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Final Dissertation

Impact of current breeding practices on the health and wellbeing of breeding mouse dam

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Madre, sei il mio basalto!
Padre, la mia ossidiana!
Oh fratello, tu sei il marmo.

Table of Contents

1 Riassunto

Si suppone che i topi utilizzati negli esperimenti di laboratorio siano nati da madri sane, ma non abbiamo nessun dato scientifico che lo dimostri. In letteratura, non ci sono dati che provino il benessere delle madri usate nei laboratori per allevare una colonia a fini sperimentali. I metodi di allevamento delle colonie sono strutturati in modo da massimizzare il successo riproduttivo e nella maggior parte dei casi sottopongono la madre a cicli concomitanti di gravidanza e allattamento. È stato provato che allattamento e gravidanza sono processi fisiologici che richiedono un elevato livello di energia (Butte & King, 2005), per questo motivo presupponiamo che gravidanze e periodi di allattamento consecutivi portino a stress metabolico e fisiologico nella madre. Sfortunatamente, non è stato effettivamente compreso l'effetto di multipli cicli consecutivi di gravidanza e allattamento.

Lo scopo principale della ricerca è determinare se gli attuali metodi di allevamento riducano il benessere della madre. La ricerca si pone l'obiettivo di studiare la salute fisiologica e il comportamento delle madri dopo 1, 2, o 4 cicli di gestazione e allattamento. Il peso della madre e della progenie è stato monitorato durante l'intero esperimento. Un *Pup Retrieval Test* è stato effettuato durante i giorni postparto tre, sei e nove. Infine, è stata svolta l'immunoistochimica sul cervello della madre per studiare la sensibilità alla leptina e per quantificare l'espressione di ossitocina nell'ippocampo.

Un effetto di numero di cicli di gestazione è stato osservato per quanto riguarda il peso corporeo della madre e la sua quantità di cibo ingerita quotidianamente. Madri che hanno avuto più di una gravidanza sono significativamente più pesanti rispetto a madri che hanno avuto una sola gravidanza e a vergini della stessa età. Tuttavia, è stato trovato che madri che hanno avuto più di una gravidanza ingeriscono giornalmente una quantità di cibo non sempre superiore rispetto a madri che hanno avuto una sola gravidanza. Il *Pup Retrieval Test* non ha mostrato nessun effetto di numeri di cicli di gravidanza. Tuttavia, è stato trovato che il comportamento materno durante il *Pup Retrieval Test* è più influenzato dal ceppo a cui appartiene la madre piuttosto che alla sua esperienza riproduttiva.

2 Summary

In Switzerland, 64% of experimental animals are mice, amounting to more than 369 436 mice used for experimental purposed in 2021 (Bundesamt für Lebensmittelsicherheit und Veterinärwesen BLV, 2021). We assume that these mice are born from healthy breeders, but we do not have solid evidence to support this notion. The current breeding methods are structured to maximise the reproductive success and can thus result in the breeding dam experiencing multiple cycles of concurrent pregnancy and lactation. We presume that concurrent pregnancy and lactation leads to some metabolic and physical strain on the dam, yet whether it affects the wellbeing of the dam is poorly understood.

The aim of this study was to determine whether the current breeding methods reduce the welfare of the mouse dams. The physiological health and behaviour of the dams were analysed after 1, 2, or 4 pregnancy cycles. Dam body weight and food intake were monitored during the entire experiment. Pup retrieval tests were conducted on postpartum day three, six, and nine. Finally, immunohistochemistry of dam brains was performed to analyse leptin sensitivity and quantify oxytocin expression in the hypothalamus.

An effect of number of pregnancy cycles was observed in the body weight and food intake parameters. Multiparous dams were significantly heavier than primiparous dams and female virgins and had a lower food intake than primiparous dams. We found that maternal behaviour during pup retrieval test is more influenced by strain than by reproductive experience. Immunostaining of mouse dam hypothalamus demonstrated the presence of neurons expressing oxytocin and leptin-responsiveness, yet further analysis is required to draw conclusions about the impact of concurrent pregnancy and lactation on these measures.

Keywords: Animal welfare; Pregnancy; Maternal behaviour; Breeding methods; Mouse dams

3 Introduction

3.1 Dam Welfare

The relatively little data that we have on the health and welfare of breeding rodent dams comes from threes field of research: there is the research that is directly geared toward improving experimental animal welfare , research inspired by the Developmental Origins of the Health and Disease (DOHaD) theory (Barker, 2007; T. P. Fleming et al., 2018), which used rodent models to investigate how maternal behaviour and the early environment impact the long-term health of the offspring (Ito et al., 2006; Kikusui et al., 2004), and ecological studies investigating maternal and reproductive health and behaviour in rodents living in more natural setting (König & Markl, 1987; Weber & Olsson, 2008). However, in each of these scenarios, the breeding mouse dam remains in the background of the biomedical research question.

For this reason, the effect of current breeding methods on the welfare of the dam is largely unknown. It may be that extent current breeding schemes affect their physical and psychological wellbeing. It has been proven that lactation and pregnancy are highly energy-demanding physiological processes (Butte & King, 2005), therefore we presume that their concurrent activity leads to some metabolic and physical stress on the dam. Unfortunately, while the effect of breeding protocols on the offspring (Foldi et al., 2011) and reproductive success (Firman & Simmons, 2008) has been documented, to date few studies have explored the impact of the current practices of mouse breeding on the health and wellbeing of the mother.

3.1.1 Gestational Stress

Gestational stress is the stress that a mother experiences during her pregnancy. It can be chronic, associated with long term stressors, or acute, related to rapid unexpected events. While there is no evidence on how breeding methods could influence the wellbeing of the mouse dam, there is a broad literature that shows how gestational stress, either chronic or acute, affects the welfare of the dam on ha behavioural (Darnaudéry et al., 2004) and physiological (Vanmierlo et al., 2018) level. One study demonstrated that chronic stress, as defined as three 45-min periods of restraint under intense illumination daily from days 14 to 21 of pregnancy, influenced the emotional reactivity of the dam, showing a reduction of exploratory behaviour in a new environment (Darnaudéry et al., 2004). Stress can also directly alter maternal care, for

instance licking and grooming (LG) behaviour in high LG mothers were reduced when exposed to gestational stress (Champagne & Meaney, 2006).

Stress experienced during pregnancy also lead to physiological alterations and disruptions in behaviour (Baker et al., 2008) and cognitive ability (Lordi et al., 2000) in offspring. In one study, female pups born from a stressed dam show elevated anxiety-like behaviour and attenuated weight gain (Baker et al., 2008). Similarly adult rats that were stressed prenatally showed high anxiety-like behaviour (Vallè et al., 1997). In another study, long-term spatial memory was altered in the offspring of chronically-stressed females (Lordi et al., 2000).

3.2 Differences between breeding in nature and in laboratory

Undomesticated house mice usually live in social groups with one dominant male and several reproducing females (Lidicker, 1976). Females generally reach sexual maturity earlier than males, typically at 5 to 6 weeks of age (van Zeegeren, 1980), or later under crowded or cold conditions (Crowcroft & Rowe, 1957). In females, oestrus cycle lasts between 4 and 6 days (Berry, 1970). Wild-living mice have a finite breeding season (Berry, 1968) and gestation lasts between 18 and 21 days (Berry, 1970). The first day of gestation is considered to be the day after the vaginal plug is seen (Hardy, 2004). Parturition usually happens overnight and about 14 to 28 hours after giving birth the dam has a post-partum oestrus cycle with an ovulation (Runner & Ladman, 1950). In a study by Ferrari et al. (2019), female mice of population of free-living house mice nurse and rear pups in the absence of the male mating partner and most (92%) choose to use communal nursing at some point during their reproductive lifespan. However, depending on the situation at birth, dams often switch between solitary and communal breeding, with older females more likely to use the first strategy and younger females more likely to use the latter. In the same study, on average, if the female mates again, she has a subsequent litter 67 days later and produces a total of 2.9 litter during her lifespan. Finally, the dam gradually weans the litter after 3 weeks and is done by day 25 (Williams & Scott, 1953).

In laboratory settings, the breeding mouse dam can be permanently housed with a male and become pregnant again during the first oestrus after parturition. This can result in concurrent cycles of pregnancy and lactation with an interbirth interval as short as 19 days. Thus, one consequence of permanent breeding techniques, which is one methos often used with laboratory mice, is that the dam is continuously experiencing pregnancy or lactation, or both

simultaneously. Another method is temporary breeding: the male is housed with the female and as soon as a vaginal plug is present, the impregnated female is removed from the cage and replaced by a new breeding female. It is possible to further categorise these techniques of breeding by the ratio of male to female breeders. In a monogamous cage, the ratio is 1:1, and in a polygamous cage, the ratio is 1:2-4.

In general, choice of breeding strategy depends on maximise the reproductive success in order to achieve research goals, research questions and evaluated from the perspective of colony productivity and maintenance (Dorsch et al., 2020). However, when conducting breeding protocols in laboratory settings, it is important to have a good knowledge of natural biology and behaviour of the species. Understanding and considering the natural setting of the experimental animal is important for its own sake and for the reliability of the research.

3.3 Maternal Behaviour

Maternal behaviour is essential for the healthy physical, emotional and social development of the offspring (Fleming et al., 1999). The mouse dam begins to develop maternal behaviours already in early gestation (Weber & Olsson, 2008), she will start constructing her maternal nest during the final three days of pregnancy (Brown, 1953), and continues to show acts of maternal care during the entire period of lactation (König & Markl, 1987).

Maternal behaviour can be divided in two categories: young-directed behaviours and non-directed maternal behaviours. The first refers to the direct interaction with pups and includes behaviour such as retrieval of the pups to the nest, pup-licking and grooming, crouching, and nursing. The second refers to any behaviours that positively influence the young without the direct contact of the mother, such as maternal aggression (i.e. protection of the pups and the nest), nest building, increasing food consumption, and reduced anxiety in exploratory context (Bridges, 2015). The dam is also capable of distinguishing her young from friendly or unfriendly conspecifics (Kinsley, 1994). Reproductively experienced females find pup odour and calls attractive and reinforcing (Meek et al., 2001), and these cues can indeed indict specific form of maternal behaviours, for example, the brief and higher frequency ultrasonic vocalisations of cold pups elicit retrieval behaviour (Smith, 1981). These responses, however, are not observed in inexperienced nulliparous female rats, because it seems that pup cues, as odours, actively inhibit pup-directed behaviour in adult rats that have not recently given birth (Fleming & Rosenblatt, 1974).

3.3.1 The regulation of Maternal Behaviour

The onset of maternal behaviour is regulated by several hormones and endocrine changes during pregnancy and parturition. Progesterone, oestradiol, and prolactin have a key role in promoting the induction of maternal care. One of the main functions of progesterone is controlling the timing of increased responsivity to pup stimuli. Circulating levels of progesterone are elevated throughout the first half of pregnancy and decline either just prior to parturition or at birth (Bridges, 1984). It has been shown that female rats become more primed to respond maternally to foster rat pups shortly before the onset of parturition, when circulating levels of progesterone decline (Slotnick et al., 1973). Oestradiol levels start rising during the second half of pregnancy, when the decline of progesterone starts (Morishige et al., 1973). In one study, injections of virgins rats with higher doses of oestradiol benzoate (EB) stimulated maternal care towards foster young (Siegel & Rosenblatt, 1975). Similarly, subcutaneous injections of ovine prolactin elicited a faster onset of maternal care towards foster pups (Bridges et al., 1985). This evidence shows that these above-mentioned hormones can individually affect the expression of the maternal behaviour, but it is also noteworthy that they work in combination to influence the onset of maternal care, as they are simultaneously changing to affect the physiology and behaviour of the dam.

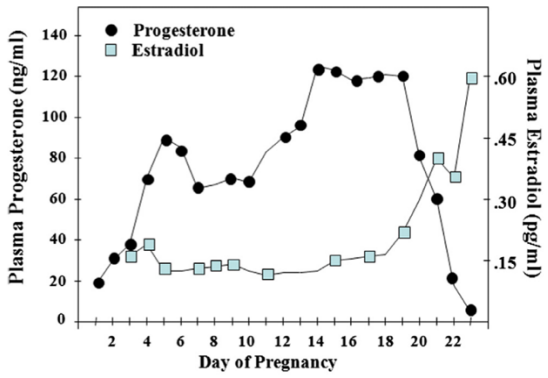

Figure 1: Oestradiol and Progesterone. Hormone profiles of oestradiol and progesterone during pregnancy in the rat. Adapted from Bridges R.S., 1990. Endocrine regulation of parental behaviour in rodents. In: Krasnegor, N.A., Bridges, R.S. (Eds.), Mammalian Parenting: Biochemical, Neurobiological, and Behavioral Determinants, Oxford University Press, New York, pp. 93–117, with permission; and taken from Bridges, R.S., 2015. Neuroendocrine regulation of maternal behavior. Frontiers in Neuroendocrinology, volume 36, pp. 178-179.

3.3.2 Neural pathway of Maternal Behaviour

Maternal behaviour is regulated through several neural pathways. The medial preoptic area (MPOA), a region in the hypothalamus, plays a key role in the induction of maternal behaviours (Numan, 1974). The MPOA contains receptors for the hormones that activate both the motor and motivational aspects of maternal care (Morgan et al., 1997). This region of the brain goes through substantial changes with pregnancy and maternal experience, and these alterations facilitate the onset of the maternal behaviour. the hormones oestradiol and prolactin prime the

brain of the dam, especially the MPOA, and make it responsive to pup stimuli. Hence, pup stimuli engage the MPOA neurons which project to the ventral tegmental area (VTA), which is the origin of mesolimbic dopamine (DA) neurons and part of the mesolimbic dopaminergic system. Stimulation of the VTA thus activates the dopaminergic projections to the nucleus accumbens (NA) (Numan & Stolzenberg, 2009). The VTA-DA to NA neural pathway is known to be important for reward seeking behaviours (Kelley & Berridge, 2002) and regulates maternal attraction and approach towards pups (Numan, 2012).

