity.

Average speed of the male **a** before and **b** after the female entry until the first consummatory event. **c** Average male speed projected towards the female **d** average distance between the pair. Metrics were taken from couples that reached ejaculation in both session from bromocriptine assay BL6 n= 9, PWK n

=13 (Fig. 3). Each line represents data of an individual; MI [modulation index (bromo-veh)/(bromo+veh)]. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test. Significance was accepted at $P < 0.05$. ** $P < 0.001$, * $P < 0.01$.

Supplementary data 5



Supplementary data 5. Additional analysis of the sexual behavior assay.

Adult sexually trained BL6 and PWK males were pre-treated with vehicle or Bromocriptine (total number of subjects used: BL6 $n = 18$; PWK, $n = 13$; with ejaculation in both sessions BL6 $n = 10$; PWK, $n = 13$).

Quantification of **a** number of mounts, **b** mount success index [mounts/(mount attempt + mounts)], **c** mount mean duration (total mount duration/#mounts), **d** Inter-mount interval (IMI), **e** number of intromissions and **f** rate of intromissions (#intromissions/total mount duration). Each animal underwent two behavioral assays: one with vehicle and one with domperidone. Administrations were contra balanced between animals. Each line represents data of an individual; Unconnected dots represent data of an individual that did not ejaculate in the matching trial (data not used for statistics). MI [modulation index (bromo-veh)/(bromo+veh)]. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test. Significance was accepted at $P < 0.05$.

Appendix A

Articles published in peer-reviewed journals

Valente, S., Marques, T. & Lima, S.Q.

No evidence for prolactin's involvement in the post-ejaculatory refractory period.
Commun Biol 4, 10 (2021). <https://doi.org/10.1038/s42003-020-01570-4>

ARTICLE



<https://doi.org/10.1038/s42003-020-01570-4>

OPEN

No evidence for prolactin's involvement in the post-ejaculatory refractory period

Susana Valente ^{1,2}, Tiago Marques ^{3,4,5} & Susana Q. Lima ¹✉

In many species, ejaculation is followed by a state of decreased sexual activity, the post-ejaculatory refractory period. Several lines of evidence have suggested prolactin, a pituitary hormone released around the time of ejaculation in humans and other animals, to be a decisive player in the establishment of the refractory period. However, data supporting this hypothesis is controversial. We took advantage of two different strains of house mouse, a wild derived and a classical laboratory strain that differ substantially in their sexual performance, to investigate prolactin's involvement in sexual activity and the refractory period. First, we show that there is prolactin release during sexual behavior in male mice. Second, using a pharmacological approach, we show that acute manipulations of prolactin levels, either mimicking the natural release during sexual behavior or inhibiting its occurrence, do not affect sexual activity or shorten the refractory period, respectively. Therefore, we show compelling evidence refuting the idea that prolactin released during copulation is involved in the establishment of the refractory period, a long-standing hypothesis in the field of behavioral endocrinology.

¹Champalimaud Research, Champalimaud Centre for the Unknown, Av. Brasília, s/n Lisboa, Portugal. ²Graduate Program in Areas of Basic and Applied Biology (GABBA), University of Porto, 4200-465 Porto, Portugal. ³Department of Brain and Cognitive Sciences, MIT, Cambridge, MA 02139, USA. ⁴McGovern Institute for Brain Research, MIT, Cambridge, MA 02139, USA. ⁵Center for Brains, Minds and Machines, MIT, Cambridge, MA 02139, USA. ✉email: susana.lima@neuro.fchampalimaud.org

Sexual behavior follows the classical sequence of motivated behaviors, terminating with an inhibitory phase after ejaculation: the post-ejaculatory refractory period (PERP)¹. The PERP is highly conserved across species and includes a general decrease in sexual activity and also inhibition of erectile function in humans and other primates². This period of time is variable across and within individuals and is affected by many factors, such as age^{3,4} or the presentation of a new sexual partner^{5,6}. The PERP is thought to allow replacement of sperm and seminal fluid, functioning as a negative feedback system where by inhibiting too-frequent ejaculations an adequate sperm count needed for fertilization is maintained^{7,8}.

Several lines of evidence have suggested the hormone prolactin (PRL) to be a key player in the establishment of the PERP^{9,10}. PRL is a pleiotropic hormone, first characterized in the context of milk production in females, but for which we currently know several hundred physiological effects in both sexes^{11,12}. The association of PRL to the establishment of the PERP in males is based on several observations. First, it was shown that PRL is released around the time of ejaculation in humans and rats^{13–21}. Anecdotally, no PRL release has been observed in a subject with multiple orgasms²². Second, chronically abnormal high levels of circulating PRL are associated with decreased sexual drive, anorgasmia, and ejaculatory dysfunctions^{23,24}. Finally, removal of PRL-producing pituitary tumors or treatment with drugs that inhibit PRL release reverse sexual dysfunctions^{25,26}. Taking these observations into consideration, it has been hypothesized that the PRL surge around the time of ejaculation plays a role in the immediate subsequent decrease of sexual activity, the hallmark of the PERP. In fact, this idea is widespread in behavioral endocrinology textbooks²⁷ and the popular press (https://en.wikipedia.org/wiki/Refractory_period; <https://www.humanitas.net/treatments/prolactin>).

PRL is primarily produced and released into the bloodstream from the anterior pituitary^{11,28} and consistent with its functional diversity, PRL receptors are found in most tissues and cell types of the body^{29,30}. Therefore, PRL may depress sexual activity directly, via PRL receptors present in the male reproductive tract. In fact, PRL has been shown to impact the function of accessory sex glands and to contribute to penile detumescence³¹. PRL can also affect central processing, as it can reach the central nervous system either via circumventricular regions lacking a blood–brain barrier³² or via receptor-mediated mechanisms³³, binding its receptors which have widespread distribution, including in the social brain network³⁴. Hence, circulating PRL can impact the activity of neuronal circuits involved in the processing of socio-sexual relevant cues and thus sexual performance. Circulating PRL reaches the central nervous system on a timescale that supports the rapid behavioral alterations that are observed immediately after ejaculation (in <2 min)³⁵. Through mechanisms that are not yet well established, PRL is known to elicit fast neuronal responses³⁶ besides its classical genomic effects³⁷. In summary, circulating PRL can impact several systems involved in sexual behavior on a timescale compatible with the establishment of the PERP.

However, despite data supporting the involvement of the ejaculatory PRL surge in the establishment of the PERP, this hypothesis has received numerous critics^{2,3,38–40}. While in humans it is well established that chronically high levels of PRL reduces libido²⁴, some authors suggest that those results were erroneously extended to the acute release of PRL around ejaculation^{2,3,38–40}. Furthermore, there is controversy in relation to PRL dynamics during sexual behavior, since in most studies PRL levels were quantified during fixed intervals of time, and not upon the occurrence of particular events, such as ejaculation. In fact, some reports in rats suggest that PRL levels are elevated through the entire sexual interaction^{41,42}. Finally, formal testing of the impact of acute PRL manipulations on sexual activity and

performance is still missing (but see ref. ⁴³ for an acute manipulation in humans).

In the present study, we tested the role of PRL in sexual activity and in the establishment of the PERP in the mouse. The sequence of sexual behavior in the mouse is very similar to the one observed in humans⁴⁴, making it an ideal system to test this hypothesis. Also, we took advantage of two strains of inbred mice that are representative of two different mouse subspecies (C57BL/6J: laboratory mouse, predominantly *Mus musculus domesticus* and PWK/PhJ: inbred wild-derived, *Mus musculus musculus*⁴⁵) and exhibit different sexual performance. Through routine work in our laboratory, we observed that while most BL6 males take several days to recover sexual activity after ejaculation, a large proportion of PWK males will re-initiate copulation with the same female within a relatively short period of time. This difference in PERP duration can be taken to our advantage, widening the dynamic range of this behavioral parameter and increasing the probability of detecting an effect with pharmacological manipulations.

First, we show that there is PRL release during sexual behavior in male mice. Second, using a pharmacological approach, we show that acute manipulations of prolactin levels, either mimicking the natural release during sexual behavior or inhibiting its occurrence, do not affect sexual activity or shorten the refractory period, respectively.

Results

Prolactin is released during sexual behavior in male mice. We first asked if PRL is released during copulation in the two strains of male mice. To monitor PRL dynamics during sexual behavior we took advantage of a recently developed ultrasensitive ELISA assay that can detect circulating levels of PRL in very small volumes of whole blood (5–10 µl), allowing the assessment of longitudinal PRL levels in freely behaving mice⁴⁶. Sexually trained laboratory mice (C57BL/6J, from here on BL6) and inbred wild-derived mice (PWK/PhJ, from here on PWK) were paired with a receptive female and allowed to mate (see “Methods” for details). During the sexual interaction males were momentarily removed from the cage to collect tail blood after which they returned to the behavioral cage, resuming the sexual interaction with the female. We collected blood samples upon the execution of pre-determined, easily identifiable, behavioral events that correspond to different internal states of the male: before sexual arousal (baseline, before the female was introduced in the cage), at the transition from appetitive to consummatory behavior (MA, mount attempt, immediately after the male attempted to mount the female), during consummatory behavior (mount, after a pre-determined number of mounts with intromissions, BL6 = 5 and PWK = 3), and after ejaculation (ejaculation, after the male exhibited the stereotypical shivering, falling to the side and separating from the female) (Fig. 1a, see “Methods” for details).

Baseline levels of circulating PRL in male mice were low for both strains (BL6 0.86 ± 0.46 ; PWK: 2.31 ± 1.37 ng/ml; please see ref. ⁴⁶ for BL6), but are significantly increased during sexual interaction (BL6: $F_{3,7} = 21.26$, $P < 0.0001$; PWK: $F_{3,8} = 17.18$, $P < 0.0001$; RM one-way ANOVA) (Fig. 1a). While in the case of BL6 males PRL levels only increased during the consummatory phase, PRL levels in PWK males are significantly increased already at the transition from appetitive to consummatory behavior (Baseline vs MA 16.30 ± 6.67 ng/ml, $P = 0.001$, Tukey’s multiple comparisons test) (Fig. 1a). In both strains, PRL levels after ejaculation are similar to the levels reached during consummatory behavior (BL6 $P = 0.71$ vs PWK $P = 0.95$, Tukey’s multiple comparisons test), in marked contrast to humans, where PRL seems to be released only around the time of ejaculation¹⁵.

