

Laboratory mouse breeding: is maternal responsiveness impaired when new pups are born in the presence of an older litter in the cage?

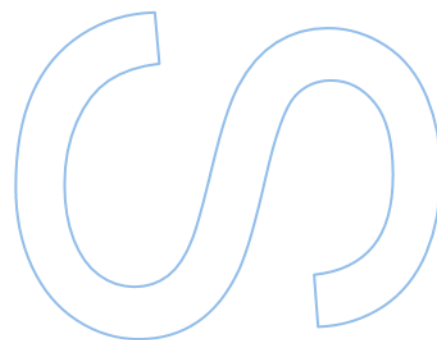
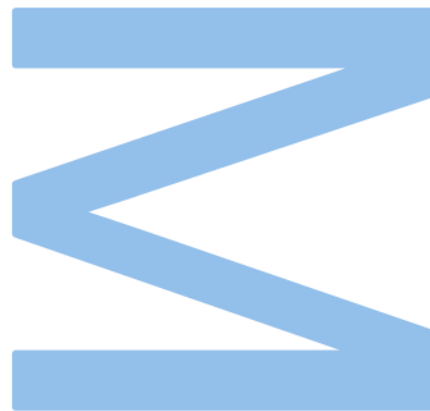
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"The creatures too, must become free"
Thomas Münzer

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Resumo

Os níveis de mortalidade pré-desmame continuam a ser um problema persistente na criação de murganhos de laboratório. Entre os fatores de risco de mortalidade pré-desmame, foi recentemente identificado o *Litter Overlap* (a presença de uma ninhada mais velha na caixa, i.e. ninhadas sobrepostas). Neste estudo, o objetivo era revelar se o *Litter Overlap* afeta a responsividade maternal, os níveis de mobilidade das crias e um repertório de comportamentos maternos. Assim, as hipóteses foram: 1) O *Litter Overlap* afeta a responsividade maternal (*Pup Retrieval* (recuperação das crias)), construção do ninho, amamentação, limpeza das crias); 2) O *Litter Overlap* afeta o comportamento materno durante o período de isolamento mãe-cria; e 3) O *Litter Overlap* afeta a atividade das crias recém-nascidas. Para testar as hipóteses, foi desenvolvido um protocolo adaptado do *Pup Retrieval Test* (PRT) para testar mães com ninhadas sobrepostas e não sobrepostas para avaliar sua responsividade maternal. As observações dos testes foram divididas em análise do comportamento da mãe durante o PRT e o período de isolamento (realizado antes do início do PRT) e análise da atividade das crias recém-nascidas durante o PRT. Mães com ninhadas sobrepostas levaram mais tempo para se aproximar, agarrar e trazer as crias de volta ao ninho. Durante o período de isolamento, as fêmeas com ninhadas sobrepostas passaram mais tempo em locomoção e autolimpeza, e as crias de ninhadas sobrepostas passaram mais tempo inativas e *pivoting* (a girar).

Palavras-chave: comportamento materno, mortalidade de crias, atividade de crias, vigor de crias, comportamento da mãe, reprodução de murganhos de laboratório, sobreposição de ninhadas, mortalidade pré-desmame, recuperação de crias, sobrevivência das crias

Abstract

Pre-weaning mortality levels remain a persistent problem in laboratory mouse breeding. Among pre-weaning mortality risk factors, it was recently identified Litter Overlap (the presence of an older litter in the cage when new pups are born). In this study, the goal was to reveal if Litter Overlap affects maternal responsiveness, pup activity levels and a repertoire of maternal behaviours. Therefore, this study's hypotheses were: 1) Litter Overlap affects maternal responsiveness (Pup Retrieval, Nest Building, Nursing, Grooming Pups); 2) Litter Overlap affects maternal behaviour during dam-pup isolation period; and 3) Litter Overlap affects new-born pup activity. To test the hypotheses, an adapted Pup Retrieval Test (PRT) protocol was developed to test dams with Overlapped and Non-Overlapped litters to evaluate their maternal responsiveness. The tests' observations were divided into Dam Behaviour analysis during the PRT and isolation period (performed before the start of PRT), and analysis of New-born Pup Activity during the PRT. Dams with Overlapped litters took longer to approach, grab, and bring pups back to nest. During the isolation period, females with Overlapped litters spent more time in Locomotion and Self-Grooming, and pups from Overlapped litters spent more time inactive and pivoting.

Keywords: maternal behaviour, pup mortality, pup activity, pup vigour, dam behaviour, mouse breeding, laboratory mice, litter overlap, mouse pre-weaning mortality, pup retrieval, pup survivability

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List of Abbreviations

B6	C57BL/6J AND C57BL/6Babr
DNA	DEOXYRIBONUCLEIC ACID
EU	EUROPEAN UNION
FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
F1	FIRST GENERATION
F2	SECOND GENERATION
HEPA	HIGH EFFICIENCY PARTICLE ABSORBANCE
IVC	INDIVIDUAL VENTILATED CAGE
i3S	INSTITUTO DE INVESTIGAÇÃO E INOVAÇÃO EM SAÚDE
NMRI	NAVAL MEDICAL RESEARCH INSTITUTE
PRT	PUP RETRIEVAL TEST
UK	UNITED KINGDOM
UP	UNIVERSITY OF PORTO

1. Introduction

Animal experimentation has played a key role in biomedical research since the beginning of biomedical science research (Franco, 2013). Discoveries such as surgical procedures, pain relievers, insulin, pacemakers, and organ transplants have been developed and tested in animals (NAP, 1991).

Growing awareness of how animals experience pain and suffering has led to strong opposition to animal research (Kiani et al. 2022). In 1959, the 3R's principle: *Replacement, Reduction and Refinement*, was described for the first time by Professor William Russell and Dr. Rex, balancing science with animal welfare (Russel & Rex, 1959). Later, aiming to promote compliance with and reinforcement of the 3Rs policy, the European Union approved Directive 2010/63/EU (Knudsen & Ritskes-Hoitinga, 2021), later transposed in Portugal to the law 113/2013.

Laboratory mice (*Mus musculus*) are an important study animal model, since it allows researchers to study complex physiological systems shared among mammals with a genetic background similar to that of humans (Breschi et al., 2017). Successfully breeding laboratory animals is crucial not only to supply enough animals for experiments but also from an animal welfare perspective. High and variable pre-weaning mortality levels remain a persistent problem. Among pre-weaning mortality risk factors, was recently identified Litter Overlap (the presence of older pups in the cage when a new litter is born; Brajon et al., 2019; Morello et al., 2020), which occurs due to the particularity of the short length of the mice' oestrus cycle (Bertolin & Murphy, 2014), which is repeated every 4 to 5 days unless interrupted by pregnancy or anestrus (Byers et al., 2012), or when one female is housed with more than one male (a common setting in breeding facilities). A higher age gap between litters leads to increased pup mortality levels (Morello et al., 2020). Possible explanations for Litter Overlap-associated mortality may be sibling competition for access to milk and impaired maternal care (maternal care is crucial for pup survival, as pups are fully dependent on dams for nutrition and thermoregulation; Brajon et al., 2019; Weber & Olsson, 2008). New-born pups have extreme difficulties in moving around the cage, due to their lack of locomotion abilities, therefore dam's ability to retrieve the pups back to the nest is important, especially because if pups are outside the nest, they may rapidly lose body temperature (Latham & Mason, 2004). Good maternal responsiveness (responding to a pup in distress; D'Amato et al., 2005) is one of the components of good maternal care.

In this study, the hypotheses are that Litter Overlap affects:

1. maternal responsiveness (Pup Retrieval, Nest Building, Nursing, Pup Grooming);
2. maternal behaviour during dam-pup isolation period;
3. new-born pup activity;

To test these hypotheses, an adapted Pup Retrieval protocol (PRT) was developed to test seven and six dams of Overlapped and Non-Overlapped litters, respectively, as a measure of their maternal responsiveness. The tests' observations were divided into three components:

1. Dam Behaviour during PRT: Frequency of dam's behaviours during PRT.
2. Dam Behaviour during the isolation period: Dam Behaviour was analysed during three out of the five-minute dam-pup isolation period.
3. New-born Pup Activity: Frequency of the five-day-old pups' behaviour during PRT.

The following study was divided into three components: observation of dam behaviour during isolation from pups, evaluation of maternal responsiveness through the PRT, and observation of pup behaviour during PRT, all tested in both Litter Overlap contexts (no overlap vs. overlap).

1.1. Animals in Biomedical Research: An Ethical Approach

Due to the taboos regarding human dissection, the first anatomical studies were performed on dissected live animals (vivisections) (Von Staden, 1989). Amidst the list of notable discoveries using vivisections of animals is the description of blood circulation and heart function (Franco, 2013). Later, between the 1800s and early 1900s, the findings of anaesthesia and analgesia combined with the rise of anti-vivisection movements, culminated in the first laws controlling the use of animals in research, for example, Great Britain's Cruelty to Animals Act of 1876. The Act stated that an experiment involving the infliction of pain upon animals should only be conducted when "the proposed experiments are essential for the due instruction of the persons to save or prolong human life". Furthermore, throughout the experiment, the animal must be anaesthetised, used only once per experiment (meaning that an experiment with several procedures could be performed using the same animals repeatedly), and killed as soon as the study was over (Lely, 1894).

In the future, animal research will be no less important than it was before. In fact, it may be even more important, since the existent remaining questions involve more complex diseases and injuries, requiring whole organisms to be studied. Many of nowadays leading killers and other debilitating conditions such as cancer and a variety of mental disorders are still poorly understood (NAP, 1991).

However, the growing awareness among the general public about the sentience of animals and their experience of pain and suffering has led to strong opposition to animal research throughout the years (Kiani et al. 2022). Scientists and ethicists have broadly acknowledged that animals can also experience distress and pain. Potential sources of such harm from their use in research include disease, basic physiological needs deprivation and invasive procedures (Kiani et al. 2022). Social deprivation and lack of the ability to carry out their natural behaviours are other causes of animal harm (Langford, 2010). Even in response to gentle handling and management, animals still can show marked stress-related physiological and hormonal alterations (Kiani et al. 2022). Some philosophical currents believe that it's possible to have a respectful attitude towards animals and non-human nature in general while understanding the need to use animals in science, especially the ones that try to define human "essence" based in comparisons between humans with other animals (Wilde, 2000).

One of the most common arguments against animal experimentation is the claim that this is a matter of speciesism (a term used when an individual is discriminated against solely due to the species it belongs to; Horta, 2010). This is a very sensitive and complex subject because we (as a society) tend to generally consider some animals "inferior" to others and to humans because we exploit them (Maurizi, 2011). For example, pets (domestic animals), usually included in the sphere of morality, are considered members of the family, with their interests normally met, their emotions and subjectivities considered and their comfort and safety normally guaranteed (Donaldson & Kymlicka, 2011).

The most accepted idea regarding animal experimentation nowadays is that the "cost/benefit" balance is a determinant factor to ethically justify animal use in research (Olsson & Sandøe, 2021). If the animals are, for example, used in a study focused on potentially life-saving treatments for humans, most people find the sacrifice of those animals justified. However, there is no justification for experiments conducted on animals for purposes such as cosmetics (Wilde, 2000). Since the relationship between humans

and animals probably will never be entirely non-conflictual (Wilde, 2000), especially in the current scientific context where animal models are still necessary to perform reliable scientific research (Freires et al., 2017), in 1959, trying to find an equilibrium between scientific needs and animal welfare, it was described for the first time the 3R's principle: *Replacement*, *Reduction* and *Refinement*, by Professor William Russell and Dr. Rex Burch in *The Principles of Humane Experimental Techniques* book. Over the years, the definitions of these three concepts have undergone several transformations, in order to keep up with the evolution of knowledge and to be more appropriate to the scientific methods currently applied (Hubrecht, 2019). Thus, today it can be defined that: *Replacement* is switching from animal models to non-animal models/non-sentient animal models that continue to proportionate the same reliability on results (Rossi & Oliveira, 2020). According to Russell & Burch (1959), it was the most important of the 3R's, since if the use of animals could be replaced, the remaining R's would become irrelevant. However, the various existing alternative pathways, such as the best use of existing literary information, predictions using mathematical models, in vitro studies (cell cultures, organoids), among others, with current technological and knowledge levels, are unable to respond to all the different existing research pathways (Knudsen & Ritskes-Hoitinga, 2021). *Reduction* implies reducing the number of animals used to the minimum possible without compromising the level of confidence (Knudsen & Ritskes-Hoitinga, 2021). Better literature search and the publication of studies regardless of the results obtained are techniques that can prevent duplication of work. Reuse (which should not be confused with continuous use) can also contribute to reducing the number of animals used in laboratories (Knudsen & Ritskes-Hoitinga, 2021). Several alternatives, which have replacement as their ultimate goal, are already used to promote the reduction of the number of animals used. For example, organoids are being developed to ultimately replace the use of living beings, however, it is currently only used as a means of reduction (Melo, 2021). *Refinement* is the attempt to cause the least possible suffering to animals throughout their lifetime (CCAC, n.d.). Although some consider it to be the least impactful of the Rs (Russell & Burch, 1959), it is still ethically relevant. Changes to experimental protocols, such as anaesthetising animals (as long as it does not interfere with the study) and providing them with the best possible living conditions (e.g., providing adequate cages), among others, may be sufficient to alleviate the stress and suffering normally associated with animals subjected to scientific experimentation (NC3Rs, n.d.).

On September 22, 2010, the European Union approved Directive 2010/63/EU, (later transposed in Portugal in the law 113/2013) which aimed to promote the reinforcement of the 3Rs policy (Knudsen & Ritskes-Hoitinga, 2021). Under this Directive, animals may be used in research when the potential scientific benefits are imperative and there is no viable alternative method. However, for research using animals to be ethical, scientifically rigorous and authorised under the Directive, it must comply with the welfare standards required and incorporated into the 3Rs (EU Monitor, n.d.). A better application of the 3Rs policy depends on the evolution of knowledge in various areas of science (ethology, cell culture, etc.), and will lead to a notable evolution in the quality of experiments from the animal perspective and partially contribute to the mitigation of all the existing controversy surrounding science and the costs that its evolution entails.

1.2. Mice in Biomedical Research

As previously stated, suitable alternatives to animal models are not available in many areas of biomedical research, since formally accepting these methods requires time, prior validation and financial and scientific investment. Alternative models represent, so far, an unconventional pathway to obtain reliable results. The accuracy of generated data and correlation with the findings observed in mammals varies among the models (Freires et al., 2017). However, these methods can be used to perform pilot tests and pre-trials before animal testing (Takahashi, 2019).

Animals are used to study human diseases because of their genetic, anatomical, and often physiological similarities to humans (Bruter et al., 2024). Although organisms, such as yeasts, worms and flies, are excellent models for studying basic biological processes, laboratory mice (*Mus musculus*) are far better at recreating the complex physiological systems shared among mammals and share a similar (95% similarity) genetic background with humans (Breschi et al., 2017). Besides that, mice are easy to handle, easy to perform genetic manipulation (there are many knock-out strains available in cryopreservation banks), many human diseases affect them too (Freires et al., 2017), have an unlimited supply, their pregnancy lasts for 3 weeks, on average they have around 7 pups per litter. They reach sexual maturity at 4 to 7 weeks old, which means high reproductive rates, and relatively low cost of use (Simmons, 2008).

To obtain scientifically valid research, the conditions associated with an experiment must be highly controlled. For example, in situations where researchers need to manipulate only one variable while keeping others constant, to observe the consequences of that

exact change. This is possible when using inbred mice strains, where the individuals are genetically very similar to each other, paving the way to annul as many other variables as possible. Also, to test hypotheses, an adequate number of subjects must be used to test the experiment's results statistically. Therefore, scientists cannot research just one animal or human, and animals allow scientists to use sufficiently large numbers of individuals to attain significant results in a short time range (Simmons, 2008).

So, despite the differences in body size and lifespan, the laboratory mouse is considered the best model for many types of biomedical research (Bruter et al., 2024).