Additionally, the NA-ventral palladium (VP) circuit receives olfactory, gustatory, tactile, auditory, and visual stimuli form the basolateral and basomedial amygdala (BLA / BMA), another significant region for the regulation of maternal behaviour (Numan et al., 2010). The VP receives projections form the BLA / BMA, NA and the prefrontal cortex (PFC) and projects to the brainstem and forebrain motor areas (Numan, 2012).

Finally, the paraventricular nucleus (PVN) of the hypothalamus is also involved in the regulation of maternal behaviour. This region has oxytocin (OXT) neurons that project to several brain regions, including MPOA, VTA, and NA. it is possible that the reciprocal projections between the MPOA and the PVH both facilitate OXT release and enhance the effect of OXT neurons in their projected regions of the brain (Numan, 2012).

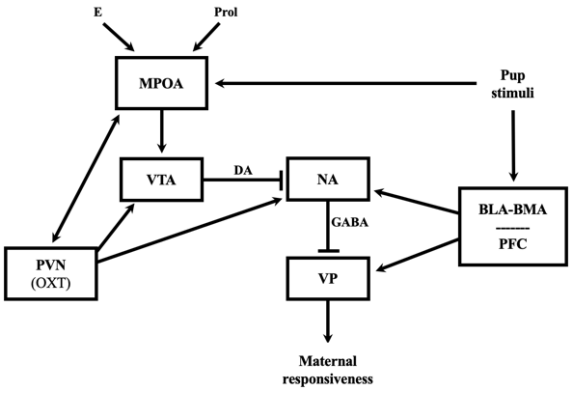

Figure 2: The Maternal Behaviour Circuit. Schematic representation of key neural regions and connections that constitute the maternal neural network. Arrows ending in a bar or arrow represent inhibition and excitation respectively. BLA-BMA, basolateral/basomedial amygdala; DA, dopamine; E, oestradiol; MPOA, medial preoptic area; NA, nucleus accumbens; PVN, paraventricular nucleus of the hypothalamus; Prol, prolactin; VP, ventral pallidum; VTA, ventral tegmental area. Adapted from Numan, M., 2012. Maternal Behavior: Neural Circuits, Stimulus Valence, and Motivational Processes. Parenting, volume 12(2-3), pp. 105-114.

3.4 Analyses of Maternal Behaviour

Experimental studies investigating maternal behaviour of mice typically focus on young-directed behaviours. When analysing maternal behaviour, there is a broad repertoire of maternal behaviours to look at: carrying, retrieving, licking and grooming pups, nursing, building the nest, and quantifying the time spent on the nest. Key readouts for the quality of

maternal care include latency to retrieve pups to the nest, representing appetitive maternal behaviour, while licking and grooming behaviour (LG) and arched-back nursing (ABN) are consummatory behaviours of particular interest (Bridges, 2015; A. S. Fleming et al., 1999). For example, in a study conducted by Menard et al. (2004), adult rats born from a high LG mother showed lower levels of behavioural fearfulness. Additionally, it has been shown that variations in both LG and ABN modify hippocampal glucocorticoid receptors in offspring, resulting in an altered function of the hypothalamic-pituitary-adrenal (HPA) axis, and in cognitive and emotional impairment (Fish et al., 2004).

During a maternal behaviour test, many factors can influence the behaviour of the dam, including age, size, and sex of the pups, age of the mother, and size of the testing cage (Caldji et al., 1998; Francis & Kuhar, 2008). For this reason, housing conditions and litter size and composition should be kept consistent across all experimental animals to avoid these confounding factors. This thesis will focus on the pup retravel test.

3.4.1 Pup retrieval test

Unlike undisturbed observation of home-cage maternal behaviour, the pup retrieval test commences with a short separation of the pups from the dams. The pups are then returned to the home-cage opposite of the maternal nest, and latencies to sniff and retrieve the pups are recorded, as is the time the dam spends on the nest. The tests are usually conducted during the first two weeks postpartum to determine whether experimental manipulations affect the natural display of maternal behaviours. The main purpose of maternal behaviour tests is to see if the time spent taking care of the pups and the behavioural pattern of the experimental dam differ from that observed in a control dam.

Latency to retrieve the pups is considered an indication of maternal motivation. Newly parturient dams under the influence of maternal hormones are more motivated to engage in maternal care and show efficient retrieval of all pups in the cage (Rees et al., 2004). However, as mentioned above, maternal responses towards pups can be altered if the dam goes through stressful events or if critical hormonal changes around parturition are blocked (Bridges, 2015). In one study, the latency to retrieve the first pup was always higher in stressed dams compared to control dams (Patin et al., 2002), indicating reduced maternal motivation.

3.5 Importance of hormones during pregnancy and lactation

During pregnancy and lactation, changes in circulating hormone levels and sensitivity to them alter the body and behaviour of the mother, thus facilitating the expression of maternal behaviour and allowing the mother to sustain the extreme metabolic demand of gestation and lactation. The thesis will focus on two such hormones: leptin and oxytocin.

3.5.1 Leptin and p-STAT3

Leptin is a hormone produced by white adipose tissue. Leptin levels in the blood positively correlate with the body mass index (BMI) and the percent total body fat (Schwartz et al., 1996). It activates the arcuate (ARC), ventromedial (VMH) and dorsomedial (DMH) hypothalamic nuclei and the brainstem neural circuits implicated in the regulation of feeding behaviour and energy balance (Elmquist, 2001; Elmquist et al., 1997, 1998). The ARC is a key controller of energy homeostasis and is considered a main target of leptin action (Hübschle et al., 2001).

Leptin alters food intake, controls energy expenditure, and manages the balance between food intake and energy used (Friedman, 2019; Hwa et al., 1997). When leptin levels are high, thus signalling to the brain about the status of increasing body energy stores, food intake decreases and energy expenditure increases in order to maintain an optimal level of body fat stores (Halaas et al., 1995; Pelleymounter et al., 1995). Some studies show that leptin modifies behaviours related to food intake, such as time spent looking for food hidden inside the cage after a period of food deprivation, but not related to the actual consumption of the food (Figlewicz et al., 2001; Getchell et al., 2006). Leptin also mobilises lipid stores and stimulates the oxidation of fatty acids to regulate energy availability in mammals (Minokoshi et al., 2002; Siegrist-Kaiser et al., 1997).

It is important to mention that leptin activated leptin receptors in the hypothalamus through several pathways, JAK-STAT3 pathway is considered to mediated the major action of leptin in energy regulation (Liu et al., 2021). The long-form leptin receptors are located in the ARC, VMH, and DMH and colocalise with neuropeptides that mediate leptin action, such as neuropeptide Y (NPY) and proopiomelanocortin (POMC) (Baskin et al., 1999; Elmquist et al., 1998; Mercer et al., 1996; Schwartz et al., 1996). Leptin-activated phosphorylation of STAT3 (signal transducer and activator of transcription 3), as demonstrated in the hypothalamus of lean wild-type animals (Vaisse et al., 1996), is a marker frequently used in the assessment of central leptin signalling. Notably, leptin-induced phosphorylated STAT3 (pSTAT3) is reduced

in the case of leptin resistance (Morris & Rui, 2009). In one study using pSTAT3 immunohistochemistry, leptin-activated pSTAT3 increased in the ARC of lean mice, but not in that of DIO (diet induced obese) mice (Münzberg et al., 2004). pSTAT3, as a transcription factor, binds to and regulates its target gene such as POMC gene, playing the physiological function of leptin (Xu et al., 2007).

Additionally, an increase in levels of circulating leptin are also correlated with the advent of reproductive maturity and fertility (Henson & Castracane, 2003). Furthermore, concentrations of serum leptin are elevated throughout human pregnancy, notably increasing during the first trimester (Henson & Castracane, 2006). During pregnancy, leptin is also produced by the placenta trophoblasts (Masuzaki et al., 1997), and is synthetised by the mammary epithelium, secreted in colostrum, and absorbed by the foetus (Casabiell et al., 1997). Studies have also demonstrated a relationship between leptin and early embryonic development, implantation, and the regulation of foetal growth (Christou et al., 2001; Hoggard et al., 2001).

Last, pregnancy is a state of positive energy balance, and similar to obesity (Levin & Dunn-Meynell, 2002), it is characterized as a state of leptin resistance (Ladyman et al., 2012; Ladyman & Grattan, 2005): the brain does not respond to rising leptin levels, and consequently the caloric intake increases. During gestation and lactation, food intake is increased to meet the extreme metabolic demands of the growing fetus and then to produce milk to supply offspring. However, despite the dramatic changes observed during pregnancy and lactation, the leptin resistant state associated with pregnancy is temporary, in fact the long-term food intake after the period of gestation and lactation does not differ from age-matched virgin controls (Ladyman et al., 2018). Still, exactly when leptin resistance is reversed after pregnancy, and whether number of pregnancies or maternal body weight impact the reversal is not known.

3.5.2 Oxytocin

OXT is a neuropeptide hormone that is synthesised in the PVN and supraoptic (SON) hypothalamic nuclei, and released form the posterior pituitary gland into the blood stream and via nerve terminals into the brain (Wang et al., 1995). Additionally, OXT is synthesised in various peripheral tissues, including the uterus, placenta, amnion, and corpus luteum (Arrowsmith & Wray, 2014). In the brain, the MPOA and VTA are targets of OXT action (Pedersen et al., 1994).

Key functions of OXT are the stimulation of uterus contraction during parturition and milk ejection during lactation (Cunningham et al., 1991). The synthesis and the release of OXT are stimulated by suckling stimuli and parturition (Douglas et al., 1998; Zingg & Lefebvre, 1988). OXT is also important for the expression of maternal behaviour. It has been shown that an intraventricular injection of OXT in the brain of a virgin female rat induced a rapid onset of maternal behaviour (Pedersen et al., 1982). In one study, single-housed virgin females injected with OXT showed pup retrieval behaviour (Marlin et al., 2015). Moreover, the injection of OXT-antagonist into the cerebral ventricles suppresses the rapid onset of postpartum maternal behaviour. in one study, parturient dams treated with an infusion of an OXT-antagonist directly into the MPOA and VTA did not show pup retrieval behaviour and nor did they assume a nursing position (Pedersen et al., 1994).

It is possible that OXT directly alters neural pathway and behavioural output to facilitate the expression of maternal behaviour. A positive correlation between better maternal care and oxytocin levels in the PVN and the MPOA was in fact reported in one study (Shahrokh et al., 2010). The same study also showed that blockade of OXT receptors in the VTA reduces pup licking and grooming. Finally, in order to study the OXT levels in the brain, most of the study conduct immunostaining on the PVN (Carcea et al., 2021; Marlin et al., 2015), which reflects the levels of central OXT production.

3.6 Aim of the Study

As we established in the precedent sections, we do not know if current mouse breeding practices preserve the welfare of the dam or if the dam could indeed benefit physically or psychologically from a specific breeding configuration. It may be that the current breeding strategies have a negative impact on the welfare of the reproductive dam, thereby it is important to understand whether certain breeding conditions are better than others in respect to the dam's wellbeing. Thus, the aim of the greater project is to investigate the impact of continuous breeding practices on the health and wellbeing of mouse dam by analysing behavioural and physiological outcomes. Within the context of this research thesis, dam body weight and food intake were monitored and analyzed in C57BL/6 and BALB/c female mice after 0 (age-matched virgin controls), 1, 2, or 4 consecutive cycles of pregnancy and lactation. Pup retrieval tests were conducted on postpartum day three, six, and nine, and the presence of differences in behaviour was assessed as a function of reproductive experience or mouse strain. Finally,

immunohistochemistry of dam brains was conducted to analyse central leptin sensitivity and quantify oxytocin expression in the hypothalamus.

Overall, the findings of the greater study will help determine whether there is a need to formulate new recommendations for mouse breeding strategies. However, the goal of the project is not only to assess animal welfare and wellbeing, but also to promote and apply 3R principles (Russell & Burch, 1959) to breeding rodents.

4 Material and Methods

4.1 Study protocol

To study the impact of consecutive periods of pregnancy and lactation on the wellbeing and health of the mouse dam, multiple endpoints related to the dams' metabolic, nutritional, and behavioural status will be analysed (See Figure 3A, green and blue boxes). This large study is conducted across four runs; this thesis will include the analysis of a portion of the endpoints collected during the first two runs. Figure 3 shows the timeline of experimental Aims 1 and 2 (see "Study objectives"). Figure 3A shows the timeline for periods of pregnancy (P) and lactation (L) for a single run of Aims 1 and 2, which will be conducted in parallel. The data collected from dams that wean their first litter at three weeks (3W), will be used for comparison in both Aims. Since we combined the data of primiparous dams with different weaning time (3W and 4W), the thesis focuses on the impact of 1, 2, and 4 cycles of pregnancy, and an additional analysis will be conducted on the impact of the lactation duration. Figure 3B shows how the four runs that are required to complete Aims 1 and 2 are temporally aligned. By conducting the experiments in multiple batches, we aim to improve reproducibility, which was previously demonstrated (von Kortzfleisch et al., 2020). Furthermore, the timing of the batches allows the inclusion of male and female offspring as breeders in subsequent runs, minimizing the total number of surplus mice generated by the study.