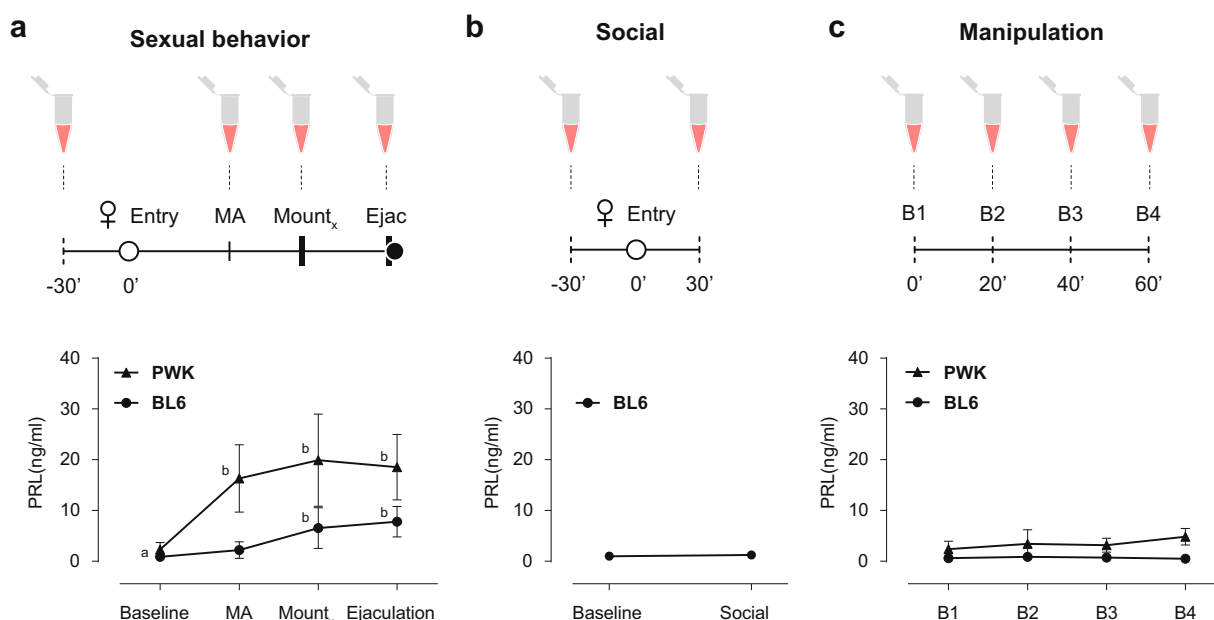


Fig. 1 Prolactin is released during sexual behavior in male mice. a Timeline for blood collection and $[PRL]_{\text{blood}}$ during sexual behavior (MA-mount attempt; BL6_{Xmounts} = 5; PWK_{Xmounts} = 3). RM one-way Anova for BL6 ($n = 8$) $F_{3,7} = 21.26$, $P < 0.0001$ and PWK ($n = 9$) $F_{3,8} = 17.18$, $P < 0.0001$, followed by Tukey's multiple comparison test $abP \leq 0.01$. **b** Timeline for blood collection and $[PRL]_{\text{blood}}$ during social behavior in BL6 males ($n = 15$) $P = 0.282$, two-tailed paired t -test. **c** Timeline for blood collection and $[PRL]_{\text{blood}}$ during repeated sampling in resting condition. RM one-way Anova for BL6 ($n = 8$) $F_{3,7} = 2.08$, $P = 0.18$ and PWK ($n = 8$) $F_{3,7} = 2.94$, $P = 0.11$. Data represented as mean $\pm$ SD.

Contrary to PWK males, which in the presence of a receptive female always engaged in sexual behavior and ejaculated, a large percentage of BL6 males never attempted to mount the female (15 out of 23, Supplementary Data 1). In such case, the session was aborted after 30 min of social interaction and a blood sample was collected (Fig. 1b). In this case, PRL levels of BL6 males did not differ from baseline (baseline 0.99 ± 0.67 vs social 1.25 ± 0.63 ; $P = 0.282$, paired t -test), further suggesting that PRL is only released in the context of a sexual interaction.

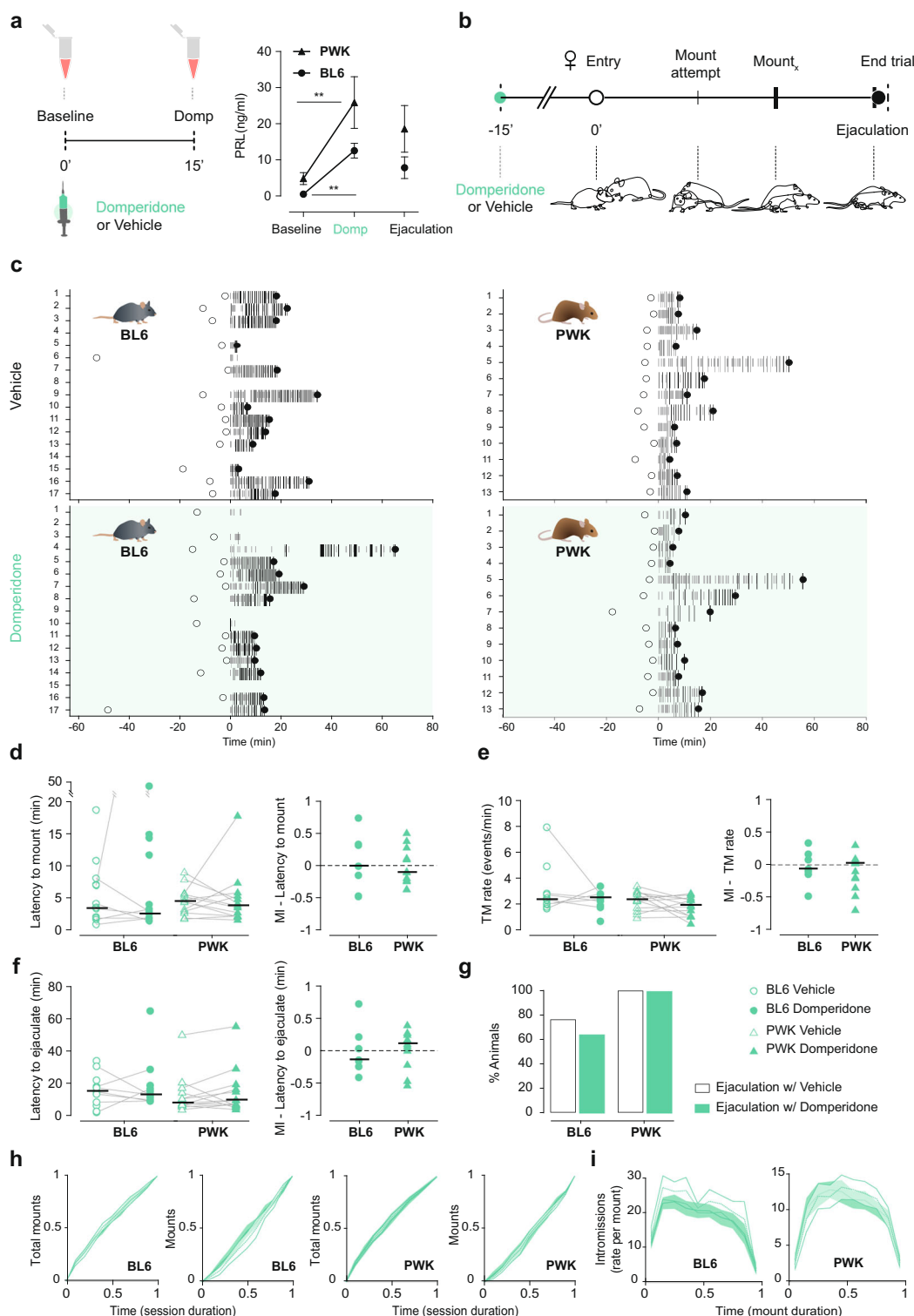
Because PRL is known to be released under stress⁴⁷ and to ensure that the changes observed in circulation are not a result from the blood collection procedure itself, all animals were initially habituated to the collection protocol in another cage, alone. To ensure that the habituation protocol was effective, in a separate experiment we measured PRL levels in the absence of any behavior. Four blood samples were collected 20 min apart from BL6 and PWK males in their home cage (Fig. 1c). In both cases, circulating PRL levels were not altered, ensuring that the observed increases were not caused by the manipulation (BL6: $F_{3,7} = 2.08$, $P = 0.18$; PWK $F_{3,7} = 2.94$, $P = 0.11$, RM one-way ANOVA).

Together, these results demonstrate that PRL is released during sexual behavior in male mice, but not during a social interaction or due to the blood collection protocol, prompting us to examine the role of PRL release during sexual behavior.

Acute prolactin release does not induce a refractory period-like state. To investigate if the increase in circulating levels of PRL that occurs during mating is sufficient to decrease sexual activity, a hallmark of the PERP, we employed a pharmacological approach to acutely elevate PRL levels before the animals became sexually aroused and assess if the male mice behave as if in a PERP-like state. PRL is produced in specialized cells of the anterior pituitary, the lactotrophs, and its release is primarily controlled by dopamine originating from the hypothalamus¹¹. Dopamine binds D2 receptors at the membrane of the

lactotrophs, inhibiting PRL release. Suppression of dopamine discharge leads to disinhibition of lactotrophs, which quickly release PRL into circulation^{48,49}. To acutely elevate PRL levels, we performed an intraperitoneal injection of the D2 dopamine receptor antagonist domperidone, which does not cross the blood–brain barrier^{50,51}, and measured PRL levels 15 min after the procedure. As expected, domperidone administration lead to a sharp rise in the levels of circulating PRL (BL6: baseline 0.4957 ± 0.4789 vs Domp 12.54 ± 2.032 , $P < 0.0001$; PWK: baseline 4.82 ± 1.645 vs Domp 25.87 ± 7.154 , $P < 0.0001$, paired t -test) (Fig. 2a) of similar magnitude to what is observed during copulation (ejaculation time point from Fig. 1a).