1.3. Laboratory Mouse Breeding

Mice are polyoestrous mammals, and females ovulate spontaneously. Weaning in mouse breeding is normally done at 20 days old, while mice become sexually mature around 7-8 weeks of age, when females and males are normally housed together for mating. Gestation lasts approximately 21 ± 2 days and mice are commonly allowed to breed continuously until retirement age (which varies across breeding facilities). A particularity in the mouse is the short length of its oestrous cycle (Bertolin & Murphy, 2014), which repeats every 4 to 5 days unless interrupted by pregnancy or anestrus (Byers et al., 2012). The oestrous cycle in mice is divided into 4 stages (proestrus, oestrus, metestrus, and dioestrus; Byers et al., 2012), and it is characterized by changes in the anatomy, endocrinology, physiology, and behaviour, that coordinate to achieve the apex of fecundity. It is crucial for the rhythm of the cycle that the hypothalamus–pituitary–gonadal axis is fully synchronized. During each phase, specific hormones drive the changes that occur and induce transformations in the mouse's anatomy. The first phase, proestrus, is the period in which pre- and peri-ovulatory development occur in the ovary, leading to the synthesis and secretion of oestrogens. Oestrus, the second phase, corresponds to the brief interval during which the female accepts the male and ovulation occurs. The third phase metestrus, is the early luteal phase that is followed by the fourth and last phase dioestrus, where progesterone is the dominant hormone. These variations render identification of the individual phase possible by examination of the vaginal appearance and of exfoliated vaginal cells (Bertolin & Murphy, 2014).

These short oestrus cycles allowed researchers to create several types of breeding systems for mice, in a short period, whether they needed genetically diverse or identical mice for their experiments. Genetically diverse, or outbred, mice are called stocks. Swiss Webster, ICR, and CD-1 are examples of the most used stocks. On the other side,

genetically identical mice are called strains (Suckow et al., 2023). There are many different types of defined strains, such as Inbred Mice that result from a minimum of 20 consecutive generations of related mice matings, being homozygous to almost all loci (e.g. C57BL/6, BALB/c, C3H, FVB, 129, DBA, and CBA); Hybrid Mice that are first generation (F1) crosses between two different inbred strains (e.g. B6D2F1 is a cross between a C56BL/6 female and DBA/2 male); When two F1 hybrids (resulting from the same cross) are mated together, it results in second generation (F2) mice (Hedrich, 2004). To create Recombinant Inbred Strains, it is necessary to cross 20 consecutive generations related mice starting with the F2 mice; Coisogenic Strains are strains that differ from each other only at one gene due to a spontaneous mutation that occurred in that gene. After the appearance of the mutation, the mutant animals are maintained as a separate strain from the original inbred strain; Mice that carry induced mutations include Transgenic mice (mice that carry foreign DNA that was inserted on purpose into their DNA), Knockout and targeted mutation mice (mice with a gene that was made non-functional), and Congenic mice (the genetic dissimilarity between the supposed identical strains is created through breeding rather than being result of a mutation. This is accomplished by breeding animals with the desired gene(s) with animals of a selected inbred host strain, then breeding their offspring back to the same host strain (backcrossing), and so on for at least ten generations; Suckow et al., 2023 & Hedrich, 2004).

Besides breeding systems, there are also mating systems (ways to set the breeding group of male and female(s)). They divide into 3 types: Pairs (monogamous mating of one male and one female), trios (polygamous, one male and two females), and harem (colony mating, one male and multiple females). Pair housing allows a more precise monitoring of the offspring, including the detection of their exact birth date, and it is one of the most used systems for inbred strains (Hubrecht & Kirkwood, 2010). Besides that, it also allows for the post-partum oestrus to be used. However, it requires more room space (more cages) and breeding resources. Trio housing is suitable for both in- and outbreeding (The Jackson Laboratory, 2024a). It more likely leads to Litter Overlap situations than pairs (both females produce offspring simultaneously), with a less accurate detection of which litter corresponds to which dam. Nevertheless, in this system, pups are raised by the two females (and the male), which is closer to their behaviour in the wild (Hubrecht & Kirkwood, 2010). Harem housing generates more offspring per male. When the females get pregnant, they should be moved to a littering

cage to avoid disturbance from the other females. The problem of detecting corresponding dams with their litters remains in the harem system, making it even harder to determine the exact age of pups. Although this system tries to take the largest number of pups from the least number of breeder animals, it is also where it is observed the highest pup mortality levels (Hubrecht & Kirkwood, 2010).

Colonies can be maintained at the research institute's animal facility, or by companies and the institute purchases the animals from the company (The Jackson Laboratory, 2024b). Therefore, in-house breeding is nowadays mostly confined to stocks that are not commercially available (Hubrecht & Kirkwood, 2010).

There are four different cage sizes commonly used to house laboratory rodents: type I, II, III (with two different heights: conventional and higher), and IV. These different cage sizes differ in their floor area and height. Type II and III are more used for mice. The type of cage being chosen varies according to the number, age, weight, familiarity, and reproductive conditions of the mice that will be housed (CCAC, 2019). Besides these, there are other "special" cage types, such as metabolic cages and Individual Ventilated Cages (IVCs). Metabolic cages are used in studies where the separation of urine and/or faeces is necessary. To do this, the cage floor is a metallic grid to let the components get through. Since the animals are not comfortable on that type of floor, being isolated and without environmental enrichment, it is recommended that the animals spend the least time possible in these cages (Kalliokoski et al., 2013). IVC (Individual Ventilated Cages) produce a cage environment different from the one in the animal room to protect mice and workers from infections. Each cage receives high-efficiency particle absorbance (HEPA)-filtered air which is supplied under positive pressure. The exhaust air is also HEPA-filtered before being returned to the room environment (Brielmeier et al., 2006).

Routines at animal facilities can affect mice behaviour. Cage changes are essential for appropriate laboratory mouse husbandry. However, it also can be disruptive to mice. It disrupts their scent cues and arrangements of nesting materials while placing them into a "new environment" at an interval that changes according to the facility management to meet general guidelines. This variation may adversely affect scientific studies. Factors such as the type of caging, type and quantity of bedding, strain, and sex can influence microenvironmental parameters. In addition, cage changes may affect the behaviour and health of mice (Rosenbaum et al., 2009), although this is still controversial (Wharton et

al., 2024). Water and food are available *ad libitum*. The water needs to be changed at least once a week and the food rack needs to be kept full, so the mice can grab the food pellets with the help of the weight of the top pellets and eat (Hubrecht & Kirkwood, 2010).

1.3.1. Mortality in Laboratory Mouse Breeding

Despite ongoing efforts to reduce the number of animals used in research and to improve their welfare according to the *Replacement, Reduction, and Refinement* (3R's) principle for research (Russell & Burch, 1959) high and variable pre-weaning mortality levels remain a persistent problem. Reports of laboratory mouse pre-weaning mortality are as high as 49%, and often above 20% for C57BL/6 mice, one of the most used mice strains (Inglis et al., 2004; Morello et al. 2020). Pre-weaning mortality typically occurs within the first 72 hours post-partum, a period during which animal care-takers usually avoid inspecting breeding cages to prevent disturbing new-born pups. Since adults tend to eat pups that are dead, even cage inspections that occur within the first 24 hours after birth may underestimate pup mortality (Brajon et al., 2021), as these casualties will not enter the breeding facilities' records. Such high mortality rates in mouse breeding may be an indication that the 3Rs principle have not been efficiently applied in breeding.

Mortality rates vary greatly among different species, but they share similar causes of death in breeding (e.g. in farm animals) such as hypothermia, underfeeding, inappropriate maternal behaviour, infections and injuries (Mellor & Stafford 2004). Similarly, several environmental, management and behavioural factors such as the thermal and social environment of the cage have been identified as possible causes for early mortality in mice (Svere & Broida, 1982; Wharton et al., 2024).

In the case of altricial species such as the mouse (species in which the young are underdeveloped at the time of birth), infants are highly dependent on their mother for nutrients and thermoregulation, thus if the mother fails to provide appropriate care, infants are likely to die. Strain differences found in pup survival rates may be due to differences in maternal ability or differences in pup viability (Weber et al., 2013). Thus, disturbed maternal behaviour may play a role in pup mortality in mouse breeding.

Among mortality risk factors in mouse breeding is also Litter Overlap, which has recently been identified as a factor that increases the probability of C57BL/6 newborns to die (Brajon et al., 2019; Morello et al., 2020).

Two of the most common configurations for housing mice in breeding facilities are pairs and trios. Although Litter Overlap is more frequent in trios, due to the presence of two breeding females, Litter Overlap in pair cages still occurs when the dam gives birth just before weaning its previous litter. Thus, the age gap between litters in pair cages of breeding C57BL/6, for example, is often above 18d, the average gestation length for this strain.

A retrospective analysis of breeding records of over 219 000 pups (Morello et al., 2020) demonstrated that an increased number and age of older pups in the cage when new pups are born leads to a higher probability of the new pups dying, especially in situations where the older litter is more than one week old at the time of the birth of the new litter (Figure 1). Therefore, in trios, we can classify as Risky Overlap the age gap between newborns and older litters that is higher or equal to eight days and classify as Non-Risky Overlap the age gap between newborns and older litters that is equal or less than five days (Morello et al., 2020).

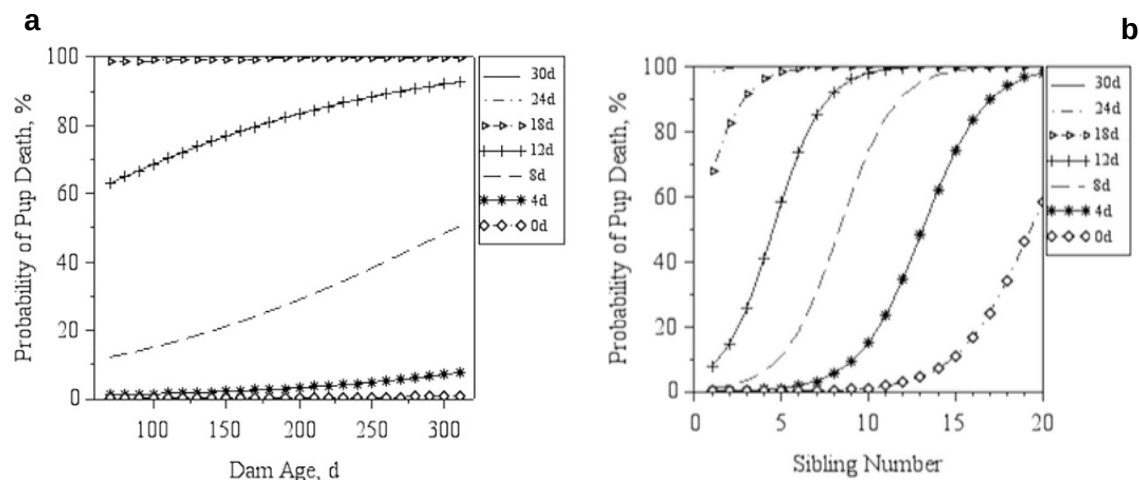


Figure 1 – Litter Overlap effects. **(a)** Predicted probabilities (least-squares means) of a pup to die as a function of Dam Age. **(b)** Predicted probabilities (least-squares means) of a pup to die as a function of Sibling Number (number of older pups in the cage at the birth of the focal litter), from Morello et al., (2020).

Possible explanations include sibling competition for access to milk and impaired maternal care and nursing behaviour.

The amount of milk consumed by a pup can affect some of their future characteristics as adults, such as weight and aggressiveness, specifically in male pups, where their weaning weight can have long-term effects on their ability to become dominant adults and affect the age at which they attain sexual maturity (Latham & Mason, 2004). The

amount of food pups obtain is a limiting factor in their growth (Falconer, 1947), thus their survival, if pups do not receive milk during their first day of life, they lose approximately 8% of their birth weight (Konig et al., 1988).

In a Litter Overlap context, the older litter is heavier, more developed and more mobile than the younger one. It may interfere with the access of the new-borns to milk by stealing it or displacing the lighter new-born pups from the dam's nipples (Morello et al., 2020). Also, Morello et al. (2020) showed that a smaller age gap between the new-born and the older litter lowers the pup's probability of death. Thus, older pups less than a week older than the younger siblings, may not be able to interfere with the youngest access to appropriate amount and quality of nutrition.

During the first 12 days after birth, pups are fully dependent on their mother for nutrition, temperature regulation and to stimulate defecation. During this period, the dam spends most of its time close to the pups, either gathering them in the nest or crouching over them (König & Markl, 1987). Brajon et al., (2019) showed that in trio cages with two litters, the three adults care for their newborn pups a total of 20% less time than adults with one single litter in the cage. Thus, reduced parental care in cages with more than one litter can be one of the mechanisms through which Litter Overlap interferes with the newborns' survival (Morello et al., 2020).

1.4. Maternal Behaviour

Maternal care is essential for pup welfare and survival, as pups completely depend on the dam for nutrition and thermoregulation (Brajon et al., 2019; Weber & Olsson, 2008). Both "active" (nest building and pup retrieval) and "passive" (resting with, crouching over, and nursing pups) maternal behaviour have been associated with litter survival (Weber, 2015). If a dam fails to provide appropriate care, infants are likely to die, for example, from hypothermia or starvation. Moreover, the dams' responsivity to stressors or anxiety levels has also been linked to pup mortality (Carter et al., 2002). Soon after birth, nursing is initiated by dams, while suckling, initiated by pups, is often observed only on day 13 post-partum (Weber & Olsson, 2008). Complications in maternal care may lead to negligent nursing behaviour by the dam and increase unequal competition for access to milk between litters (since new-born pups are less mobile than older ones). Therefore, in a Litter Overlap context, maternal care can be hampered by the following factors: The presence of the older pups in the cage, possibly leading to unequal competition between older pups and new-borns; Increased cage stocking density, which may also contribute

to increased severity of the consequences of competition, as trauma caused by trampling and stepping of new-borns either by adults or older pups may occur (Morello et al., 2020); Dam advanced age when having the new-borns, that according to Morello et al., (2020), increases the probability of pup death, perhaps due to the age-related hearing loss that is common to females of C57BL/6 mice. Pup calls are important signs of stress and affect maternal behaviour such as pup retrieval. Thus, if older dams are not capable of hearing their pups correctly, pup chances of survival could be reduced through a reduction in maternal care (Latham & Mason, 2004). Ultrasounds and Wriggling calls elicit different patterns of maternal behaviour: Wriggling calls release maternal behaviour and 'attention' responses in the mother (Ehret & Bernecker, 1986), where Licking pups is the more frequent maternal response, followed by changes in suckling position and Nest Building. On the other hand, USVs are used to indicate a kind of discomfort, distress or pain which is terminated by an appropriate response from an adult (Ehret & Bernecker, 1986). Wriggling calls keep these maternal behaviours at a high level. Otherwise, their spontaneous rates of occurrence would decrease rapidly after the birth of the pups (Ehret & Bernecker, 1986), since after delivery, the mothers lose maternal interest in their pups very rapidly (Scudder et al. 1967), and maternal behaviour is kept at a high level only by external stimuli from the pups which may function as an indicator of the vitality of the young (Ehret & Bernecker, 1986). Thus, ultrasounds release mainly pup approach and retrieving in adults (Ehret & Haack, 1984). When wriggling calls are produced, USVs do not occur (Ehret & Haack, 1984).

Only after 23 days of life, nursing gets replaced by resting with body contact without offspring trying to suckle, nevertheless, litters still require maternal care at this age (Weber & Olsson, 2008).

So, new-born pups have extremely limited abilities to move, therefore the mother's ability to retrieve the pups if they fall outside the nest is important for survival, since, without the insulation from the nest and the mother, pups rapidly lose body temperature (Latham & Mason, 2004).

1.4.1. Pup Retrieval

Maternal behaviour is regulated by the combination of factors of the central nervous, endocrine systems, and sensory stimuli from the offspring and the environment (Stern, 1989). It is also influenced by genetic factors where different strains of mice show different patterns of maternal behaviour (Carlier & Cohen-Salmon, 1982). Since C57BL/6

mothers are better than other strains in performing nest building and pup retrieval behaviours (Brown et al., 1999), when questioning C57BL/6 dams if Litter Overlap context affects their ability to provide maternal care, researchers should aim at these behaviours.

Pup Retrieval is a natural behaviour of maternal care where a female (that can be or not the mother) finds and then carries a pup back to the nest by taking a part of its body into her mouth (Brown et al., 1999). A Pup Retrieval Test (PRT) consists of the simulation of a situation where the dam needs to retrieve the pups (that were artificially placed outside the nest) back to the nest, thus it is used to quantify maternal care quality (Winters et al., 2022). It can be used in a variety of applications, such as research on disorders that affect mother-infant interactions (autism, chronic prenatal stress, postpartum depression, and schizophrenia; Winters et al., 2022).

Attachment 1 summarises the main characteristics of the PRTs performed over the past 42 years. Not surprisingly, the methodology for PRTs among these papers is substantially variable. Such variation demonstrates the need for more studies to standardize PRTs and methods for evaluating maternal care.