Figure 3A

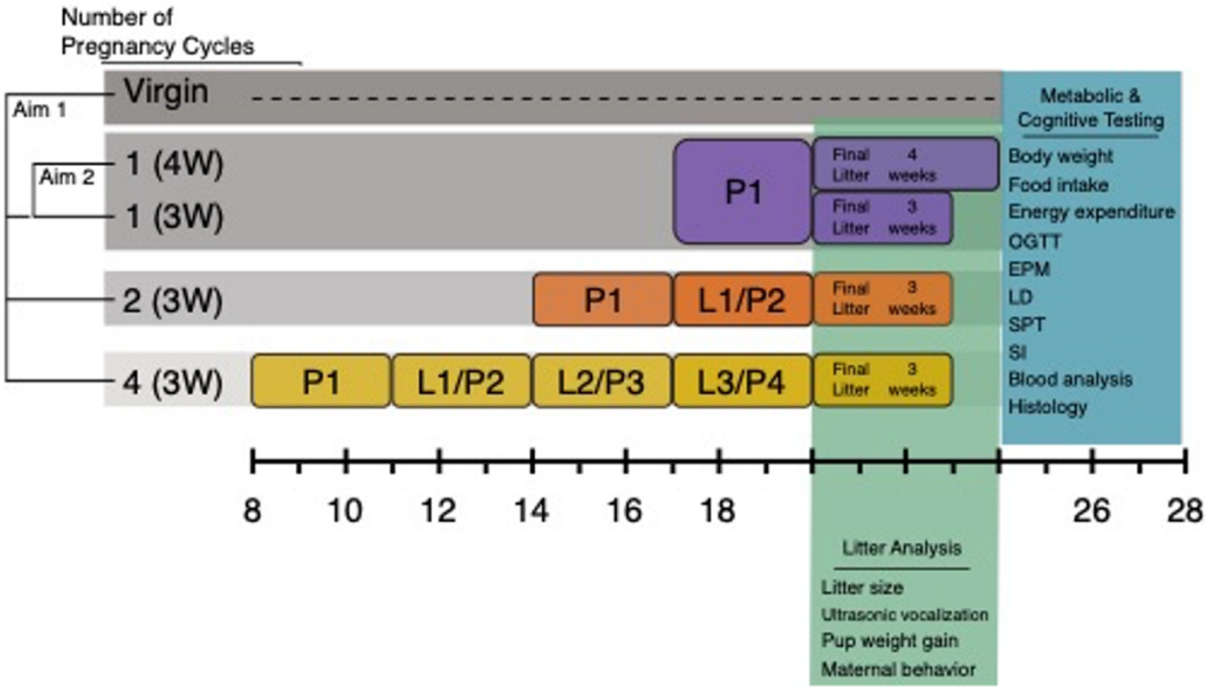

Figure 3B

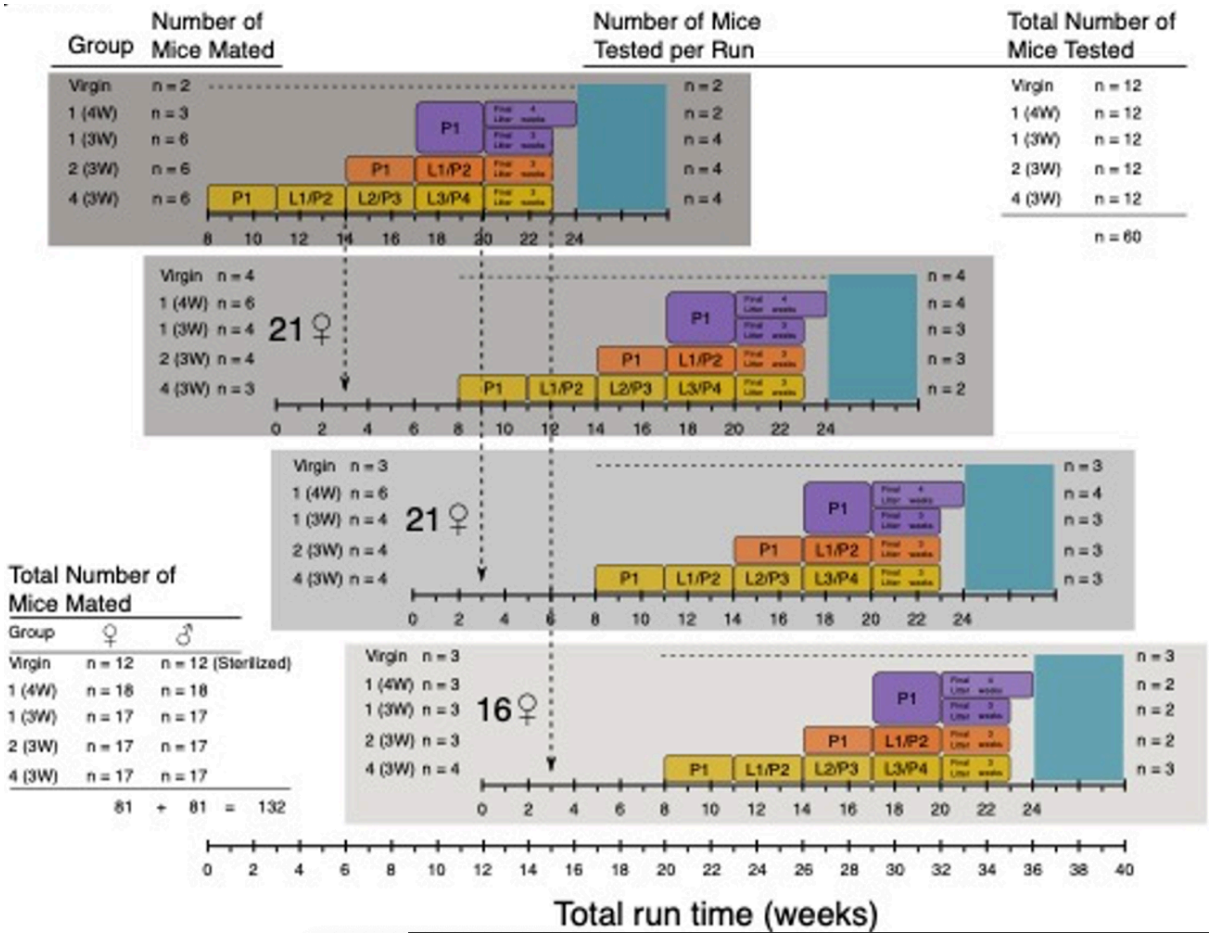

Figure 3: Timeline of Experimental Aims 1 and 2. Figure 1A depicts the structure of one of the four runs to complete Aims 1 and 2; Figure 1B shows how the four runs will be aligned to maximise use of offspring for subsequent breeding. For Aim 1, the primary variable is the number of pregnancy and lactation cycles (P and L1, 2, or 4), and for Aim 2, the primary variable is weaning time after 3 (3W) or 4 weeks (4W). Following the final weaning, metabolic and cognitive parameters will be assessed in postpartum dams over a 4-week testing period. Dams will be single-housed for one week to measure food intake and energy expenditure, but will be otherwise group-housed until the time of sacrifice and collection of blood and tissue. Abbreviations: OGTT – oral glucose tolerance test; EPM – elevated plus maze; LD – light-dark box; SPT – sucrose preference test; SI – social interaction test. The figure depicts the full-length of the project and indicates all the experiments conducted un the project. For this thesis, body weight, food intake, maternal behaviour and brain histology will be the main variable of interest.

4.2 Study objectives

The primary objective of the project is to determine whether current permanent breeding practices reduce the welfare of female breeding dams. The thesis focuses on the impact of number of breeding cycles [1, 2, or 4] determined in BALB/cByJ and C57BL/6J female mice randomly assigned to a breeding condition or age-matched virgin control condition. Addressing the following experimental aim will complete this goal.

Aim 1: Analyse the physiological health and behaviour of age-matched breeding dams after the weaning of one, two, or four consecutive litters and compare to virgin females.

We hypothesise that four rounds of consecutive pregnancy and lactation will have a negative impact on the health and wellbeing of the dams, when compared to age-matched virgin females or primiparous dams. However, due to lack of empirical evidence on how breeding practices affect the dams' health, the study is largely exploratory.

4.3 Animals

The experiment was performed in BALB/cByJ (n = 12) and C57BL/6J (n = 17) mice. To avoid unforeseen bias, all experimental breeders were born in-house (UZH LASC animal facility in Schlieren ZH) to founding breeders that were purchased from Charles River Laboratories (France). Mice were screened and entered the experiment when they were clear of any physical abnormalities, demonstrated stable or increasing bodyweight, and did not demonstrate stereotypies. Mice were housed in standard IVC cages (Allentown T 2L) with tissue, crinkles and a red house for nesting, a wooden stick for gnawing, and ad libitum access to the facility's standard breeding chow (Kliba 3338) and water. Mice were housed in a temperature-controlled environment (21 ± 2°C) under a 12/12-h light-dark cycle (lights off at 11:00 am). Mice were housed in pairs (either female-female or female-male) or with their offspring. The female mice in the virgin group were housed in pairs. Housing conditions were designed to match the standard housing conditions of breeding animals on the OHB breeding floor at the LASC Schlieren facility and used a similar schedule and conditions of routine animal husbandry during the breeding phase of the experiment used. To identify group-housed mice, tails were marked with non-toxic marker, which was reapplied as needed.

Due to limited available data on breeding dam welfare, the empirical experiments are exploratory. We have thus applied the Fermi methods of approximation to estimate sample size (Reynolds, 2019), based on feasibility and resources, in order to estimate how many dams are needed not only for welfare evaluation but also to populate the females subjects for the ensuing run or experiment.

For all the analyses described, mice were randomly assigned at the start of each run to one of the following breeding conditions: (1) 1 cycles of pregnancy and lactation with weaning at 4 weeks (n = 2), (2) 1 cycle of pregnancy and lactation with weaning at 3 weeks (n = 10), (3) 2 cycles of pregnancy and lactation with weaning at 3 weeks (n = 9), (4) 4 cycles of pregnancy and lactation with weaning at 3 weeks (n = 8). Group sizes given here reflect the animals that were analysed in the context of this thesis, which represent only a portion of those analysed for

the entire experiment. The identity cards of the breeding females, their biological samples and behavioural recording were numerically coded and analysed by researchers blind to the group identity.

The female and male breeders were not used beyond this project, yet a portion of the offspring produced by the experimental breeding was used as breeders in subsequent batches of the experiment. For remaining surplus offspring, we offer them internally to other research groups in the same animal facility, and on the Swiss AniMatch animal sharing platform. When these are not viable options, we seek to offer the cadavers of surplus offspring to Zurich Zoo.

4.4 Monitoring of pregnancy and assessment of food intake and body weight

Dams were checked three times a week. On Monday, Wednesday and Friday, females, males, and food were weighed manually. Around expected birth days, cages were checked each morning between 8.00 and 11.00 for litters. Criteria for defining a pregnant mouse were a weight gain of at least 20% and the presence of two visible lateral lumps on the belly of the dam. Following confirmation of pregnancy, the male breeder was removed from the cage on estimated gestation day 17, to avoid the possibility of another pregnancy.

If pups were observed in the cage or the dam was in the process of giving birth (with some pups in the nest), this day was designated as postpartum day 1 (P1); dams and pups were left undisturbed. On P2, the litter was weighted, and the number and sex of the pups were recorded. On P3, P6, P9, P12, P15, P18, and P21 weights of the dams, litters, and food were recorded starting around 11.00. Litter parameters were not analysed for this bachelor thesis but will be analysed for the larger project.

4.5 Pup retrieval test

To assess maternal motivated behaviour, a pup retrieval test was conducted in the early dark phase (between 11.00 and 13.00) on P3, P6, and P9, and each session was recorded with an infrared webcam (Raspberry Pi NoIR camera RPi-CAM-V2 Night Vision connected to a Raspberry Pi 3 Model B+ board). Before the start of the test, the food, the dam, and the litter (in this order) were weighed, without moving the nest. To reduce any influence of the experimenter on the scent of the pups or the dam's behaviour, gloved hands were rubbed with bedding from the cage before taking out the pups. When possible, four pups (two female and two male pups) were removed from the litter and kept under warming red light for ten minutes.

If there were fewer than two male and two female pups in the litter, the experimenter removed up to four pups, regardless of the ratio between male and female. The food grid was removed from the cage, the position of the nest was noted, and the cage was moved to the corresponding rack for the video recording. The video was always taken from the long side of the cage. Ten minutes after the pups were separated for the dam, the video recording was started. Pups were then placed in the two furthest corners from the nest: female pups were put in one corner and male pups in the other corner. The corner position of the female and male pups was switched from left to right for every test and noted each time. The video was stopped six minutes after the first pup was added to the cage.

4.5.1 Analysis of the pup retrieval videos

The video were analysed using the Behavioural Observation Research Interactive Software (BORIS), a free, versatile open-source event-logging software for video coding and live observations (Friard & Gamba, 2016). Five minutes of each video were coded. The video analysis started once the hands of the experimenter were completely off the cage. If the dam had already approached the pups when experimenters' hands were still in the cage, the moment the pups were added to the cage was considered as the start point for the coding of the video. The behavioural parameters analysed are shown in Table 1.

Behaviour	Definition
Latency to retrieve first pup	Time from adding pups until the retrieval of the first pup to the nest
Latency to retrieve all pups	Retrieving time of pups from first pup grabbed until the last pup in the nest
Latency to sniff the pups	Time from adding the pups until the dam started sniffing the pups
Time spent sniffing the pups	Time spent sniffing the pups before starting the retrieval
Time spent on nest	Time spent overall on nest within 5 minutes
Time spent on nest pre retrieval	Time spent on nest before retrieving all pups
Time spent on nest post retrieval	Time spent on nest after retrieving all pups

Table 1: Definition of maternal behaviours analysed in the pup retrieval test.

An individual behaviour was initiated as soon as the behaviour was observed. In the case of time spent on the nest, the beginning and the end of the behaviour was set once the upper body of the dam was respectively inside and outside the nest. If the dam was seen building the nest, this was coded as time spent on the nest even if the upper body of the dam was outside of the nest.

