UNIVERSITY OF COPENHAGEN
DEPARTMENT OF EXPERIMENTAL MEDICINE

The influence of a refined handling protocol on welfare and anaesthetic parameters in C57BL/6JRj mice

Master Thesis

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Title The influence of a refined handling protocol on welfare and anaesthetic parameters in C57BL/6JRj mice

Subject description The purpose of this study is to investigate the effect of tunnel handling, a refined handling method, on the welfare of C57BL/6JRj mice, when injection anaesthesia is applied. In addition, the effect of handling method on the anaesthesia is assessed. Anaesthetic parameters include loss of righting reflex and recovery of righting reflex. Nest building activity, time-to-integrate-into-nest test, voluntary interaction with handler, faecal corticosterone metabolites and automatic cage monitoring is used to assess welfare impact.

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Submission date April 2nd 2024

Acknowledgements A special thanks to my supervisors Peter Bollen and Dorte Bratbo Sørensen for their great help throughout the process. Thanks to Klas Abelson, professor, and Otto Kalliokoski, associate professor, for their help with experimental planning and statistical analyses. Thanks to Morten Malmberg, DVM, Christine Broholm, Trine Marie Ahlman Glahder, Helle Runchel Porsdal and the animal technicians at 16.4 for helping with the practical aspects of the project. Lastly, thanks to Anna Merne, Frederik Damsgaard, DVM, Pernille Colding-Sørensen, DVM, Janne Kjeldsen, Birit Buhr and Henriette Soja for support during the project and help with proofreading.

Petra Buhr

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Abstract

Introduction/background: Millions of laboratory mice are used in research every year in the European Union, and an inevitable part of research is handling of the mice. Handling stresses laboratory mice, and because of this, tunnel handling has been developed as a less stressful handling method compared to traditional tail handling. Studies have demonstrated that tunnel handling decreases stress and anxiety in mice when measuring voluntary interaction and anxiety tests.

Materials & methods: Sixty-four C57BL/6JRj mice (male and female) were housed in single-sex pairs and randomised into either tail or tunnel handling. After arrival mice were only handled by the assigned handling method. After two weeks of handling, tests were conducted. The tests used in this study were voluntary interaction with handler, nest building activity and time-to-integrate-into-nest test. Following these tests all mice were subjected to injection anaesthesia, and the voluntary interaction with handler, nest building activity and time-to-integrate-into-nest test were repeated. Levels of faecal corticosterone metabolites were analysed at two time points: mid study and at termination.

Results: No significant differences due to handling methods were seen in this study in voluntary interaction with handler, nest building activity, time-to-integrate-into-nest test, anaesthetic parameters or faecal corticosterone metabolites. Female mice had significantly higher levels of faecal corticosterone metabolites compared to male mice. Time until recovery of righting reflex in anaesthesia was analysed and female mice had a significantly shorter recovery of righting reflex time compared to male mice.

Conclusion: Overall, no significant differences in behavioural tests, anaesthesia or faecal corticosterone were found between tail and tunnel handled mice in this study. The fact that no significant differences were found may be due to the sample size being too small, the tests not being appropriate or sensitive enough or that the mice did not have different stress levels in the different handling groups. Further research is needed to improve our understanding of handling stress in laboratory settings.

Resume

Introduktion: Millioner af laboratoriemus bruges hvert år i forsøg, og håndtering af musene er generelt en uundgåelig del af forskning. Håndtering stresser laboratoriemus, og derfor er tunnelhåndtering blevet udviklet som et mindre stressende alternativ til traditionel halehåndtering. Flere studier har demonstreret at tunnelhåndterede mus udviser mindre stress og angst i frivillig interaktionstest og tests for angst.

Materialer & metoder: Fireogtres C57BL/6JRj mus (hanner og hunner) blev randomiseret til enten hale- eller tunnelhåndtering og blev opstaldet i enkeltkønspar. Efter ankomst blev musene kun håndteret med den tildelte håndteringsmetode. Efter to ugers håndtering blev der udført adfærdstests. De tests, der blev anvendt i studiet var: frivillig interaktion med forsøgsperson, redebygningstest og tid til integration af nyt redemateriale. Efter disse tests blev alle dyr udsat for injektionsbedøvelse, og frivillig interaktion med forsøgsperson, redebygningstest og tid til integration af nyt redemateriale blev gentaget. Niveauer af fækale kortikosteronmetabolitter blev analyseret på to tidspunkter, midt i studiet og ved afslutningen.

Resultater: Der blev ikke observeret signifikante forskelle på baggrund af håndteringsmetoder i dette studie i frivillig interaktion med forsøgsperson, redebygningstest og tid til integration af nyt redemateriale, anæstetiske parametre eller fækale kortikosteronmetabolitter. Hunmus havde signifikant højere niveauer af fækale kortikosteronmetabolitter sammenlignet med hanmus. Tiden fra anæstesiinduktion til musen vågnede blev analyseret, og her havde hunmus signifikant kortere anæstesitid sammenlignet med hanmus.

Konklusion: Overordnet set blev der ikke fundet signifikante forskelle i adfærdstests, anæstesiresultater eller fækale kortikosteronniveauer mellem halehåndterede og tunnelhåndterede mus i dette studie. Manglen på signifikante forskelle kan tilskrives faktorer såsom en lille stikprøvestørrelse, utilstrækkelig følsomhed af testene eller at musene i de forskellige håndteringsgrupper havde ensartede stressniveauer. Yderligere forskning er påkrævet for at forbedre vores forståelse af håndteringsstress i laboratoriemiljøer.

Abbreviations

3R/3Rs – replacement, reduction, refinement

ACTH – adrenocorticotrophic hormone

BP – blood pressure

Bpm – beats per minute

BW – Bodyweight

DVC® – Digitally ventilated cage

EPM – Elevated plus maze

HR – heart rate

HPA – hypothalamic-pituitary-axis

IP – Intraperitoneal

IVC – Individually ventilated cage

LORR – Loss of righting reflex

NBA – Nest building activity

OFT – Open Field Test

RORR – Recovery of righting reflex

SC – Subcutaneous

SpO_2 – Oxygen saturation

TINT – Time-to-integrate-into-nest test

VI – Voluntary interaction

1 Introduction and background

Around 3.8 million mice are used in biomedical research every year in countries of the European Union (European Commission, 2023). Researchers in the European Union are legally bound to implement the 3R (replacement, reduction and refinement) principles as described by Russel & Burch (1959) when conducting experiments involving laboratory animals, in order to ensure the lowest possible level of distress in the animals (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes, 2010). Handling is stressful to laboratory mice, which makes this an obvious point for refining methods (Rasmussen et al., 2011).The focus of this thesis is the refinement of laboratory mouse handling by using tunnel handling instead of tail handling.

1.1 Handling methods

1.1.1 Handling elicits stress response

Handling of laboratory mice is necessary when conducting experiments, but the method of handling is of great importance to the welfare and stress levels of the mice, as seen in a range of behavioural and physiological measures (Gouveia & Hurst, 2013; Henderson, Dani, et al., 2020; Hull et al., 2022; Hurst & West, 2010). It has been documented that regular laboratory procedures such as handling and cage change, cause stress to laboratory mice (Balcombe et al., 2004; Rasmussen et al., 2011). A study by Rasmussen et al. (2011) showed that regular laboratory procedures, such as cage change, cause stress to laboratory mice when tail handled either by forceps or by hand. The handled mice had elevated corticosterone levels compared to the undisturbed control mice. In contrast to this, the corticosterone levels of mice which were allowed a passive transfer from one cage to another, did not differ significantly from the control group, indicating that handling caused the stress response (Rasmussen et al., 2011). In the study by Rasmussen et al. (2011), the levels of corticosterone were normalised at 60 minutes after cage change.

1.1.2 Alternative handling methods

The traditional handling method of catching or handling mice by lifting them by the base of the tail with either fingers or forceps is a method still widely used in research facilities (Deacon, 2006b; Henderson, Smulders, et al., 2020; Wahlsten et al., 2003). In recent years, alternative handling methods have been developed to mitigate the negative effects of handling (Hurst & West, 2010). Alternative handling methods include tunnel handling, cupping, a combination of tunnel and cupping,

and ladder handling (Hurst & West, 2010; Sandgren et al., 2021). Tunnel handling is generally done with a home cage tunnel (clear or coloured acrylic tunnel) by placing the tunnel with one hand along the cage side, while the opposite hand is used to guide the mouse into the tunnel, after which the tunnel is lifted to allow transfer of the mouse. Cup handling is performed by scooping the mouse up in one or both hands, sometimes with the hands closed loosely over the mouse to prevent it from jumping out of the hands. A combination of tunnel handling and cup handling is sometimes done, where the mouse is tipped out of the tunnel and on to a hand after lifting the mouse out of the cage with the tunnel (Hurst & West, 2010). Handling the mouse with a ladder is done by placing a cage ladder on cage bottom, guiding the mouse onto the ladder, after which the ladder can be lifted to allow transfer (Sandgren et al., 2021).

1.2 Assessing welfare, stress and anxiety

1.2.1 Voluntary interaction and anxiety tests

A popular way to assess the effect of different handling methods is a voluntary interaction (VI) test. Using this method, the time the mice spend in proximity to a hand or handling device in the cage is recorded. This is viewed as a measure of anxiety towards the handler or handling device (Gouveia & Hurst, 2013; Henderson, Dani, et al., 2020; Hurst & West, 2010). Different approaches to VI exist. Some studies have tested all mice regardless of handling method with a gloved hand in the cage to assess anxiety towards the handler (Henderson, Dani, et al., 2020; Roughan & Sevenoaks, 2019), whereas other studies have tested interaction with the handling device, meaning gloved hand for tail handled mice and a gloved hand and tunnel for tunnel handled mice (Gouveia & Hurst, 2013, 2017; Hurst & West, 2010).

Several studies have demonstrated that tunnel handled mice have increased VI (with either device or just a gloved hand) compared to tail handled mice, suggesting that tunnel handling leads to less anxiety for the mice (Gouveia & Hurst, 2013, 2019; Henderson, Dani, et al., 2020; Hurst & West, 2010; Roughan & Sevenoaks, 2019). It has furthermore been demonstrated that tunnel handled mice continue to have increased VI following either unpleasant procedures such as repeated restraints and subcutaneous (SC) injections, intraperitoneal (IP) injections or isoflurane anaesthesia, suggesting that the positive effect of tunnel handling is greater than the negative effect of mentioned procedures (Gouveia & Hurst, 2019; Henderson, Dani, et al., 2020).

A study performed on C57BL/6JRj and BALB/c mice showed that the method of testing VI (with hand or handling device) matters, as the mice in the study, regardless of handling method, had increased VI when presented with a tunnel in hand compared to a gloved hand alone. Furthermore, the study found that while tunnel handled BALB/c mice did have an increased VI when tunnel handled, C57BL/6JRj mice did not. This suggests that VI results depend on the test method as well as mouse strain (Novak et al., 2022).