2. Materials and Methods

2.1. Animals

Two groups of mice were used to accomplish the proposed MSc project. The first group of mice (Group 1) consisted in 13 C57BL/6J (B6) mouse litters from the i3S (project host)'s own breeding colony that produces mice for experimental purposes, thus no new animals were generated solely for the purposes of this project. The B6 litters of Group 1 were housed either in pairs (i.e. one female and one male mouse; $n=3$) or trios (i.e. two female and one male mice; $n=10$). Food and water were provided ad libitum, under a standard 12-h light-dark cycle (lights on at 8h), and room temperature was maintained at a fixed level of 21°C (Table 2). Two pups were used per litter ($n=26$) in this project, which were four ($n=10$), five ($n=12$), six ($n=2$) and seven ($n=2$) days old. Selected litters were based on the number of B6 litters with four-to-seven-day-old new-borns (focal pups) and on the presence of an older litter in the cage during the data collection phase of this project (June to August of 2024). Litters with focal pups born in the presence of an older litter in the cage were classified as Overlapped Litters ($n=7$), whereas litters with focal pups born without the presence of an older litter were classified as Non-Overlapped Litters ($n=6$). Five of the Overlapped Litters had an age gap of zero to two days of age with the older litter in the cage (i.e. non-risky overlap), whereas the remaining two litters had age gaps of nine and 15 days of age (i.e. risky overlap). Details about daily breeding routines in i3S's B6 breeding colony can be found in Table 1.

Table 1. Mouse breeding routine in the C57BL/6J colony at the i3S Animal Facility, adapted from Morello et al., 2024.

Mouse Strain	C57BL/6J
Housing configuration	Trios (two adult females and one male) /pairs (one adult female and one male)
Type of cages	Standard Type II (pair-housing, 268 x 215 x 141 mm) and III (trio-housing, 425 x 276 x 153 mm) Tecniplast Filter-Top Static Cages
Bedding	Corncob bedding (grade 12, ULTRAGENE, Portugal)
Nest material	Compacted shredded virgin craft paper (E-Cube, Allentown LLC, New Jersey, USA)
Enrichment	Shelter (GLP Semi-detached Dome Home, LBS Biotechnology, United Kingdom)
Water	Ad libitum from a drinking bottle with nipple, sterilised by reverse osmosis, filtered with a Polyethersulfone filter (SPECTRUM Filtration, England) and ultraviolet light disinfection
Food	Ad libitum as 12 mm dry pellets in a hopper (2018S – Mucedola Tecklad for Inotiv, Italy)
Cage change routine	Once a week on a fixed weekday for each room
Room temperature (target)	21°C (20°C-22°C)
Light schedule	Cycles of 12 hours of light (7:00h to 19:00h/ 8:00h to 20:00h between end of March and end of October) and 12 hours of dark
Weaning age (target)	21 days (delayed if pups were too small or if completion of 21 d was on a weekend or holiday)
Breeding start age (target)	Dam between eight to nine weeks of age
Retirement age (target)	Dams are retired at one year of age or sooner if presenting welfare/health problems
Facility pup-counting routine	Pups are counted once between their birth and day seven post-partum by a trained technician, with minimal handling, during cage-change.
Study pup-counting routine	Pups were counted daily by a trained scientist at 09.00h, and by the animal care-taker at 16,00h if litters were born either past 09.00h, or on weekends.

The second Group (Group 2) consisted of mice recorded at the Biological Support Unit (BSU) of the Babraham Institute (Cambridge, UK), from mid-March to mid-June 2017, for Brajon et al.’s study (2019), which used a total of 109 wild-type litters derived from C57BL/6J parent stock (C57BL/6Babr), being 35 and 20 Overlapped and Overlapped Litters, respectively. Five Overlapped Litters (three risky and two in non-risky) and five Non-Overlapped Litters were selected from Brajon et al.’s study (2019) to form Group 2, all housed in trios for use in the current MSc project. Litter selection was based on whether at least three four-to-seven-day-old pups were displaced away from the nest, creating the conditions for pup retrieval to take place. The Babraham Institute’s animal facility’s routines can be found in Table 2.

Table 2. Mouse breeding routines at the C57BL/6Babr mouse colony at the Babraham Institute's Biological Support Unit, adapted from Morello et al., 2020.

Mouse Strain	C57BL/6Babr
Housing Configuration	Trios
Type of Cages	IVC (individually ventilated cage) Tecniplast GM500 transparent polysulphone
Ventilation Rate	65 to 75 air changes/hour
Air handling unit	Tecniplast DGM80 and DGM160
Bedding	5mm deep soft-wood-flake bedding (ECO6, Datesand Group, Manchester, UK)
Nest	7g of white paper rolls (Enrich-n ´Nest, Datesand Group, Manchester, UK)
Enrichment	Tecniplast Mouse Pouch Loft, a second level flooring within the cage
Water	<i>Ad Libitum</i> , sterilized through reverse osmosis and provided through automatic drinking valves (Edstrom A160/QD2, Avidity Science LLC, Waterford, WI, USA)
Food	<i>Ad Libitum</i> , in the form of 9.5mm diameter dry pellets (CRM(P), Special Diet Services, Witham, Essex, UK)
Cage Change Routine	Cages are changed once every second week; cages with large litters are sometimes cleaned every week
Room Temperature (target)	20 to 21°C
Room Relative Humidity (target)	50%
Light Schedule	12h of light (7:00 - 19:00) and 12h dark
Weaning Age (target)	21 days
Breeding Start Age (target)	8 to 9 weeks
Retirement Age (target)	24 to 32 weeks; longer if productive
Pup Counting Routine	Pups are counted once between their birth and day 7 post-partum with minimal handling

2.2. Pup Retrieval Test (PRT)

A PRT protocol was developed based on a background literature review. This literature review also served as a starting point to determine all the behavioural repertoire that should be observed from the PRTs as a measure of maternal responsiveness, with some adaptation to the context of Litter Overlap. The developed PRT Protocol used 20 scientific papers (Table 1) that used PRTs in their methodologies to test their respective hypotheses. Based on these publications, a set of PRT-relevant criteria was selected for building the protocol and evaluating potential PRT response variables suitable to

investigate the current project's hypotheses. The selected criteria were: "Study's Goals", "Year of Publication", "Mouse Strain", "Pup Age when Tested", "Dam-Pup Isolation Time" (period of separation of dams from their litters), "Type of Container/Room for the Dam-Pup Isolation Period", "Acclimatization Time before PRT", "Heating-pad Temperature" (for isolating the pups), "Number of Pups Isolated", "Location of the Animals (dam and pups) During the Isolation Period"; "Post-Isolation Protocol" (protocol after the isolation period began), "Number of Pups Used" (number of pups tested), "Pup Placement Order" (order by which the pups were placed inside the test cage during PRT), "Post-PRT Protocol" (protocol post-PRT); "Schedule" (time of the day when the test was performed); "PRT Test Duration"; "List of All Measured Variables" (variables that were measured in PRT).

PRT-selected response variables are listed as follows:

1. Time Taken to First Approach a Pup: For the 1st pup, it's the time since the last pup (3rd) was placed in the cage, that the dam took to have the 1st contact with it. For the other 2 pups is the time since the dam leaves the nest after retrieving the previous pup until it approaches the pup. The 1st contact is considered when the dam elongates the body towards the pup to sniff it carefully. This was measured from video observations for each of the two pups used for the PRT test in each litter. The total time taken to approach both pups (summation of time to approach pup 1 and pup 2) and the average time taken to approach a pup were used as the response variables in the present study;
2. Time spent Doing Other Activities: Time spent performing behaviours that were not predicted to be part of the PRT observation. This was measured from video observations during the PRT of the two pups used for the PRT test in each litter. The total time spent in other activities (summation of time spent in other activities during PRT of pup 1 and 2) and the average time spent in other activities per pup were used as the response variables in the present study;
3. Time Between First Approach and Taking Pup into the Mouth: Time that the dam took since the first contact to grab the pup. Time starts counting when the dam tilts its head to take the pup into her mouth or forepaws. This was measured from video observations of each of the two pups used for the PRT test in each litter. The total time taken since approach to mouth (summation of time to pick pup 1 and 2 with the mouth since approach) and the average time taken to grab a pup with the mouth were used as the response variables in the present study;

4. Time Between Pup in Mouth and Nest: Time since the mother initiates the retrieval (retrieving initiates when the dam tilts its head to take the pup into her mouth or forepaws and ends when the pup is no longer in the mouth or forepaws of the mother) of the pup, until placing it in the nest. The retrieve is considered complete when the female places 50% of the pup's body inside the nest/inside the dome. This was measured from video observations of each of the two pups used for the PRT test in each litter. The total time taken since mouth to the nest (summation of time to place pup 1 and 2 in the nest since being picked with the mouth) and the average time taken to place a pup in the nest since being picked with the mouth were used as the response variables in the present study;

5. Time Spent Carrying: Total time that the dam spent carrying the pups until placing them in the nest. This was measured from video observations of each of the two pups used for the PRT test in each litter. The total time carrying (summation of time carrying pup 1 and 2) and the average time carrying a pup were used as the response variables in the present study;

6. Pup Investigation Without Retrieval: The number of times the female drew away from one of her pups, situated outside the nest, without their being transported. This was measured from video observations of each of the two pups used for the PRT test in each litter. The total number of times the dam drew away (summation of times dam drew away from pup 1 and 2) and the average number of times drew away per pup were used as the response variables in the present study;

7. Time Spent in the Nest with Pups: Time the dam spent inside the nest with the pups, interacting with them, until it leaves the nest for the first time after retrieving the pup or stops physically interacting with the retrieved pup. This was measured from video observations of each of the two pups used for the PRT test in each litter. The total time spent in the nest (summation of time spent in the nest with pup 1 and 2) and the average time spent in the nest with each pup were used as the response variables in the present study;

8. Interval between retrievals. Time since the dam places a pup in a nest until it grabs with the mouth the next one. This was measured from video observations of each of the two pups used for the PRT test in each litter.

9. Unusual Behaviours. Number of times that the dam performed behaviours that are considered to be unusual when performing Pup Retrieval such as: removing a previously returned pup from the nest; and extensive disruption of the nest shavings. This was

measured from video observations of each of the two pups used for the PRT test in each litter. The total number of times the dam performed an unusual behaviour (summation of times dam performed unusual behaviours to pup 1 and 2) and the average number of times the dam performed unusual behaviours per pup were used as the response variables in the present study;

10. Time Spent Doing Maternal Behaviours. Time spent by the dam performing the maternal behaviours described in the ethogram (except for complete and incomplete retrieval). This was measured from video observations of each of the two pups used for the PRT test in each litter. The total time spent in the nest (summation of time spent doing maternal behaviours to pup 1 and 2) and the average time spent doing maternal behaviours to each pup were used as the response variables in the present study;

2.2.1. PRT Protocol for Evaluating Group 1, from i3S

For evaluating maternal responsiveness using Group 1, the developed PRT Protocol was used, as presented below:

A Go-Pro video camera (HERO 3+ model, GoPro Inc., USA) and an Android-operated smartphone (used as a chronometer and as a backup recording device) were wrapped in plastic film and disinfected with a potassium peroxymonosulfate-based disinfectant (Virkon, Lanxess AG, Germany), prior to conducting each of the PRTs. A three-dimensional-printed camera support (Figure 2) was fumigated with hydrogen peroxide before the study and remained in the B6 breeding room during the entire period of data collection. This support was disinfected with a 70% ethanol solution was sprayed on the support every test day.

Prior to each PRT, the breeding room's animal caretaker placed the study cage(s) on the workbench (with closed lid(s)) with lights and fans on for 30 minutes for acclimation.



Figure 2 - Simulation of the setup used in the i3S's animal facility experiments. The three-dimensional-printed support allowed the camera to film the cage from above, without human interference.

The 30-minute acclimation period is enough to avoid stress-related bias (Aung et al., 2022; Cohen et al., 2011; Kohl et al., 2018; Meek et al., 2000; Zimprich et al., 2017). While preparing for the PRT, a clean cage with food, water, bedding and nest material was placed on the workbench, along with a heating mat set at approximately 35°C surface temperature (Wei-Li Wu et al., 2009; Winters et al., 2022). For isolating the dam, the mating female (of trio-housed mice), the male and the older litter (if present) were placed in the clean cage (Figure 3). The adults were handled with the help of a transparent plastic tube, while the pups were picked by hand. The decision to record this section instead of recording the acclimatisation period was due to animals eventually falling asleep during this period, therefore it was decided to check if the data from the five-minute separation time would be more relevant to the analysis.



Figure 3 – Male, mate-female and older litter in the clean cage during the dam-pup isolation period. The nest material (paper towel sheets) was only briefly removed for photographic purposes.

The new-born pups were then carefully placed inside a shelter on top of the heating mat (Figure 4). The heating mat was disinfected between PRTs with a 70% ethanol solution.



Figure 4 – Tested litter during the five-minute separation time. It is not directly on top of the heating pad (protected by a paper sheet), and also protected by the igloo. In B the pups are only without the igloo for photographic purposes.

The dam was isolated from her pups and cage-mates in her original cage for five minutes. Dams were maintained in their original cage during the isolation time because they take longer to perform the PRT in a novel cage (Aung et al., 2022). During the isolation period, the nest was scored according to its “height and closure of the walls surrounding the nest cavity” (Hess et al., 2008; Attachment 2), as illustrated in Figure 5.



Figure 5 – Nest photo for nest scoring. Example of nest photo taken before PRT to perform nest scoring (Hess et al., 2008), during the dam isolation period.

When the separation time was completed, three pups were picked by hand and placed at the opposite cage end from the nest (Figure 6) which were approximately equally distant to each other.



Figure 6 – Screenshot of the beginning of one of the PRT trials. The three pups are aligned, and equally distant to each other.

When the three pups were placed inside the home cage, it marked the beginning of the nine-minute test (three minutes per pup; based on previously validated protocols three minutes is enough time for the female to retrieve the pups; Aung et al., 2022; Carcea et al., 2021; Ng et al., 2023; Okabe et al., 2017; Wei-Li Wu et al., 2009). Because two of the Overlapped Litters used only had two pups, the response variables were only observed for the first two pups retrieved in all litters. The pup retrieval test ended after six (two-pup litters) to nine minutes (for litters with three or more pups) or when the dam finished retrieving all the pups back to the nest (in this case, the test pups were placed back in the core of the nest). The nest was considered to be either inside the white igloo (if it existed) or in the area with a higher concentration of nest material. A complete retrieval was considered when at least 50% of the pup's body was on the nest area, characterized by a virtual circle containing more than 50% of the nest material.

2.2.2. PRT Protocol for Evaluating Group 2, from the Babraham Institute

The video recordings of Group 2 (Figure 7) were not originally meant to be used in PRTs, thus it was necessary to make some adjustments to the developed PRT protocol so that the analysis could be performed in the most consistent way possible. For each litter, a scan sampling was made every 30 seconds of the videos and selected the videos of the first three pups that were completely displaced to outside the nest (independently of how

far they go). Since the context of these PRT was different, the variable “Interval between Retrievals” was not measured because some of the trials were separated (pup 1 and 2 in different trials). Thus, the start of each Babraham-PRT test was considered to be when the pup was visibly fully out of the nest. The maximum duration of the test was also three minutes maximum per pup. If after this period the dam didn’t retrieve the pup(s), they were considered non-retrievals and excluded from the analysis.



Figure 7 – Screenshot of one of the videos from the Babraham Institute (Trio with two females and a male, where one of them is inside the red tunnel, and a Non-Overlapped litter).

2.3. Maternal Behaviour

2.3.1. Ethogram

The ethogram was built both for PRT observation as well for Dam-Pup Isolation Period observations. The ethogram (Attachment 3) resulted from a literature review process (Table 1) plus a pre-trial test to validate the ethogram by observing 22 PRT recordings from the papers cited in the review. The protocol used to build the proposed ethogram was based on Gutierrez-Gomez et al. (2021). Here, the aim was to build an ethogram that could, not only, describe the interaction between a dam and its pups (specifically during a PRT in the context of Litter Overlap) but also dams' solo behaviours. The ethogram was adapted from the pre-existing ethograms from Brajon, et al., (2019), Winters, et al., (2022) and unpublished refined ethograms from the LAS (i3S, IBMC, UP)

team to allow to perform PRTs. The adapted ethogram was tested for its applicability to PRTs. Continuous observations were performed on PRT videos, and the observed behaviours were grouped into functional categories (i.e., categorizing behaviours that serve a common function together). This step aids in the interpretation of the ethogram and allows for a more systematic analysis of the data. The final step was to validate the ethogram. The process of validation (the visualization of the 22 PRT recordings mentioned above) allowed to add/remove or combine relevant behaviours and ensured that behaviours' descriptions were accurate for the reader (even for readers outside the area). For that, a person from the area who knows the behaviour studied read the descriptions and guided us throughout the refining process. Once this was done, the final Ethogram was used in PRT from 63 openly published videos corresponding to 11 litters from Winters et al., 2022 and Zimprich et al., 2017. Minor necessary changes were applied to the ethogram which became a standardized PRT ethogram, which can be used for PRTs in the context of Litter Overlap.