The observer coding the videos was blinded from the identity of the dam. After rating all videos from P3, P6, and P9 and after rating all videos, the first three videos from each test day were reevaluated to control for intra-observer reliability. 95% agreement was reached between scoring sessions. After coding, the time budget was exported from BORIS, output data were analysed with Microsoft Excel and R softwer. Data were plotted using R software.

4.6 Sacrifice and Perfusion

The day of the sacrifice, at 8.00 the food was removed from the cage of the dam. Starting from 10.15, a single intraperitoneal (i.p.) dose of leptin (2 mg / kg; Murine Leptin; 5 mg / ml; i.p.; Lot # 012176 A2821; PeproTech; UK; Diluted in 2.5 ml phosphate-buffered saline (PBS), 0.01, pH 8.0) was injected in one dam every ten minutes. Forty minutes after leptin injection, dams were injected with a single i.p. injection of 300-350 mg / kg Pentobarbital (Pentobarbital-Na 50 mg/ml, Formula Magistralis, Apotheke Tierspital Zürich, Winterthurerstrasse 260, 8057 Zürich). Five minutes after pento injection, after pinch reflexes were lost, collection of tissues started. First, an ear sample was taken and stored in dry ice. Then, the chest and the rib cage of the dam were opened with scissors and approximately 1 ml of blood was collected from the right atrium with a 25G needle. In the case this was not possible due to sampling complications, intracavity blood was collected and noted. Blood was directly cooled on ice. Following the blood sample, two tissue pieces from different lobes of the liver were taken, frozen in liquid nitrogen and stored at -80°C. To restrict the perfusion of flush and fixative to the upper body, the descending aorta was clamped. A 24G needle was inserted into the left ventricle of the heart. The animal was first flushed with 0.1 M ice-cold phosphate buffer (PB) for 1.5 minutes, followed by 2.5 minutes fixation with ice-cold 2% paraformaldehyde (PFA).

After the perfusion, the abdominal cavity was open to collect visceral fat and uterus, the tail tip was taken, and finally the brain was extracted. Visceral fat and uterus were immediately frozen in liquid nitrogen and stored at -80°C. Tail samples were directly stored in dry ice. Brains were put into 2% PFA on ice and then soaked in 30% sucrose solution in 0.1 M PB, pH 7.4 (Sucrose; ACS reagent; S5016-2.5KG; SIGMA-ALDRICH; Switzerland) at 4°C until the brains had sunk. Brains were blocked into fore- and hindbrain, snap-frozen in hexane (Hexane; Fluka Chemie GmbH; Switzerland) on dry ice for four times and stored at -80°C until further processing. Femur, tibia, and fibula of both legs from each dam were extracted and stored in 4% PFA on ice. Blood samples were centrifugated for 10 minutes at 2000 rpm, plasma was transferred into clean tubes and immediately frozen on dry ice and stored at -80°C.

Samples of ear, blood, liver, abdominal fat, tail, and bones were not analyzed for this bachelor thesis, but frozen for the purpose of the whole project.

4.7 Immunohistochemistry

The brains of the dam were sliced in coronal sections (30 µm) collecting the following regions: nucleus accumbens – NAc – (Bregma +1.54 to +0.86), then medial preoptic area of the hypothalamus– MPOA – (Bregma +0.74 to -0.58) and finally the paraventricular hypothalamic nucleus – PVN – and the arcuate nucleus – ARC – (Bregma -1 to -2.7). Sections were collected into 4 series using cryostat (Leica CM3050 S; Biosystems; DE) and mounted directly onto Superfrost® glass slides (Superfrost® Plus; Thermo Scientific; DE). Regions of the brain were defined based on Paxinos and Franklin's the mouse brain in stereotaxic coordinates (Franklin & Paxinos, 2013). The slides were then stored in slide holders containing cryoprotectant (20% glycerol, 30% ethylene glycol, 50% 0.02 M phosphate buffer (PBS), pH 7.4) at -20°C until further processing. For the purpose of this research thesis, only the PVN and the ARC were immunostained.

A double-staining protocol was performed on the slides. On day 1, slides were rinsed in 0.02 M KPBS, blocked in 4% normal donkey serum (NDS), 0.4% Triton and 1% bovine serum albumin (BSA) in KPBS for 20 min at room temperature. Then, sections were incubated in α-pSTAT3 (1:500; Cell Signaling; Rabbit mAb #9145) & α-Oxytocin (1:1000; Chemicon; Mouse Ab #MAB5296) in 1% NDS, 0.4% Triton and 1% BSA in KPBS for 44 hours at 4°C shielded from the light. On day 3, sections were incubated in donkey-α-rabbit-Alexa555 (1:100; Jackson ImmunoResearch) & donkey-α-mouse-Alexa-488 (1:100; Jackson ImmunoResearch) in 1% NDS and 0.3% Triton for 2 hours at room temperature. Slides were then rinsed again in 0.02 KPBS, then counterstained with DAPI (40 µl in 250 ml of KPBS) for 4 minutes at room temperature and finally washed in KPBS. Slides were covered with Vectashield and stored at 4°C, shielded from light until further analysis.

4.7.1 Image acquisition

All brain tissue sections analysed were viewed on a Zeiss Axios Scan.Z1 (Axio Scan.Z1, Carl Zeiss Microscopy GmbH, 37081 Göttingen, Germany). Using QuPath software (Bankhead et al., 2017), regions of interest were identified and photographed. Neurons positive for pSTAT3 and oxytocin will be quantified and analysed at a later time for the purpose of the whole project.

4.8 Statistical Analysis

For all statistical analyses R software was used. Body weight and food intake data are represented as mean ± SD. Pup retrieval parameters are represented as Interquartile Range (IQR). Two-way-ANOVA was used to assess the statistical significance of body weight, food intake and pup retrieval test. P-values ≤ 0.05 were defined as statistically significant.

5 Results

5.1 Postpartum dam body weight increases with number of pregnancy cycles

As shown in the Figure 4A, dams have generally higher body weight compared to virgin control mice. Multiparous dams have higher postpartum body weight than primiparous dams, notably the group of dams that experienced 4 cycles of pregnancy had the highest body weight compared to dams with 1 cycle or 2 cycles of pregnancy. From the graph, we can see that body weight increases from P3 to P15, and while it then decreases after P15, interestingly, the dams remain heavier on P21 compared to the initial postpartum body weight of P3. An overall effect of time (Df = 6, $F_{162.5,\ 27.1}$ = 1.153e+29, P < 0.0001), number of pregnancy cycles (Df = 3, $F_{1642.7,\ 547.6}$ = 2.330e+30, P < 0.0001) and time x number of pregnancy cycles interaction (Df = 18, $F_{29.1,\ 1.6}$ = 6.876e+27, P < 0.0001) on body weight was found (Figure 4).

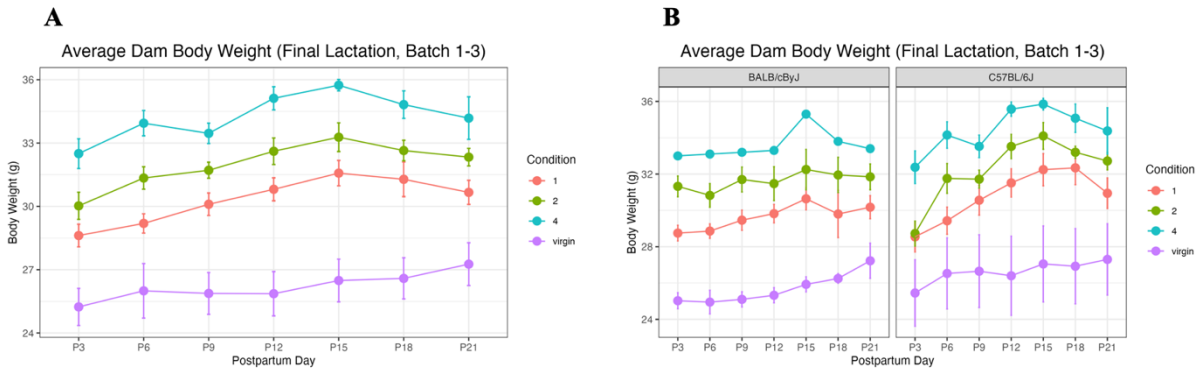

Figure 4: Body weight during postpartum. A) Body weight (g) changes in dams that experienced 1, 2, or 4 cycles of pregnancy and in virgin control mice on postpartum day (P) 3, P6, P9, P12, P15, P18, P21 and **B)** the same data separated by strain: BALB/cByJ and C57BL/6J. **(A-B)** Data are represented as mean ± SD.

As shown in the Figure 4B, the same tendencies were observed when analysing the body weight in both BALB/cByJ [effect of time (Df = 6, $F_{59.1,\ 9.85}$ = 1.529e+30, P < 0.0001), number of pregnancy cycles (Df = 3, $F_{623.3,\ 207.77}$ = 3.228e+31, P < 0.0001) and time x number of pregnancy cycles interaction (Df = 18, $F_{12.0,\ 0.66}$ =1.032e+29, P < 0.0001)] and C57BL/6J dams [effect of time (Df = 6, $F_{106.5,\ 17.8}$ = 6.219e+28, P < 0.0001), number of pregnancy cycles (Df = 3, $F_{988.0,\ 329.3}$ = 1.153e+30, P < 0.0001) and time x number of pregnancy cycles interaction (Df = 18, $F_{16.6,\ 0.9}$ = 3.239e+27, P < 0.0001)].

5.2 Postpartum food intake is higher in primiparous dams compared to multiparous dams

As shown in the Figure 5A, virgin control mice kept a relatively constant daily food intake from P3 to P21. In the mouse dam, daily food intake increases during the first two week of the postpartum period, it decreases after P15, and it seems to normalise around P18. Looking at the graph, we can say that dams that experienced 4 cycles of pregnancy had the highest daily food intake compared to dams that experienced 1 or 2 cycles of pregnancy. Additionally, dams that experienced 2 cycles of pregnancy had a lower daily food intake compared to dams that experienced 1 cycle of pregnancy. An overall effect of time (Df = 6, $F_{541.0,\ 90.2}$ = 5.500e+29, P < 0.0001), number of pregnancy cycles (Df = 3, $F_{1924.2,\ 641.4}$ = 3.912e+30, P < 0.0001) and time x number of pregnancy cycles interaction (Df = 15, $F_{191.4,\ 10.6}$ = 6.488e+28, P < 0.0001) on daily food intake was found (Figure 5A).

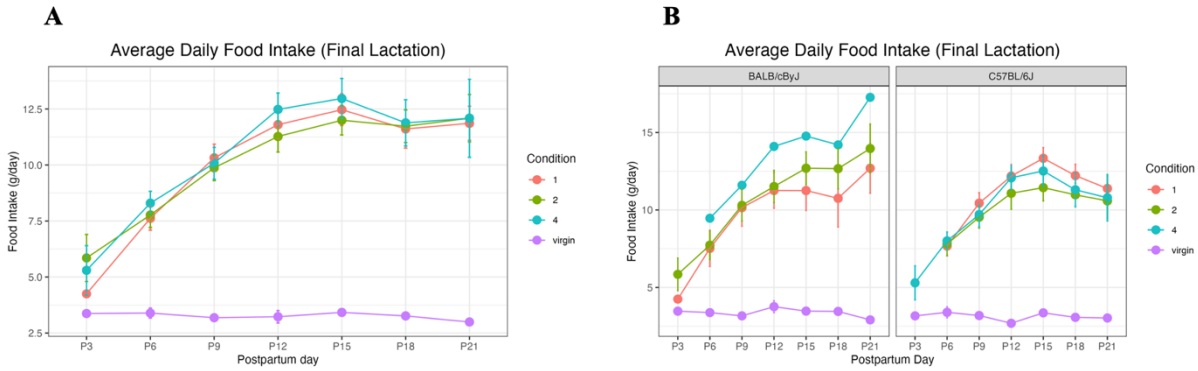

Figure 5: Food intake during postpartum. A) Overview of food intake (g/day) changes in dams that experienced 1, 2, or 4 cycles of pregnancy and in virgin control mice on postpartum day (P) 3, P6, P9, P12, P15, P18, and P21, and **B)** the same data separated by strain: BALB/cByJ and C57BL/6J. **(A-B)** Data are represented as mean ± SD.

When the data were then separated by strain, we observed a remarkable difference between conditions in BALB/cByJ [Fig 5B; effect of time (Df = 6, $F_{250.3,\ 41.72}$ = 2.305e+30, P < 0.0001), number of pregnancy cycles (Df = 3, $F_{787.5,\ 262.51}$ = 1.450e+31, P < 0.0001) and time x number of pregnancy cycles interaction (Df = 14, $F_{100.0,\ 5.88}$ = 3.249e+29, P < 0.0001)]. As shown in the Figure 5B, BALB/cByJ dams that experienced 4 cycles of pregnancy had the highest daily food intake. Moreover, BALB/cByJ dams that experienced 2 cycles of pregnancy clearly had a higher daily food intake than primiparous BALB/cByJ dams. On the other hand, C57BL/6J dams that experienced 4 cycles had a higher daily food intake compared to C57BL/6J dams that experienced 2 cycles of pregnancy. However, it seems that C57BL/6J primiparous dams had the highest daily food intake during postpartum period [Fig 5B; effect of time (Df = 6, $F_{288.7,\ 48.1}$ = 8.949e+29, P < 0.0001), number of pregnancy cycles (Df = 3, $F_{1121.9,\ 374.0}$ =

6.954e+30, P < 0.0001) and time x number of pregnancy cycles interaction (Df = 14, $F_{89.2, 5.6}$ = 1.037e+29, P < 0.0001)].