Therefore, on a separate experiment we investigated how domperidone-treated male mice behave with a receptive female. If PRL is sufficient to induce a PERP-like state, treated males should exhibit decreased sexual activity, which could be manifested in distinct manners, such as on the latency to initiate consummatory behavior or on the vigor of copulation. Each male from the two strains was tested twice, once with vehicle and another time with domperidone, in a counterbalanced manner and all the annotated behaviors are depicted over time on Fig. 2b, c (see “Methods” for details). Some BL6 males did not reach ejaculation in one of the sessions. A similar frequency was observed among unmanipulated animals (Supplementary Data 1). Despite differences in the dynamics of sexual behavior between BL6 and PWK males, administration of domperidone does not seem to affect sexual performance, as we could not detect any significant difference in the latency to start mounting the female, frequency of attempts to mount the female, time taken to ejaculate, or proportion of animals that reached ejaculation (Fig. 2d–g). Domperidone administration also does not seem to affect the dynamics of the sexual interaction across the session or within each mount (Fig. 2h, i, respectively) or other measures of sexual behavioral performance (Supplementary Data 2). Moreover, the majority of males reached ejaculation (latency to ejaculate since female entry: BL6: 16.2 ± 7 with a maximum of 62 min; PWK: 12.3 ± 6.7 with a



maximum of 59 min, Supplementary Data 2) within the time window where PRL levels remain elevated after domperidone administration⁴⁶.

In summary, domperidone administration, which causes an acute elevation of circulating PRL levels similar to what is observed at the end of copulation, does not have an inhibitory effect on any behavioral parameter related to sexual activity on the two strains of mice tested, that is, it does not induce a PERP-like state.

Blocking prolactin release during copulation does not decrease the duration of the refractory period. The release of PRL which is observed during sexual behavior has been proposed to be crucial in the establishment of the PERP⁹. To test this hypothesis, we acutely inhibited PRL release during sexual behavior by taking advantage of bromocriptine, a D2 receptor agonist. Bromocriptine's activation of D2 receptors on the lactotrophs' membrane blocks PRL release, a well-established procedure to inhibit the discharge of this hormone from the pituitary^{34,52}. If PRL is

Fig. 2 Acute prolactin release does not induce a refractory period-like state. **a** Timeline for blood collection and $[PRL]_{\text{blood}}$ after Vehicle (Veh—black) or Domperidone (Domp—green) administration. Domp administration lead to a sharp rise in the levels of circulating PRL (Baseline vs Domp: BL6 $n = 8$, $P < 0.0001$; PWK: $n = 9 < 0.0001$, paired t -test) of similar magnitude of PRL release during copulation. Values from the ejaculation time point (Fig. 1a) were included as a reference. **b** Timeline for sexual behavior assay using sexually trained BL6 and PWK males pre-treated with Veh or Domp ($t = -15$ min). Each animal was tested twice, in a counterbalanced manner: once with Veh and once with Domp. **c** Raster plot aligned to the first consummatory event (first mount attempt or mount), representing the sexual behavior executed by the male, with mount attempts represented in small gray bars, mounts in long black bars (width correlated with mount duration), and ejaculation with a black circle. Time of female entry in the apparatus represented with an open circle. BL6 $n = 17$; PWK $n = 13$. Quantification of **d** latency to mount (first mount attempt or mount, BL6 $P = 1.0$, PWK $P = 0.6355$), **e** rate of total mounts (TM, mount attempts + mounts) (BL6 $P = 0.9375$, PWK $P = 0.3054$) and **f** latency to ejaculate (from **d**, BL6 $P = 0.8125$, PWK $P = 0.21631$). With BL6 represented by circles and PWK by triangles (Veh—open, Domp—solid). Each line represents data of an individual. Only animals that ejaculated in both sessions were considered in the statistics ($n_{\text{BL6}} = 7$, $n_{\text{PWK}} = 13$). Individuals that did not ejaculate in one of the trials are represented as unconnected dots (not used in statistics). MI [modulation index (Domp – Veh)/(Domp + Veh)] between the two conditions for both strains. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank-sum test. **g** Percentage of animals that reached ejaculation in the Veh (open) and Domp (solid) condition. **h** Cumulative distributions of total mounts and mounts along the behavioral assay. Histogram aligned to the first consummatory event with 0.1 min bins. Time normalized for the duration of the session from female entry to ejaculation. **i** Rate of intromissions executed during the mount. Histogram for all mounts of each session, aligned to beginning of the mount with 0.1 min bins. Time normalized for the duration of the mount.

indeed necessary for the establishment of the PERP, we expected that after ejaculation, drug-treated males would regain sexual activity faster than controls.

To test bromocriptine's efficiency in blocking PRL release during sexual behavior, we first injected a group of males with bromocriptine (or vehicle) and measured PRL levels at three time points: (i) before the drug or vehicle injection, (ii) before the female was inserted in the cage, and then (iii) after ejaculation (Fig. 3a). As shown in Fig. 3a, bromocriptine administration efficiently blocked PRL release in both subspecies of mice, since PRL levels after ejaculation are not different from baseline (BL6: B1 vs Ejac, $P = 0.3$, B2 vs Ejac $P = 0.99$; PWK: B1 vs Ejac, $P = 0.97$, B2 vs Ejac $P = 0.99$; Tukey's multiple comparisons test after RM two-way Anova).

In a separate experiment we then tested the effect of the pharmacological manipulation on the establishment of the PERP and its duration. In this case, the male and female were allowed to remain in the cage undisturbed for a period of up to 2 h after ejaculation. Each male from the two strains was tested twice, once with vehicle and a second time with bromocriptine, in a counterbalanced manner. Each session ended once the male performed the first attempt of copulation after ejaculation (red diamonds) or after 2 h if no attempt was made (Fig. 3b, see "Methods" for details). All the annotated behaviors are depicted over time in Fig. 3b, c (see "Methods" for details).

As shown in Fig. 3d, inhibiting PRL release during sexual behavior did not change the proportion of male mice of the two strains that reached ejaculation (Supplementary Data 1) or regained sexual activity in the 2 h after ejaculation (corresponding to the proportion of red diamonds in Fig. 3c). In addition, and contrary to what was expected, we observed a significant increase in the PERP of PWK males (Fig. 3e, veh: 21.7 ± 4.18 vs bromo: 35.4 ± 16.3 , $P = 0.007$ by Wilcoxon signed rank test). Administration of bromocriptine also seems to affect the initial sexual arousal, as we could detect a decrease in the latency to start mounting the female (Fig. 3f, trend for B6 males and significant for PWK, veh: 4.06 ± 4.35 vs bromo: 1.93 ± 1.13 , $P = 0.06$; and veh: 2.78 ± 1.7 vs bromo: 1.48 ± 0.55 , $P = 0.01$, respectively, by Wilcoxon signed rank test). This observation was not due to an increase in activity/locomotion of the bromocriptine-treated males, as the average male speed before and after the female entry was not affected by the manipulation, nor the distance between the pair (Supplementary Data 3). Despite the locomotor activity being the same, the average male speed projected towards the female increased significantly for PWK males treated with bromocriptine as they moved in a goal-directed way, directionally towards the female (Supplementary Data 3).

Once consummatory behavior was initiated, control and bromocriptine-treated males exhibited similar levels of sexual performance, as we could not detect any difference in the frequency of attempts to mount the female or time taken to reach ejaculation (Fig. 3g, h). Other aspects of the sexual interaction were also not altered (Supplementary Data 4). Furthermore, bromocriptine administration does not seem to affect the dynamics of the sexual interaction across the session or within each mount (Fig. 3i, j, respectively).

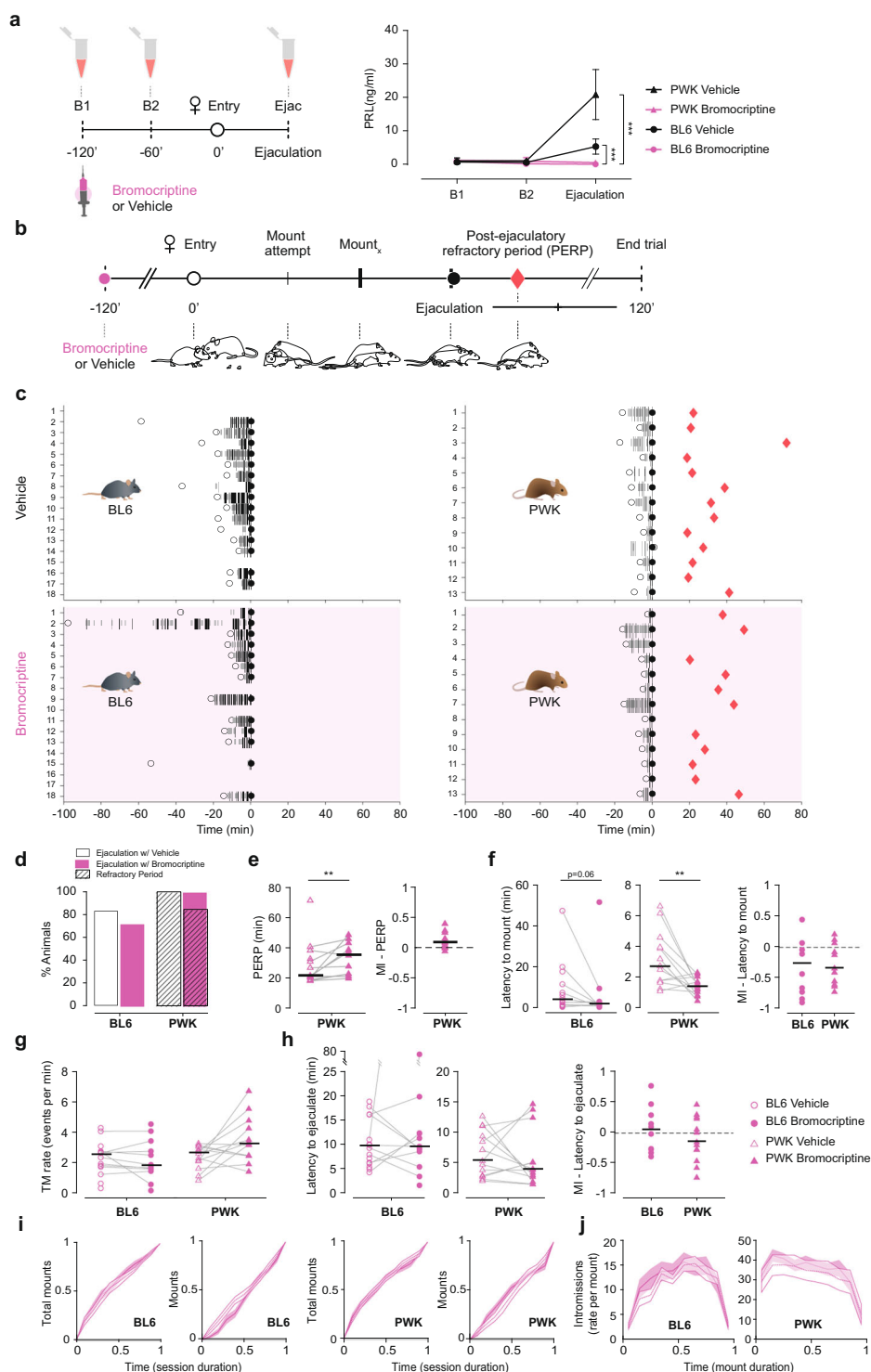
In summary, blocking PRL release during copulation does not affect the proportion of animals that regain sexual activity within 2 h after ejaculation and contrary to what was expected, bromocriptine leads to an increase in the duration of the PERP of PWK males. Except for a decrease in the latency to start mounting, maintaining circulating PRL low, at levels similar to what is observed prior to the sexual interaction, does not affect any of the parameters of sexual performance analyzed in both strains of mice.