2.3.2. Dam-Pup Isolation Period Observation

Dam behaviour was coded from recordings of the five-minute isolation period from i3S experiments, using Boris software (v.8.27). From these five-minute videos, a scan analysis of the three minutes following the first 26 seconds of each video was performed. Since it was decided to study maternal behaviour after the PRT trials had already begun, not all of the litters had the whole five-minute isolation period recorded. Behaviours were compared between cages with and without Litter Overlap, and the observer was blind for the group each video belonged to. The variables that were analysed are:

1. Time Spent in Locomotion: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.
2. Time Spent Sniffing: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.
3. Time Spent in Nest Building: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.
4. Time Spent in Other Behaviours: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.
5. Time Spent in Self Oriented Behaviours: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.
6. Time Spent Digging: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.

7. Time Spent Inactive: This was measured from video observations of the dam-pup isolation period, prior to the PRT in each litter.

These variables were chosen from the literature review process, where only the variables that could provide information potentially useful to answer the research question were selected (Table 1).

2.4. Newborn Pup Activity Observation

Pup activity was continuously observed during the PRT for each pup, starting from the moment that the last (third) pup was placed in the cage until the pup that was being scored was successfully retrieved. Due to the lack of ethograms for the evaluation of young (four to seven-day-old) pups, there was a need to develop an ethogram to quantify the level of activity of pups during PRT (Figure 8). To build the ethogram for pup behaviour during PRT, descriptions from unpublished data of LAS, Heyser, 2004 and Feather-Schussler et al., 2016 were combined.

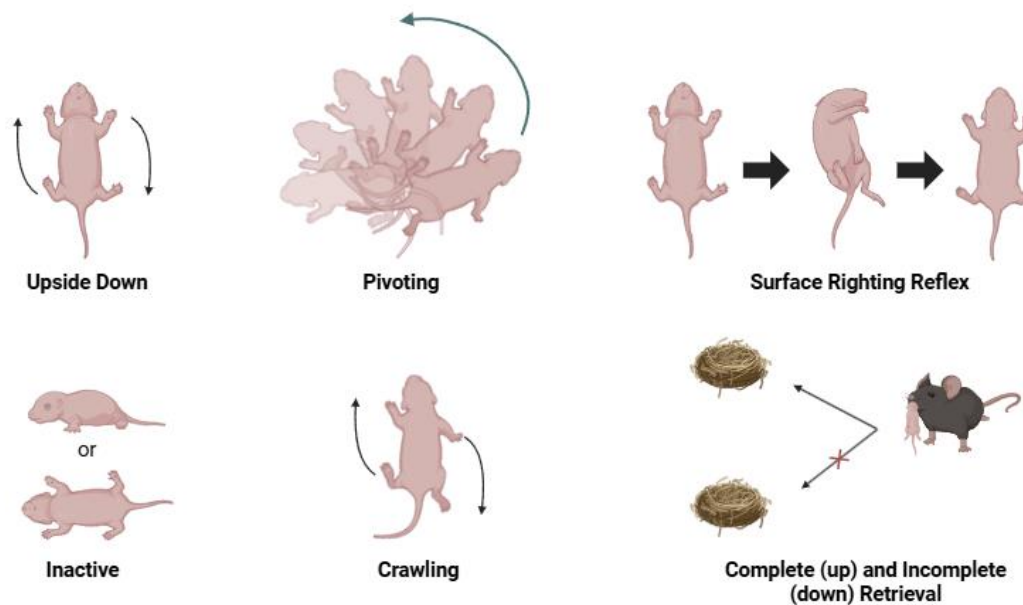


Figure 8 – Newborn Pup Activity Ethogram Illustration during Pup Retrieval Test. The figure was illustrated with BioRender.

The variables that were analysed are:

1. Time Spent Pivoting: The hindlimbs are not coordinated with the forelimbs. Combined with the pelvis remaining anchored to the ground, and forelimb movements, pup moves in a circular motion (Heyser, 2004). This was measured from video observations of the dam-pup isolation period, before the PRT in each litter.

2. Time Spent Crawling: Paddling movements of the paws make the pup drag itself forward or push itself backwards. Often the hindlimbs fail to keep up with the forelimbs leading them to be dragged in an extended position with the soles of the feet facing upward (Heyser, 2004). This was measured from video observations of the dam-pup isolation period, before the PRT in each litter.

3. Time Spent in Other Behaviours: Time spent performing behaviours that were not predicted to be part of the PRT observation. This was measured from video observations during the PRT of the two pups used for the PRT test in each litter.

4. Time Spent Inactive: The pup remains motionless, regardless of its body posture or position within the cage, either facing up or down. This was measured from video observations of the dam-pup isolation period, before the PRT in each litter.

5. Number of Times Performing Surface Righting Reflex: Motor ability for a mouse pup to be able to flip onto its feet from a supine position. This was measured from video observations of the dam-pup isolation period, before the PRT in each litter.

6. Time Spent Being Upside Down: Pup in a supine/ventral position not being inactive. This was measured from video observations of the dam-pup isolation period, before the PRT in each litter.

7. Number of Incomplete Retrievals: Number of times the dam picks up a pup that is outside the nest area but does not complete the full retrieval of the pup back to the nest, leaving it outside the nest. This was measured from video observations of the dam-pup isolation period, before the PRT in each litter.

2.5. Statistical Analysis

PRT response variables were regressed as functions of the presence of an older litter in the cage (two-level categorical fixed effect; Overlapped vs. Non-Overlapped Litter), Institution of Data Collection (two-level categorical fixed effect; the Babraham Institute vs. i3S), the interaction between the two categorical variables, and pup age as a co-variate (numerical fixed effect), since it varied from four to seven days, with an average of 4.7 ± 0.7 days. Response variables from pup and dam behaviours were only measured in i3S, thus modelled solely as functions of the presence of an older litter in the cage and pup age. Multicollinearity was checked by regressing each of the independent variables as a function of the remaining ones in a numerical format, but no multicollinearity issues were detected. Procedure GENMOD was used on SAS (2018

University Edition, SAS Institute Inc., USA), considering a Poisson data distribution with robust standard errors, as residuals failed to reach normality. Least-square means were compared considering a 95% confidence interval (i.e. significance at $p < 0.05$), applying a Tukey-Kramer adjustment for multiple pairwise comparisons. Pup retrievals observed from video records from the Babraham Institute were spontaneous, without dam and pups isolation period, and performed in a cage with two adult cage-mates and an older litter in Overlapped litters. Thus, pup retrievals from the Babraham Institute are not comparable with those of i3S, which were performed in a controlled way. Nevertheless, if Institution interacted with Overlap in the statistical model, results were presented separately, by Institution. Otherwise, differences between Overlapped and Non-Overlapped litters are presented altogether, including data from both institutes.

3. Results

As mentioned in the Materials and Methods section, 13 litters were tested from i3S, while 10 litters were selected from Brajon et al.'s study (2019), five being Overlapped and five Non-Overlapped. Litters from the Babraham Institute were selected based on the occurrence of new-borns being displaced away from the nest, creating the conditions for the pup retrieval tests. However, one of the Non-Overlapped litters ended up being excluded from the analysis, as pups were not retrieved back to their nests within the six-minute test length limit.

3.1. Pup Retrieval Test (PRT)

The Total Time taken to Approach the two pups tested in the PRT was affected by Litter Overlap ($X^2_1 = 53.73$, $p < 0.0001$), Institution ($X^2_1 = 6.52$, $p = 0.0107$), and the interaction between the two categorical variables ($X^2_1 = 5.78$, $p < 0.0162$), as depicted in Figure 9a (i3S: No-Overlap = 11,1s, Overlap = 35,7s; Babraham: No-Overlap = 11,7s, Overlap = 20,6s). Total Time to Approach the two pups decreased as the Age of the Focal Litter increased ($X^2_1 = 16.62$, $p < 0.0001$). Dams of Non-Overlapped Litters of both Institutions took less time to approach the two pups ($Z = -2.97$, $p = 0.0156$ in i3S, and $Z = -7.60$, $p < 0.0001$ in the Babraham Institute) than those of Overlapped Litters. In Average Time Taken to Approach a Pup, it was affected by Litter Overlap ($X^2_1 = 35.00$, $p < 0.0001$) and Institution ($X^2_1 = 9.13$, $p < 0.0025$), and the p-value for interaction between the two categorical variables that was $>0,0050$ (Figure 9b and 9c, respectively). Since there was

no interaction between the two categorical variables, Overlap and Institution are presented separately.

The Total Time taken from Approach to Mouth for the two pups tested in the PRT was affected by Institution ($X^2_1 = 374.81$, $p < 0.0001$), and the interaction between the two categorical variables ($X^2_1 = 15.38$, $p < 0.0001$), as depicted in Figure 9d (i3S: No-Overlap = 35,4s, Overlap = 76,5s; Babraham: No-Overlap = 6,7s, Overlap = 76,5s). The total time taken from Approach to Mouth for the two pups increased as the Age of the Focal Litter increased ($X^2_1 = 77.64$, $p < 0.0001$). Dams of Non-Overlapped litters of i3S took less time to pick the pups to mouth since the approach than the Overlapped ones. $p\text{-value} > 0,050$ for overlap between Babraham Institute litters. Similar results were obtained for Average Time From Approach to Mouth (Figure 9e; i3S: No-Overlap = 17,7000s, Overlap = 38,2679s; Babraham: No-Overlap = 3,3450s, Overlap = 2,2330s).

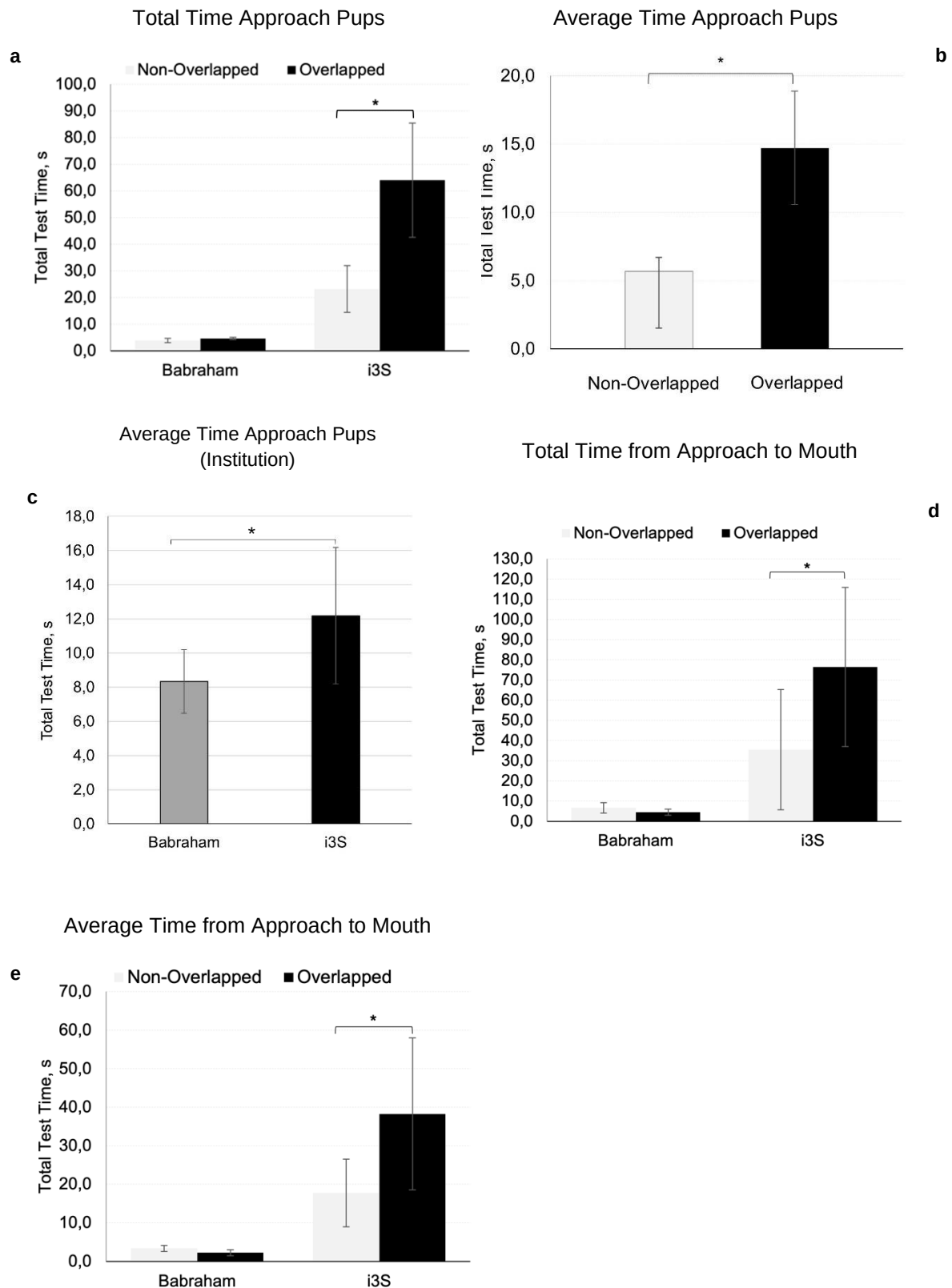


Figure 9 – Pup Retrieval Test First Two Stages Time Budget. **(a)** Total Time the females took to Approach two pups during Pup Retrieval Test. Overlapped females from i3S took more time to approach pups than Non-Overlapped females at the same institution; **(b)** Average Time each female took to Approach two pups during Pup Retrieval Test. Overlapped females took longer to approach pups; **(c)** Average Time females from each Institution took to Approach two pups during Pup Retrieval Test. Females from i3S took more time to approach the pups. **(d)** Total Time the females took from Approach to

Mouth for the two pups during Pup Retrieval Test. Overlapped females from i3S took more time to approach pups, followed by non-overlap females at the same institution; **(e)** Average Time each female took from Approach to Mouth on two pups during Pup Retrieval Test. Females from i3S with Overlapped litters took longer than those with Non-Overlapped ones. "*" refer to statistical significance ($P < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

The Total Time taken from Mouth to Nest for the two pups tested in the PRT was affected by Litter Overlap ($X^2_1 = 12.65$, $p = 0,0004$), Institution ($X^2_1 = 292.88$, $p = 0,0004$), and the interaction between the two categorical variables ($X^2_1 = 5.70$, $p < 0.0001$), as depicted in Figure 10a (i3S: No-Overlap = 23,1s, Overlap = 64,1s; Babraham: No-Overlap = 3,8s, Overlap = 4,6s). Dams of Non-Overlapped Litters from i3S took less time to approach the two pups ($Z = -10.57$, $p < 0.0001$) than the Overlapped ones. Results obtained for Average Time From Mouth to Nest showed no interaction between the two categorical variables and were affected by Litter Overlap ($X^2_1 = 6.32$, $p = 0,0119$) and Institution ($X^2_1 = 146.44$, $p < 0,0001$) as shown in Figures 10b and 10c, respectively. Since there was no interaction between the two categorical variables, Overlap and Institution are presented separately.