When surveying the obstacles for implementation of tunnel handling, increased time consumption and difference in scientific outcomes compared to historical data were some of the worries the responders had (Henderson, Smulders, et al., 2020). A study revealed that tunnel handling was initially more time-consuming during cage change but within a few weeks this difference was diminished in NOD SCID, athymic nude, and C57BL/6 mice (Arnott et al., 2023).

Table 1 provides an overview of studies, where the effect of tunnel handling has been tested. As it appears, the enrichment levels vary in the studies, and none of the studies have the same level of enrichment in the cages as the present study (a score of 5, details regarding enrichment available in cage is seen in 3.3 Housing). The housing rooms, in regard to activity level in the rooms have not been described in the studies. Only a few studies describe the number and gender of handlers.

1.2.2 Nest building

1.2.2.1 Natural nesting behaviour

Building nests is an important behaviour intrinsic in mice, as nests are important in terms of thermal protection, protection of pups and to avoid predators (Barnett, 1956; Latham & Mason, 2004). Both male and female mice exhibit nest building behaviour (Robert Lisk et al., 1969). Because of the biological importance of nests, nesting behaviour is highly motivated, and is used to assess welfare and impact of different procedures in mice (Jirkof, 2014).

*Table 1 Studies with VI tests on tail and tunnel handled mice. Enrichment score: 1 point given per type of enrichment (nesting material, house, tunnel, gnawing blocks etc.). *From Harlan, **Different sub-experiments had different number of handlers, ***From Charles River.*

Study	VI test method		Strain/stock	Sex of mice	No. of handlers	Gender of handler	Enrichment score
	Device	Hand					
Hurst & West (2010)	x		BALB/c*, ICR (CD-1)*, C57BL/6*	Female	One/ Multiple*	Unknown	2
Gouveia & Hurst (2013)	x		Hsd:ICR (CD-1), C57BL/6JOlaHsd	Both	One	Unknown	1 or 2
Gouveia & Hurst (2017)	x		BALB/cOlaHsd	Female	One	Unknown	2
Clarkson et al. (2018)	x		C57BL/6J	Male	One	Unknown	3
Nakamura & Suzuki (2018)	x		Jcl:ICR	Both	One	Unknown	0
Roughan & Sevenoaks (2019)		x	BALB/cAnCrl	Both	One	Female	3
Gouveia & Hurst (2019)	x		C57BL/6JolaHsd, BALB/cOlaHsd	Both	One	Unknown	2
Clarkson et al. (2020)	x		C57BL/6***	Male	Unknown	Unknown	3
Henderson, Dani et al. (2020)		x	BALB/c***	Both	One	Female	3
Sensini et al. (2020)	x		C57BL/6NCrl	Male	Unknown	Unknown	1
Sandgren et al. (2021)	x		C57BL/6NRj	Both	Multiple	Female	4
Novak et al. (2022)	x	x	C57BL/6JRj, BALB/cRj	Both	One	Female	3

1.2.2.2 Nest building activity

One way to assess nest building behaviour is to do a nest building activity (NBA) test by scoring the nest after complexity (Deacon, 2006a; Gaskill et al., 2011, 2013; Gjendal et al., 2020; Hess et al., 2008; Jirkof, 2014; Jirkof et al., 2013). Nest building behaviour is affected by many factors such as ambient temperature, type of nesting material, and the absence or presence of pain and stress (Gaskill et al., 2011; Hess et al., 2008; Jirkof et al., 2013). The quality of the nest mice build is influenced by the type of nesting material, as more natural nesting material allows the mice to build better nests (Hess et al., 2008). The presence of painful conditions or stress alter the quality of the nest built. In a study by Jirkof et al. (2013), female C57BL/6 mice were used to assess the effect of anaesthesia and/or postsurgical pain on nest building. Both anaesthesia and anaesthesia combined with surgery resulted in significantly lower nest scores than the baseline scores. This change in nest score after anaesthesia has also been shown by Gjendal et al. (2019, 2020) where a 15-minute exposure to isoflurane anaesthesia resulted in a lower nest score in female C57BL/6NTac mice. Daily IP injections for three days, however, did not influence the nest score in female C57BL/6NTac mice (Gjendal et al., 2019). Similarly, a study on handling methods and acute restraint stress in male CD-1 mice showed no difference in nest score between tail and tunnel handled mice, before or after they were subjected to restraint stress (Redaelli et al., 2021).

1.2.2.3 Time-to-integrate-into-nest test

Time-to-integrate-into-nest test (TINT) is a test where it is determined if mice integrate new nesting material into their existing nest within a set timeframe of 10 minutes. This binary test was developed by Rock, Karas, Gartrell Rodriguez, et al. (2014) as a non-invasive way to evaluate pain behaviour in mice. They showed a correlation between postsurgical pain and lower willingness to integrate new nesting material. Furthermore, some strains of mice were less willing to integrate new nesting material, but it was concluded that the test needed to be validated further in order to use it as a tool for welfare assessment in mice.

A study on CD-1 mice revealed that post-surgical pain as well as buprenorphine treatment reduced the chance of a positive TINT response (Gallo et al., 2020). In another study on CD-1 mice, it was evaluated whether mice responded differently to TINT if they were group- or single-housed. Although not significant, all the failed TINT's were by single housed mice. Single housed mice were

in general slower to integrate new nesting material, suggesting that social isolation stress may result in less expression of nesting behaviour (Rock, Karas, Gallo, et al., 2014).

1.3 Anaesthesia

General anaesthesia is defined as loss of conscience, loss of pain perception and muscle relaxation (Flecknell, 2023). Loss of conscience is often measured by loss of righting reflex. Loss of pain response is assessed by response when a painful stimulus is applied, for example the pedal withdrawal reflex test. A well-balanced general anaesthesia sufficient for surgery has a low risk of side effects, short induction time to minimise the period of excitation, loss of reflexes and analgesic properties. It is beneficial to use a multimodal approach of sedatives and anaesthetic drugs, as the adverse effects are often dose-dependent, so low doses of multiple drugs will minimise the risk of adverse effects (Flecknell, 2023; Navarro et al., 2021).

The route of anaesthesia administration is important. IP injections are widely used in mice but pose a risk of pain or administering the compound into the gastroenteric tract, liver, bladder, or other abdominal organs. To avoid this risk, SC administration can be used, and in addition it is generally considered to be less painful than IP injections, which results in refinement of the anaesthesia induction (Turner et al., 2011).

Ketamine, xylazine and midazolam is used as an anaesthetic combination for mice (Buhr et al., 2023). Ketamine is a NMDA agonist with dissociative anaesthetic effect as well as analgesic properties, with only mild depression of respiration and an increase in blood pressure. Xylazine is an α-2 adrenergic agonist with sedative and analgesic properties and can induce cardiovascular and respiratory depression. Midazolam is a benzodiazepine with sedative and anxiolytic properties. Both xylazine and midazolam increases the effect of anaesthetic drugs. (Flecknell, 2023; Navarro et al., 2021).

During anaesthesia, whether inhalant or injection, supplementing anaesthetised mice with oxygen is important, as mice will otherwise develop severe hypoxia. In a study by Blevins et al. (2021) mice were anaesthetised with different doses of ketamine/xylazine or ketamine/xylazine/acepromazine, some with oxygen supplementation and some without. Without oxygen supplementation the mortality rate was 100%, whereas it was 0% when mice were supplemented with oxygen. In another study on ketamine/xylazine and ketamine/xylazine/midazolam anaesthesia by Buhr et al. (2023) all mice not

supplemented with oxygen developed severe hypoxia, whereas the mice had significantly higher levels of peripheral oxygenation when oxygen was supplied.

The effect of an anaesthetic protocol may vary based on strain, sex and age, why it is important to adjust dosage in relation to the specific mice needing to be anaesthetised (Hildebrandt et al., 2008; Navarro et al., 2021). Restraint and IP injections are acute stressors in mice, causing spikes in corticosterone in urine as well as an increase in heartrate (HR) and restraint stress can result in higher doses of anaesthetic needed for induction due to physiological changes (Hildebrandt et al., 2008; Meijer et al., 2005, 2006). A study on male C57BL/6J mice found that acute restraint stress resulted in prolonged recovery from isoflurane anaesthesia, compared to mice that had not been subjected to restraint stress (Xu et al., 2023).

Anaesthesia in mice is associated with subsequent weight loss. This has been described both in ketamine/xylazine based injection anaesthesia and in short isoflurane anaesthesia. In a study with male CD-1 mice all mice had lost weight two days after anaesthesia with either ketamine/xylazine or ketamine/xylazine/lidocaine (Dholakia et al., 2017). In a study with female C57BL/6NTac mice a weight loss was also found in the two days following a 15-minute isoflurane anaesthesia (Gjendal et al., 2019).

1.4 Corticosterone levels in blood and faeces

Stressful events activate the hypothalamic-pituitary-axis. When this happens, adrenocorticotrophic hormone is released from the anterior pituitary, which stimulates the cortex of the adrenal gland to release corticosterone into the blood stream. The corticosterone is later eliminated through the urine and faeces (Han et al., 1983; Kolber et al., 2008). There is a delay between an increase of corticosterone in blood and when it is evident in faeces of approximately 8-9 hours (Kalliokoski et al., 2010; Siswanto et al., 2008).

Levels of corticosterone in either serum or faeces are used to assess the impact of procedures and conditions on stress levels in laboratory mice (Bailoo et al., 2018; Balcombe et al., 2004; Ghosal et al., 2015; Gjendal et al., 2020; Harper & Austad, 2000; Oatess et al., 2021; Rasmussen et al., 2011). Female mice have a larger adrenal gland and higher levels of serum corticosterone than male mice (Aoki et al., 2010; Oatess et al., 2021).

A study performed on male C57BL/6 mice found that tail handling resulted in enlarged adrenal glands compared to when mice were tunnel handled (Clarkson et al., 2020). Adrenal gland size is influenced by chronic stress, so an increase in adrenal gland size is indicative of a chronic stress condition (Borrow et al., 2019), suggesting that tail handling leads to increased chronic stress compared to tunnel handling. Another study with BALB/c and C57BL/6JRj mice found no difference in either plasma corticosterone levels or adrenal gland weight between tunnel and tail handled mice after nine days of handling, but females did have higher levels of corticosterone and larger adrenal glands than males (Novak et al., 2022).

Levels of faecal corticosterone metabolites (FCM) are influenced by storage time, as bacteria and enzymes present in the faecal matter will naturally decompose the corticosterone metabolites over time (Khan et al., 2002).

1.5 Other measures

A relationship between tunnel handling, increased VI and less anxious behaviour in elevated plus maze (EPM) and Open Field test (OFT) has been demonstrated. Hurst & West (2010) found that tunnel handled mice had increased interaction time with handling device as well as more entries into the open arms of an EPM. This corresponds with the findings of Henderson, Dani, et al. (2020) who found that tunnel handled mice interacted more with a gloved hand, entered the open arms of the EPM and entered the centre in an OFT significantly more than tail handled mice. A study conducted with BALB/c and C57BL/6JRj mice, tunnel handled BALB/c, but not C57BL/6JRj, had increased time in open arms of an EPM compared to when tail handled (Novak et al., 2022). See *Appendix 1 – EPM & OFT* for a description of EPM and OFT.