Discussion

The PERP is highly conserved across species and is characterized by a general decrease in sexual activity after ejaculation². The pituitary hormone PRL is released during copulation and has been put forward as the main player in the establishment of the PERP⁹. However, the involvement of PRL in the establishment and duration of the PERP is controversial and has not been formally tested². Here we show that despite being released during copulation as previously shown in other taxa, PRL is neither sufficient nor necessary for the establishment of the PERP.

We first showed that PRL is released during copulation in male mice. The proportion of BL6 males that engaged in sexual behavior when tail blood is collected is lower than the proportion of animals that copulated in the rest of the experiments, suggesting that the procedure affects their sexual activity. However, once they start mating, all animals reached ejaculation. Importantly, once habituated, the procedure itself does not lead to PRL release. This opens up the possibility to perform such type of experiments using an "within-animal" design, a very important point particularly when there is a large interindividual variability, while decreasing the number of animals used.

Despite being released during sexual behavior in mice, PRL dynamics are quite different from what has been observed in humans. In men, PRL seems to only be released around the time of ejaculation^{15,16}, and only when ejaculation is achieved¹⁶. Indeed, the fact that PRL surge was only observed when ejaculation was achieved was one of the main results that lead to the idea that PRL may play a role in the acute regulation of sexual



activity after orgasm in humans⁵³. In contrast, in mice we observed an increase in circulating levels of PRL in sexually aroused PWK males and in BL6 males during the consummatory phase. The discrepancy between our results and the results published by others might be a result of the sampling procedure. Despite the fact that in human studies blood was continuously collected, PRL detection was performed at fixed time intervals and not upon the occurrence of particular events, such as ejaculation. Therefore, when averaging PRL levels across individuals, each participant might be in a slightly different internal state. Also, because PRL concentration is determined over fixed

intervals of time, it is difficult to pinpoint the PRL surge to the time of ejaculation (even though the human studies show that sexual arousal per se is not accompanied by an increase in PRL levels)⁹. To our knowledge, a single study assessed PRL levels during sexual behavior in male mice, stating that PRL is released after ejaculation⁵⁴. In this case, blood was also continuously sampled at fixed intervals of time. In contrast, in our study the blood was collected upon the execution of particular events, such as the first MA, a pre-defined number of mounts and ejaculation. Thus, even though the intervals between PRL measurements are different for each mouse, we ensure that PRL levels are measured

Fig. 3 Blocking prolactin release during copulation does not decrease the duration of the refractory period. **a** Timeline for blood collection and [PRL]_{blood} after Vehicle (Veh—black) or Bromocriptine (Bromo—pink) administration. RM two-way Anova with treatment (Veh or Bromo) as between subject's factor and time (B1, B2, and Ejac) as the within subject's factor, followed by Tukey's multiple comparison test: BL6 ($n = 5$ each) Treatment $F_{1,8} = 21.08$, $P = 0.0018$, $Ejac_{veh}$ vs $Ejac_{bromo}$ $P < 0.0001$; Time $F_{2,16} = 18.41$ $P < 0.0001$, $B1_{bromo}$ vs $Ejac_{bromo}$ $P = 0.3$; and PWK ($n_{veh} = 6$, $n_{bromo} = 4$) Treatment $F_{1,8} = 28.43$, $P = 0.0007$, $Ejac_{veh}$ vs $Ejac_{bromo}$ $P < 0.0001$; Time $F_{2,16} = 23.97$, $P < 0.0001$, $B1_{bromo}$ vs $Ejac_{bromo}$ $P = 0.97$. **b** Timeline for sexual behavior assay using sexually trained BL6 and PWK males pre-treated with Veh or Bromo ($t = -120$ min). Each animal was tested twice in a counterbalanced manner. **c** Raster plot aligned to ejaculation, representing the sexual behavior performed by the male, with mount attempts represented in small gray bars, mounts in long black bars (width correlated with mount duration), ejaculation with a black circle and PERP (latency to the first consummatory event (mount attempt or mount) after ejaculation represented with a red diamond). Time of female entry in the apparatus represented with an open circle. BL6 $n = 18$; PWK, $n = 13$. **d** Percentage of animals that reached ejaculation (solid color) and re-initiated the consummatory behavior after ejaculating (PERP, dashed color) in Veh (white) and Bromo (pink) conditions. Quantification of **e** PERP duration ($P = 0.007$), **f** latency to mount (first MA or mount) (BL6 $P = 0.0644$, PWK $P = 0.0132$), **g** rate of total mounts (TM, MA + mounts) (BL6 $P = 0.32$, PWK $P = 0.0681$) and **h** latency to ejaculate (from **f**, BL6 $P = 0.92$, PWK $P = 0.68$). With BL6 represented by circles and PWK by triangles (Veh—open, Bromo—solid). Each line represents data from an individual; only animals that ejaculated in both sessions were considered in the statistics ($n_{BL6} = 10$, $n_{PWK} = 13$). Individuals that did not ejaculate in one of the trials are represented as unconnected dots (not used in statistics). Data presented as median $\pm$ MAD (median absolute deviation with standard scale factor) following Wilcoxon rank-sum test. MI [modulation index (Bromo—Veh)/(Bromo + Veh)] between the two conditions for both strains. **i** Cumulative distributions of total mounts and mounts along the behavioral assay. Histogram aligned to the first consummatory event with 0.1 min bins. Time normalized for the duration of the session from female entry to ejaculation. **j** Rate of intromissions executed during the mount. Histogram for all mounts of each session, aligned to beginning of the mount with 0.1 min bins. Time normalized for the duration of the mount.

for all individuals in a similar internal state. Independently of the differences in the dynamics of circulating PRL levels, the increase we observe seems to be specific to a sexual encounter, since PRL levels in BL6 males that never attempt copulation remain unaltered from baseline.

In order to test if PRL by itself is sufficient to decrease sexual activity, we injected domperidone to induce an artificial PRL surge. In this case, the male mouse should behave like a male that just ejaculated: for example, exhibit longer latency to initiate the sexual interaction, which in the case of BL6 mice should take days. Even though domperidone administration leads to circulating levels of PRL that are similar to the ones observed at the end of a full sexual interaction, this manipulation did not cause any alteration in terms of sexual performance, as all behavioral parameters remained unaltered for both strains of mice. Although most likely other neuromodulatory systems were affected by our manipulation (via PRL), the fact that the domperidone manipulation did not cause a PERP-like state might still be due to the fact that the full repertoire of neuromodulators and hormones accompanying an ejaculation was not present⁵⁵. Further experiments could test this idea by examining if combinations of different neuromodulators and hormones administered together with PRL can induce a PERP-like state.

Last, we asked if the elevation in PRL levels during sexual behavior is necessary for the establishment and duration of the PERP. For that we took a complementary pharmacological approach, where we injected bromocriptine, a D2 receptor agonist that temporarily inhibits the release of PRL. PRL levels after ejaculation in bromocriptine-treated males are similar to pre-copulatory levels. If PRL is indeed necessary to establish the PERP, we would expect a decrease in its duration that should easily be observed in the PWK males (since they regain sexual activity on average 30 min after ejaculation) or even in the BL6 (which take days). We observed a decrease in the latency to start mounting the female and, contrary to our expectation, a significant increase in the PERP duration of PWK males. We believe these effects may be mediated by the direct effect of bromocriptine, rather than an effect of PRL itself. First, baseline PRL levels are already very low in male mice and therefore the manipulation most likely did not affect them. Second, systemic administration of dopamine agonists has shown that anticipatory measures of sexual behavior are more sensitive to disruption than

are consummatory measures of copulation^{56,57}. This agrees with our results, where we observed a significant decrease in the latency to initiate mounting with bromocriptine, while no other parameter of sexual performance was affected. Interestingly, bromocriptine-treated PWK males seem more ballistic in their approach to the female, suggesting a more goal-directed behavior towards the female. Bromocriptine (and domperidone) might also have an effect outside the central nervous system as D2 receptors are expressed in the human and rat seminal vesicles⁵⁸. It is not known if direct manipulation of these receptors in the seminal vesicles has an impact on the PERP, which could explain our results.

In this study we investigated the role of PRL in the PERP of two different strains of mice that belong to the two main subspecies of house mouse, *Mus musculus musculus* (PWK) and *Mus musculus domesticus* (BL6), for three main reasons. As already presented, the two strains have very different PERP duration, widening the dynamic range of this behavioral parameter and increasing the probability of detecting an effect of the manipulations. Second, in addition to differences in PERP duration, BL6 and PWK males have very different sexual performance, reflected in several behavioral parameters as, for example, the number of mounts needed to reach ejaculation. Despite the differences (which were not explored as they are outside the scope of the present study), the effects of the pharmacological manipulations were similar across the two strains, causing either no effect or changing the behavior in the same direction (shorter latency to start mounting the female in bromocriptine-treated males). This is an important point that strengthens our conclusions. Finally, although fundamental for many present-day discoveries, the usage of the common inbred strains of mice comes at a cost, due to the limitations in their genetic background that sometimes leads to results that are specific to the strain of mouse used^{59–61}. Wild-derived strains of mice are valuable tools that can complement the genetic deficiencies of classical laboratory strains of mice^{45,62}. Despite the fact that larger numbers of animals are used (because experiments are repeated on each mouse strain), this approach is already routinely used in other fields, such as in immunological studies⁶³, providing greater confidence to the results obtained from the effect of pharmacological manipulations on behavior, for example.