5.3 Maternal behaviour during pup retrieval test is more influenced by strain than by reproductive experience

To visualise the range of maternal behavioural responses during the pup retrieval test, data were plotted in two ways; the first plot depicts data from all dams, including those that fails to retrieve their pups to the nest (Figure 6A, 6C, 6E, and 6G, and Figure 7A, 7C, 7E, and 7G), and the second plot only included data from dams that successfully completed the retrieval of al pups within the 5-min test (Figure 6B, 6D, 6F, and 6H, and Figure 7B, 7D, 7F, and 7H). Statistical analyses were conducted only on the dataset that included dams that completed the retrieval test. Time spent on the nest was plotted both in second and as a percentage of total test time. Time spent on nest pre- or post-pup retrieval was plotted as a percentage of pre- or post-retrieval time, respectively. A two-way ANOVA was conducted to analyse first the effect of postpartum day (PD) and the number of cycles of pregnancy on maternal behaviour, and then the effect of strain and number of cycles of pregnancy on the maternal behaviour. For the ANOVA analysing the impact of strain and number of pregnancy cycles as factors, data from each dam was averaged across postpartum days and analysed.

There was no effect of time (PD), number of pregnancy cycles, nor PD x number of pregnancy cycles interaction on **latency to sniff pups** [Figs 6A and B; PD (Df = 2, $F_{277, 138.26}$ = 1.998, P = .143), number of pregnancy cycles (Df = 2, $F_{59, 29.52}$ = .427, P = .654), PD x number of pregnancy cycles (Df = 4, $F_{184, 46.11}$ = .666, P = .618)], **time spent sniffing pups before starting the retrieve** [Figs 6C and D; PD (Df = 2, $F_{198, 98.97}$ = 1.458, P = .240), number of pregnancy cycles (Df = 2, $F_{95, 47.54}$ = .701, P = .500), PD x number of pregnancy cycles (Df = 4, $F_{124, 30.95}$ = .456, P = .768)], **latency to retrieve the first pup** [Figs 6E and F; PD (Df = 2, $F_{361, 180.67}$ = 1.404, P = .252), number of pregnancy cycles (Df = 2, $F_{124, 61.82}$ = .481, P = .758), PD x cycles of pregnancy (Df = 4, $F_{242, 60.39}$ = .469, P = .758)], **time spent to retrieve all pups** [Figs 6G and H; PD (Df = 2, $F_{307, 153.6}$ = 0.062, P = .940), number of pregnancy cycles (Df = 2, $F_{3892, 1945.8}$ = .782, P = .461), PD x number of pregnancy cycles (Df = 4, $F_{11632, 2908.0}$ = 1.169, P = .332)], **total time spent on the nest** [Figs 7A and B; PD (Df = 2, $F_{4404, 2202}$= .616, P = .543), number of pregnancy cycles (Df = 2, $F_{8049,}$ 4025 = 1.126, P = .330), PD x number of pregnancy cycles (Df = 4, $F_{12719, 3180}$ = .889, P = .475)] and **time spent on the nest after retrieving all pups** [Figs 7G and H; PD (Df = 2, $F_{1845, 922.3}$= .296, P =.745), number of pregnancy cycles (Df

= 2, $F_{4226, 2112.8}$ = .678, P = .511), PD x number of pregnancy cycles (Df = 4, $F_{6378, 1594.5}$ = .512, P = .727)].

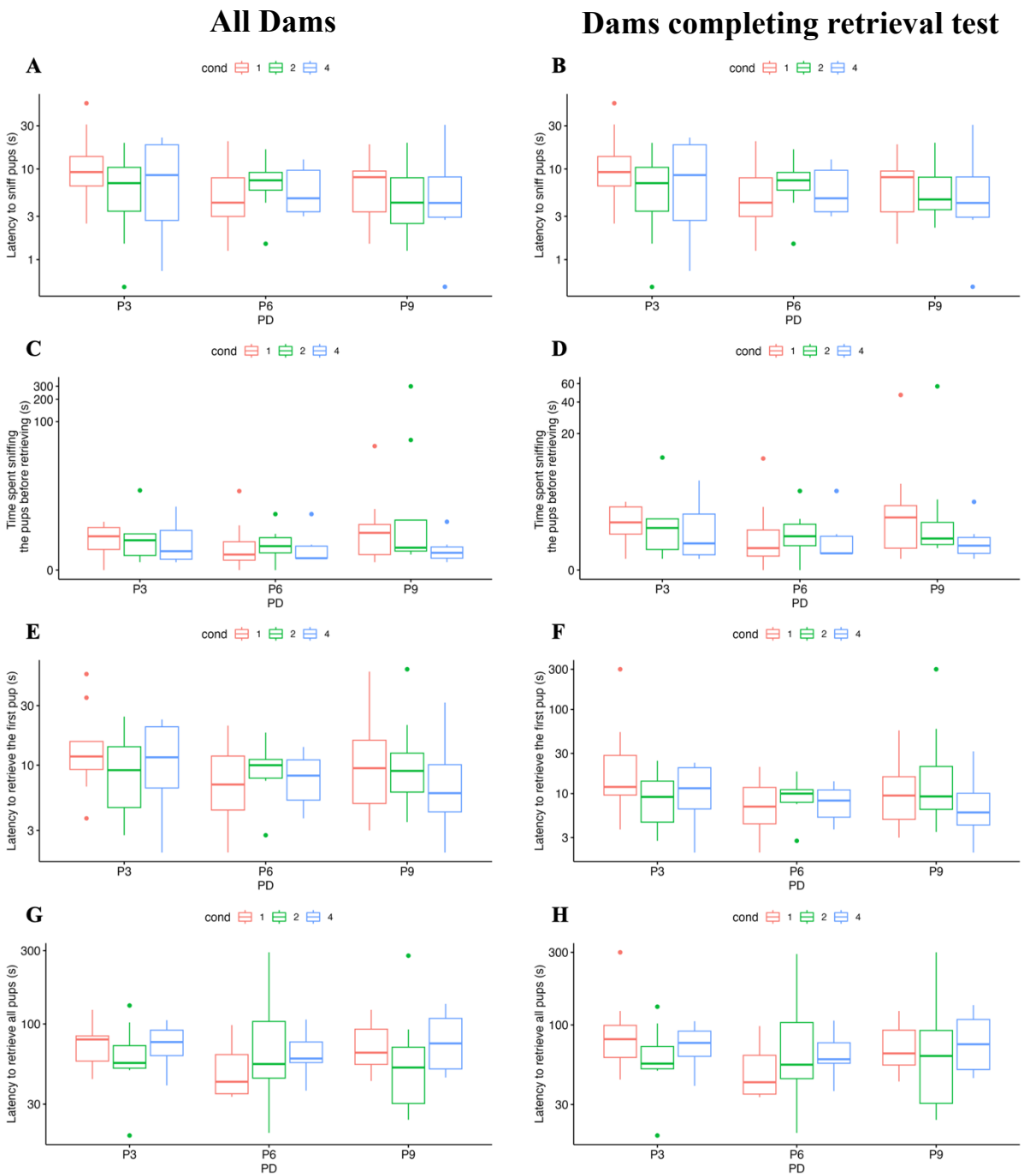

Figure 6. Two-way-ANOVA analysing postpartum days (PD) and number of pregnancy cycles (cond) as main factors impacting pup-directed behaviours in mouse dams. Latency to sniff pups (A-B), time spent sniffing the pups before retrieving pups (C-D), latency to retrieve first pup (E-F), latency to retrieve all pups (G-H). (A, C, E, G) Data from all experimental dams are plotted. **(B, D, F, H)** Only data from dams that completed the retrieval test are plotted. A logarithmic scale was used to show the data.

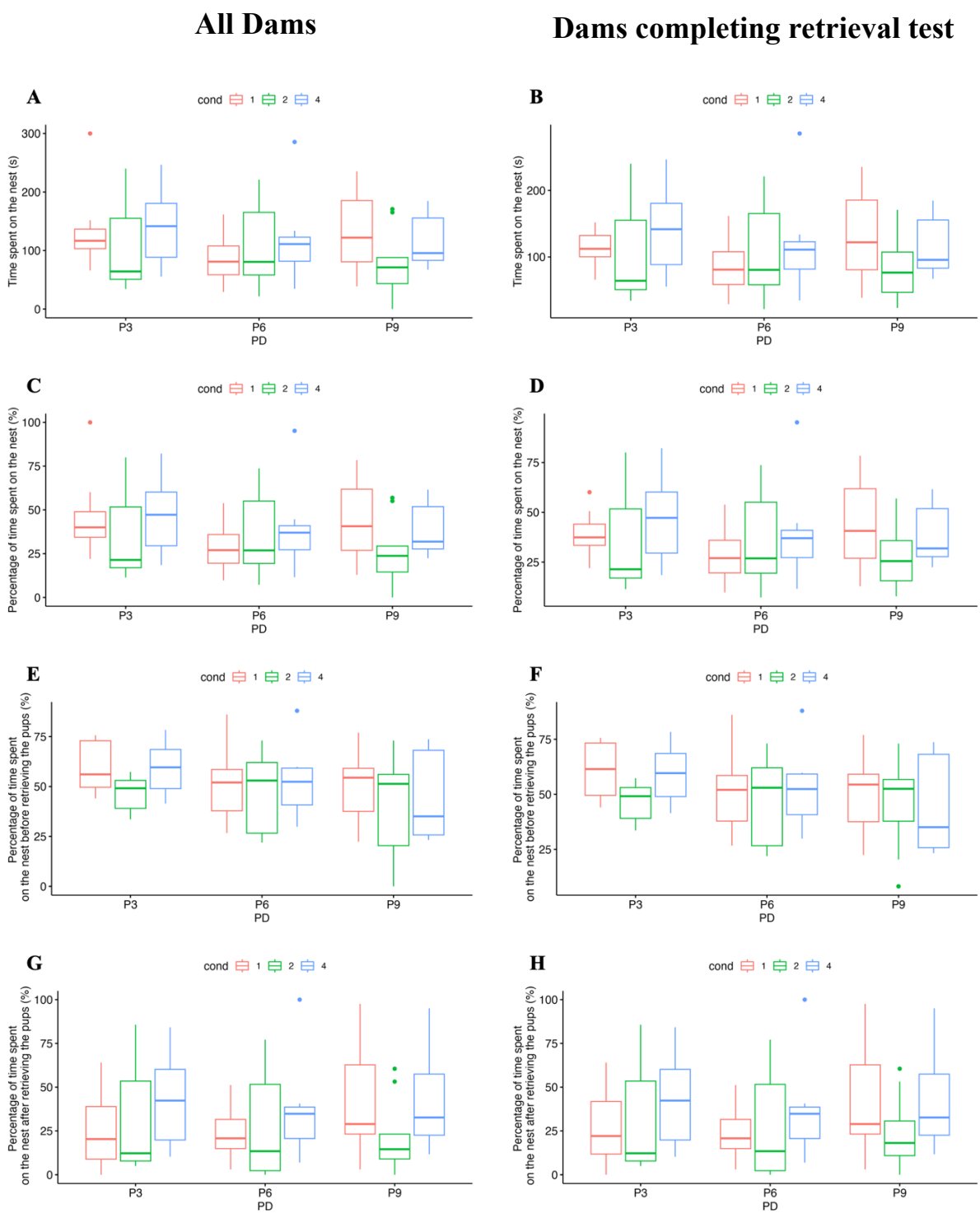

Figure 7. Two-way-ANOVA analysing postpartum days (PD) and number of pregnancy cycles (cond) as main factors impacting time the mouse dams spent on the nest. Total time (s) spent on nest (A, B), time spent on nest as a percentage (C, D), time spent on nest before retrieving all pups as a percentage (E, F), time spent on nest after retrieving all pups as a percentage (G, H). (A, C, E, G) Data from all experimental dams are plotted. **(B, D, F, H)** Only data from dams that completed the retrieval test are plotted.

A small effect of time (Df = 2, $F_{2872,\ 11436.1}$= 3.983, P = 0.0230) and PD x number of pregnancy cycles interaction (Df = 4, $F_{3862,\ 965.4}$ = 2.678, P = 0.0387) was found on **time spent on the nest before retrieving all pups** (Figure 7E and F). As shown in the Figure 7F, on P3, dams spend more time on the nest before retrieving all pups compared to P6 and P9. Moreover, on P3, dams that had 2 cycles of pregnancy spent less time on the nest before retrieving all pups compared to primiparous dams and dams that had 4 cycles of pregnancy. On P9, dams that had 4 cycles of pregnancy spent less time on the nest before retrieving all pups compared to primiparous dams and dams gone through 2 cycles of pregnancy. Lastly, there was no effect of the number of pregnancies (Df = 2, $F_{1241,\ 620.7}$ = 1.722, P = .1863) **on time spent on nest before retrieving all pups**.