A study by Wilde et al. (2017) explored whether the sex of the handler or handling method (tail, tunnel and cup) had an effect on the HR and blood pressure (BP) when measured in a tail-cuff and by telemetry. No difference in handling method or sex of handler were found in either HR or BP, and the authors concluded that the restraint stress of the cuff may have exceeded the effect of the stress of handling method or sex of handler.

1.6 Cage activity

To monitor cage activity non-invasively a Digitally Ventilated Cage (DVC®) (Tecniplast, Buguggiate, Italy) can be used in combination with individually ventilated cages (IVC). The DVC® system consists of a sensing board underneath the cage with 12 electrodes, where the electrical capacitance is measured continuously (4 times/second), providing accurate information about the level of cage activity. This monitoring can be used to compare activity between groups or be used as a welfare measure (Iannello, 2019). DVC® has previously been used to detect changes in locomotor activity in mice after stressful events, like cage changes (Fuochi et al., 2021).

2 Aim and hypotheses

2.1 Aim

The aim of this thesis is to investigate the effect of a refined handling method, tunnel handling, as an alternative to tail handling, while applying a stressor, anaesthesia.

Both handling and anaesthesia have an impact on the welfare of laboratory mice, such as the C57BL/6 strain. The C57BL/6 strain was chosen for this study, as it is one of the most commonly used mouse strains in research (Johnson, 2023). The stress and anxiety in C57BL/6 will be evaluated by analysing voluntary interaction with handler, as well as nesting behaviour before and after the mice have been anaesthetised. In addition, faecal corticosterone metabolites will be measured. Anaesthetic parameters will be assessed to evaluate if handling method has an effect on anaesthesia.

2.2 Working hypotheses

1. **Voluntary interaction:** Tunnel handled mice have increased contact time with the handler compared to tail handled mice.
2. **Anaesthesia:**
    a. Tunnel handled mice have a shorter time to loss of righting reflex compared to tail handled mice.
    b. Tunnel handled mice have a shorter time until recovery of righting reflex compared to tail handled mice.
3. **Time-to-Integrate-Into-Nest Test:** The fraction of tunnel handled mice (cages) that integrate new nesting material before the 10-minute cut-off is larger than for tail handled mice.
4. **Nest Building Activity:** The tunnel handled mice will have an increased nest building score (0-5) compared to the tail handled mice.
5. **Faecal corticosterone metabolites:** Tunnel handled mice will have a lower amount of faecal corticosterone metabolites compared to tail handled mice.

3 Methods and materials

3.1 Animals

For the study 64 C57BL/6JRj mice (Janvier-Labs, France) 6 weeks old, equal parts male and female were used. A power analysis of VI in similar studies showed a required sample size of 16 per group (power analysis done on 2 groups).

3.2 Groups

Mice were randomised to cage, earmark (left or right ear) and group upon arrival. The mice were housed in single-sex pairs. Experimental groups can be seen in Table 2.

Table 2 Experimental groups

	Tail handling	**Tunnel handling**
♀ **C57Bl/6JRj**	16	16
♂ **C57Bl/6JRj**	16	16
Total	32	32

3.3 Housing

The mice were housed two per cage in EM500 cages (Tecniplast, Buguggiate, Italy) in a DVC® rack. Each cage contained Aspen bedding (Tapvei, Estonia), one red house (University of Copenhagen, Denmark), a clear plexiglass handling tunnel (50 x 130 mm) (Datesand, UK) and two small Aspen Bricks (Datesand, UK). For nesting, one Sizzle-pad of 8g (Brogaarden, Lynge, Denmark) and 3 g of Paperwool (Datesand, UK) were given to each cage. Food pellets (SAFE® D40, Safe, France) were placed in food trough as well as on cage bottom to ensure all mice having equal access to food. Cage setup can be seen in Figure 1. Water (tap water) was available at all times in a drinking bottle. Cages were changed once after two weeks, and water bottles changed weekly. During cage change, some of the old

Figure 1 Cage setup

nesting material was transferred to the new cage, as well as both the tunnel and red house. Cages were placed in a room with no other cage racks or mice apart from the ones in this study. The mice were on a 12-hour light/dark cycle with light on from 6 AM to 6 PM. Room temperature was kept at 21±1°C. Relative humidity in cages was 36-40%.

3.4 Experimental design

The study was carried out as a case control study, with handling method and sex as the independent variables. The method of handling considered control was traditional tail handling.

Due to logistics, the study was conducted in four consecutive sub-experiments with 16 mice per sub-experiment, as seen in Table 3.

Table 3 Overview study weeks

Sub-experiment /study week							
1	Study week 1	Study week 2	Study week 3	Study week 4			
2		Study week 1	Study week 2	Study week 3	Study week 4		
3			Study week 1	Study week 2	Study week 3	Study week 4	
4				Study week 1	Study week 2	Study week 3	Study week 4

3.5 Ethical considerations

The study was ethically reviewed and approved by the Danish competent authority; the Animal Experiment Council and was conducted under license 2021-15-0201-00901 at Department of Experimental Medicine, University of Copenhagen.

3.6 Timeline

Upon arrival the mice were randomly allocated to cage, earmark (left or right) and handling method (day 1). The earmarking was done by experienced animal technicians during the transfer from the transport cage to the new cage, and the transfer was done using tunnel handling on all mice. After an acclimatisation period of five days, the mice were handled and weighed by the assigned handling method three times weekly for two weeks, with a cage change on study day 15. On days 20 and 22 the mice were handled and weighed, a VI test was done, and an NBA test was initiated. On day 21 a TINT was done followed by a scoring of the nest and anaesthesia thereafter. On day 23 a TINT was

done followed by a scoring of the nest. On day 24 the mice were weighed and euthanised under anaesthesia. The timeline can also be seen in Table 4.

Table 4 Timeline over study activity. VI = voluntary interaction, NBA = nest building activity, TINT = Time-to-integrate-into-nest test.

Study day (week no.)	**Events**
1 (1)	Arrival, earmarking and randomisation
6 (2)	1st handling session
8 (2)	2nd handling session
10 (2)	3rd handling session
13 (3)	4th handling session
15 (3)	5th handling session and cage change
17 (3)	6th handling session
20 (4)	7th handling session, VI test and start of NBA
21 (4)	TINT, NBA score, anaesthesia
22 (4)	8th handling session, VI test and start of NBA
23 (4)	TINT, NBA score
24 (4)	9th handling session and euthanasia

3.6 Experimental procedures

3.6.1 Handling sessions

After an acclimatisation period of five days, the mice were handled three times weekly, by either tail or tunnel depending on the group. In the handling sessions, nesting material and the red house were removed from the cage to facilitate handling. Mice were moved from home cage into an empty cage on a scale for body weight measurement. After weighing, the mice were moved back into their home cage, and the nest and house were placed back into the cage. On days with VI test, the mice were marked on the tail with either a black or red permanent marker, depending on the earmarking placed in the right or left ear, respectively.

Tail handling was done by lifting the mouse by the tail, with no support by the opposite hand, to imitate the usual way of transferring mice when tail handling (Deacon, 2006b; Leach & Main, 2008). Tunnel handling was done by holding the clear plexiglass tunnel (home cage tunnel) at the side of the cage with one hand and guiding the mouse into the tunnel with the opposite hand. When the mouse was in the tunnel, the tunnel was lifted from the cage, and the animal was tipped backwards out of the tunnel. These methods were chosen to keep the handling method strictly to either tail handling or tunnel handling, thereby avoiding any form of cupping.

All handling sessions were done in a randomised order. Randomisation was done using the =RAND() function in Excel. The mice were handled by the same handler in all handling sessions.

3.6.2 Voluntary interaction with handler

To assess willingness to interact with the handler, a VI test was done. This test was done within an hour after a handling session. The handler removed nesting material, house and handling tunnel from the cage, and sat still in front of the cage for 60 seconds. After the 60 seconds in front of the cage, the handler put a gloved hand into the front part of the cage for 60 seconds. The VI setup can be seen in Figure 2. The test was video recorded from above the cage for later analysis. Interaction was defined as time spent in the front third part of the cage, closest to the handler. Time spent in the front third part of the cage was defined as any part of the mouse over the threshold, except the tail. Interaction percent of the 60 seconds were analysed for each mouse in the cage, after which a cage average was calculated. The cage average was used to avoid pseudo replication, as the behaviour of the two mice could arguably not be seen as independent of each other (Gouveia & Hurst, 2019; Henderson, Dani, et al., 2020). The mice were marked with either red or black on the tail to facilitate differentiation during analysis. This method of evaluating VI is similar to the method used by Henderson, Dani, et al. (2020).

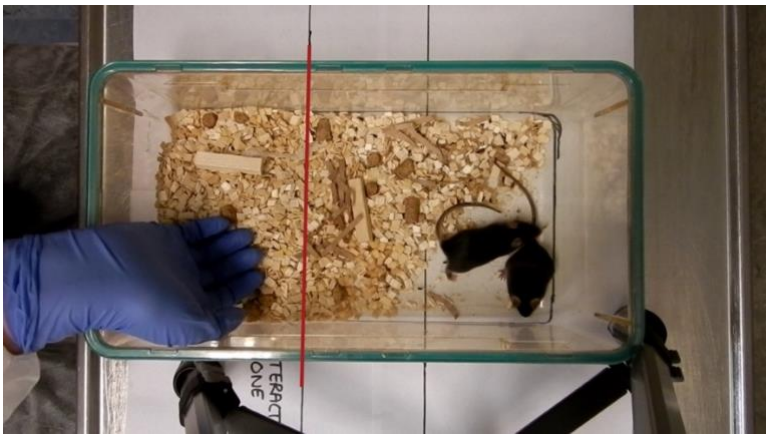

Figure 2 VI setup. The red line is the threshold for interaction.

The order of testing was done in a randomised manner, using Research Randomizer (Urbaniak & Plous, 2013). Testing and analysis of VI was done in a blinded manner by removing the cage identifier and assigning each cage a letter in a randomised order. VI tests were done by the same handler throughout the study. Editing and analysis of VI videos were done using DaVinci Resolve (*DaVinci Resolve*, 2023) and Adobe Premiere Pro (*Adobe Premiere Pro*, 2024).

3.6.3 Nest building activity

To assess if nest quality was influenced by handling method, nest quality was assessed twice for each cage. The two tests were initiated in the morning, the day before and after anaesthesia. The experimenter removed existing nesting material, house and tunnel from the cage, and introduced new nesting material, one sizzle-pad and 3 g of paperwool, to each cage. Approximately 24 hours after the new nesting material was introduced, nest quality was assessed. The experimental unit for this test was "cage".