As initially discussed, PRL can affect peripheral and central pathways that have been implicated in sexual performance and

motivation. Even though our manipulations indiscriminately affect both, our results refute the idea that PRL decreases sexual activity, the hallmark of the PERP. What could be the role of copulatory PRL? PRL release may be the “side-effect” of the neuromodulatory changes that occur during sexual behavior; this is merely the result of reduction in DA levels (DA inhibits PRL release) and/or the increase in oxytocin and serotonin (known stimulating factors of PRL release) instead of having the principal role in the establishment of PERP^{64–66}. The fact that PRL levels are already elevated during sexual interaction in BL6 and PWK males further suggests that PRL cannot promote by itself reduced sexual activity, at least in male mice. Other studies point towards a role of PRL in the establishment of parental behavior^{67–69}. New behavioral paradigms will be fundamental in unraveling this mystery.

Methods

Animals. BL6 (*Mus musculus domesticus*, C57BL/6J) and Wild (*Mus musculus musculus*, PWD/PhJ and PWK/PhJ) mice were ordered from The Jackson Laboratories and maintained in our animal facility. Animals were weaned at 21 days and housed in same-sex groups in stand-alone cages (1284L, Techniplast, 365 × 207 × 140 mm) with access to food and water ad libitum. Mice were maintained on a 12:12 light/dark cycle and experiments were performed during the dark phase of the cycle, under red dim light. All experiments were approved by the Animal Care and Users Committee of the Champalimaud Neuroscience Program and the Portuguese National Authority for Animal Health (Direção Geral de Veterinária).

Females were kept house grouped and males were isolated before the sexual training. Both males and females were sexually experienced and habituated to be handled and to the assay routine. To enter the study, a male had to ejaculate three times (in four sessions). Males interacted with different females in each sexual encounter. Each manipulation was performed in a different group of males and, within each manipulation, experiments were conducted in parallel for both BL6 and PWK. Trials were conducted in the male home cage (1145T, Techniplast, 369 × 156 × 132 mm) stripped from nesting, food, and water: covered with a transparent acrylic lid. The trial started with the entry of the female in the setup ($t = 0$ min).

Ovariectomy and hormonal priming. All females underwent bilateral ovariectomy under isoflurane anesthesia (1–2% at 1 L/min). After exposing the muscle with one small dorsal incision (1 cm), a small incision was made in the muscle wall, at the ovary level, on each side. The ovarian arteries were cauterized and both ovaries were removed. The skin was sutured, and the suture topped with iodine and wound powder. The animals received an i.p. injection of carprofen before being housed individually with food supplemented with analgesic (MediGel, 1 mg carprofen/2 oz cup) for 2 days recovery and then re-grouped in their home cages.

Female mice were primed subcutaneously 48 h before the assay with 0.1 ml estrogen (1 mg/ml, Sigma E815 in sesame oil) and 4 h before the assay with 0.1 ml progesterone (5 mg/ml, Sigma 088K0671 in sesame oil).

Blood collection. Tail-tip whole-blood samples were collected from the male tail, immediately diluted in PBS-T (PBS, 0.05% Tween-20), and frozen at -20°C where it was stored until use⁴⁶.

To profile $[\text{PRL}]_{\text{blood}}$ during sexual behavior (Fig. 1a), baseline blood was collected 30 min before ($t = -30$ min) the female entry ($t = 0$ min). From this point on, blood collection was locked to the onset of specific behaviors: once the male did the first MA, after executing a fixed number of mounts (Mx) and after ejaculation (after the male exhibited the stereotypical shivering, falling to the side and decoupling from the female). We choose Mx = 5 for BL6 and Mx = 3 for PWK to ensure that the males would have sufficient sexual interaction without reaching ejaculation. Contrarily to PWK males that, in the presence of a receptive female, the majority engages in sexual behavior, BL6 do not. Thus, after 30 min interacting with the female without displaying sexual interest, we collected a blood sample and terminated the trial (Fig. 1b, social). Because blood collection is an invasive procedure and PRL is also released under stress, we evaluated if the manipulation itself could induce PRL release. For that we collected blood every 20 min for 1 h from males resting in their home cage (Fig. 1c).

Domperidone is a D2 dopamine receptor antagonist that was previously used to study the inhibitory tone of dopamine on PRL release from the pituitary, inducing a PRL peak 15 min after i.p. injection⁴⁶. To test the magnitude of the PRL release of the two mouse strains under domperidone (Fig. 2a), we conducted a pilot study where we collected a blood sample before (baseline) and 15 min after Domp injection (20 mg/kg; Abcam Biochemicals). We opted to manipulate $[\text{PRL}]_{\text{blood}}$ through domperidone instead of injecting PRL directly to induce a PRL release similarly to a natural occurring instead of adding a recombinant form.

Bromocriptine is a D2 dopamine receptor agonist known to inhibit endogenous prolactin release³⁴. To test its efficacy on blocking PRL release during sexual behavior (Fig. 3a), we conducted a second pilot study where the males were injected (100 µg bromo or vehicle) 2 h before the trial started. Blood samples were collected just before injection, 1 h after injection and after ejaculation.

Prolactin quantification. $[\text{PRL}]_{\text{blood}}$ quantification was done as previously described⁴⁶. Briefly, a 96-well plate (Sigma-Aldrich cls 9018-100EA) was coated with 50 µl capture antibody anti-rat PRL (anti-rPRL-IC) (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), AFP65191 (Guinea Pig), NIDDK-National Hormone and Pituitary Program (NHPP, TORRANCE, CA) at a final dilution of 1:1000 in PBS of the antibody stock solution, reconstituted in PBS as described in the datasheet (Na_2HPO_4 7.6 mM; NaH_2PO_4 2.7 mM; and NaCl 0.15 M; pH 7.4). The plate was protected with Parafilm® and incubated at 4°C overnight in a humidified chamber. The coating antibody was decanted and 200 µl of blocking buffer (5% skimmed milk powder in PBS-T) was added to each well to block nonspecific binding. The plate was left for 2 h at room temperature on a microplate shaker. In parallel, a standard curve was prepared using a twofold serial dilution of Recombinant mouse Prolactin (mPRL; AFP-405C, NIDDK-NHPP) in PBS-T with BSA 0.2 mg/ml (bovine serum albumin; Millipore 82-045-1). After the blocking step, the plate was washed (three times for 3 min at room temperature with PBS-T), 50 µl of quality control (QC), standards or samples were loaded in duplicate into the wells, and incubated for 2 h at room temperature on the microplate shaker. The plate was washed, and the complex was incubated for another 90 min with 50 µl detection antibody (rabbit alpha mouse PRL, a gift from Patrice Mollard Lab) at a final dilution of 1:50,000 in blocking buffer solution. Following a final wash, this complex was incubated for 90 min with 50 µl horseradish peroxidase-conjugated antibody (anti-rabbit, IgG, Fisher Scientific; NA934) diluted in 50% PBS and 50% blocking buffer. One tablet of O-phenylenediamine (Life Technologies SAS 00-2003) was diluted into 12 ml citrate-phosphate buffer pH 5, containing 0.03% hydrogen peroxide. One hundred microliters of this substrate solution was added to each well (protected from light), and the reaction was stopped after 30 min with 50 µl of 3 M HCl. The optical density from each well was determined at 490 nm using a microplate reader (SPECTROstar^{Nano}, BMG LABTECH). An absorbance at 650 nm was used for background correction.

A linear regression was used to fit the optical densities of the standard curve vs their concentration using samples ranging from 0.1172 to 1.875 ng/ml, using the former as the lower limit of detection. Appropriate sample dilutions were carried out in order to maintain detection in the linear part of the standard curve, and PRL concentrations were extrapolated from the OD of each sample. To control for reproducibility of the assay, trunk blood of males injected with domperidone was immediately diluted in PBS-T and pulled to be used as QC. Loading of the wells was done vertically left to right and QC was always loaded on the top row. The formula $\text{OD}(\text{Co}, t) = \text{OD}(\text{Ob}) + \alpha(\text{QC}, t)$ was used to correct the ODs for loading dwell time (OD: optical density, Co: corrected, t: well number, Ob: observed, α : QC linear regression'). Coefficient of variability was kept to a maximum of 10%.

Behavioral assays. Each male underwent two trials: one with vehicle and one with drug (domperidone or bromocriptine). Administrations were counterbalanced between animals and spaced 7 days. In the first assay, for pharmacological induction of acute PRL release (Fig. 2b), the male was injected i.p. with domperidone or vehicle 15 min before the trial started ($t = -15$ min). Animals were allowed to interact until the male reached ejaculation or 1 h in the case the male did not display sexual behavior. Conversely, for pharmacological blockage of PRL release (Fig. 3b), a second group of males were pre-treated with bromocriptine or vehicle with a subcutaneous injection 2 h before the beginning of the trial ($t = -120$ min). Animals were allowed to interact until a maximum of 2 h after the male reached ejaculation or 1 h in the case the male did not display sexual behavior. For the BL6 unmanipulated group, the female was added to the male home cage without disturbing the male (Supplementary Data 1). Animals were allowed to interact until the male reached ejaculation or 1 h in the case the male did not display sexual behavior.