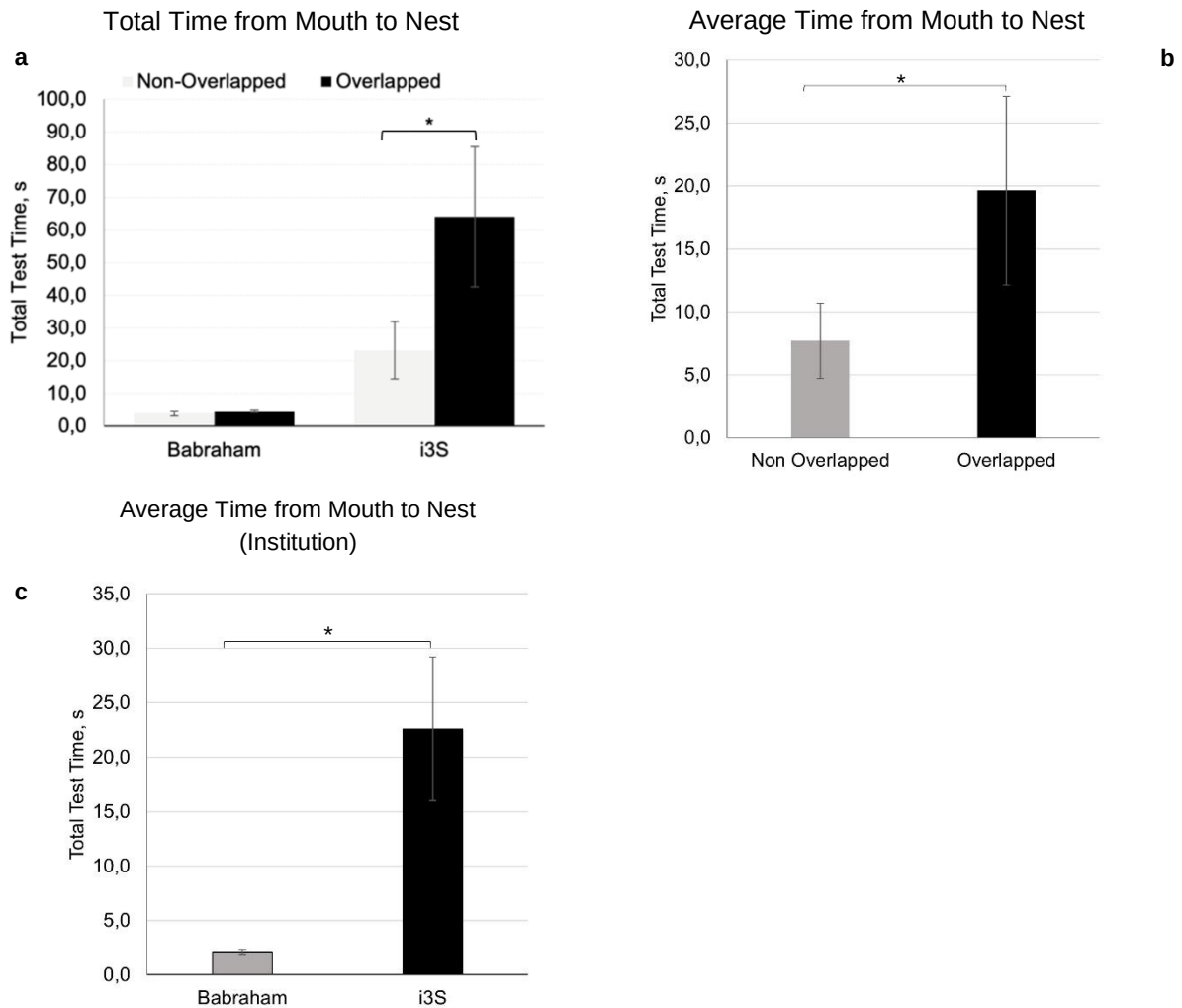


Figure 10 – Pup Retrieval Test Last Stage Time Budget. **(a)** Total Time the females took from Mouth to Nest for the two pups during Pup Retrieval Test. Overlapped females from i3S took more time to take the pups to the nest, followed by Non-Overlap females at the same institution; **(b)** Average Time each female took from Mouth to Nest for the two pups during Pup Retrieval Test. Overlapped females took longer to take pups to the nest; **(c)** Average Time females from each Institution took from Mouth to Nest. Females from i3S took more time to bring the pups back to the nest. "*" refer to statistical significance ($P < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

The Total Time the dams Spent Doing Maternal Behaviours for the two pups tested in the PRT was affected by Institution ($X^2_1 = 459.18$, $p < 0.0001$), and the interaction between the two categorical variables ($X^2_1 = 5.70$, $p < 0.0001$), as depicted in Figure 11a (i3S: No-Overlap = 0s, Overlap = 3,7s; Babraham: No-Overlap = 84,7s, Overlap = 16,1s). Time spent in Maternal Behaviours increased as the Age of the Focal Litter increased ($X^2_1 = 86.67$, $p < 0.0001$). Dams of Overlapped Litters from Babraham Institute spent more time performing maternal behaviours to their pups ($Z = 12.44$, $p < 0.0001$) than the Non-Overlapped ones. Similar results were obtained for Average Time Performing

Maternal Behaviours (Figure 11b; i3S: No-Overlap = 0s, Overlap = 1,9s; Babraham: No-Overlap = 42,3s, Overlap = 8,1s).

The Number of Times that the females Drew Away from the two pups tested in the PRT was affected Litter Overlap ($X^2_1 = 16.77$, $p < 0.0001$) and Institution ($X^2_1 = 28.28$, $p < 0.0001$), as depicted in Figure 11c and 11d, respectively. Females with Non-Overlapped Litters drew away fewer times from the pups ($Z = -2.56$, $p = 0,0105$) than Overlapped ones. Since there was no interaction between the two categorical variables, Overlap and Institution are presented separately. Similar results were obtained for Average Number of Times Female Drew Away (Figure 11e and 11f).

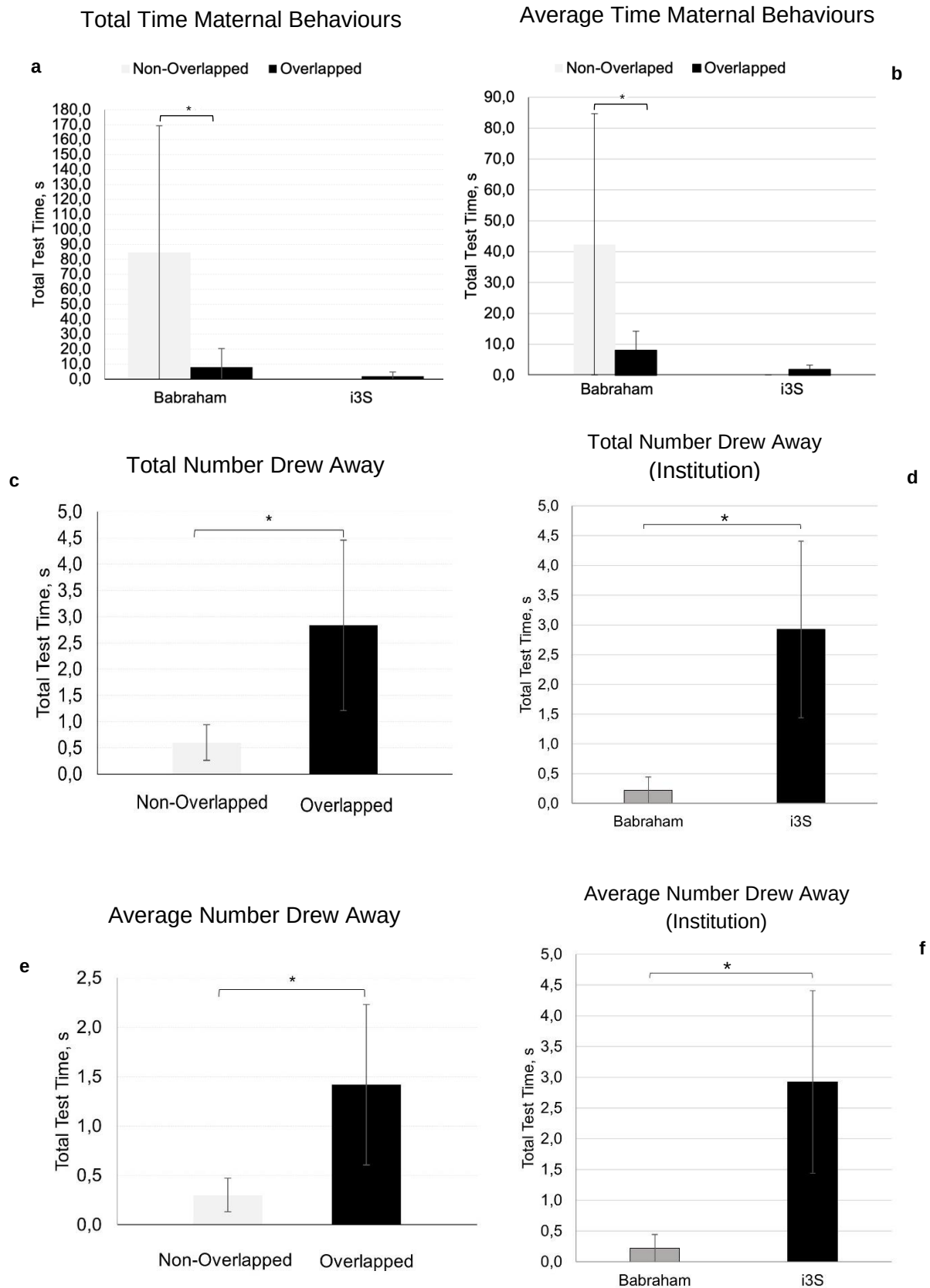


Figure 11 – Maternal Behaviours and Drew Away Time Budget. **(a)** Total Time dams spent in Maternal Behaviours during Pup Retrieval Test. Non-Overlapped females from Babraham Institute spent more time in Maternal Behaviours than those with Overlapped litters; **(b)** Average time dams spent in Maternal Behaviours during Pup Retrieval Test. Non-Overlapped females from Babraham Institute spent more time in Maternal Behaviours than those with Overlapped litters; **(c)** Total

number of times that the females Drew Away from the two pups during Pup Retrieval Test. Females with Non-Overlapped litters drew away fewer times from their pups; **(d)** Total number of times females of each Institution Drew Away from the two pups during Pup Retrieval Test. Females from i3S Drew Away more times than females from Babraham Institute. **(e)** Average number of times female Drew Away from their pups during Pup Retrieval Test. Females with Non-Overlapped litters drew away fewer times; **(f)** Average number of times females from each Institution Drew Away during Pup Retrieval Test. Females from i3S Drew Away more times, than the ones from i3S. "*" refer to statistical significance ($p < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

The Total Time that Females Spent in the Nest with the two pups during the PRT was affected by Litter Overlap ($X^2_1 = 71.88$, $p < 0.0001$), Institution ($X^2_1 = 108.26$, $p < 0.0001$), as depicted in Figure 12a and 12b, respectively. However, there was no interaction between the two categorical variables ($X^2_1 = 0.22$, $p > 0.6408$). Females with Non-Overlapped Litters spent more time in the nest with the pups ($Z = 103.14$, $p = < .0001$) than the Overlapped ones. Similar results were obtained for Average Time Spent in the Nest (Figure 12c and 12d).

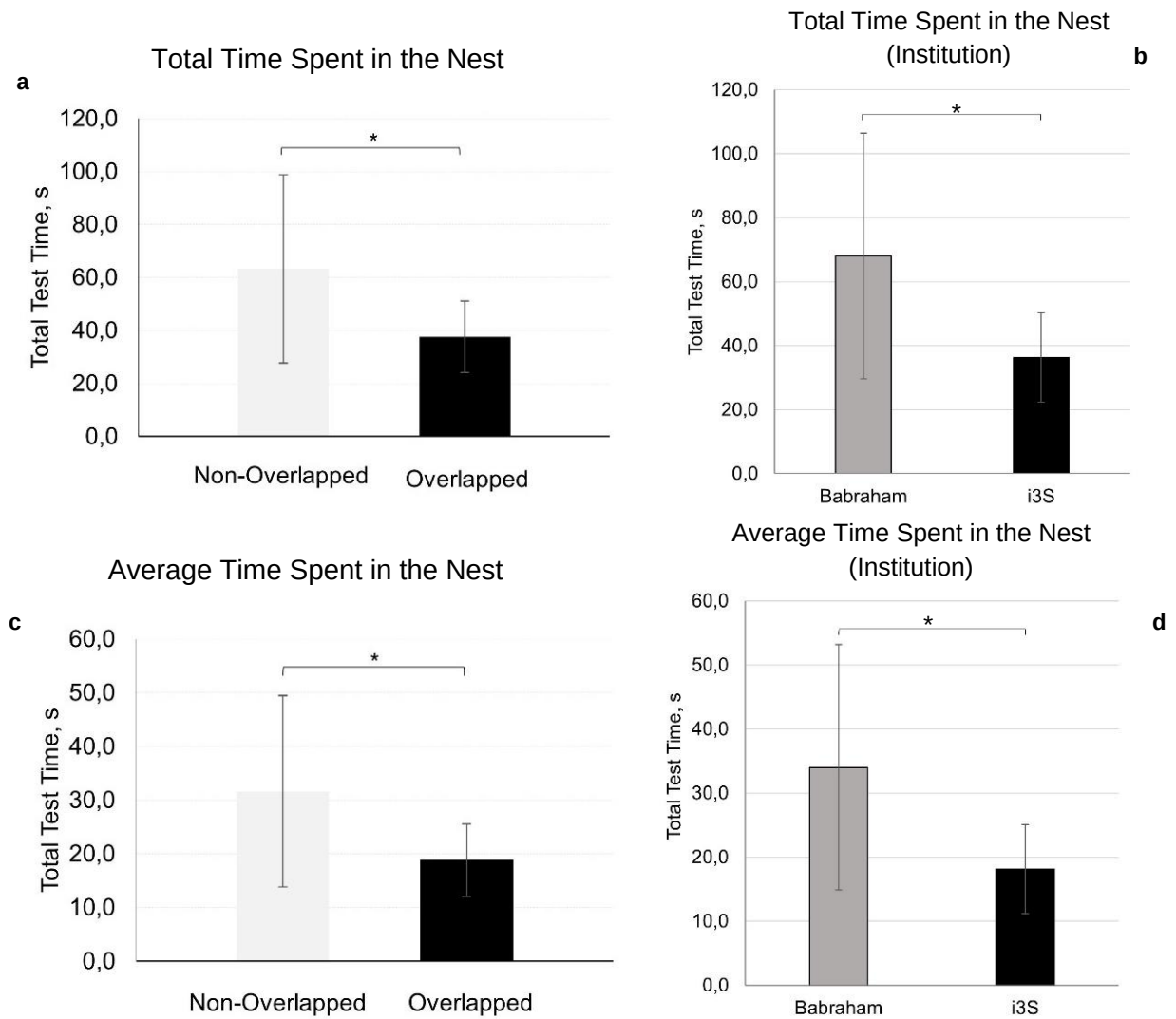


Figure 12 – Time in the Nest Time Budget. **(a)** Total time the females Spent in the Nest with the two pups during Pup Retrieval Test. Females with Non-Overlapped litters spent more time with their pups in the nest; **(b)** Total time females from each Institution Spent in the Nest with the two pups during Pup Retrieval Test. Females from Babraham Institute spent more time in the Nest with the pups. **(c)** Average time each Female Spent in the Nest with the two pups during Pup Retrieval Test. Females with Non-Overlapped litters spent more time with their pups in the nest; **(d)** Average time each female from each Institution Spent in the Nest with the two pups during Pup Retrieval Test. Females from Babraham Institute spent more time in the Nest with the pups. "*" refer to statistical significance ($p < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

The Total Time Between the Retrievals of the two pups tested in the PRT (only at i3S, since in Babraham Institute the PRT occurred separately) was affected by Litter Overlap ($X^2_1 = 5.42$, $p = 0.0199$) as depicted in Figure 13a. Time Between Retrievals decreased as the Age of the Focal Litter increased ($X^2_1 = 79.69$, $p < 0.0001$). Dams with Non-Overlapped litters had a higher Interval Between Retrievals ($Z = 2.33$, $p = 0.0198$).

The Total Time to Approach the First Pup tested in the PRT by Litter Overlap ($X^2_1 = 23.83$, $p < 0.0001$) as depicted in Figure 13b. Time to Approach Pup 1 decreased as the Age of the Focal Litter increased ($X^2_1 = 79.69$, $p < 0.0001$). Dams with Non-Overlapped litters took less time to Approach the First Pup ($Z = -4.77$, $p \leq 0.0001$).

The Total Time to Approach the second pup tested in the PRT was affected by Institution ($X^2_1 = 60.48$, $p < 0.0001$), and the interaction between the two categorical variables ($X^2_1 = 59.03$, $p < 0.0001$), as depicted in Figure 13c (i3S: No-Overlap = 4,1s, Overlap = 13,6s; Babraham: No-Overlap = 41,7s, Overlap = 12,6s). Time to Approach Pup 2 decreased as the Age of the Focal Litter increased ($X^2_1 = 79.69$, $p < 0.0001$). Dams with Non-Overlapped litters from Babraham Institute took more time to Approach the Second Pup ($Z = 8.33$, $p \leq 0.0001$).

Total Time Spent Carrying was not affected by Litter Overlap ($p > 0,0500$) but was affected by Institution ($X^2_1 = 20.49$, $p < 0.0001$), as depicted in Figure 13d. Similar results were obtained for Average Time Spent Carrying (Figure 13e).