The assessment was made by scoring the nest by the method described by Hess et al. (2008). The nest received a score between 0 and 5 on four sides, corresponding with the cage sides, and an average score was calculated. Scoring was done in the following manner: 0; nesting material is completely undisturbed, 1; nesting material is manipulated but a nest site has not been formed, 2; nest is flat with no or incomplete walls formed, 3; nest is cup-shaped with wall no higher than 50% of potential sphere, 4; nest is an incomplete dome with walls higher than 50% of a potential sphere, 5; nest is a complete dome with only a mouse-sized hole in the side or on top of the nest. Nest placement was recorded. If there were two nests in one cage, this was noted, and the nest with the tallest walls scored. After the scoring of nest quality, the house and tunnel were replaced in the cage. The same house and tunnel were reintroduced into each cage as before testing, which were stored in separate plastic bags for each cage to minimise unnecessary cross-contamination with scents from the cages.

The order of testing was done in a randomised manner, using Research Randomizer (Urbaniak & Plous, 2013). The observer was blinded to group and sex, as cage identifier was removed before the test and replaced by a randomly assigned letter. NBA was done by the same experimenter throughout the study.

3.6.4 Time-to-integrate-into-nest test

To assess if willingness to integrate new nesting material was influenced by handling method, TINT was performed a total of two times per cage, before and after anaesthesia. The experimental unit for this test was "cage". TINT was done within 3 hours of beginning of the light period and was done with 3 strips of Paperwool rolled loosely into a ball. The TINT was done by removing the cage from rack, lifting cage lid slightly, placing the nesting material in opposite end of existing nest, replacing cage in cage rack and starting a timer. TINT setup can be seen in Figure 3. Time until integration (moving the material to the existing nest) was recorded. If mice had not integrated the new nesting material after 10 minutes, they were assessed as non-responders. This test was not done in a blinded manner, as a pilot study for the present study showed that moving the cages to a different room before TINT resulted in no response to the test in half the cages (unpublished results). The method is similar to the one described by Rock et al. (2014). TINT was done by the same experimenter throughout the study. For analysis purposes, the data was converted to yes/no to integration into nest within 10 minutes, due to risk of bias if there were non-responders.

Figure 3 TINT setup. New nesting material can be seen in front left part of cage and existing nest in back right of cage.

3.6.5 Anaesthesia

To assess if induction time and length of anaesthesia was influenced by handling method, all mice were anaesthetised once after two weeks of handling. The mice were removed from their home cage by assigned handling method, placed on cage grid, restrained with a 3-finger-scruff (Norecopa, 2023) and dosed SC with anaesthetic solution in the lower abdominal region. Injection was done by an experienced animal technician, and the needle (27G) and syringe (1 ml) were replaced for each animal. Both mice from each cage were anaesthetised within a few minutes to minimise stress for the last mouse to be injected. After injection the mouse was placed in a cage with bedding, a red house

and cage lid with an absorbent pad on top, to shelter as much as possible from light and noise. This was done to ensure as smooth an induction as possible. The induction cage was placed on a heating mat to ensure adequate heat to the mice.

Figure 4 Anaesthetised mice in oxygen masks. Temperature, HR and oxygen saturation is being measured on the mouse on the right side.

The first sub-experiment (16 mice, four from each group/sex) of mice unfortunately had no heating during induction due to an error.

Time from injection to loss of righting reflex (recumbency) was recorded. 10 minutes after injection, the mouse was transferred from the induction cage onto a heating pad with an absorbent pad with and an oxygen mask. Here eye ointment was applied (Øjensalve Neutral Ophtla, Actavis). The fur on the thigh (right leg) was clipped using an electric hair clipper (Aesculap Isis, Aesculap, Germany) to facilitate pulse oximetric measurements. At 15 and 30 minutes after injection temperature, HR and peripheral oxygenation were measured using a thermometer (BAT-12 Microprobe Thermometer, Physitemp Instruments LLC, United States) and a pulse oximeter (MouseOx Plus, Starr Life Sciences, United States). The setup and how the temperature, HR and SpO_2 was measured is seen in Figure 4. Presence of pedal withdrawal reflexes were checked using forceps. After the last measurement, each mouse was placed back into the home cage, which was placed on a heating pad, and the animal was monitored until the righting reflex was regained, which was recorded. The mice were not supplemented with oxygen after being returned to home cage for recovery due to logistics. When all mice were awake, the cages were placed back into the cage rack.

The anaesthesia was a combination of 100mg/kg ketamine (1 ml Ketaminol Vet. 50mg/ml, MSD Animal Health), 4.3mg/kg xylazine (0.1 ml Xylavet 20mg/ml, Biovet ApS or Xysol vet. 20mg/ml, CP-Pharma), 3.76mg/kg midazolam (0.35 ml Midazolam Hameln 5mg/ml, Hameln) and sterile water (3.2 ml Sterilt vand Mini-Plasco, B Braun). Dosage was based on previous experiences from Buhr et al. (2023), where this combination resulted in surgical anaesthesia and a safe recovery of the mice.

The order of testing was done in a randomised manner, using Research Randomizer (Urbaniak & Plous, 2013). The observers were blinded to group. The same two experimenters did all observations on anaesthesia, and the same animal technician anaesthetised all mice throughout the study.

3.6.6 Faecal corticosterone metabolites

To assess if handling method influenced levels of faecal corticosterone metabolites, faecal pellets were collected from each cage after cage change as well as after termination. Collection times were chosen to avoid disturbing the mice unnecessarily as the collection could then be done without additional cage changes.

The faecal samples were analysed using a corticosterone ELISA kit (EIA-4164, DRG Instruments GmbH, Germany). Results were recorded at 450 nm in a plate reader (HiPo MPP-96 Microplate Reader, BioSan, Latvia). Details regarding sorting and extraction can be found in *Appendix 2 – FCM method*.

3.6.7 Euthanasia

The mice were euthanised by cervical dislocation under isoflurane anaesthesia.

3.7 Statistical methods

All statistical analyses were performed in GraphPad Prism 10.1.1.

A 2-way ANOVA with handling method and sex as independent variables was used to analyse voluntary interaction, time until loss of righting reflex, time until recovery of righting reflex and faecal corticosterone metabolites. To analyse TINT Fischer's exact test was used, with analysis of females and males separately and Bonferroni correction of p-values to account for multiple tests. To analyse NBA, Mann-Whitney U tests were used, with analysis of females and males separately and Bonferroni correction of p-values to account for multiple tests. Significance level (α) set to 0.05.

4 Results

4.1 Voluntary interaction

The sample size was n=8 for all groups at both first and second VI except tail handled males at second VI where n=6. This was because two male tail handled mice died during anaesthesia, and their cages were excluded from all analyses after the anaesthesia.

No significant differences were found in interaction time between handling method (p-values 0.4646, 0.9128) or sex (p-value 0.9545, 0.9694) at either first VI or change in means from first to second VI. Results from VI tests are presented in Figure 5.

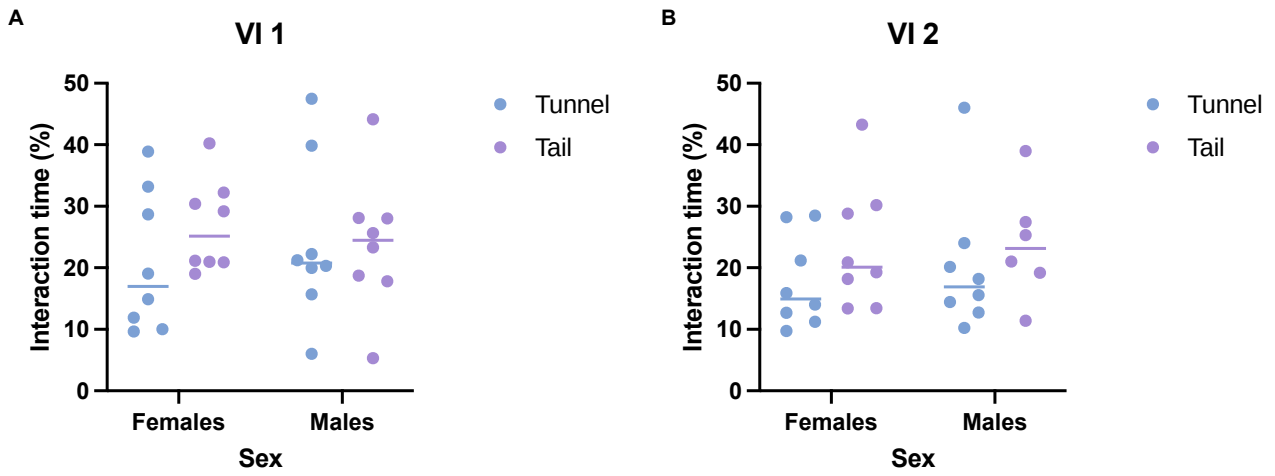

Figure 5 VI in C57BL/6JRj mice before (A) and after (B) anaesthesia. Sample size n=8 for all groups except male tail handled mice with n=6. VI = voluntary interaction

4.2 Nest building activity

The sample size was n=8 for all groups at both first and second NBA, except tail handled males at second NBA where n=6. This was because two male tail handled mice died during anaesthesia, and their cages were excluded from all analyses after the anaesthesia.

No significant differences were found in nest scores between handling methods were found (p-values 0.2894, 0.5571, 0.9999, 0.8422). Results are presented in Figure 6.

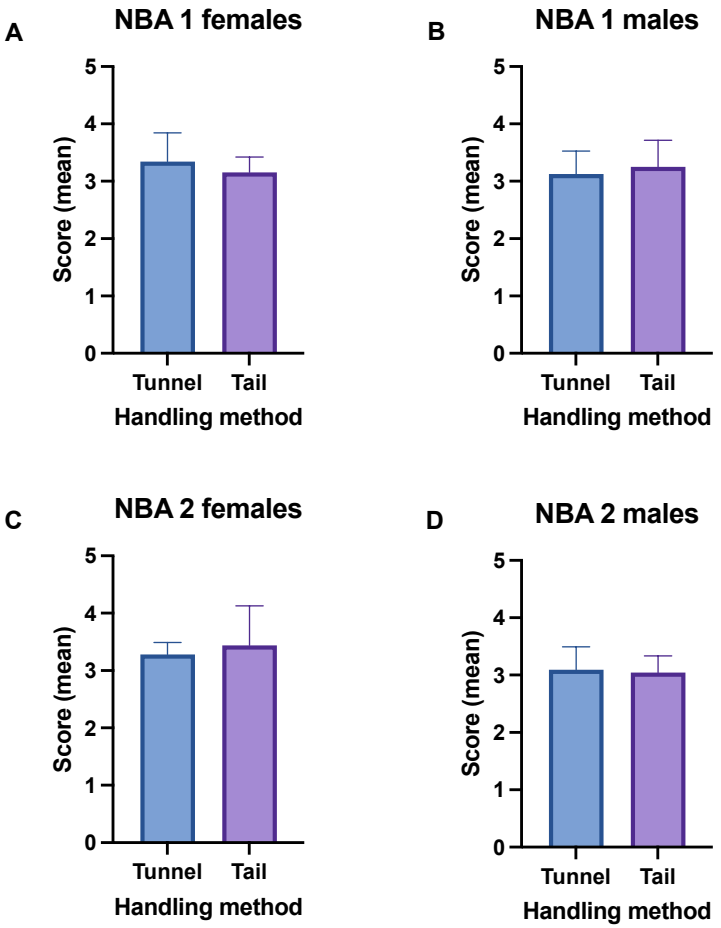

Figure 6 NBA (nest building activity) scores in C57BL/6JRj mice before (A & B) and after (C & D) anaesthesia. Sample size n=8 for all groups except male tail handled mice with n=6. Results displayed as mean values + SEM.