Behavior analysis. The behavior was recorded from the top and side with pointgrey cameras (FL3- U3-13S2C-CS) connected to a computer running a custom Bonsai software⁷⁰. Behavior was manually annotated using the open source program Python Video Annotator (<https://pythonvideoannotator.readthedocs.io>) and analyzed using Matlab. The number of MA (mount without intromission), mounts (mounts with intromission), latency to mount (first MA or mount), latency to ejaculation and PERP (latency between ejaculation and the next mount) was calculated. Total number of mounts (TM) was calculated as the sum of MA and mounts and TM rate was calculated as TM/latency to ejaculate. The percentage of animals that reached ejaculation and regain sexual activity under 2 h (refractory period) were also calculated. The modulation index (MI) was calculated as $(X_{\text{drug}} - X_{\text{vehicle}})/(X_{\text{drug}} + X_{\text{vehicle}})$. The centroid position and individual identity of each pair was followed offline using the open source program idtracker.ai⁷¹ and used to calculate male velocity and interindividual distance with Matlab (Supplementary Data 3).

Statistical analysis. The statistical details of each experiment, including the statistical tests used and exact value of n , are detailed in each figure legend. Comparisons were always performed within the same strain and not across strains. Data related to prolactin quantification were analyzed using GraphPad Prism 7 software and presented as mean $\pm$ SD. For comparison within strain (Fig. 1a, c) an RM one-way Anova followed by a Tukey's multiple comparison test was used. Comparison of paired samples comparing two groups, statistical analysis was performed by using a paired-sample two-tailed t -test (Figs. 1b and 2a baseline-Domp). For the effect of treatment (veh or bromo) and time of sampling (B1, B2, and Ejac) (Fig. 3a) an RM two-way Anova followed by Tukey's multiple comparison test was used. Data related to animal behavior were analyzed with MATLAB R2019b and presented as median $\pm$ MAD (median absolute deviation with standard scale factor). Animals were randomized between treatments and comparison between the two conditions were done with Wilcoxon rank-sum test (Figs. 2d–f and 3e–h and Supplementary Data 2–4). Only animals that ejaculated in both sessions were included in the statistical analysis (Domperidone: $n_{BL6} = 7$, $n_{PWK} = 13$; bromocriptine: $n_{BL6} = 10$, $n_{PWK} = 13$). Significance was accepted at $P < 0.05$ for all tests.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated to support the findings of this study are available from the corresponding author upon reasonable request.

Received: 18 August 2020; Accepted: 4 December 2020;

Published online: 04 January 2021

References

- Masters, W. H. & Johnson, V. E. *Human Sexual Response* (Bantam Books, New York, 1966).
- Seizert, C. A. The neurobiology of the male sexual refractory period. *Neurosci. Biobehav. Rev.* **92**, 350–377 (2018).
- Levin, R. J. Revisiting post-ejaculation refractory time—what we know and what we do not know in males and in females. *J. Sex. Med.* **6**, 2376–2389 (2009).
- McGill, T. E. Sexual behavior of the mouse after long-term and short-term postejaculatory recovery periods. *J. Genet. Psychol.* **103**, 53–57 (1963).
- Rodriguez-Manzo, G. Blockade of the establishment of the sexual inhibition resulting from sexual exhaustion by the Coolidge effect. *Behav. Brain Res.* **100**, 245–254 (1999).
- Wilson, J. R., Kuehn, R. E. & Beach, F. A. Modification in the sexual behavior of male rats produced by changing the stimulus female. *J. Comp. Physiol. Psychol.* **56**, 636 (1963).
- Rojas-Durán, F. et al. Correlation of prolactin levels and PRL-receptor expression with Stat and Mapk cell signaling in the prostate of long-term sexually active rats. *Physiol. Behav.* **138**, 188–192 (2015).
- Hernandez, M. E. et al. Prostate response to prolactin in sexually active male rats. *Reprod. Biol. Endocrinol.* **4**, 28 (2006).
- Kruger, T. H. C., Haake, P., Hartmann, U., Schedlowski, M. & Exton, M. S. Orgasm-induced prolactin secretion: feedback control of sexual drive? *Neurosci. Biobehav. Rev.* **26**, 31–44 (2002).
- Drago, F. Prolactin and sexual behavior: a review. *Neurosci. Biobehav. Rev.* **8**, 433–439 (1984).
- Freeman, M. E., Kanyicska, B., Lerant, A. & Nagy, G. Prolactin: structure, function, and regulation of secretion. *Physiol. Rev.* **80**, 1523–1631 (2000).
- Grattan, D. R. & Kokay, I. C. Prolactin: a pleiotropic neuroendocrine hormone. *J. Neuroendocrinol.* **20**, 752–763 (2008).
- Brody, S. & Krüger, T. H. C. The post-orgasmic prolactin increase following intercourse is greater than following masturbation and suggests greater satiety. *Biol. Psychol.* **71**, 312–315 (2006).
- Egli, M., Leeners, B. & Kruger, T. H. C. Prolactin secretion patterns: basic mechanisms and clinical implications for reproduction. *Reprod. Camb. Engl.* **140**, 643–654 (2010).
- Exton, M. S. et al. Coitus-induced orgasm stimulates prolactin secretion in healthy subjects. *Psychoneuroendocrinology* **26**, 287–294 (2001).
- Exton, N. G. et al. Neuroendocrine response to film-induced sexual arousal in men and women. *Psychoneuroendocrinology* **25**, 187–199 (2000).
- Krüger, T. et al. Neuroendocrine and cardiovascular response to sexual arousal and orgasm in men. *Psychoneuroendocrinology* **23**, 401–411 (1998).
- Krüger, T. H. C. et al. Serial neurochemical measurement of cerebrospinal fluid during the human sexual response cycle. *Eur. J. Neurosci.* **24**, 3445–3452 (2006).
- Exton, M. S. et al. Cardiovascular and endocrine alterations after masturbation-induced orgasm in women. *Psychosom. Med.* **61**, 280–289 (1999).
- Kruger, T. et al. Specificity of the neuroendocrine response to orgasm during sexual arousal in men. *J. Endocrinol.* **177**, 57–64 (2003).
- Oaknin, S., Castillo, A. R. D., Guerra, M., Battaner, E. & Mas, M. Changes in forebrain Na,K-ATPase activity and serum hormone levels during sexual behavior in male rats. *Physiol. Behav.* **45**, 407–410 (1989).
- Haake, P. et al. Absence of orgasm-induced prolactin secretion in a healthy multi-orgasmic male subject. *Int. J. Impot. Res.* **14**, 3900823 (2002).
- Svare, B. et al. Hyperprolactinemia suppresses copulatory behavior in male rats and mice. *Biol. Reprod.* **21**, 529–535 (1979).
- Buvat, J. Hyperprolactinemia and sexual function in men: a short review. *Int. J. Impot. Res.* **15**, 3901043 (2003).
- Sato, F. et al. Suppressive effects of chronic hyperprolactinemia on penile erection and yawning following administration of apomorphine to pituitary-transplanted rats. *J. Androl.* **18**, 21–25 (1997).
- Melmed, S. et al. Diagnosis and treatment of hyperprolactinemia: an Endocrine Society clinical practice guideline. *J. Clin. Endocrinol. Metab.* **96**, 273–288 (2011).
- Grattan, D. R. & Bridges, R. S. in *Hormones, Brain and Behavior* 2nd edn, 2471–2504 (Academic Press, 2009).
- Riddle, O., Bates, R. W. & Dykshorn, S. W. The preparation, identification and assay of prolactin—a hormone of the anterior pituitary. *Am. J. Physiol.* **105**, 191–216 (1933).
- Nagano, M. & Kelly, P. A. Tissue distribution and regulation of rat prolactin receptor gene expression. Quantitative analysis by polymerase chain reaction. *J. Biol. Chem.* **269**, 13337–13345 (1994).
- Aoki, M. et al. Widespread cell-specific prolactin receptor expression in multiple murine organs. *Endocrinology* **160**, 2587–2599 (2019).
- Aoki, H. et al. Suppression by prolactin of the electrically induced erectile response through its direct effect on the corpus cavernosum penis in the dog. *J. Urol.* **154**, 595–600 (1995).
- Ganong, W. F. Circumventricular organs: definition and role in the regulation of endocrine and autonomic function. *Clin. Exp. Pharmacol. Physiol.* **27**, 422–427 (2000).
- Grattan, D. R. et al. Feedback regulation of PRL secretion is mediated by the transcription factor, signal transducer, and activator of transcription 5b. *Endocrinology* **142**, 3935–3940 (2001).
- Brown, R. S. E., Kokay, I. C., Herbison, A. E. & Grattan, D. R. Distribution of prolactin-responsive neurons in the mouse forebrain. *J. Comp. Neurol.* **518**, 92–102 (2010).
- Kirk, S. E., Grattan, D. R. & Bunn, S. J. The median eminence detects and responds to circulating prolactin in the male mouse. *J. Neuroendocrinol.* **31**, e12733 (2019).
- Georgescu, T., Ladyman, S. R., Brown, R. S. E. & Grattan, D. R. Acute effects of prolactin on hypothalamic prolactin receptor expressing neurones in the mouse. *J. Neuroendocrinol.* **32**, e12908 (2020).
- Bole-Feysot, C., Goffin, V., Edery, M., Binart, N. & Kelly, P. A. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev.* **19**, 225–268 (1998).
- Corona, G., Jannini, E. A., Vignozzi, L., Rastrelli, G. & Maggi, M. The hormonal control of ejaculation. *Nat. Rev. Urol.* **9**, 508 (2012).
- Levin, R. Is prolactin the biological “off switch” for human sexual arousal? *Sex. Relatsh. Ther.* **18**, 237–243 (2003).
- Turley, K. R. & Rowland, D. L. Evolving ideas about the male refractory period. *Bju Int.* **112**, 442–452 (2013).
- Kamel, F., Right, W. W., Mock, E. J. & Frankel, A. I. The influence of mating and related stimuli on plasma levels of luteinizing hormone, follicle stimulating hormone, prolactin, and testosterone in the male rat 1. *Endocrinology* **101**, 421–429 (1977).
- Kamel, F. & Frankel, A. J. Hormone release during mating in the male rat: time course, relation to sexual behavior, and interaction with handling procedures. *Endocrinology* **103**, 2172–2179 (1978).
- Kruger, T. et al. Effects of acute prolactin manipulation on sexual drive and function in males. *J. Endocrinol.* **179**, 357–365 (2003).
- Lenchow, C. & Lima, S. Q. In the mood for sex: neural circuits for reproduction. *Curr. Opin. Neurobiol.* **60**, 155–168 (2020).
- Gregorová, S. & Forejt, J. PWD/Ph and PWK/Ph inbred mouse strains of *Mus m. musculus* subspecies—a valuable resource of phenotypic variations and genomic polymorphisms. *Folia Biol. Prague* **46**, 31–41 (2000).
- Guillou, A. et al. Assessment of lactotroph axis functionality in mice: longitudinal monitoring of PRL secretion by ultrasensitive-ELISA. *Endocrinology* **156**, 1924–1930 (2015).
- Gala, R. R. The physiology and mechanisms of the stress-induced changes in prolactin secretion in the rat. *Life Sci.* **46**, 1407–1420 (1990).