Though we did not observe a dramatic effect of number of pregnancy cycles, maternal behaviour seemed to be more influenced by the strain of the dam. As shown in the Figure 8D, BALB/cByJ dams spend more **time sniffing the pups** compared to C57BL/6J [effect of strain (Df = 1, $F_{359.7,\ 359.7}$ = 17.813, P < 0.0001), number of pregnancy cycles (Df = 2, $F_{85.9,\ 43.0}$ = 2.127, P = .1266), strain x number of pregnancy cycles (Df = 2, $F_{102.8,\ 51.4}$ = 2.546, P = 0.0853)]. Moreover, as shown in the Figure 8H, BALB/cByJ dams take more **time to retrieve all pups** compared to C57BL/6J [effect of strain (Df = 1, $F_{13623,\ 13623}$ = 18.809, P < 0.0001), effect of number of cycles (Df = 2, $F_{3704,\ 1852}$ = 2.557, P = .0845)]. An effect of strain x number of pregnancy cycles interaction (Df = 2, $F_{48147,\ 24073}$ = 33.236, P < 0.0001) was also found on **time spent to retrieve all pups**. Looking again to Figure 8H, it seems that BALB/cByJ dams that experienced 2 cycles of pregnancy are slower to retrieve compared to primiparous BALB/cByJ or those with 4 cycles of pregnancy. However, C57BL/6J dams seem to be faster to retrieve after experiencing 2 cycles of pregnancy, compared to group of dams that experienced 1 or 4 cycles of pregnancy. Finally, there was no effect of strain, number of pregnancy cycles, nor strain x number of pregnancy cycles interaction on **latency to sniff pups** [Figs 8A and B; strain (Df = 1, $F_{96.7,\ 96.70}$ = 2.990, P = 0.088), number of pregnancy cycles (Df = 2, $F_{46.3,\ 23.15}$ = .716, P = .492), strain x number of pregnancy cycles (Df = 2, $F_{67.7,\ 33.85}$ = 1.046, P = .356)], and **latency to retrieve the first pup** [Figs 8E and F; strain (Df = 1, $F_{84,\ 83.75}$ = 1.226, P = .272), number of pregnancy cycles (Df = 2, $F_{104,\ 52.06}$ = .762, P = .470), strain x number of pregnancy cycles (Df = 2, $F_{170,\ 84.90}$ = 1.243, P = .295)].

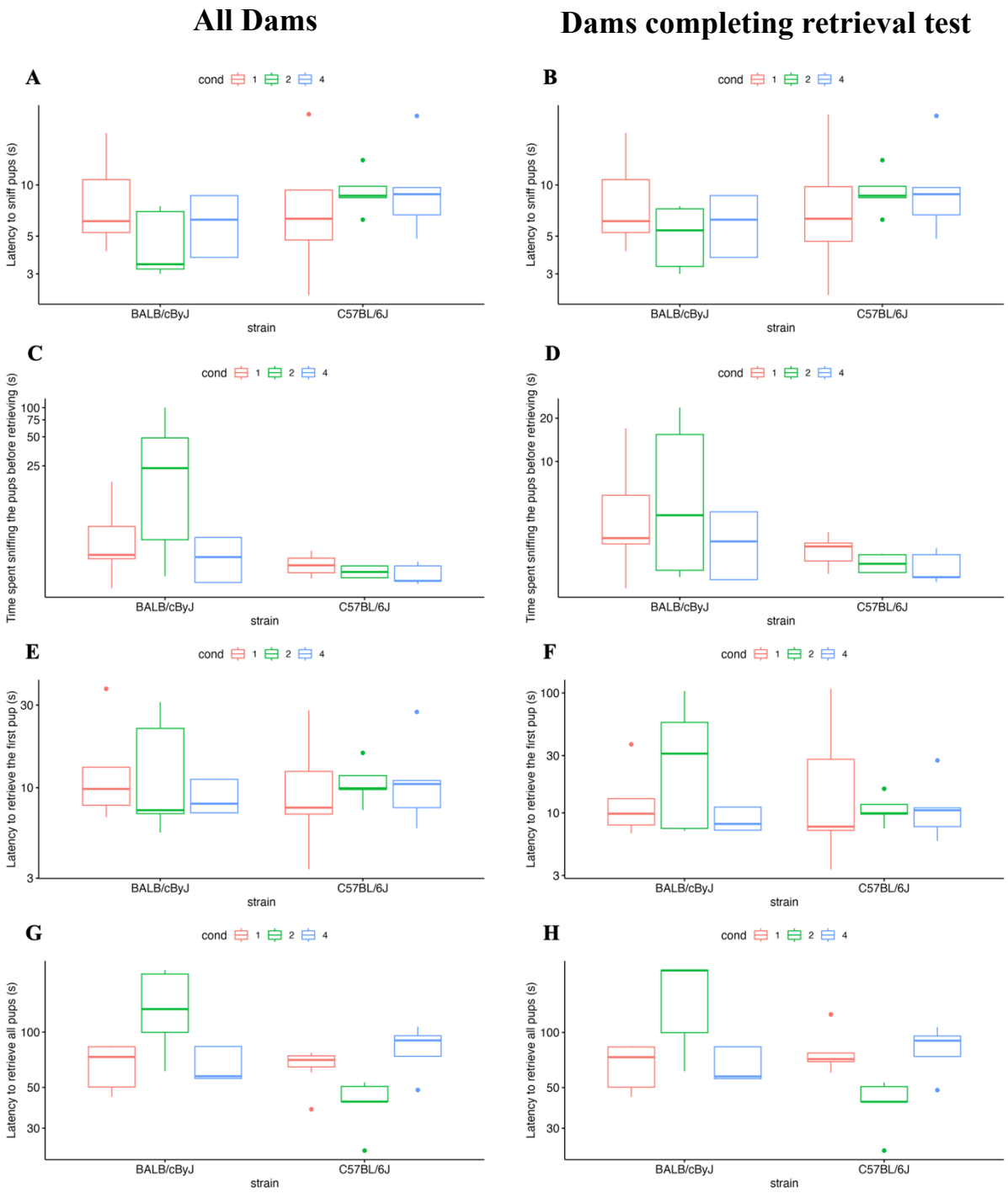

Figure 8. Two-way-ANOVA analysing dam strain and number of pregnancy cycles (cond) as main factors impacting pup-directed behaviors. Latency to sniff pups (A-B), time spent sniffing the pups before retrieving pups (C-D), latency to retrieve first pup (E-F), latency to retrieve all pups (G-H). (A, C, E, G) Data from all experimental dams are plotted. **(B, D, F, H)** Only data from dams that completed the retrieval test are plotted.

Additionally, as shown in the Figure 9B and 9D, C57BL/6J spend more **time on the nest** compared to BALB/cByJ [effect of strain (Df = 1, $F_{8732, 8732}$ = 76.764, P < 0.0001), effect of number of pregnancy cycles (Df = 2, $F_{813, 407}$ = 3.574, P = 0.033), strain x number of pregnancy cycles (Df = 2, $F_{199, 99}$ = .874, P = .422)]. As shown in the Figures 9F and 9H, this difference seems to be preserved both in **time spent on the nest before retrieving all pups** [Fig 9E and F; effect of strain (Df = 1, $F_{454, 453.7}$ = 4.668, P = 0.03401)] and **time spent on the nest after retrieving all pups** [Fig 9G and H; effect of strain (Df = 1, $F_{14246, 14246}$ = 73.464, P < 0.0001)]. Both primiparous BALB/cByJ and primiparous C57BL/6J spend more **time on the nest before retrieving all pups** compared to dams after 2 cycles of pregnancy [Fig 9E and F; effect of number of pregnancy cycles (Df = 2, $F_{1138, 569.1}$ = 5.856, P = 0.00438)]. Moreover, it seems that primiparous C57BL/6J also spend more **time on the nest before retrieving all pups** also compared to C57BL/6J dams after 4 cycles of pregnancy [Fig 9E and F; effect of strain x number of pregnancy cycles (Df = 2, $F_{2573, 1376.7}$ = 14.165, P < 0.0001)]. However, dams that experienced 4 cycles of pregnancy spend more **time on the nest after retrieving all pups** compared to dams that experienced 1 or 2 cycles of pregnancy both in BALB/cByJ and C57BL/6J [Fig 9G and H; effect of number of pregnancy cycles (Df = 2, $F_{2046, 1023}$ = 5.276, P = 0.00724)]. Lastly, there was no effect of strain x number of pregnancy cycles interaction (Fig 9G and H; Df = 2, $F_{867, 434}$ = 2.236, P = .11416) on **time spent on the nest after retrieving all pups**.

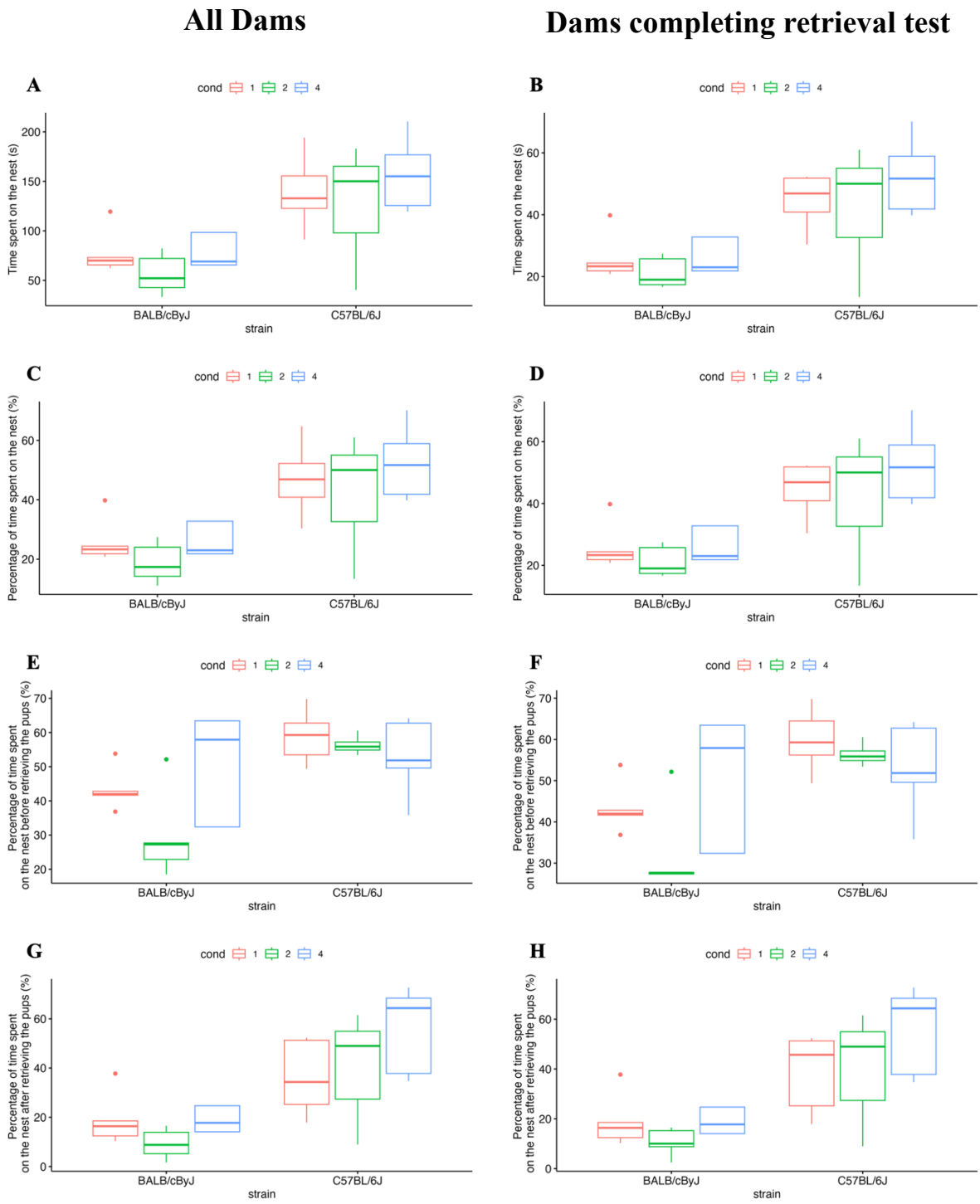

Figure 9. Two-way-ANOVA analysing strain and number of pregnancy cycles (cond) as main factors impacting the pup-directed behaviours. Total time (s) spent on nest (A, B), time spent on nest as a percentage (C, D), time spent on nest before retrieving all pups as a percentage (E, F), time spent on nest after retrieving all pups as a percentage (G, H). (A, C, E, G) Data from all experimental dams are plotted. **(B, D, F, H)** Only data from dams that completed the retrieval test are plotted.

5.4 Immunohistochemistry for oxytocin in the PVN

Due to the fact that the experiment is run in several batches over the course of approximately one year, I was only present to section the brains from Batch 1, and then conducted immunohistochemistry for the detection of oxytocin. At the completion of all batches, the brains will be processed to count and analyse the number of positive oxytocin cells in the PVN. Only then will we be able to make a conclusion about whether the number of pregnancy cycles influences oxytocin expression. However, as shown in the Figure 10, we were able to capture some images of immunostained brains to show the positive outcome of the immunohistochemistry. At the end of the experiment, the number of oxytocin-positive neurons in the PVN and MPOA will be counted and analysed.

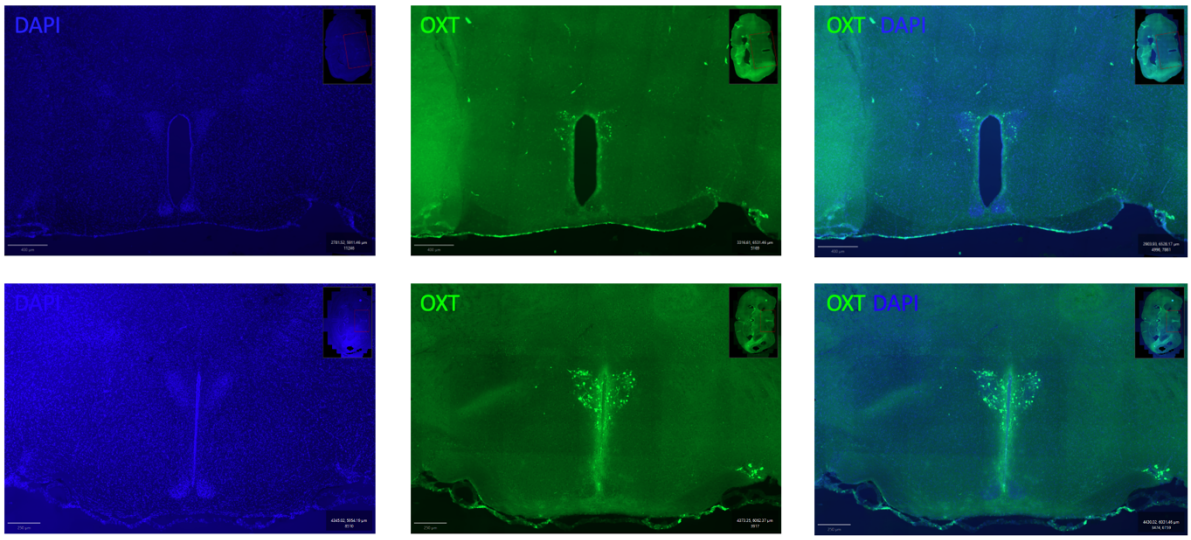

Figure 10: representative images of oxytocin-positive neurons in the rostral (top) and medial (bottom) levels of the PVN. Images of PVN were captured from the brain of two mice. Sections were immunostained for DAPI (blue labelling) and oxytocin (Alexa-555: green labelling). Scale bars represent 400 µm.