4.3 Time-to-integrate-into-nest test

The sample size was n=8 for all groups at both first and second TINT, except tail handled males at second TINT where n=6. This was because two male tail handled mice died during anaesthesia, and their cages were excluded from all analyses after the anaesthesia.

No significant differences were found in positive TINT results between handling methods (p-values 0.6084, >0.9999, 0.4667, >0.9999). Results are presented in Figure 7.

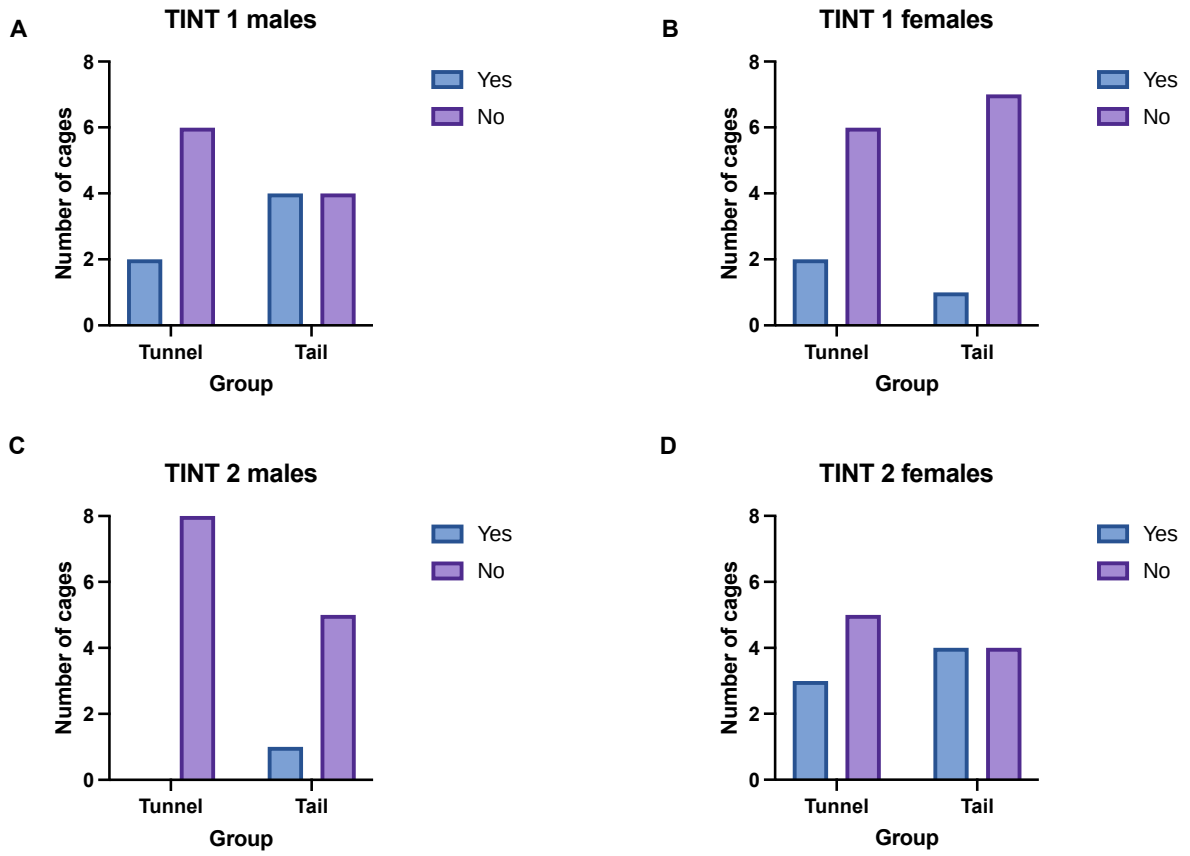

Figure 7 TINT (Time-to-integrate-into-nest test) response (yes/no) in C57BL/6JRj mice before (A & B) and after (C & D) anaesthesia. Sample size n=8 for all groups except male tail handled mice with n=6.

4.4 Anaesthesia

4.4.1 Loss of righting reflex

The sample size was n=16 for all groups. Two male mice (tail handled) died under anaesthesia after all measurements, these are included in LORR.

No significant differences were found in time from injection until LORR in either handling method (p-value 0.2875) or sex (p-value 0.1503), see Figure 8.

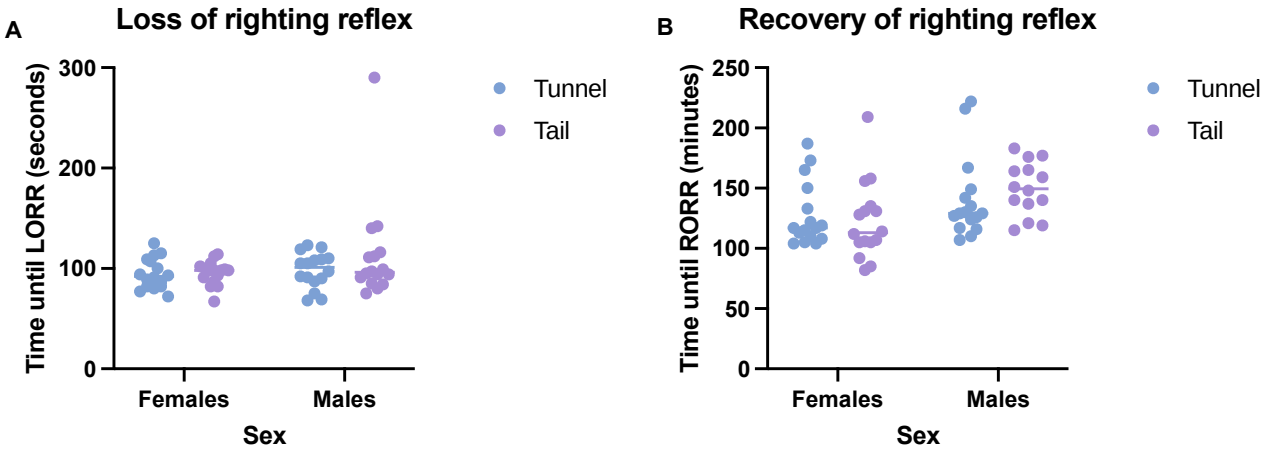

Figure 8 (A) Time from injection until loss of righting reflex (LORR) in tunnel handled and tail handled mice, n=16 for all groups and (B) Time from injection until recovery of righting reflex (RORR) in mice from tunnel handled group (females n=16, males n=16) and tail handled group (females n=16, males n=14) under ketamine/xylazine/midazolam anaesthesia. *Two tail handled males died under anaesthesia.*

4.4.2 Loss of pedal withdrawal reflexes

All mice anaesthetised had absent pedal withdrawal reflexes at both 15 and 30 minutes after injection.

4.4.3 Recovery of righting reflex

A total of 62 out of 64 mice anaesthetised recovered from the anaesthesia without issues. Two tail handled males died under anaesthesia more than 30 minutes after injection (after they were moved back to their home cage for recovery). This resulted in a sample size of n=16 for all groups except tail handled males. Tail handled males had a sample size of n=14.

Females had a significantly shorter time until RORR than males (p-value 0.0093), but no significant difference in RORR time between handling methods was observed, see Figure 8. The female mice had an average RORR time of 125 minutes and the males 145 minutes.

4.4.4 Heart rate, peripheral oxygen saturation and temperature

Mean HR, SpO_2 and temp can be seen in *Table* 5 and are visually presented in Figure 11 in *Appendix 3 – HR, SpO2 & Temperature*. All groups had a lower body temperature 30 minutes after induction compared to 15 minutes after induction.

*Table 5 HR, SpO_2 and temperature of C57BL/6JRj mice 15 and 30 minutes after induction of anaesthesia. *n=15 in SpO_2 at 30 minutes due to a technical error.*

Group	**Sex**	**Sample size**	**HR (bpm)**		**SpO_2 (%)**		**Temperature (°C)**	
			15 min	**30 min**	**15 min**	**30 min**	**15 min**	**30 min**
Tail	Female	n=16	260.9	262.8	98.2	98.6	34.8	34.4
	Male	n=16*	243	235.6	98.1	98.6	34.6	34.0
Tunnel	Female	n=16	257.8	254.1	98.6	98.5	34.8	34.4
	Male	n=16	275.1	264.5	98.6	98.4	34.8	34.6

4.4.5 Bodyweight

Following anaesthesia, a weight loss was observed across all groups (from handling session 7 to 8) as seen in Figure 9.

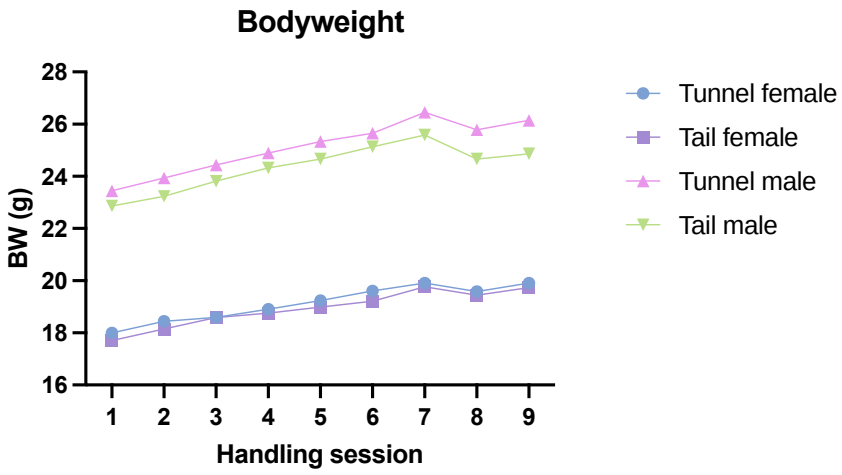

Figure 9 Bodyweight of C57BL/6 mice at handling sessions. Results displayed as mean values.

4.5 Faecal corticosterone metabolites

The sample sizes at cage change were n=8 in all groups except tunnel handled males with n=7, as one sample was lost due to a laboratory error. Sample sizes at termination were n=8 in all groups except n=6 in tail handled males, as two males died under anaesthesia; their cages were excluded from analyses as only one mouse was left in each cage.

There were no significant differences in FCM between handling methods at either cage change (p-value 0.8182) or termination (p-value 0.2216). Female mice had significantly higher levels of FCM at both time points (p-values <0.0001), as seen in Figure 10.