48. Lyons, D. J. & Broberger, C. TIDAL WAVES: network mechanisms in the neuroendocrine control of prolactin release. *Front. Neuroendocr.* **35**, 420–438 (2014).
49. Grattan, D. R. 60 years of neuroendocrinology: The hypothalamo-prolactin axis. *J. Endocrinol.* **226**, T101–T122 (2015).
50. Cocchi, D. et al. Prolactin-releasing effect of a novel anti-dopaminergic drug, domperidone, in the rat. *Neuroendocrinology* **30**, 65–69 (1980).
51. Costall, B., Fortune, D. H. & Naylor, R. J. Neuropharmacological studies on the neuroleptic potential of domperidone (R33812). *J. Pharm. Pharmacol.* **31**, 344–347 (1979).
52. Molik, E. & Błasiak, M. The role of melatonin and bromocriptine in the regulation of prolactin secretion in animals—a review. *Ann. Anim. Sci.* **15**, 849–860 (2015).
53. Krüger, T. H. C., Hartmann, U. & Schedlowski, M. Prolactinergic and dopaminergic mechanisms underlying sexual arousal and orgasm in humans. *World J. Urol.* **23**, 130–138 (2005).
54. Bronson, F. H. & Desjardins, C. Endocrine responses to sexual arousal in male mice. *Endocrinology* **111**, 1286–1291 (1982).
55. Pfaus, J. G. Pathways of sexual desire. *J. Sex. Med.* **6**, 1506–1533 (2009).
56. Pfaus, J. G. & Phillips, A. G. Role of dopamine in anticipatory and consummatory aspects of sexual behavior in the male rat. *Behav. Neurosci.* **105**, 727–743 (1991).
57. Hull, E. M., Muschamp, J. W. & Sato, S. Dopamine and serotonin: influences on male sexual behavior. *Physiol. Behav.* **83**, 291–307 (2004).
58. Hyun, J.-S. et al. Localization of peripheral dopamine D1 and D2 receptors in rat and human seminal vesicles. *J. Androl.* **23**, 114–120 (2002).
59. Puglisi-Allegra, S. & Cabib, S. Psychopharmacology of dopamine: the contribution of comparative studies in inbred strains of mice. *Prog. Neurobiol.* **51**, 637–661 (1997).
60. Cabib, S., Puglisi-Allegra, S. & Ventura, R. The contribution of comparative studies in inbred strains of mice to the understanding of the hyperactive phenotype. *Behav. Brain Res.* **130**, 103–109 (2002).
61. Welker, E. & Loos, H. Vder Quantitative correlation between barrel-field size and the sensory innervation of the whiskerpad: a comparative study in six strains of mice bred for different patterns of mystacial vibrissae. *J. Neurosci.* **6**, 3355–3373 (1986).
62. Fernandes, C. et al. Behavioral characterization of wild derived male mice (*Mus musculus musculus*) of the PWD/Ph inbred strain: high exploration compared to C57BL/6J. *Behav. Genet.* **34**, 621–630 (2004).
63. Deschepper, C. F., Olson, J. L., Otis, M. & Gallo-Payet, N. Characterization of blood pressure and morphological traits in cardiovascular-related organs in 13 different inbred mouse strains. *J. Appl. Physiol.* **97**, 369–376 (2004).
64. McIntosh, T. K. & Barfield, R. J. Brain monoaminergic control of male reproductive behavior. I. Serotonin and the post-ejaculatory refractory period. *Behav. Brain Res.* **12**, 255–265 (1984).
65. McIntosh, T. K. & Barfield, R. J. Brain monoaminergic control of male reproductive behavior. II. Dopamine and the post-ejaculatory refractory period. *Behav. Brain Res.* **12**, 267–273 (1984).
66. Carmichael, M. S. et al. Plasma oxytocin increases in the human sexual response. *J. Clin. Endocrinol. Metab.* **64**, 27–31 (1987).
67. Brown, R. E. Hormones and paternal behavior in vertebrates. *Am. Zool.* **25**, 895–910 (1985).
68. Schradin, C. & Anzenberger, G. Prolactin, the hormone of paternity. *Physiology* **14**, 223–231 (1999).
69. Stagkourakis, S. et al. A neuro-hormonal circuit for paternal behavior controlled by a hypothalamic network oscillation. *Cell* **182**, 960–975.e15 (2020).
70. Lopes, G. et al. Bonsai: an event-based framework for processing and controlling data streams. *Front. Neuroinformatics* **9**, 7 (2015).
71. Romero-Ferrero, F., Bergomi, M. G., Hinz, R. C., Heras, F. J. H. & Polavieja, G. Gde idtracker.ai: tracking all individuals in small or large collectives of unmarked animals. *Nat. Methods* **16**, 179–182 (2019).

Acknowledgements

We thank Patrice Mollard and his team for welcoming S.V. in their facilities, teaching the uElisa technique, and for providing antibodies. We also express our gratitude to Francisco Romero for all the support with IdTracker, Gil Costa for the figure design, the Champalimaud Vivarium for their support with the wild derived animals, and the whole Lima lab for critical input. This work was supported by the Champalimaud Foundation, UIDB/04443/2020, LISBOA-01-0145-FEDER-022170, LISBOA-01-0145-FEDER-022231, ERC Consolidator Grant (772827, to S.Q.L.), Fundação para a Ciência e Tecnologia SFRH/BD/51011/2010 (to S.V.), and PhRMA Foundation Postdoctoral Fellowship in Informatics (to T.M.).

Author contributions

S.Q.L. and S.V. designed the study. S.V. performed the experiments, annotation of the behavior, and IdTracker. T.M. wrote the Matlab code. S.V. analyzed the data with input from S.Q.L. and T.M. S.Q.L. and S.V. wrote the paper with contributions from others.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s42003-020-01570-4>.

Correspondence and requests for materials should be addressed to S.Q.L.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021

Supplementary Data

No evidence for prolactin's involvement in the post-ejaculatory refractory period

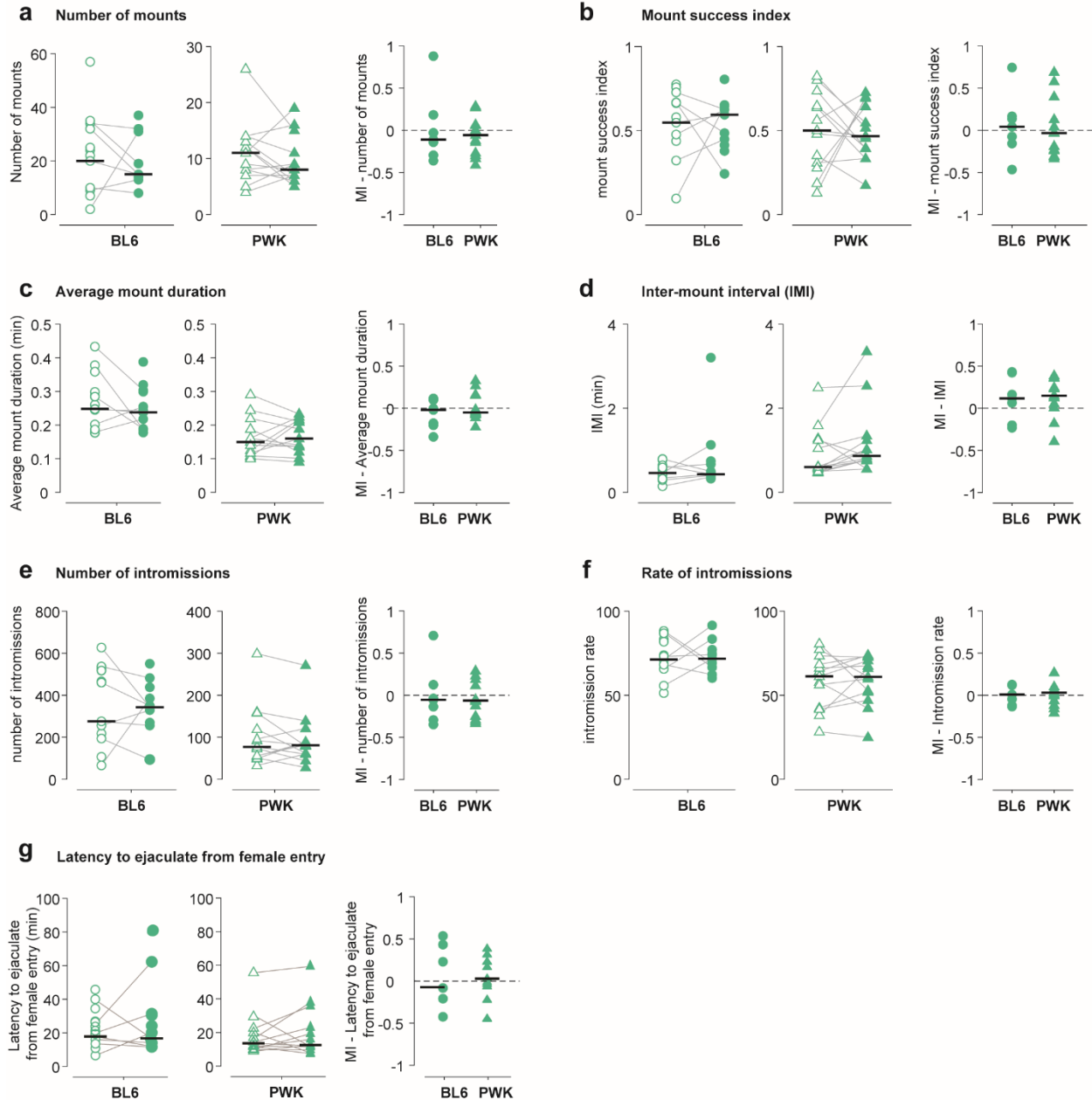
Valente et al.