5.5 Immunohistochemistry for pSTAT3 in the MPOA

Similar to the immunohistochemistry for oxytocin, only brains form Batch 1 dams were sectioned and stained pSTAT3, so there was not sufficient time to count and analyse positive cells for the purpose of this thesis. Representative images of hypothalamus from a mouse dam stimulated with leptin and then immunostained for pSTAT3 is shown in the Figure 11A-C. in the Figure 11A, regions of interest (medial basal hypothalamus, including the arcuate nucleus and ventromedial nucleus of hypothalamus) were identified and marked by the software to be analysed later in time. A digital zoom of the arcuate nucleus is shown in Figure 11B (20x magnification). Additionally, to optimise the microscopy protocol, images were captured with

(on the right) and without z-stack analysis (on the left), and a sample how the program will count individual pSTAT3-positive cells is shown (Figure 11C).

Figure 11A

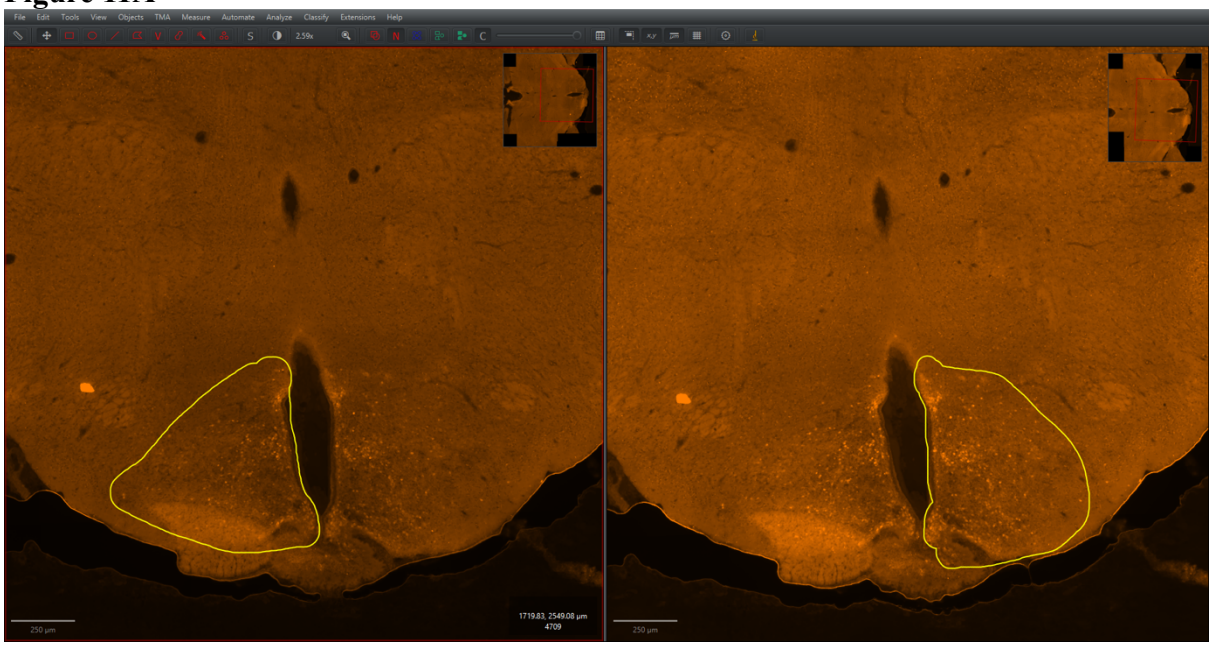

Figure 11B

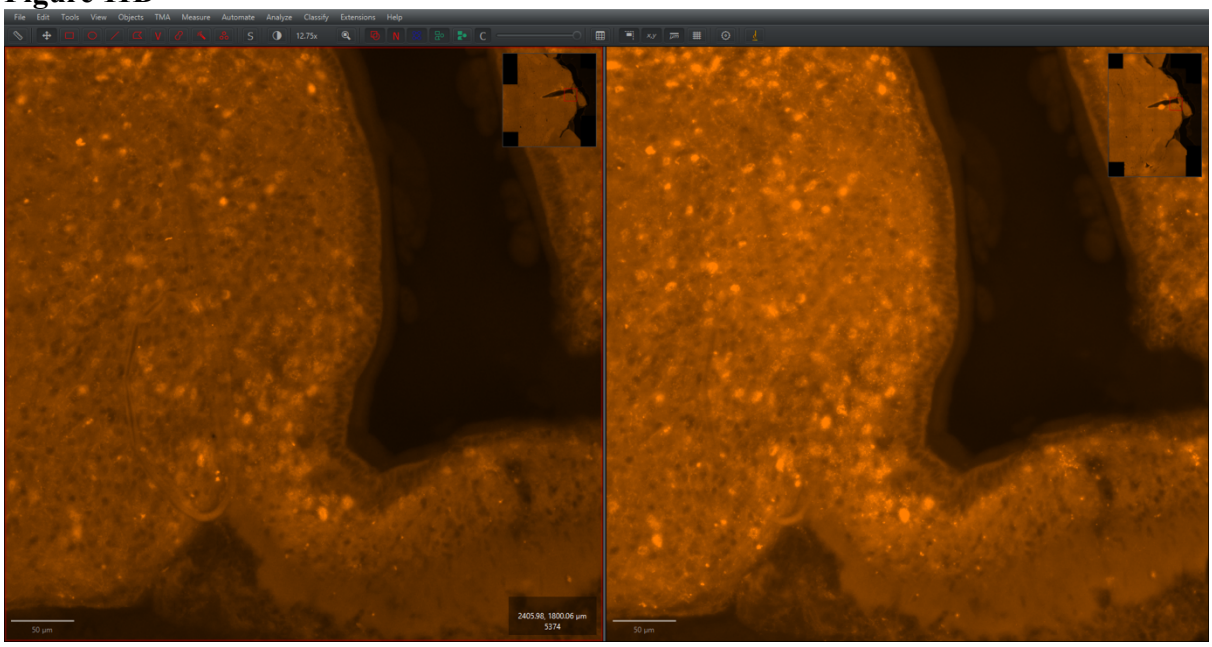

Figure 11C

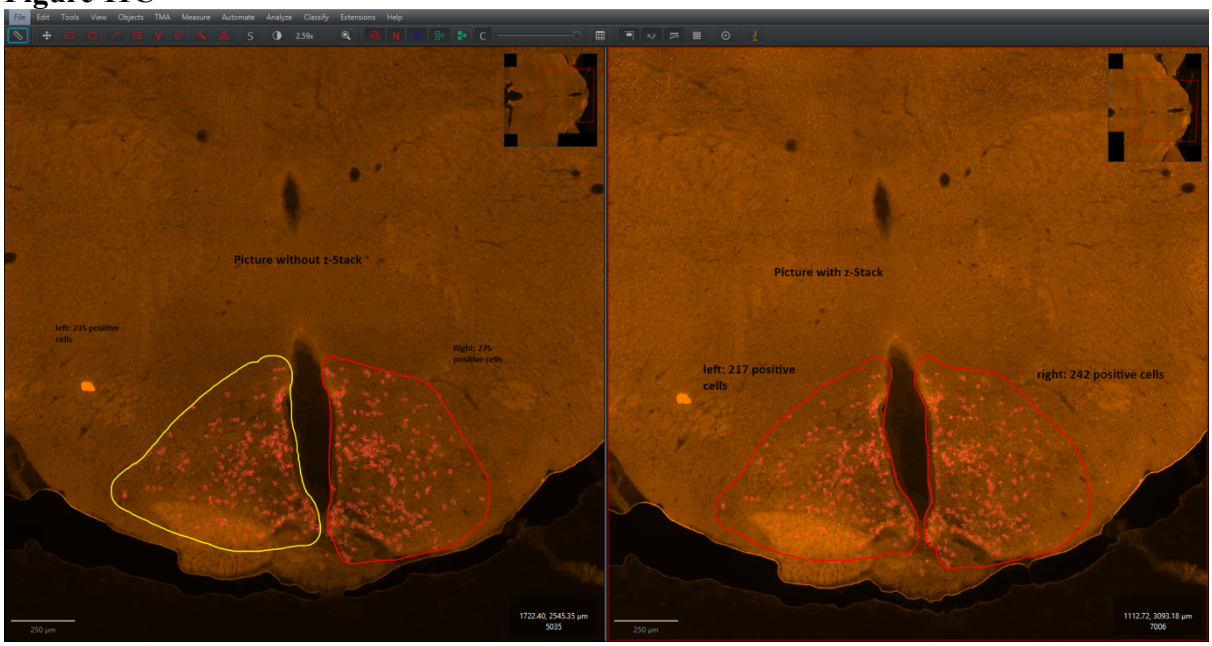

Figure 11: Cell count of pSTAT3-positive neurons in the caudal hypothalamus. Arcuate and ventromedial nucleus of the hypothalamus after immunostaining for pSTAT3 (Alexa-488). Scale bars represent **A)** 250 µm and **B)** 50µm. In **(A)**, regions of interest are circled. **C)** Example of automated counting of pSTAT3-positive cells with (right) and without (left) z-stack. Scale bars represent 250 µm.

6 Discussion

While the impact of maternal stress during pregnancy on physiological welfare and behaviour of the offspring has been well documented (Baker et al., 2008; Contu & Hawkes, 2017; Lordi et al., 2000; Menting et al., 2019), to date, few studies have explored the effect of gestational stress on the welfare of the dam (Darnaudéry et al., 2004). However, it is important to state that this study focused on the effect of a paradigm of induced stress, either chronic or acute, on the mouse dam during pregnancy, rather than analysing whether consecutive cycles of pregnancy and lactation can per se influence the wellbeing of the dam. our study aimed to find out if multiple cycles of pregnancy and lactation, due to current breeding methods, are indeed stressful for the dam, and if that is so, we wanted to study if stress is severe enough to have consequences on the physiology or behaviour of the mother.

6.1 Body weight of the dam

It has been described in both humans and rodents that pregnancy and lactation can lead to increased maternal body weight (Harris et al., 1997; Ladyman et al., 2018). One study showed that mouse dams maintain a significantly higher body weight than age-matched controls for up to 8 weeks following a single round of pregnancy and lactation after weaning pups (Ladyman et al., 2018). It was also shown that multiple cycles of pregnancy and lactation causes weight gain in mice (Rebholz et al., 2012). Additionally, one study shows that lactating dams with concurrent pregnancy did not have a larger increase in food intake when compared to lactating dams that were not pregnant (Johnson et al., 2001). Similarly, in a study conducted on rats, lactating females with concurrent pregnancy did not increase their food intake compared to dams that were simply lactating (Leon & Woodside, 1983).

In our study, body weight during the postpartum period of mouse dams experiencing 4 cycles of pregnancy and lactation was significantly higher compared to the body weight of dams after 1 or 2 cycles of pregnancy and lactation and age-matched female virgin mice. However, the food intake per day of dams experiencing 4 cycles of pregnancy and lactation was not always higher than food intake per day of dams experiencing 1 or 2 cycles of pregnancy and lactation. For instance, it seems that dams that experienced 4 cycles of pregnancy had a higher daily food intake compared to dams that experienced 2 cycles of pregnancy and primiparous dams later in the postpartum period (after P9). Moreover, it seems that primiparous dams had the highest food intake when analysing C57BL/6 dams. The fact that multiparous dams had a higher body

weight compared to primiparous and virgin female mice, but not always a higher food intake, makes us hypothesize that there may be some metabolic processes that regulate differently the energetic demand during multiple cycles of pregnancy and lactation (see “Leptin”). While beyond the scope of this thesis, the mouse dams are also being assessed for changes in energy expenditure following multiple cycles of pregnancy and lactation, and these data will provide additional information about what aspects of metabolic control are modified with increasing reproductive experience.

6.2 Pup retrieval test

Maternal behaviour is influenced by the wellbeing of the dam and can be so altered if she is not healthy or if she is stressed. numerous studies have shown that being stressed during pregnancy causes deficits in maternal care (Meek et al., 2001; Patin et al., 2002). In one study, dams exposed to chronic unpredictable stressors during pregnancy spent significantly less time grooming and nursing their pups and were less likely to spend time on the nest than non-stressed dams (Meek et al., 2001). Another study shows that stressed dams retrieved 96% to 98% of their pups into the nest compared to 100% in control dams (Patin et al., 2002).

In our study, we investigated whether concurrent cycles of pregnancy and lactation can indeed cause stress to the dam and affect its wellbeing, and consequently affect the expression and quality of maternal care. In order to answer this question, we compared the results of a pup retrieval test between dams that experienced one, two or four cycles of consecutive pregnancy. As it has been shown that pregnancy and lactation are highly energy-demanding processes (Butte & King, 2005), we the hypothesised that multiparous dams would experience more physiological stress than primiparous dams, since they possible experience greater metabolic and physical demand. Following from this hypothesis is the idea that a dam experiencing more physiological stress when display deficiencies in maternal behaviour.