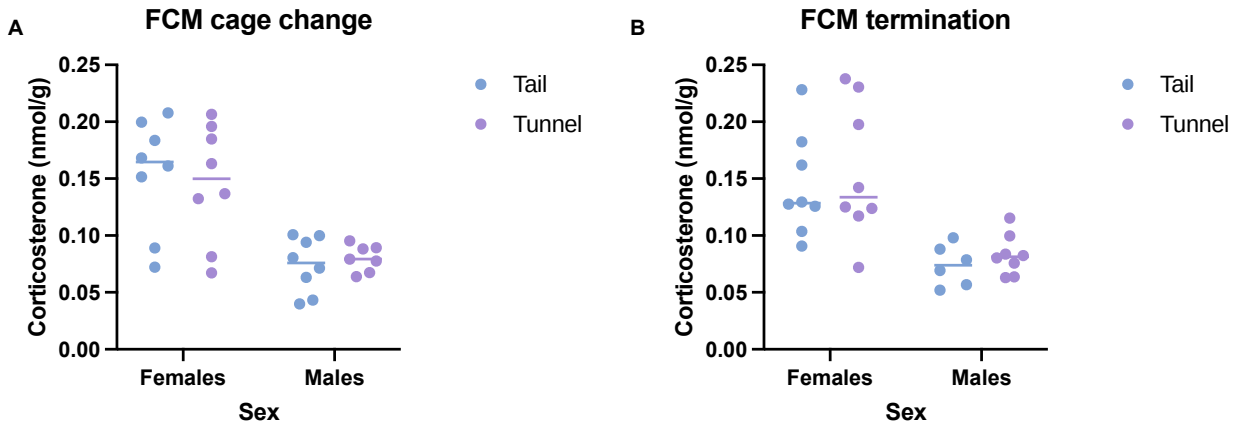

Figure 10 Levels of FCM (nmol/g) for C57BL/6 mice at cage change (A) and termination (B). Female mice had significantly higher levels of FCM than male mice.

4.6 Cage activity (DVC)

Activity levels did not seem to be affected by group. See *Appendix 4 – Cage activity* for graphs showing daily cage activity (Figure 12) and cage activity following sixth handling (Figure 13).

5 Discussion

5.1 Voluntary Interaction

The results showed no difference in VI caused by handling method. This is conflicting with multiple other studies published (Gouveia & Hurst, 2013; Henderson, Dani, et al., 2020; Hurst & West, 2010), which found increased contact time in tunnel handled C57BL/6JOlaHsd, CD-1, and BALB/c mice. A study by Novak et al. (2022) found, similarly to the present results, that tunnel handled C57BL/6JRj mice did not have increased VI compared to tail handled mice, indicating that strain might have a greater impact in VI than handling method. The study by Novak et al. (2022) was the only one with the same sub-strain as was used in the present study, so C57BL/6JRj mice may not be inclined to interaction with hands generally, regardless of handling method.

Gouveia & Hurst (2013, 2019) and Hurst & West (2010) tested VI with the handling device, i.e. either tunnel and hand or only a hand, thereby performing different tests on tail handled and tunnel handled mice. This difference in method between groups can potentially be considered misleading, as it does not directly show difference in anxiety/curiosity to handlers, but rather anxiety/curiosity towards a hand or a tunnel (or both). Furthermore, this difference in test could pose a risk of bias in analysis, as the groups would not be blinded to the handlers and the ones doing the analysis. Henderson, Dani, et al. (2020) have tested VI with only a gloved hand to portray the interaction with the handler and not the handling device. They found that tunnel handled mice had increased contact time compared to tail handled mice, which supports the idea that tunnel handling decreased the anxiety towards people. Novak et al. (2022) found that all mice regardless of handling method, had increased VI when a tunnel was used compared to when VI was done with a gloved hand only. The findings by Novak et al. (2022) indicate that testing VI with handling device and not a hand only, might be confounding to the results found in other studies.

A potential publication bias must be considered, as some studies with no difference between tunnel and tail handled mice may never have been published. This could explain the vast difference in the present study compared to several of the published studies.

As seen in Table 1 the number of handlers, the gender of handlers, and the level of enrichment present in the cage vary greatly in the studies that have tested VI in tail and tunnel handled mice. Some studies did not disclose the number and gender of handlers. Furthermore, most of the studies used the VI

device method and not the hand only. All these factors could potentially influence the stress levels of the mice and their performance in the VI test and may explain the difference in the effects seen. These unknown factors make it difficult to conclude definitively what factors led to the result in the present study.

A power analysis done prior to the experiments showed a required sample size of 16 mice per group, which was the number of mice chosen. During the pilot study, it became evident that the behaviour of the mice during VI was not independent of each other. Because of this it was decided to average the two mice per cage in regard to VI, which resulted in the actual sample size of eight per group, the experimental unit being "cage", instead of "mouse". This smaller sample size means that the conclusions of these tests might not accurately reflect the effect of handling method, as a smaller sample size results in lower power.

5.2 Nest building activity

No differences in NBA were seen between handling methods. Studies (Gjendal et al., 2019, 2020) have shown differences when anaesthetising mice with isoflurane, but only after the first anaesthesia, and not subsequent anaesthesias, suggesting that some forms of stressful procedures result in lower NBA scores. The reason why no difference was seen in NBA between handling groups in this study may be that different handling methods do not elicit a strong enough stress response to have an impact on nest building behaviour in C57BL/6JRj mice. Another possible explanation is that the NBA is not sensitive enough, or that nesting highly motivated due to biological importance to mice and therefore not influenced by stress of different handling methods. Lastly the sample size may have been too small to document a possible difference.

5.3 Time-to-integrate-into-nest test

No difference in TINT response was seen in handling methods. TINT is not a very validated method of evaluating welfare (Rock, Karas, Gartrell Rodriguez, et al., 2014). Similar to the NBA, TINT is a test reliant on the natural nesting behaviour of mice being disrupted, which may be an explanation of why no differences between tunnel handled and tail handled mice were seen, as handling method itself may not be stressful enough to elicit change in the important biological behaviour of nesting. As studies have shown some link between post-surgical pain and a reduced probability of a positive TINT, this test might be better suited when assessing more severe stress or painful conditions (Gallo

et al., 2020; Rock, Karas, Gartrell Rodriguez, et al., 2014). Another possible explanation for the result is that the sample size may have been too small to show a difference between groups.

5.4 Anaesthesia

This experiment found no difference in time until LORR in either sex or handling method. All mice, except one, had short induction times, which is important for good anaesthesia. All 64 mice anaesthetised had absent pedal withdrawal reflexes, which is important if the goal is a surgical anaesthesia.

Out of 64 mice, two male mice did not recover from the anaesthesia. They both died after being returned to the home cage for recovery. HR and SpO_2 were generally good in all mice at 15 and 30 minutes after induction of anaesthesia (see Table 5 and Figure 11 (*Appendix 3 – HR, SpO2 & Temperature*)). A potential explanation to the two mice that died during the anaesthesia may be severe hypoxia after oxygen was removed. This is because the mice were not planned to be supplemented with oxygen during recovery after they were returned to the home cage, due to logistical difficulties. As other studies have shown, mice develop severe hypoxia and have a higher risk of anaesthesia related death when not supplemented with oxygen during anaesthesia (Blevins et al., 2021; Buhr et al., 2023).

Time until RORR did not vary between handling groups, but female mice had a significant shorter RORR time compared to male mice, an average of 125 minutes and 145 minutes for female and male mice, respectively. This length of anaesthesia will be sufficient to do surgery or imaging procedures, which makes it applicable to several procedures. For shorter procedures, different dosages of the sedative and anaesthetic drugs might be more applicable. The difference in total RORR time between female and male mice was not surprising as the sex of the mouse influences the effect of anaesthetics (Hildebrandt et al., 2008; Navarro et al., 2021).

A weight loss was seen across all groups following the anaesthesia. This weight loss could be due to stress or the fact that the mice have a lower activity in the dark period following the anaesthesia (see Figure 12 in *Appendix 4 – Cage activity*). The lower activity levels could result in the mice possibly not ingesting as much food and water as they normally would have, resulting in body weight loss due to dehydration and a negative energy balance. All groups gained weight and almost reached pre-

anaesthetic BW levels within three days after anaesthesia, suggesting that this change in bodyweight is short term and will be normalised within few days after anaesthesia. Loss of BW has been documented in other studies and was not unexpected (Dholakia et al., 2017; Gjendal et al., 2019).

5.5 Faecal corticosterone metabolites

In this experiment, the level of FCM in tail and tunnel handled mice did not differ significantly at either cage change or termination. There were, however, a significant difference between females and males. This sex difference has been described previously and is considered natural (Aoki et al., 2010; Oatess et al., 2021). There might be further explanations of the fact, that no difference in handling was observed. One potential reason could be that handling itself, whether by tail or tunnel, is not a strong enough stressor to elicit a difference in faecal corticosterone. Another potential explanation for the results, is the large intervals of sampling. Combined with the relative short time of handling compared to time left undisturbed, even if handling method affected corticosterone, a difference might not have been evident.

If the study should be optimised, serum corticosterone could be measured shortly after handling sessions. However, this would require more handling and restraint, and maybe even pain, to obtain samples. Alternatively, a short window of time for FCM analyses could have been chosen, collecting faeces within 12 hours after a handling session. This method would probably have been more sensitive to the short-term stress immediately after handling, as changes in corticosterone in the blood are evident within 8-9 hours (Kalliokoski et al., 2010; Siswanto et al., 2008).

Clarkson et al. (2020) found that tail handling resulted in a larger adrenal gland compared to tunnel handled mice, indicative of a chronic stress condition. This finding was not, however, supported by the findings in a study by Novak et al. (2022) where handling method did not influence corticosterone levels or adrenal gland size. The difference in results in these two studies indicate that tail handling can result in chronic stress compared to tunnel handling, but not always.

Storage time affects FCM levels as bacteria and enzymes naturally decompose the corticosterone (Khan et al., 2002). As all sample periods were the same in cage change and termination, respectively, this should not be an issue in regard to analyses and conclusion.

5.6 DVC data

As the data from the DVC is only descriptive and no hypothesis was present, no statistical analyses were performed on the DVC data. Activity levels seemed to be affected by anaesthesia (study day 21) as well as VI and NBA (20 and 22) compared to days with no disturbances. On days with regular handling sessions (day 10, 13, 17) the cage activity did not seem to be disturbed other than shortly after the handling session.

When looking at cage activity following the sixth handling session, activity levels returned to regular low levels within approximately an hour. This time frame corresponds with the time it takes for plasma corticosterone levels to normalise after a cage change (Rasmussen et al., 2011). The results show that even though handling itself is a stressful event, the mice return to normal behaviour quite quickly.

Even though the activity is quite high after a cage change, it doesn't seem to affect the normal circadian rhythm in the dark period. However, there were signs that activity on the days that the VI and NBA tests were done were disturbed in the following dark period compared to the normal activity pattern.

The activity was unsurprisingly quite disturbed following anaesthesia. The activity levels in the dark period following the anaesthesia were quite low compared to the normal pattern, where higher activity levels are expected. The two dark periods after this were also disturbed across all groups, but this disturbance could be due to both the anaesthesia and the following VI and NBA tests the day after the anaesthesia.

5.7 Other aspects

The mice in the present study were housed in an animal room absent of any other mice, and all procedures were done in the same room they were housed, so the mice were not transported to unknown rooms during the study. Furthermore, only the experimenters were present in the room, as well as an animal technician doing short welfare checks, so the environment was generally quiet and calm. This might have resulted in mice that generally had low stress levels, as their environment was calm and predictable.