Supplementary Data 1 Percentage of BL6 males that reached ejaculation.

Comparison of the percentage of BL6 males that ejaculated in the different type of experiments:

a) PRL monitoring: tail tip blood collection during the trial; b) PRL manipulation: substance injection prior beginning of the trial; c) un-manipulated males.

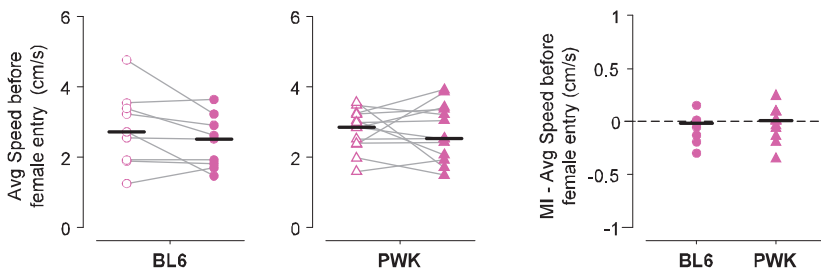
Experiment type		# males that ejaculated	Total number males	% males that ejaculated
a) PRL monitoring	Fig. 1a-b	8	23	34.8
b) PRL manipulation – Veh	Fig. 2c	13	17	76.5
b) PRL manipulation – Domp	Fig. 2c	11	17	64.7
b) PRL manipulation – Veh	Fig. 3c	15	18	83.3
b) PRL manipulation – Bromo	Fig. 3c	13	18	72.2
c) Un-manipulated	---	6	9	66.7



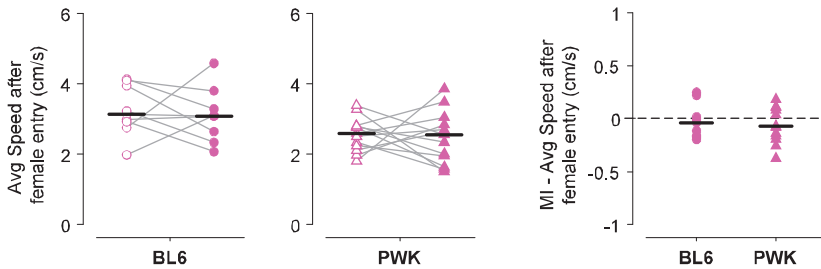
Supplementary Data 2 Additional analysis of the sexual behavior assay using adult sexually trained BL6 and PWK males pre-treated with vehicle or Domperidone (total number of subjects used: BL6 $n = 17$; PWK, $n = 13$; with ejaculation in both sessions BL6 $n = 7$; PWK, $n = 13$). Quantification of **a** number of mounts, **b** mount success index [mounts/(mount attempt + mounts)], **c** mount mean duration (total mount duration/#mounts), **d** Inter-mount interval (IMI), **e** number of intromissions, **f** rate of intromissions (#intromissions/total mount duration) and **g** latency to ejaculate from female entry. Each animal underwent two behavioral assays: one with vehicle and one with domperidone. Administration was counterbalanced between animals. Each line represents data of an individual; Unconnected dots represent data of an individual that did not ejaculate in the matching trial (data not used for statistics). MI [modulation index (domp-

veh)/(dom+veh)]. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test.

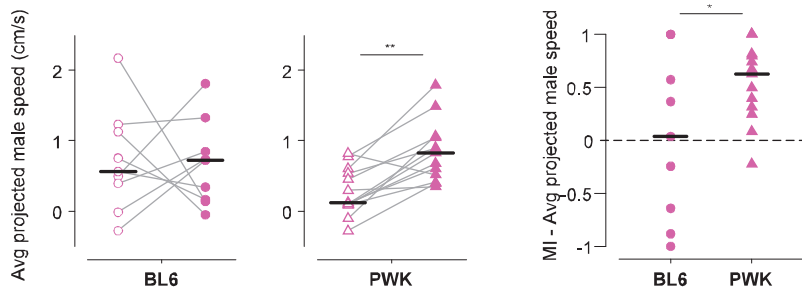
a Average speed before female entry



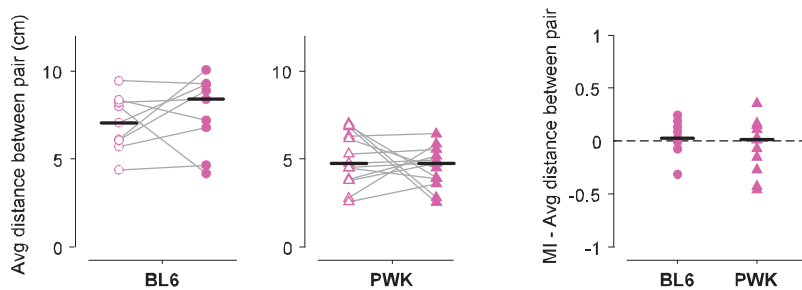
b Average speed after female entry



c Average male speed projected towards the female

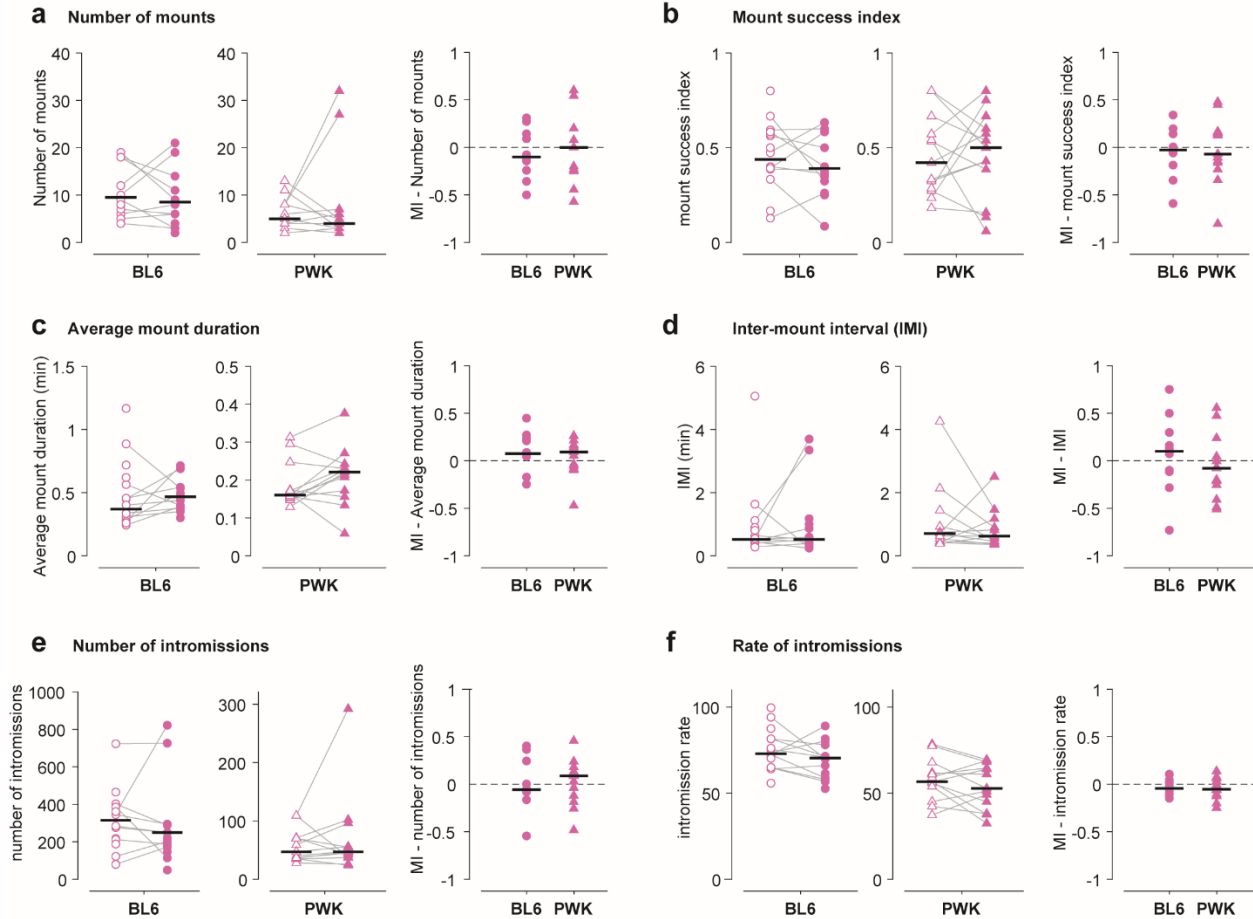


d Average distance between the pair



Supplementary Data 3 Bromocriptine treated animals do not show increased locomotion activity. Average speed of the male **a** before and **b** after the female entry until the first consummatory event. **c** Average male speed projected towards the female **d** average distance between the pair. Metrics were taken from couples that reached ejaculation in both sessions from bromocriptine assay BL6 n= 9, PWK n =13 (Fig. 3). Each line represents data of an individual; MI [modulation index (bromo-veh)/(bromo+veh)]. Data presented as median $\pm$

M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test. Significance was accepted at $P < 0.05$. * $P < 0.05$, ** $P < 0.01$.



Supplementary Data 4 Additional analysis of the sexual behavior assay using adult sexually trained BL6 and PWK males pre-treated with vehicle or Bromocriptine (total number of subjects used: BL6 $n = 18$; PWK, $n = 13$; with ejaculation in both sessions BL6 $n = 10$; PWK, $n = 13$). Quantification of **a** number of mounts, **b** mount success index [mounts/(mount attempt + mounts)], **c** mount mean duration (total mount duration/#mounts), **d** Inter-mount interval (IMI), **e** number of intromissions and **f** rate of intromissions (#intromissions/total mount duration). Each animal underwent two behavioral assays: one with vehicle and one with domperidone. Administrations were contra balanced between animals. Each line represents data of an individual; Unconnected dots represent data of an individual that did not ejaculate in the matching trial (data not used for statistics). MI [modulation index (bromo-veh)/(bromo+veh)]. Data presented as median $\pm$ M.A.D. (median absolute deviation with standard scale factor) following Wilcoxon rank sum test. Significance was accepted at $P < 0.05$.