Unfortunately, we were not able to answer this question within the time constraints of the bachelor thesis. After analysing the current data set, which consists of approximately 40% of all planned subjects, we did not observe an effect of the number of pregnancy cycles on maternal motivation. Reasonably, we think that this outcome was caused by the small size of the population sample. However, it is fair to keep in mind the possibility that multiple cycles of pregnancy may not 1) affect the welfare of the dam, 2) nor her expression of maternal behaviour, or 3) her welfare may be affected in other ways, while preserving pup retrieval

behaviour. it may also be that having more than one parity may have a positive effect on maternal behaviour and its expression, as a mother gains maternal experience. In one study, biparous mouse dams were significantly faster retrieving the first pup when compared to primiparous females (Cohen-Salmon, 1987). In another study using mice, retrieving behaviour improved during the first lactation and the improvements seemed to be maintained during later lactation periods (Beniest-Noirot, 1961). However, results in literature are inconsistent. Some studies show that time spent to retrieve pups did not differ between primiparous and previously-experienced multiparous mothers (Beach & Jaynes, 1956; Moltz & Robbins, 1965).

While we are then not able to state if concurrent cycles of pregnancy and lactation can affect the maternal behaviour in the pup retrieval test, we should nonetheless take into consideration other variables or factors that can influence the dependent variable. For example, as shown previously maternal behaviour seems to be strongly influenced by mouse strain. Several studies have shown strong differences between inbred strains of mice in pup retrieving behaviour. In one study, female C57BL/6 mice took less time to retrieve the first pup than female BALB/c mice, and they seemed to be better retrievers compare to the other strains observed in the experiment (Carlier et al., 1982). Consistent with this, in our study, C57BL/6 dams took less time to retrieve all pups compared to BALB/c dams. We also found that C57BL/6 dams spent less time sniffing the pups before retrieving them compared to BALB/c dams. However, it may be that differences observed in maternal behaviour are partly caused by differences between strains in the quality of signals produced by the pups, i.e. the frequency of pup vocalisation, or / and the ability of the dam to perceive such signals (Cohen-Salmon et al., 1985). While not performed in the context of this thesis, the pup vocalisations of the offspring born in this experiment were also assessed, so changes in vocalisations across strain and number of pregnancy cycles will be determined.

Finally, BALB/c dams spent overall less time on the nest compared to C57BL/6 dams, and these results seem to be consistent when looking at the time spent on the nest both before and after retrieving all pups. Interestingly, in one study, litter loss was associated with females spending more time outside the nest (Weber, 2015), demonstrating that offspring mortality in mice may be related to maternal behaviour or attentiveness. Usually, when we find dead pups inside the cage, we often assume that they were killed by the mother or the father, because most of the time we find them eaten. However, since newborn mouse pups are completely dependent on their mother for nutrition and thermoregulation, their death may be linked to other causes

than infanticide or cannibalism, like starvation and hypothermia. In previous studies, there was in fact no evidence that females losing their pups actively kill them (Weber, 2015; Weber et al., 2013). It may be that multiple factors lead to high pup mortality. As breeding methods may influence dam welfare and the expression of maternal behaviour, the methods may consequently influence the success of pup survival (Auclair et al., 2014; Morello et al., 2020; Wright & Brown, 2000). In one study, overlapping litters, a common phenomenon in trio-breeding cages, led to a 30% to 60% higher probability of neonatal pup mortality (Morello et al., 2020). Similarly, communal rearing of litters results in an increase in pup mortality, aggravated with the presence of each additional litter alongside the focal litter (Ferrari et al., 2019). However, another study of the same population found an increase in pup survival in litters reared communally (Auclair et al., 2014). Moreover, it has been also found a relationship between strain and pup survival in mice. It has been shown that there are higher odds of mortality in C57BL/6 mice compared to BALB/c mice (Weber, 2015). Additionally, litter loss lead to an increase in the number of breeding animals needed to supply experimental animals, which in turn increases costs and counteracts the 3R goal of reducing the number of animals used for experimental purposes. Litter loss can be actually reduced after carefully investigating all underlying causes.

The behavioural differences between mouse strains used in laboratory settings outside of the pregnancy and lactation periods are also well-documented. For example, BALB/c mice are less socially responsive than C57BL/6 mice (Sankoorikal et al., 2006), it has in fact been shown that BALB/c are more likely to avoid rather than approach a stimulus mouse (Brodkin et al., 2004). Similarly, one study demonstrates that female C57BL/6 mice show lower levels of anxiety-like behaviour and higher levels of social exploration and social contacts than female BALB/c mice (An et al., 2011).

Due to differences between strains observed in maternal behaviour, it would be important to investigate in the future whether current breeding methods have the same effect on the welfare of dams of different strains. It may be that there is a breeding method that better suits a certain strain of mice. Answering to this question is important for the welfare of the dam itself, but it is also important for the welfare of the offspring, and consequently for the research's sake. While the current study has considered strain as a factor, and therefore included C57BL/6 and BALB/c mice, future work should include additional mouse strain that are commonly used in biomedical research.

6.3 Leptin

During gestation, the body goes through remarkable hormonal changes that helps the dam to meet the metabolic demand of a growing fetus. Pregnancy is associated with a state of insensitivity to leptin (Ladyman et al., 2012). The brain exhibits leptin resistance and so maternal food intake, and thus caloric intake, increases despite increasing maternal fat reserves. However, it has been shown that the state of leptin insensitivity during pregnancy is temporary. While the precise timing of the return of leptin sensitivity is not known, in one study, primiparous mouse dams were responsive to exogenous leptin in terms of reduction of food intake and pSTAT3 expression in the brain approximately 10 weeks after weaning pups (Ladyman et al., 2018). Work from our group previously showed that the brains of rat dams exhibit leptin responsiveness already on the day of pup-weaning around postpartum day 25 (Leuthardt et al., 2021).

In our current study, we found that dams experiencing multiple cycles of pregnancy did not have always higher food intake during lactation compared to primiparous dams, even if they had a significantly higher body weight. Since we recorded the body weight of dams and their food intake during lactation period without a concurrent pregnancy, we can then presume that there may be some metabolic processes that regulate differently the energetic demand following multiple cycles of pregnancy and lactation. Furthermore, post-weaning food intake data were collected but not yet analysed, and it will be informative to assess whether food intake stabilises in the absence of suckling demand, and if this varies based on reproductive experience. We hypothesise that there are alterations in central leptin sensitivity of multiparous dams, but we cannot yet draw any conclusions because the maternal brain samples are still being collected and will be stained and processed in the coming months. We do not know at this point if multiple cycles of pregnancy and lactation could affect brain leptin sensitivity in the long term. During the period of gestation, we can presume that there is a different responsiveness to leptin in primiparous dams compared to multiparous dams. It could be that going through concurrent cycles of pregnancy lead to compounded modifications in central leptin sensitivity and consequently have an impact on the caloric intake of the dam during gestation, lactation and beyond. We will only be able to draw these conclusions when the brains collected from all the dams in the study are collected, stained and analysed.

6.4 Oxytocin

Oxytocin has a main role in the regulation of maternal behaviour and deficiencies in central oxytocin alters the expression of maternal care (Pedersen et al., 2006; Pedersen & Boccia, 2003). It has been shown that virgin female rats treated with intraventricular injection of oxytocin exhibit a rapid onset of maternal care (Pedersen et al., 1982). Moreover, oxytocin expression levels in the PVN and MPOA has a positive correlation with better maternal care (Shahrokh et al., 2010). One aim of the present study is to test the hypothesis that the number of concurrent cycles of pregnancy and lactation influences oxytocin expression in the maternal brain. We speculate that females with pregnancy experience will have increased oxytocin levels compared to virgin females. As earlier work suggests that oxytocin expression levels are positively correlated with the quality of maternal care (Shahrokh et al., 2010), and we failed to observed difference in maternal care based on number of pregnancy cycles, we hypothesise that number of reproductive cycles will not affect central oxytocin levels. An additional confounding factor is that the maternal brains were collected 4-5 weeks after pups were weaned, and whether active sucking is necessary for enhanced oxytocin expression is not known. So, while it is possible to observe differences in oxytocin expression based on maternal experience, we will only be able to draw such conclusion when all maternal brains have been collected, stained and analysed in the coming months. In the future, it will be also interesting to quantify central oxytocin expression earlier in the postpartum period, to determine if there are any differences between control virgins, primiparous, and multiparous dams when pups are actively suckling and being care for.

6.5 Impact of the project on animal welfare

The project applies the 3R principles, primarily by incorporating refinement practices with the aim to assess, and potentially improve, the welfare of breeding mouse dams. However, as the experiments of our project are designed to investigate the effects and the complex interactions of pregnancy and lactation cycles on the metabolic health and general wellbeing of mouse dams, it is impossible to replace the *in vivo* methods with *in vitro* methods. Great care was also taken when designing the experiments to reduce the number of experimental mice required to answer our experimental questions. For example, the data collected from the dams that wean their first litter at 3 weeks were used for comparison in both Aim 1 and Aim 2, which reduces the experimental animal numbers. Further, sequential blocks of the experiments were performed in such a way that pups born to primiparous dams can be incorporated into

subsequent runs, thus eliminating the need to purchase any additional breeders after the first run. We also try to refine our experiment, for example employing tunnelling handling of the mice, as an alternative at handling the mice by their tail.

While there are many 3R studies which have focused on improving the animal welfare of laboratory mice, less research had been conducted to improve the lives of the mothers of these animals. We recognize that in order to refine maternal welfare, we must first define maternal welfare and how it is influences by number of reproductive cycles. So, the main goal of the project is to define the state of wellbeing of mouse breeding dams under consecutive breeding strategies. By doing that, we are then able to determine if further refinement of these rodent breeding practices is indicated. So, we believe that the project could lead to develop breeding protocols which not only benefit the breeding facility due to improved reproductive success, but which also benefit the breeding animals themselves due to improvements in their physical and psychological health.

Additionally, as many studies have shown, the healthier the dam, the healthier the pups. Research inspired by the Developmental Origins of the Health and Disease (DOHaD) theory (Barker, 2007; T. P. Fleming et al., 2018), using rodent models, investigates how maternal behaviour and the early environment impact the long-term health of the offspring. However, it is difficult to disentangle the impact of breeding practices on the dam from the impact on the offspring. In fact, we already know that metabolic dysfunction of a rodent dam has strong effects on her offspring (Contu & Hawkes, 2017; Menting et al., 2019). We also know that deficient maternal care leads to a dysregulation in behaviour and cognitive ability. Female pups born from a stressed dam show elevated anxiety-like behaviour and attenuated weight gain (Baker et al., 2008), and long term spatial memory is altered in offspring born from females chronically stressed (Lordi et al., 2000). Yet few studies have investigated the effect of concurrent pregnancy and lactation on the health of offspring. It was previously shown that offspring from a pregnant lactating dam are smaller at weaning than those born under non-concurrent conditions (König & Markl, 1987). Consequently, these smaller pups are at a disadvantage when they need to compete with age-matched, but heavier, conspecifics for resources, such as food. These findings support the idea that a pregnant dam that is concurrently lactating will prematurely wean her pups in order to put her resources into the new pregnancy.

Our findings have the possibility to improve the welfare and stability of the offspring, and to potentially influence reproducibility of animal experiments involving mice. The more we know

about how to improve the health and wellbeing of the mothers, the healthier their offspring will be, thus improving experimental conditions of all future studies. In Switzerland alone, our study could affect the welfare of around 400,000 experimental mice (and countless surplus mice) yearly. Our research will add to the knowledge we already have on current breeding practices and may even lead to the establishment of new breeding recommendations, leading to healthier mothers and offspring.

If the results of the whole project demonstrate that the welfare of the breeding dam is jeopardized by permanent breeding conditions, these data will generate grounds to assess the necessity of new recommendations of breeding practices, at the local and national level. The project will also generate awareness by placing a spotlight on the topic of experimental breeding animal welfare and allow us to assess whether more structured guidelines or regulations are needed for breeding management. Moreover, this study will also help determine whether there is current need to change the way laboratory animals are bred in Switzerland, where there is currently no standardized breeding protocol available. It will contribute new insights into the impact that consecutive pregnancy and lactation have on mother's behavioural, metabolic, or nutritional profile. We also hope that this study could bring awareness beyond Switzerland about the importance of the welfare of the breeding animals and how their health and well-being could potentially impact how their offspring respond when used in biomedical research.

It is important to point out that the findings of the project will have the potential to impact people working at all levels with experimental rodents: animal facility managers, animal care takers, researchers, and educators. As the experiment is performed in permanently housed C57BL/6 and BALB/c breeding pairs, representing popular breeding methods and mouse strains for biomedical colonies, the results will be of interest to a broad audience of researchers managing breeding colonies, in additional to commercial and academic breeding facilities.

7 Conclusion

Based on the result of this experiment we can conclude that number of pregnancy cycles influences the metabolic processes of dams. Multiparous dams showed a higher body weight than primiparous dams but a lower daily food intake. However, we found that maternal behaviour during pup retrieval test was more influenced by mouse strain than be reproductive experience. C57BL/6 dams spent less time sniffing the pups prior to retrieving and took less time to retrieve all pups compared to BALB/c dams. Additionally, C57BL/6 dams spent overall more time on the nest compared to BALB/c dams. While we currently do not have enough evidence to conclude if multiple cycles of pregnancy and lactation have a negative impact on the health and wellbeing of the dams, the data presented within compromise only a portion of the parameters to be analysed. Only after further behavioural, metabolic and nutritional readouts are completed and additional dams are added to each experimental group will we be able to answer these questions.

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