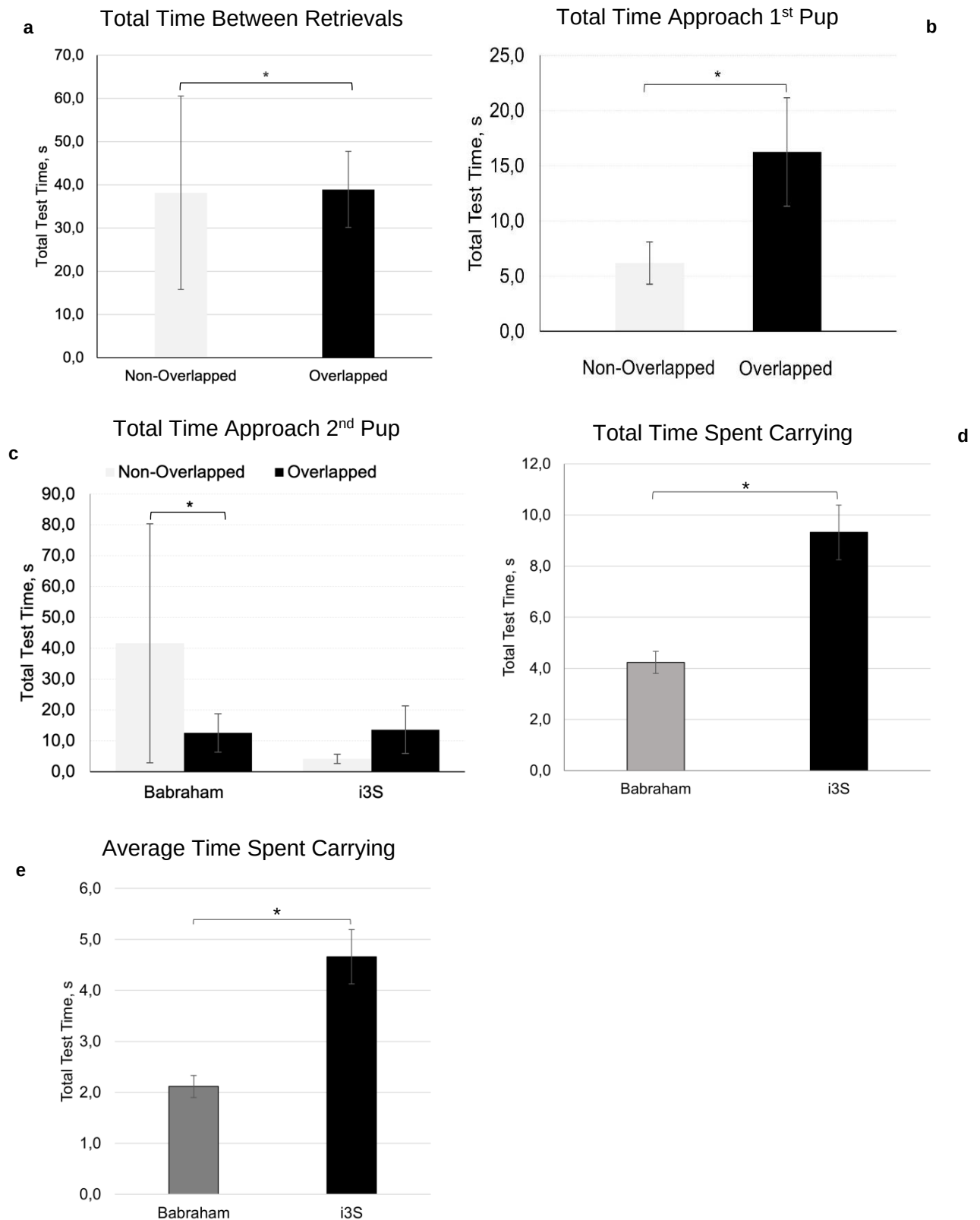


Figure 13 – Interval Between Retrievals and Approach Pups Time Budget. **(a)** Total Time Females took Between Retrievals of the two pups during Pup Retrieval Test. Females with non-overlap litters spent less time between retrievals; **(b)** Total time females took to Approach the First Pup during Pup Retrieval Test. Females with Non-Overlap litters took less time to Approach the first pup **(c)** Total Time females took to Approach the second pup during Pup Retrieval Test.

Females with Non-Overlap litters took more time to Approach the second pup. **(d)** Total time females from each Institution spent Carrying the two Pups during Pup Retrieval Test. Females from i3S spent more time Carrying their pups; **(e)** Average time females from each Institution spent Carrying the two pups during Pup Retrieval Test. Females from i3S spent more time Carrying their pups. "*" refer to statistical significance ($p < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

Time Spent Doing Other Activities and Unusual Behaviours, were mostly present in one of the institutions only, therefore did not enter the analysis.

3.2. Dam Behaviour during Isolation Period

The Time Dam Spent Inactive during isolation period (only in i3S) was affected by Litter Overlap ($X^2_1 = 161.25$, $p < .0001$), as depicted in Figure 14a. Dams with Non-Overlapped litters spent more time inactive ($Z = 8.87$, $p = <.0001$).

The Time Dam Spent in Locomotion during isolation period was affected by Litter Overlap ($X^2_1 = 71.50$, $p < .0001$), as shown in Figure 14b. Dams with Non-Overlapped litters spent less time in Locomotion ($Z = - 8.04$, $p < .0001$).

The Time Dam Spent in Other Behaviours during isolation period was affected by Litter Overlap ($X^2_1 = 9.04$, $p = 0.0026$), as shown in Figure 14c. Dams with Non-Overlapped litters spent more time in Other Behaviours ($Z = 2.93$, $p = 0.0034$).

The Time Dam Spent in Nest Building during isolation period was affected by Litter Overlap ($X^2_1 = 14.34$, $p = 0.0002$), as shown in Figure 14d. Time spent in Nest Building decreased as the Age of the Focal Litter increased ($X^2_1 = 94.14$, $p < 0.0001$). Dams with Non-Overlapped litters spent more time in Nest Building ($Z = 3.78$, $p = 0.0002$).

The Time Dam Spent in Self-Grooming during isolation period was affected by Litter Overlap ($X^2_1 = 37.11$, $P < .0001$), as shown in Figure 14e. Dams with Non-Overlapped litters spent less time in Self-Grooming ($Z = - 3.95$, $p < .0001$).

The Time Dam Spent Sniffing during isolation period was affected by Litter Overlap ($X^2_1 = 13.79$, $p = 0.0002$), as shown in Figure 14f. Dams with Non-Overlapped litters spent more time Sniffing ($Z = 3.69$, $p = 0.0002$).

For the variable "Digging", p -value > 0.050 .

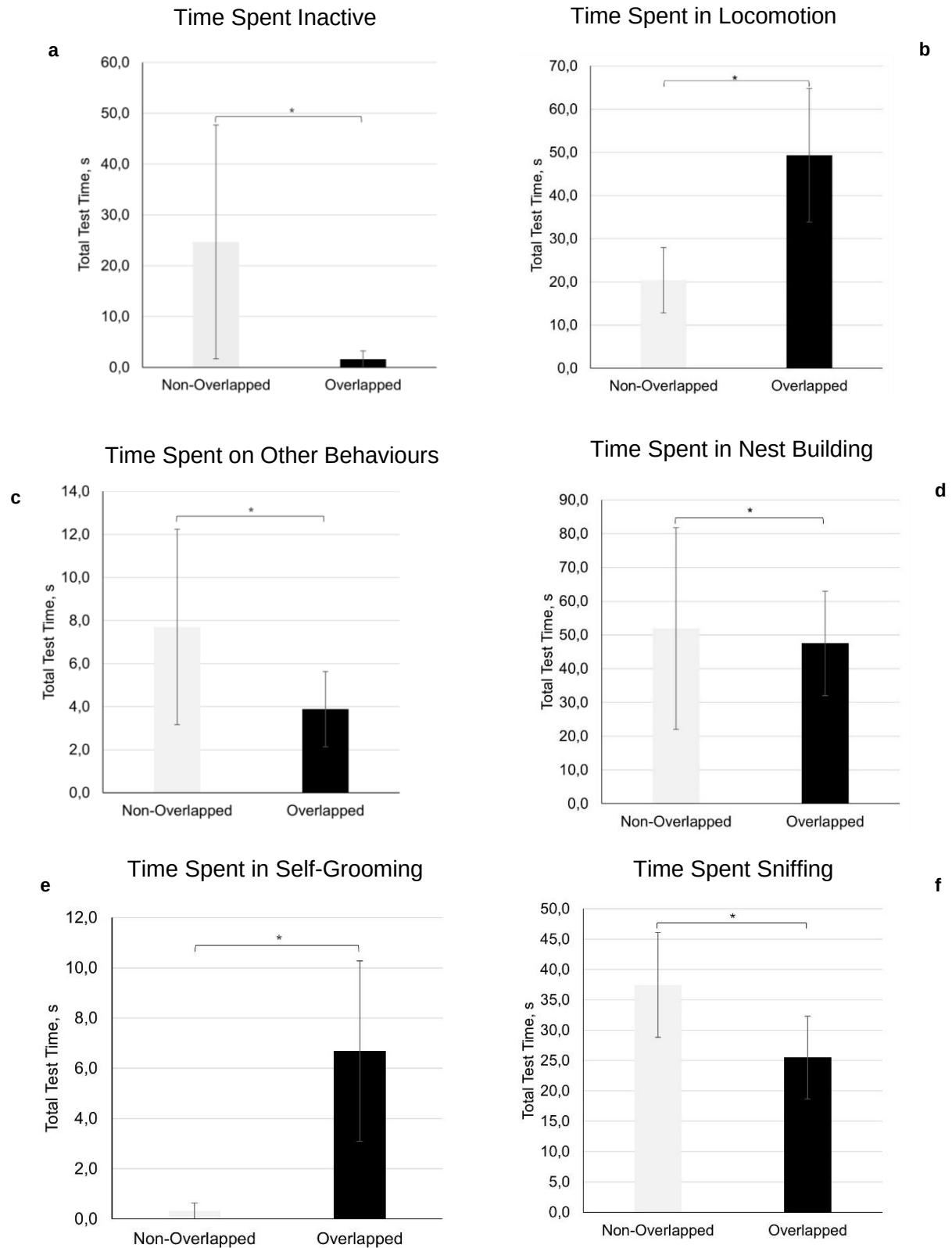


Figure 14 – Dam Behaviour Time Budget. **(a)** Time Females Spent Inactive during dam-pup isolation period. Females with Non-Overlap litters spent more time inactive; **(b)** Time Females Spent in Locomotion during dam-pup isolation period. Females with Non-Overlap litters spent less time in Locomotion; **(c)** Time Females Spent in Other Behaviours during dam-pup isolation period. Females with Non-Overlap litters spent more time in Other Behaviours; **(d)** Time Females Spent in Nest Building during dam-pup isolation period. Females with Non-Overlap litters Spent more time in Nest Building; **(e)** Time Females Spent in Self-Grooming during dam-pup isolation. Females with Non-Overlap litters spent more time in Self-Grooming; **(f)** Time Females Spent Sniffing during dam-pup isolation period. Females with Non-Overlap litters spent

more time Sniffing. "*" refer to statistical significance ($p < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

3.3. New-born Pup Activity

The Total Time Pups Spent Crawling during PRT (only in i3S) was affected by Litter Overlap ($X^2_1 = 21.44$, $p < .0001$) as depicted in Figure 15a. Time spent Crawling increased as the Age of the Focal Litter increased ($X^2_1 = 248.24$, $p < 0.0001$). Pups from Non-Overlapped litters spent more crawling ($Z = 4.51$, $p \leq 0.0001$). Similar results were obtained for Average Time Spent Crawling (Figure 15b).

The Total Time Pups Spent Inactive during PRT (only in i3S) was affected by Litter Overlap ($X^2_1 = 42.19$, $p < .0001$) as depicted in Figure 15c. Time spent Inactive decreased as the Age of the Focal Litter increased ($X^2_1 = 67.60$, $p < 0.0001$). Pups from Non-Overlapped litters spent less inactive ($Z = -6.21$, $p \leq 0.0001$). Similar results were obtained for Average Time Inactive (Figure 15d).

The Total Time Pups Spent Pivoting during PRT (only in i3S) was affected by Litter Overlap ($X^2_1 = 84.91$, $p < .0001$) as depicted in Figure 15e. Time spent Pivoting decreased as the Age of the Focal Litter increased ($X^2_1 = 6.33$, $p = 0.0119$). Pups from Non-Overlapped litters spent less pivoting ($Z = -8.81$, $p = < .0001$). Similar results were obtained for Average Time Performing Maternal Behaviours (Figure 15f).

The variables "Incomplete Retrieval" and "Pup Righting" were not affected neither by Litter Overlap or Age of the Focal Litter ($p > 0,050$).

The variable "Other Behaviours" was not affected by Litter Overlap ($p > 0,050$), and Time Performing Other Behaviours decreased as the Age of the Focal Litter increased ($X^2_1 = 51.44$, $p < 0.0001$).

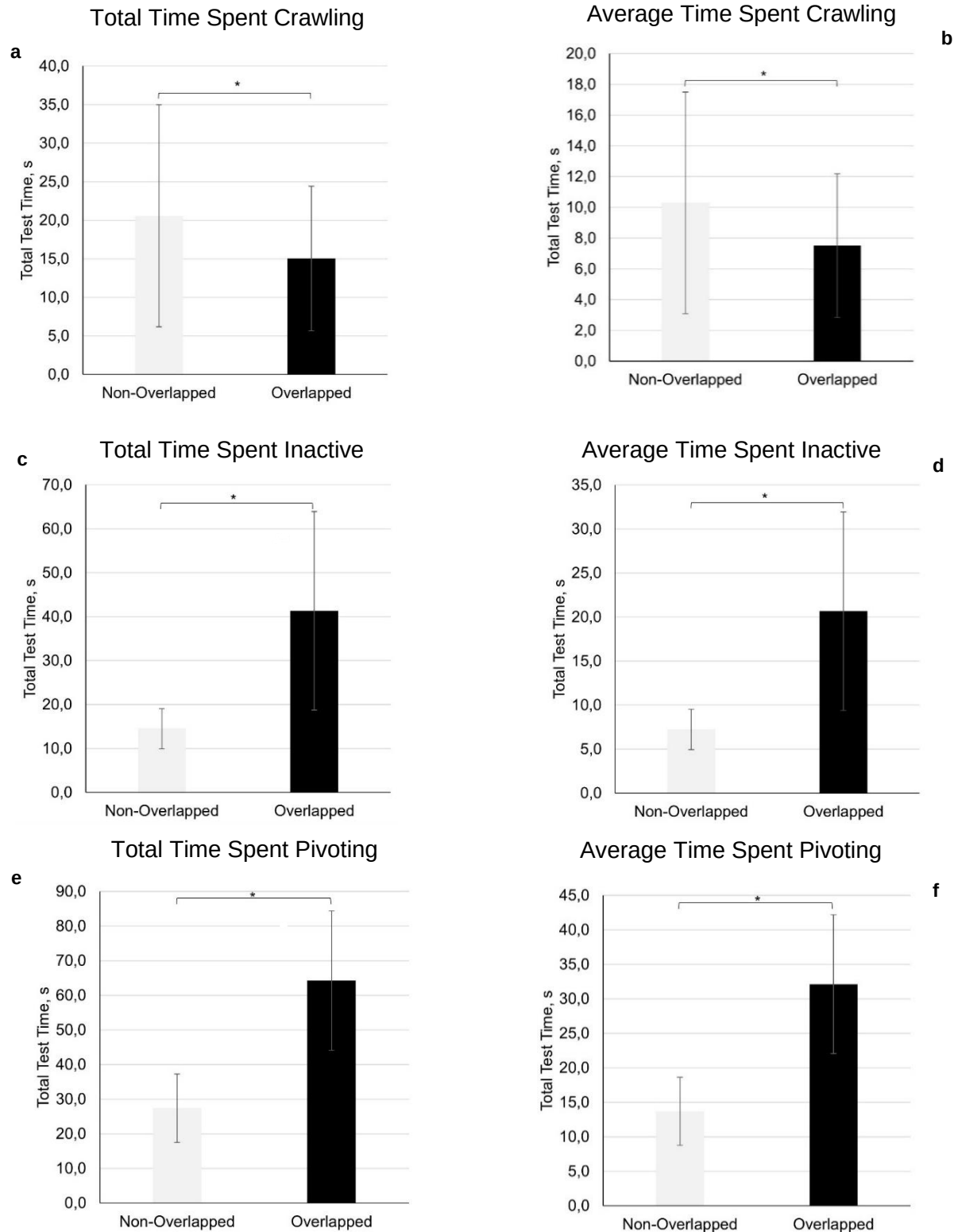


Figure 15 – Pup Activity Time Budget. **(a)** Total Time Pups Spent Crawling during Pup Retrieval Test. Pups from Non-Overlap litters spent more time Crawling; **(b)** Average Time each Pup Spent Crawling During Pup Retrieval Test. Pups from Non-Overlap litters spent more time crawling; **(c)** Total Time Pups Spent Inactive During Pup Retrieval Test. Pups from Non-Overlap litters spent less time Inactive; **(d)** Average Time each Pup Spent Inactive during Pup Retrieval Test. Pups from Non-Overlap litters spent less time Inactive; **(e)** Time Pups Spent Pivoting During Pup Retrieval Test. Pups from Non-Overlap litters spent less time pivoting; **(f)** Average Time Each Pup Spent Pivoting During Pup Retrieval Test. Pups from Non-Overlap litters spent less time Pivoting. "*" refer to statistical significance ($p < 0.0500$) from comparing Least-Squares Means, but columns represent real population means $\pm$ respective standard errors.