This study did not show differences in tunnel handled mice compared to tail handled mice in any of the tests done, even though comparable studies did find significant differences. The results of this study suggest that the mice in the present study may not have been significantly more stressed by the tail handling than the tunnel handling. As discussed, some of the tests might not have been sensitive enough, which could also be a possible explanation. That no significant differences were seen between tunnel and tail handled mice in this study may be an advantage, as implementing tunnel handling should not impact comparisons to historical data where tail handling was used. Furthermore, tunnel handling is not more time consuming than tail handling, so implementation should not influence time spent on husbandry and experiments when using tunnel handling instead of tail handling (Arnott et al., 2023).

6 Conclusion

This study aimed to investigate whether tunnel handling is less aversive for C57BL/6JRj mice compared to tail handling. No evidence supporting this hypothesis was found in the study.

However, extensive research supports that tunnel handling is less aversive than tail handling in other strains and C57BL/6 substrains. Several studies of these other strains and substrains have demonstrated less anxious behaviour in EPM/OFT along with increased VI time, showing that tunnel handling is less aversive than tail handling.

Notably, tunnel handling has been demonstrated to be equally time efficient as tail handling meaning that implementation of tunnel handling does not impose an additional time burden on researchers and animal caretakers (Arnott et al., 2023).

Tunnel handling is likely less aversive than tail handling for most strains and is not more time-consuming than tail handling. This supports implementing tunnel handling in future research to improve animal welfare and proper care.

7 Future perspectives

Future studies should investigate if the VI test is appropriate for studying anxiety towards handler or device. This could be assessed using a 2x2 factorial study design with the handling method (tail/tunnel) and VI test method (hand/tunnel) as factors to determine whether or not mice are tamed by tunnel handling. Different strains of mice should be tested to determine difference in response. Further studies could also include other measures of anxiety, to evaluate on overall anxiety levels. Care should be taken to include a large enough sample size to avoid a low power in future studies. Another perspective could be to do a study on handler effect, factoring in experience in (tunnel) handling, personality type, gender of handler and gentleness during handling to see the effect.

8 References

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Appendix 1 – EPM & OFT

Elevated Plus Maze

The Elevated Plus Maze (EPM) is a test used to assess levels of anxiety-like behaviour in mice. The EPM is a plus-shaped maze with two open and two closed arms, and the maze is elevated. The time mice spend in the open arms and the number of entries into the open arms are used to evaluate the level of anxiety-like behaviour in the mouse, as mice that spend less time and have fewer entries into the open arms are seen as having higher levels of anxiety. This is because the normal behaviour of the mouse is to hide in small, dark, closed areas to avoid dangers such as predators, so willingness to explore open areas renders the mouse vulnerable and is therefore seen as an expression of less anxious-like behaviour (Lezak et al., 2017).

Open Field Test

The Open Field Test (OFT) is an enclosed area with surrounding walls used to measure either or both anxiety-like behaviour and locomotor activity. To measure anxiety-like behaviours in the OFT number of entries into the centre, time spent in the centre and distance travelled is used. The OFT uses similar behavioural patterns as the EPM (Lezak et al., 2017).

Appendix 2 – FCM method

Soiled bedding was stored in plastic bags at -20°C until sorting. Sorting was done either manually by forceps or on a sieve-shaker (Retsch AS 400 Control) with subsequent manual sorting. A total of 15 ml faecal pellets were used from each cage. The faecal pellets were transferred into 50 ml tube and stored at -20°C until extraction. Extraction was done by adding 96% ethanol (3ml/g faeces) to the tubes and incubating the tubes at room temperature for approximately 18 hours on a rocking table. After this the tubes were centrifuged (Labogene Scanspeed 1236R) for 20 minutes at approximately 4000 RPM. The supernatant was transferred into a 15 ml tube and centrifuged again for 20 minutes at approximately 4000 RPM. 1.4 ml of supernatant was transferred to a 1.5 ml Eppendorf tube. The samples were then centrifuged (Eppendorf Centrifuge 5430) for 15 minutes at approximately 10,000 RPM. 300 µl of the supernatant was transferred to borosilicate evaporation vials and dried in centrifugal evaporator (Genevac EZ-2 personal evaporator) at room temperature. After evaporation the dried samples were dissolved in 300 µl PBS using app. 5 glass beads (diameter 2mm) and vortexed to ensure all of the sample had been resuspended. Samples were then stored at -20°C until analysis.

Appendix 3 – HR, SpO_2 & Temperature

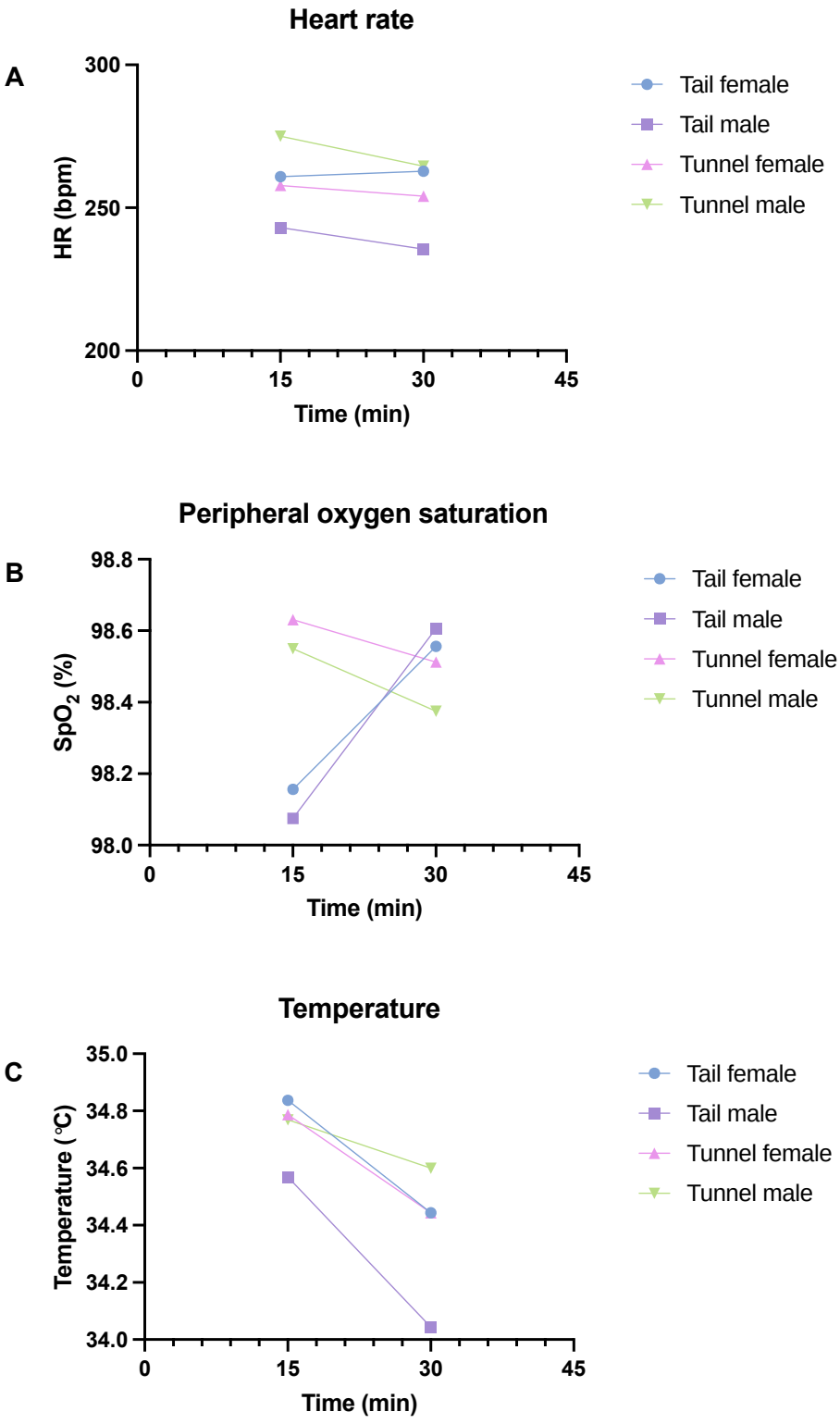
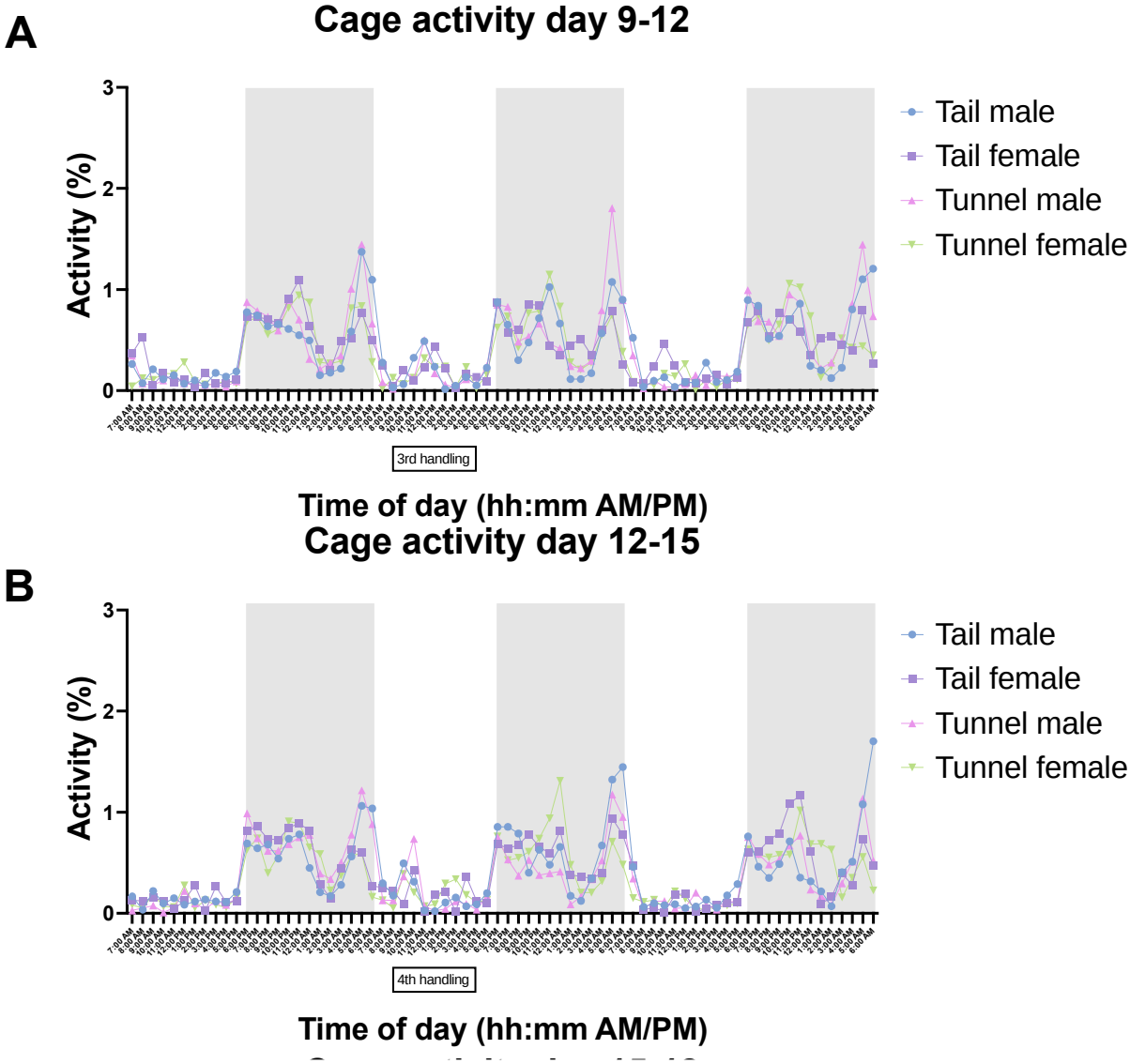