4. Discussion

4.1. Pup Retrieval Test

The PRT latency values obtained in this study are according to the range of PRT latency values reported in the literature, either for Overlapped or Non-Overlapped litters, despite the experimental background of this study being different from the majority of the other studies (e.g. the dam tested is the actual mother of the pups). The response variables Average Time to Approach, Average Time from Mouth to Nest, Number of Times the Female Drew Away, Time to Approach Pup 1, and Interval Between Retrievals were lower in Non-Overlapped litters than in Overlapped, both in Babraham Institute and i3S. Of these variables, Average Time to Approach pups, Time to Approach Pup 1 and 2 (separately), and Interval Between Retrievals decreased as Age of Focal Pup increased. The variable Time Spent in the Nest, on the other hand, was higher in Non-Overlapped litters than in Overlapped in both Institutions. For the response variables Total Time to Approach, Time from Approach to Mouth, Total Time from Mouth to the Nest and Time Spent Doing Maternal Behaviours, Time to Approach Pup 2, Time Spent Doing Other Activities and Unusual Behaviours there was an interaction between Institution and Overlap situation, thus results were presented separately. Only one litter was excluded from the analysis because there was no retrieval of one of the pups.

Weber et al. (2015) revealed that dams performing less maternal behaviours (specifically nest building and pup retrieval behaviours) is associated with increased pup mortality levels. Also, Scarola et al. (2020) found that maternal rats exposed to predator threat demonstrate increased interrupted grooming and take longer to retrieve all pups, showing the impact of stressing environmental contexts on maternal responsiveness. Therefore, in this study, the increased time taken to approach, grab, and bring pups back to nest in Overlapped litters may be an indication that dam maternal responsiveness, or maternal behaviour in general, was disturbed when older pups were present, which may lead to decreased pup survival rates in Litter Overlap contexts. Since one of the goals of the PRTs was to evaluate maternal behaviour, Time Spent in the Nest with the Pups, Time Spent Doing Maternal Behaviours, and Number of Times the Female Drew Away (interpreted as the number of failed attempts to initiate retrieval) were also taken into account. Results for these behaviours altogether may be an indication that dams with Overlapped litters have more difficulty delivering proper maternal care to their litters.

Although there is evidence that dams distinguish their pups from others (inclusively it was demonstrated that the latency to retrieve alien pups is higher than the latency to retrieve their own ones), they do retrieve them in the same frequency (Ostermeyer & Elwood, 1983). In a Litter Overlap context, dams care for both litters equally (Chantrey & Jenkins, 1982; Garner et al., 2016; Mogi et al., 2017), however, it is not known how pup recognition mechanisms function in Litter Overlap. It is possible that two litters inside the cage will overstimulate dams, leading them to neglect the younger litter, and younger pups' stimuli will be attenuated by those of the older pups. Therefore, Litter Overlap may have affected the communication between pups and dams.

Pup ultrasonic vocalisations (USVs) and pup odour are thought to be two of the communication mechanisms between dams and pups, triggering maternal behaviour in mice (Okabe et al., 2013). Pup USVs can be used not only as a cue to stimulate maternal behaviour but also as an individual signature of pups in mice (Mogi et al., 2017). The same conclusion was also reached regarding pup odour (Mogi et al., 2017) since dam distinction of its pups still occurs when auditory cues are masked. Chantrey & Jenkins (1982) suggested that mouse dams could recognize their own pups using olfactory cues and showed that mothers fail to distinguish between their own pups and alien pups when olfactory cues are removed. Previous studies have mainly shown that lesions of olfactory perception inhibited arched-back nursing and licking/grooming of pups (Okabe et al., 2013). Therefore, regarding vocal communication, it is unlikely that USV was the main cue for dams to respond to their pups because pups from such young age as the ones used in this study (4-7 days old) produce fewer USVs. Pups of this age are more likely to produce "Wriggling calls" (low-frequency calls, emitted mainly between days 2 and 15 after birth by mouse pups while pushing for the teats; Ehret & Bernecker, 1986). Thus, in this study, vocalizations (both USVs and wriggling) may not have been the primary method used by the dams and pups to trigger Pup Retrieval Behaviour. In overlapped litters, it is possible that primarily communication through odour cues were affected in a way that dams reduced their responsiveness to their pups.

During early life, a mother and her pups establish a very close bond, and the olfactory learning of the nest odour is very important for bond formation (Reis et al., 2014). During the neonatal period, olfactory learning, specifically related to urine odour (Londei et al., 1989) is a key factor in attachment establishment, and eliciting maternal behaviours such as pup licking (especially from the pup's perspective, since they are born blind and deaf;

Reis et al., 2014, Londei et al., 1989). Thus, any disturbance in olfactory cues within the first days of life could impact pup retrieval.

In summary, the increased cage stocking density of overlapped litters may lead to an overload of olfactory information to the dam, which ends up not being able to process the cues properly, which may have been reflected in the longer latency to retrieve pups in this study. Dams may not have been able to bond properly with all pups inside the cage, possibly causing a disturbance in the quality of maternal behaviours.

4.2. Dam Behaviour

During Dam-Pup Isolation Period females with Non-Overlapped litters spent more time Inactive, Nest Building, Sniffing, and performing Other Behaviours than females with Overlapped litters. On the other hand, females with Overlapped litters spent more time in Locomotion and Self-Grooming than females with Non-Overlapped litters. Of these variables, Time Spent in Nest Building decreased as Age of Focal Pup increased.

The decreased performance of maternal behaviours may be one of the causes underlying high pup mortality in Overlapped litters. Females of Non-Overlapped litters engaged more in self-directed behaviours and Nest Building, while Nest building was previously linked to pup mortality (Weber, 2015). Thus, pups of Non-Overlapped litters may have benefited from Nest Building to survive. Rodent neonates are thought to be poikilothermous (their brains are not yet developed enough to thermoregulate on their own). Therefore, pups are at risk of dying when deprived of dam's regulatory influence for too long (Aubert et al., 1997). Both Pup Retrieval and Nest Building behaviours are involved in the thermoregulation of pups. Nest building (among other functions) is useful to enhance the dam's ability to control litter's temperature (reducing the rate of heat loss and the energy required to maintain the temperature; Vogt & Lynch, 1982). In a cold ambient the nest becomes an important support of survival for litter and dams, since pups cannot survive for long when out of the nest, Pup Retrieval Behaviour has a high priority in the "individual repertoire" of the dam (Aubert et al., 1997). Breeding rooms are often kept at 21°C, which has been demonstrated to be cold stressful even for adult mice (Gaskill et al., 2009), thus reduced maternal behaviour may play a role in pup mortality in the context of breeding.

4.3. New-born Pup Activity

During PRT, pups from Non-Overlapped litters spent more time crawling than pups from Overlapped litters. Pups from Overlapped litters spent more time inactive and pivoting. The variables Number of Incomplete Retrievals, Time Spent in Pup Surface Righting, and Time Spent Upside Down were not affected either by Litter Overlap or Age of the Focal Litter, and Time Spent Performing Other Behaviours was not affected by Overlap. Of these variables, Time Spent Inactive, Time Spent Pivoting; and Time Spent Performing Other Behaviours decreased as Age of Focal Pup increased. Time Spent Crawling increased as Age of Focal Pup increased.

Crawling is a more complex behaviour than pivoting, since it demands more coordination between the fore and back limbs, allowing pups to dislocate themselves back and forth (while pivoting only allows them to move in circles, with back limbs not/barely moving) (Feather-Schussler et al., 2016). Therefore, pups from Non-Overlapped litters may have an higher ability to contribute to their return to the nest and access to protection from the nest and nutrition (from dam's milk). More access to protection and nutrition may be one of the mechanisms by which more pups survive in Non-Overlapped litters.

4.4. Limitations of the Study

This study was originally designed to use data from 14 litters from the i3S breeding facility. However, issues related to mice availability did not allow for data collection of the desired number of litters. Thus, to reach the intended number of litters and statistical power, video recordings from Brajon et al., (2019) were used. This study, therefore, was not designed to compare Institutions. Differences in components of the PRT between Institutions such as (Average Time to Approach Pups, Time from Mouth to Nest, Time Spent in the Nest With Pups, Number of Times the Female Drew Away from Pups Without Retrieval and Time Spent Carrying Pups) may be related to the highly different social contexts of the trials, as in the Babraham Institute the retrievals evaluated occurred spontaneously, where females were on their normal social environment with their cage mates and both litters inside the cage. Also, breeding routines in both institutes had different housing conditions, mice had distinct sub-stains, and behavioural videos were recorded in another period of time (2017-2018). Ideally, it would be better to evaluate the results of this study with a higher number of litters of the same Institution or to perform the same PRT protocol on both Institutions.

The mouse strain used in this study was C57BL6/(J and Babr), one of the most used strains in biomedical research, which means the findings of this study may not apply to other mouse strains.

This study was motivated by the need to understand the mechanisms by which Overlapped litters have higher pup mortality than the Non-Overlapped ones. One of the hypotheses states that Overlap disturbs maternal behaviour. Here, the only studied aspect of maternal care and responsiveness was Pup Retrieval behaviour, and maternal behaviour during the PRT isolation period, which means that although results suggest that Overlap is indeed interfering with maternal responsiveness, this is only applicable to the studied aspects of maternal behaviour.

Since the animals used were part of the i3S's B6 colony for animal experimentation, it was not possible to do a more complete evaluation of dams' pre and post-partum behaviours.

Conclusion

The present study demonstrated that dams with Overlapped litters take longer to approach, grab, and bring pups back to the nest and that during the isolation period, females with Overlapped litters spend more time in Locomotion and Self-Grooming, and that pups from Overlapped litters spend more time inactive and pivoting during PRT. Altogether, these results show that situations of Litter Overlap may disturb maternal behaviour and pup activity, potentially being two of the mechanisms underlying pup death in situations of Litter Overlap. Number of litters per Institution and period of observation were limitations of this study. In the future, therefore, overlap studies should include a higher number of participating litters, different strains and more aspects of maternal and pup behaviour. Future studies trying to understand pup death in Overlapped litters should address sibling competition, parental behaviour in asynchronized litters, and focus on creating practical husbandry routines to either avoid Litter Overlap or reduce its negative effects on pup survivability.

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Attachments

Attachment 1

Pup Retrieval Test Literature Review papers. Each line represents a paper that was included in the review, and each column represents a parameter of PRT. “____” means that the paper’s PRT didn’t require that variable and “???” means that although the paper has a reference to that variable, it does not specify the answer required.

Publication	Goal	Strain and Pups' age	Room and Acclimation	Heating-Pad Temperature and Dam-Pup Isolation Time	Location of mother and test pup	Method After Isolation	Number of pups Separated and Tested	Pup Placement Order and Test Duration	Schedule	Measured Variables
Winters et al 2002 https://doi.org/10.1038/s41598-022-05641-w	Develop a new methodology to score PRTs	C57BL/6J Rj mice At least 5 days	Test Room 1h acclim.	35°C 5 min	Mother: Home cage (that was inside a Styrofoam Box) Pups: Only the pup that is tested in that round is placed inside a heated glass cup (3.3 glass crystallizing dish)	1 - Mother back to the nest (original cage with the other 5 pups); 2 - The separated pup is placed on the opposite corner of the nest 3 - Repeat with all pups	1 pup separated at a time All pups tested	One-by-one and placed only after the dam being in the nest (the dam stays in the cage) 90s per pup	Any time as long as it is consistent to avoid circadian cycle related bias	"Latency to Retrieval; Time necessary to complete retrieval; Efficacy of retrieval; Maternal Approach; Carrying; Digging; Maternal perception of pup distress; nursing", "pup licking", "self-grooming", and "disruptive digging on the pup"
Zimprich et al 2018 https://doi.org/10.1002/cpmo.36	Assessing Sociability, Social Memory, and Pup Retrieval in Mice	???? 3 to 7 days	Test Room 20 to 30 min	————— 3 to 7 days	Mother: Home Cage Pups: ???	1. Place the behavioral cage and install the video camera above the cage (place the cage low enough to be able to watch the experiment without leaning above the cage/watch the live video on the computer screen instead) 2. Put the mouse of interest in the cage, and let it habituate 20 to 30 min 3. Start the video camera and timer. 4. Place the first pup into the cage. Choose a spot distant from the mock nest. Ideally wait until the adult animal is located in the mock nest before placing the first pup. 5. Place the remaining 4 pups in different distant places across the cage at 30s intervals.	5 pups	One-by-one (the mother stays in the cage) placed with 30 sec intervals Terminate the experiment when all pups are retrieved or until 5min	When the room has a 30 to 50 lux	Number of pups retrieved and latency for retrieval (first latency value measured from introduction of the first pup to the arena until the first retrieval of any pup (sometimes the first pup introduced is not the first pup retrieved; next latency value is measured from retrieval of the first pup until the retrieval of the second pup and so on until all pups are retrieved).
Salais-López et al 2021 https://doi.org/10.1159/000510038	Investigate maternal motivation in dams and virgin female mice, using a novel variant of the pup retrieval (motivated PRT) and to analyse the role of prolactin and of stimuli derived from a litter of pups and its mother, in the acquisition of maternal motivation.	CD1 mice 2 to 6 days	Colony Room 0 min	————— ???	??? ???	1 - The experimental female and her non-experimental partner were removed from their home cage 2 - barriers were glued to the walls, one in each corner of the cage distal to the nest. 3 - 4 pups were removed from the nest and 2 of them were carefully placed behind each of the barriers. The remaining 4 pups were left on the nest site (to prevent the experimental females from building a new nest site behind either of the barriers) 5 - the experimental female was placed back in the home cage and her behaviour was video-recorded for 10 min.	8 pups Tested 3 times throughout the days	2 pups were left behind each barrier, and 4 pups were left on the nest site 10min	——	Possible differences in locomotion; Barrier crossing; Time in the centre of the cage; Latency to approach pups, and the time that the experimental animals interacted with the pups behind the barriers. Then, using the Kaplan Meier "survival" analysis, the researchers explored differences in the distribution of the retrieval latencies for the first and the fourth pups in each trial"
Kohl et al 2018 https://doi.org/10.1038/s41586-018-0027-0	To investigate how (MPOAGal) neurons of the hypothalamus coordinate motor, motivational, hormonal and social aspects of parenting in mice"	C57BL/6 J 1 to 4 days	Colony Room 30 min	??? ???	Mother: Home Cage Pups: ???	1 - Two 1–4-day-old C57BL/6 J pups were placed in different corners opposite the nest. 2 - Once retrieval occurred, a timer was started. Each test was recorded using a multi-camera system and behaviours were scored by an individual blind to the genotype.	2 pups ???	??? ???	beginning of the dark phase, performed under dim red light	Successful/Unsuccessful Retrieval
Stolzenberg et al 2012 https://doi.org/10.1016/j.vhbeh.2012.05.012	Experience-induced changes in behaviour may be mediated by chromatin modifications, changing the expression of maternal care promoter genes	C57BL/6J 2-7 days	Test Room 24h all together	??? 15 min	Mother: Home Cage Pups: Empty Novel Cage	"4 stimulus pups (2–7 days old) were scattered in the cage, in the opposite corner from the nest"	4 pups	All pups together 2h (30min per pup)	dark phase of the light/dark cycle under dim red light.	Latencies to retrieve each pup to the nest; Group all pups inside the nest (sniff/lick all pups in the nest); Crouch overall pups inside the nest
Carlier, 1982 https://doi.org/10.1016/S0163-1047(82)91213-4	To study differences in patterns of pup care in mice between different Inbred Strains	???? 1 day	Test Room ???	————— ???	Mother: Novel Cage Pups: Home Cage (placed in a heap 20 cm from the nest)	The observation began when the experimenter placed the female in the nest.	All pups	All pups together 900s	Between 10 AM and 4 PM	Mean number of living pups on the day of the test; Duration between the moment when the female is placed on the nest and the moment when her snout contacted one of her pups (first contact); Duration between the first contact and the taking into the mouth of the first pup (first retrieval); Duration between the first retrieval and placing of the first offspring in the nest (the first pup carrying). Time spent in the nest after placing the first pup in the nest (nest with the first pup); Number of times the female drew away from one of her pups, situated outside the nest, without their being transported (move away). Percentage of females remaining in the nest with all their pups (nest with all pups) Intervals between the retrieval of the first and second pups, the second and third pups, and the third and fourth pups were recorded for females having retrieved at least four pups."

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Wu et al 2009 https://doi.org/10.1371/journal.pone.0006508	To probe the effect of caregiver hearing on maternal care and adolescent social development of mice	CD-1 mice 1 to 3 days old	??? ???	35°C 10min	Mother: Novel Cage Pups: Novel Cage	Pups were placed in each of the three corners of the virgin cage that did not contain a nest"	3 pups	All Together 2 days (30 min recorded each day)	???	Investigation Latency (Time to investigate the first pup); Pup retrieval latency (Time to retrieve each pup into the nest); Duration of licking/grooming/sniffing pups (Time spent licking/grooming/sniffing pups, regardless of whether the pup was in the nest); Crouching behaviour (When the virgin mouse used an arched-back posture to cover the pup in the nest); Crouching duration (time mice spent crouching after all three pups were retrieved)" Pup injury (After retrieval test)
Xie et al 2017 https://doi.org/10.1101/2022.03.21.485130	Track the gradual emergence of a repetitive and highly-stereotyped parental behavior and show that trial-by-trial performance correlates with the history of midbrain dopamine (DA) neuron activity	adult DAT-Cre female mice 0 to 5 days old	???	10 min	Mother and pups: Home Cage	???	5 pups for 3 sessions	All pups together (scattered around the cage) 5 min (1min per pup)	During the light phase (9h - 19h)	Retrieval latency index = $\frac{[(t1-t0)+(t2-t0)+\dots+(tn-t0)]}{(n \times L)}$ - where n = number of pups scattered in the cage initially, t0 = time of the start of a trial, tn = time of retrieving the nth pup, L = 300 s.
Okabe et al 2017 https://doi.org/https://doi.org/10.1016/j.psvneuen.2017.01.036	Investigate the role of OT for facilitating effects of adult-pup interactions on alloparental behavior via neural activity of preoptic area (POA), including MPOA and adjacent area	C57BL/6J 4-5 days old	???	2 days	Mother: Novel Cage Pups: Novel Cage	3 donor pups were placed in separate corners of each test cage over a 20-min period	3 pups	All together, one in each corner of the cage 20 min (~6.5 min per pup)	???	Retrieving latencies
Sajjaviyriya et al 2024 https://doi.org/10.1016/j.pentides.2024.171226	To know whether the mother's effort to retrieve the infants to their nest requires the vasopressin-signaling via V1b receptor	hybrid 129/Sv and C57BL/6 background 2 days old	Test Room	36°C 60 min	Mother: Home Cage Pups: Novel Cage	4 pups were randomly selected and placed at the corners of the new cage covered by a handful of home cage bedding. The dam was placed in the centre of the cage, and pup retrieval behaviour was observed."	4 pups	All together, one in each corner of the cage 15 min (~4min per pup)	???	Number of retrieved pups counted every minute;
Carcea et al 2021 https://doi.org/10.1038/s41586-021-03814-7	Continuously monitored the behaviour of female virgin mice co-housed with an experienced mother and litter	C57BL/6 1 to 4 days old	???	20 min + 2 min	Mother: Behavioural Arena Pups: Grouped in a corner of the arena and covered with nesting material	"One pup was removed from the nest and placed in an opposite corner of the arena. The experimental female was given 2 min per trial to retrieve the displaced pup and return it back to the nest; Another pup was then taken out of the nest, placed away from the nest and the next trial was begun."	10 pups	1 pup was removed from the nest and placed in an opposite corner of the arena. Repeat for all pups (varying the position of the isolated pup relative to the nest from trial to trial) 2min per pup	???	Vocalization of isolated pups during testing; time to retrieval
Meek et al 2000 https://doi.org/10.1016/S0031-9384(00)00431-5	To study the effects of stress experienced during pregnancy and raising stressed offspring on maternal behaviour	Swiss± Webster mice 3 days old	Test Room	30 min	Mother and Pups: Home Cage	The dams were then given up to 10 min to retrieve the pups to the nest	All pups (exact number unknown)	Pups were scattered randomly around the home aquarium 10 min	???	Total number of pups retrieved: the number of pups the dams retrieved to the nest within 10 min; Latency to retrieve the first pups: the interval between removal of the cardboard barrier and return of the first pup back to the nest; Latency to retrieve all pups: the interval between removal of the cardboard barrier and return of all pups to the nest.
Stevenso et al 2021 https://doi.org/10.1101/ein.19327	Systematic analysis of pup retrieval behaviour across three days in alloparental female mice	CBA/CaJ, C57BL/6J from 0 days old	???	5 min	Mother: Home Cage Pups: ???	Pups were returned to the home cage. Most WT retrieved all pups in less than 2 minutes. After testing, all adults and pups were placed in their home cages. The same procedure was performed again daily till D5."	3 to 5 pups	1 placed at each corner and at the center (the nest was left empty if there were fewer than 5 pups) 10 min	Dark phase	Latency was calculated using the following formula: latency index = $\frac{[(t1-t0) + (t2-t0) + \dots + (tn - t0)]}{n \times L}$ where n = # of pups outside of nest; t0 = start of trial; tn = time of nth pup gathered L = trial length An error was scored when the Sur interacted with the pups (licking, sniffing, etc.) but did not successfully gather them to the nest or to another location in the cage. Post-retrieval phase – time from when the last pup was retrieved to the end of the 10-minute recording session.

Publication	Goal	Strain and Pups' age	Room and Acclimation	Heating-Pad Temperature and Dam-Pup	Location of mother and test pup	Method After Isolation	Number of pups Separated and Tested	Pup Placement Order and Test Duration	Schedule	Measured Variables
Stolzenberg & Rissman, 2011 https://doi.org/10.1111/j.1365-2826.2011.02112.x	To show that naturally postpartum females are faster to retrieve pups and spend more time crouching over pups than spontaneously maternal virgin females, but these differences diminish with increased maternal experience.	C57BL/6J 2 to 7 days old	Test Room 45 min all together	???	???	???	20 times (each with 4 pups)	Placed in the areas of the cage farthest from the female's nest	Dark Phase	Hovering, sniffing and licking, or crouching over the pups were recorded. Hovering is the upright posture over pups (so that pups had access to the female's ventral surface), including actively sniffing/licking the pups or engaging in self-grooming. Crouching happens when females are in a quiescent, immobile posture, with all four limbs supporting a slightly arched or highly arched posture over the pups. Latencies to retrieve each pup to the nest, sniff/lick all pups in the nest, and crouch over all pups in the nest. Forty-five minutes after the presentation of pups, each female was observed continuously for 15 minutes
Cohen et al 2011 https://doi.org/10.1016/j.neuron.2011.08.019	To study if exposure to pups' body odor reshapes neuronal responses to pure tones and natural auditory stimuli through in vivo cell-attached recordings in the primary auditory cortex of female mice	NMRI mice 4 days old	???	???	Mother: Clean plastic chamber Pups: Clean plastic chamber	???	5 pups	Placed in the cage consecutively with 30 to 40 s intervals 5min (1min per pup)	The first 6h of the light cycle	Probability to retrieve pups; Latency of each pup retrieval was scored manually from the videotape
Gerasime nko et al 2021 https://doi.org/10.1016/j.physbeh.2021.113395	To understand the role of RAGE during the postpartum period, through examination of parental behaviour in female RAGE KO mice and ultrasonic vocalizations in pups	Ager- /- of the C57BL/6 3 days old	???	10 min	Mother: Home Cage Pups: Novel Cage	Dams were returned to the original home cage in the presence of their 5 biological pups for assessing parental retrieval behaviour	5 pups	Placed individually at remote places from the nest 10 min	Between 10h to 15h.	Retrieval (defined as positive if the dams carried all the five pups to the original nesting place or within two-third of the distance between the nest and the place in which the pups had been placed). A dam was physically restrained in a 50-mL polypropylene tube for 20 mins for examining maternal behaviour under stress. A second pup retrieval test was then performed as described above.
Aung et al 2022 "HOME CAGE PUP RETRIEVAL" https://doi.org/10.1111/jne.13206	Determine whether maternal behaviour is altered in a well-described prenatally androgenised (PNA) mouse model of PCOS	C57BL/6 inbred female mice 3 to 5 days old	Test Room 30 min	???	Mother: Home Cage Pups: Novel Cage	Pups were removed from the home cages of lactating dams during testing. Three foster pups were placed into the cage and all pup-directed behaviours were recorded	3 pups	Placed into the three corners of the cage at the opposite end from the nest 30 min	Between 9h to 11h AM	Latency to approach each pup (defined as the first time the nose of the female visually touched the pup); Latency to retrieve each pup to the nest; Grouping of pups in the nest and the time to reach kyphosis (arching of the back into a nursing position).
Aung et al 2022 "NOVEL CAGE PUP RETRIEVAL" https://doi.org/10.1111/jne.13206	Determine whether maternal behaviour is altered in a well-described prenatally androgenised (PNA) mouse model of PCOS	C57BL/6 inbred female mice 5 days old	Test Room 10 min	???	Mother: Novel Cage Pups: ???	Three pups were placed into three corners of the cage and pup-directed behaviours were recorded. After testing, female mice were returned to their home cage.	3 pups	3 pups placed into three corners of the cage 60 min in the dams and for 90 min in the virgin female groups (virgin females display lower levels of maternal motivation to interact with pups)	Between 9h to 11h AM	Latency to approach and retrieve each pup; Time taken to gather all three pups together; Time to reach kyphosis (only assessed for groups of lactating dams)
Ng et al 2023 https://doi.org/10.1111/jne.13247	To characterize the role of oxytocin in components of maternal motivations during the mid-postpartum period	C57BL/6J and ddY (a Swiss-derived non-congenic mouse strain) mice 1 to 5 days old	???	0 min	Mother: Novel Cage Pups: Home Cage	Placed in each corner of the cage except for the corner with the nest	3 pups	Placed in each corner of the cage except for the corner with the nest 15 min	Between 9h to 12h AM	Latency for the dam to first sniff a pup; Retrieve each pup into the nest; Group the pups (collection of all three pups close to each other in the nest); Crouch over all three pups in the nest for more than 1 min were observed (defined as "full-parental behaviour"). Pup retrieval counted when the mother placed the pup into the nest. Pup carrying and dropping outside of the nest, and pups crawling into the nest by themselves or falling into the nest from the edge/bank of the nest did not count.
Champagne et al 2007	To examine aspects of maternal care differentiating mice	Inbred 129Sv; C57BL/6J;	Colony Room	???	Mother: Novel Cage	Three pups from the dam's litter were randomly placed in the home cage, and the mother was then reintroduced to the cage	3 pups	Randomly placed away from the nest	???	Latency to sniff a pup, retrieve each of three pups, nest build and crouch over pups

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https://doi.org/10.1016/j.physbeh.2007.03.014	strains; Explore the relationship between postpartum behaviour	outbred Swiss day of birth	0 min	Pups: Novel Cage	end of the home cage 15 min
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Attachment 2

Nest Scoring table (Hess et al., 2008).

	Description of Nest Material	Score
Undisturbed	Nesting material has not been moved; no sign of interaction or manipulation of the material	0
Disturbed	Interaction with the nesting material is evident for example disturbed, chewed, spread around the cage) but has not been gathered to a nest site. If a nest site is present in the regular bedding material, either there is no concentration of the material in the nest site, or the material is merely piled on top of the nest cavity in the regular bedding	1
Flat Nest	Nesting material has been gathered to form a nest site in the cage, identified by a clear nest cavity in the middle of the material, or between the material and the cage wall. The nest is a flattened saucer shape with no, or incomplete, walls	2
	Flat nest with 1 side that is less than half of a sphere	2,25
	Flat nest with 2 sides that are less than half of a sphere	2,5
	Flat nest with 3 sides that are less than half of a sphere	2,75
Cup	Nesting material has been gathered to form a nest site in the cage. The nest has identifiable walls that form a 'cup' or 'bowl' (similar to a shallow soup bowl), such that the walls would not reach the widest point of an imaginary sphere that would fill the nest hollow ('half of a sphere')	3
	Cup-shaped nest with 1 side that is half of a sphere	3,25
	Cup-shaped nest with 2 sides that are half of a sphere	3,5
	Cup-shaped nest with 3 sides that are half of a sphere	3,75
Incomplete Dome	Bedding material has been gathered to form a nest site in the cage. The walls reach (and may close back over) the widest point of an imaginary sphere that would fill the nest hollow ('half of a sphere')	4
	Incomplete dome with 1 side that is more than half of a sphere	4,25
	Incomplete dome with 2 sides that are more than half of a sphere	4,5
	Incomplete dome with 3 sides that are more than half of a sphere	4,75
Complete Dome	Nesting material has been gathered to form a nest site in the cage. The walls completely enclose the nest hollow. A small (mouse-sized) exit hole may be found on the side or the top of the dome	5

Attachment 3

Pup Retrieval Test and video observation Ethogram.

Category	Behaviour	Description	Event
Unusual Behaviours	Pup separation (Exclusion)	The pup becomes visible outside the nest due to a behaviour performed by the adults. The behaviour that the adult was performing when the pup gets displaced from the nest should be written in a comment.	Point event considered when the adult leaves the pup outside the nest area.
	Pup Investigation Without Retrieval	The dam approaches or interacts with the test pup(s) that is (are) outside the nest, but does not retrieve it (them) and moves away from it (them)	Point event
General Activity	Locomotion	All locomotion behaviour performed on the cage floor, on the cage lid, or while climbing along the cage walls. It is considered locomotion when the dam moves at least one mouse distance.	State
	Sniffing	Short head movements with snout towards any part of the cage (floor, air, bars). It isn't considered sniffing if the dam is motionless or performing other behaviours.	State
	Self Oriented Behaviours	The dam is performing grooming, scratching, brushing, or licking its own body surface.	State
	Digging	"The dam uses snout, front- or hindlimbs to displace bedding material." (Winters et al., 2022)	
Maternal Behaviours	Incomplete Retrieval	The dam picks up a pup that is outside the nest area, but does not complete the full retrieval of the pup back to the nest, leaving it outside the nest	Point event
	Complete Retrieval	The dam picks up the pup either with her mouth or forepaws and places it back within the nest area (the area with the most nest material within the cage)	Point event
	Sniffing Pup(s)	Short head movements with snout towards the pup(s). It isn't considered "Sniffing Pups" when the dam is motionless or when it is performing other behaviours.	State
	Pup Grooming	The head of the dam moves up and down while touching one or more pups and/or when the dam handles the pups with her forepaws. Grooming is also considered when it is possible to see the dam cleaning the afterbirth (eating the placenta and/or umbilical cord).	State
	Nursing	The dam may be in different locations and body postures while the pups are close to its belly. The dam may be attempting to move the pups closer to its belly (with the head in a depressed posture) or just laying. If it's not completely visible consider "Unclear".	State
	Nest Building	The dam digs, carries, pushes, and moves nesting material either with its mouth or paws. This behaviour also includes situations in which the dam seems to be destroying the nest (this should be specified in a comment).	State
	Nest Building for Isolated Pup(s)	The dam digs, carries, pushes or moves nesting material either with its mouth or paws towards an isolated pup.	State
Invisible	Other Behaviours	When it is not possible to distinguish the behaviour performed by the Dam. Different from unclear as here we can see the dam but don't understand the behaviour performed.	State
	Unclear	The Dam is in a position where it is not possible to distinguish the type of behaviour that is performing. "Unclear" should be marked in cases of doubt, if either the nest material is covering visibility or the video has a bad quality. Exclude behaviour "Other Behaviours".	State

Maintenance	Drinking	The dam rears up and touches the nipple bottle.	State
	Eating	The dam is either handling (with its paws or snout) or ingesting the food pellets from the food hopper or those that are on the cage floor.	State
Inactive	Inactive	The dam remains motionless for at least 3 seconds, regardless of its body posture or position within the cage, either facing up or tucked into its own body.	State