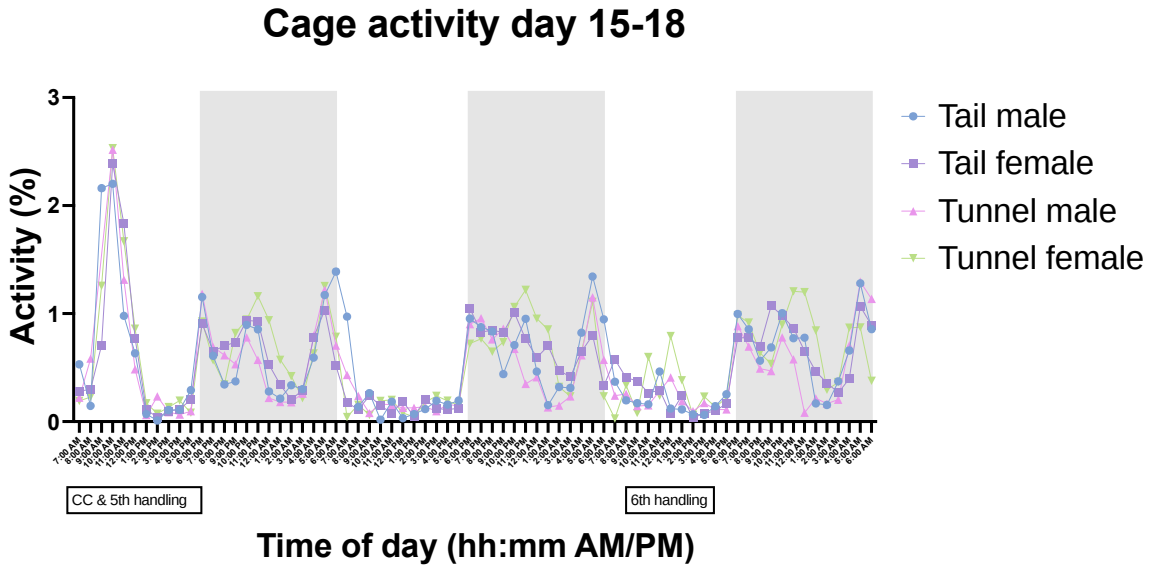
Figure 11 Heart rate (A), peripheral oxygen saturation (B) and temperature (C) in C57BL/6JRj mice 15 and 30 minutes after induction of anaesthesia. Results displayed as mean values.

Appendix 4 – Cage activity

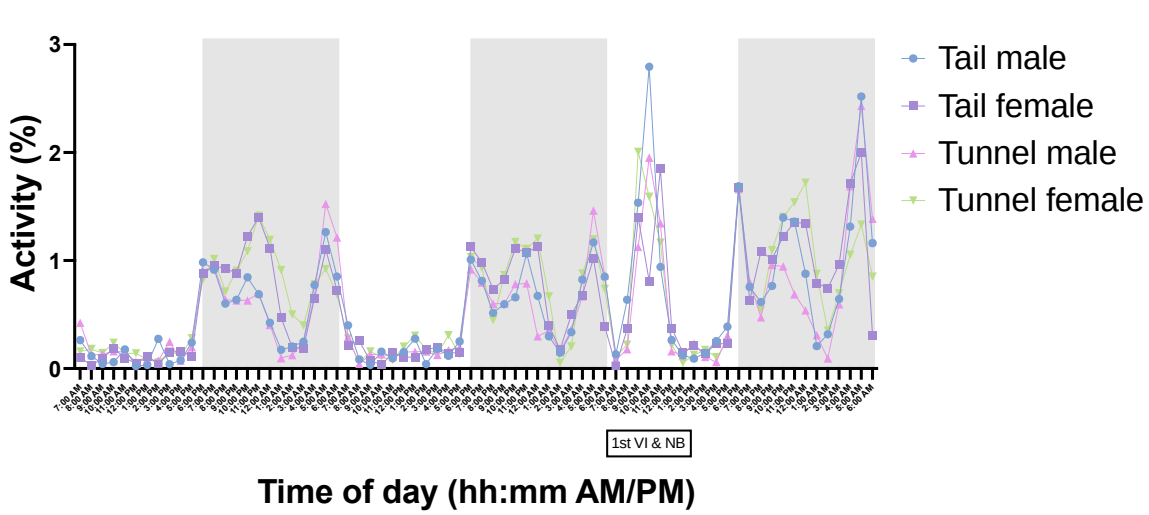
Figure 12 Cage activity on days 9-12 (A), 12-15 (B), 15-18 (C), 18-21 (D) and 21-24 (E). Grey areas are dark periods (6 PM to 6 AM). Results displayed as mean values. Figure continued on next page.



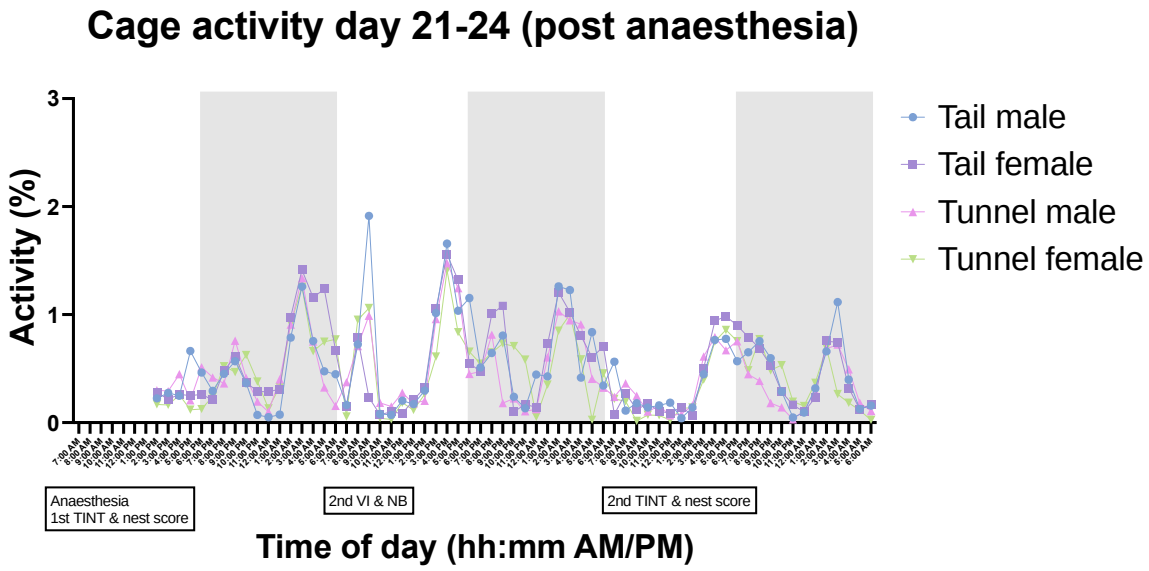
C


Cage activity day 15-18
Activity (%)
Tail male
Tail female
Tunnel male
Tunnel female
CC & 5th handling
6th handling
Time of day (hh:mm AM/PM)

D


Cage activity day 18-21
Activity (%)
Tail male
Tail female
Tunnel male
Tunnel female
1st VI & NB
Time of day (hh:mm AM/PM)

E


Cage activity day 21-24 (post anaesthesia)
Activity (%)
Tail male
Tail female
Tunnel male
Tunnel female
Anaesthesia
1st TINT & nest score
2nd VI & NB
2nd TINT & nest score
Time of day (hh:mm AM/PM)

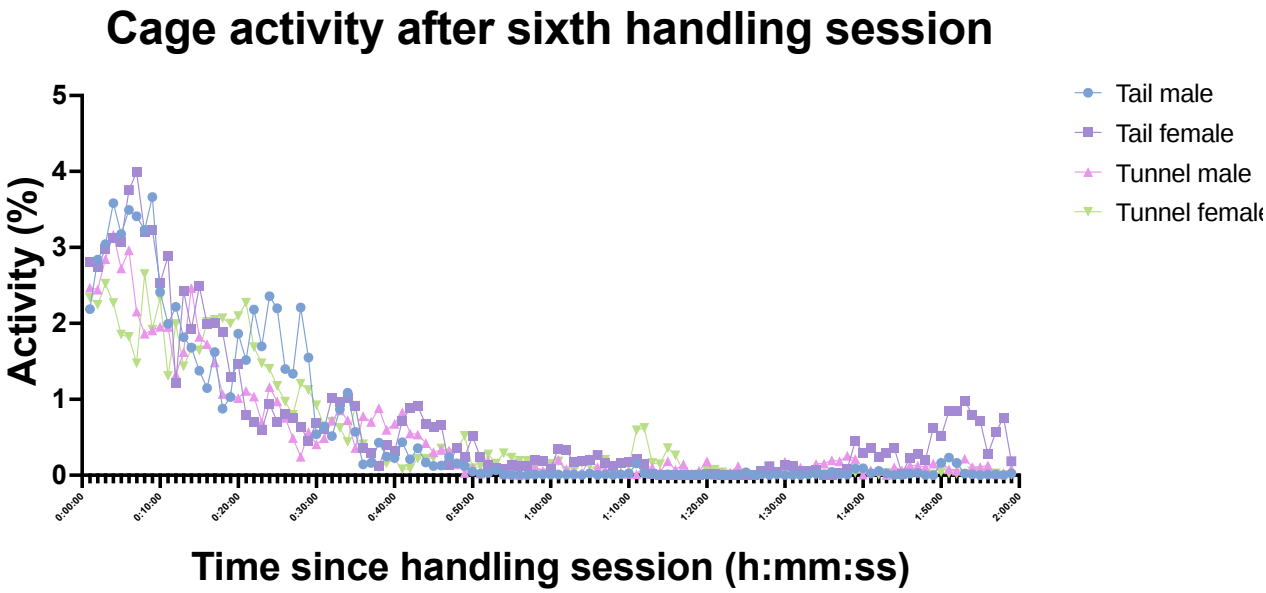


Figure 13 Cage activity following the sixth handling session on study day 17. Results displayed as mean values.