Refinement is but a Click Away: Exploring Clicker Training as a Stress-Reduction Method for Mice Undergoing Minimal-Restraint Intraperitoneal Injections

Veterinary Medicine Utrecht University
Department Population Health Sciences; Division: Animals in Science and Society (DWM)

Author: L.M.L. Derksen (6128041)
Tutor: Dr. E.M.A. Langen
Deadline: 09-02-2024

Table of contents

Abstract

This study investigated the effectiveness of clicker training as a refinement method to reduce stress in mice undergoing minimal-restraint intraperitoneal injections. This minimal-restraint injection method required only for their rear end to be lifted by the base of the tail. 10 mice were trained to stand still during the injection process, without actually undergoing injections. Following training, intraperitoneal injections were administered, and the duration mice stood still during injection and the total injection duration were measured. Post-injection, a reward acceptance test and a voluntary approach test were conducted to assess the durations of contact seeking behaviour towards the researcher and risk assessment behaviours. Results were compared with those of two control groups: the exposed control group (n=10), familiarised with the reward, researcher, and environment but not the injection handling method, and the unexposed control group (n=9). Statistical analysis revealed no significant differences between treatment groups for standing still during injection, the total injection duration, contact seeking or risk assessment behaviours. These findings failed to provide conclusive evidence of the efficacy of clicker training regarding stress reduction. However, exposed control mice showed a notable trend of increased touching behaviour towards the researcher, which was categorised as a contact seeking behaviour. This suggests that exposure alone has the potential to decrease aversion towards the researcher. Despite study limitations, including a small sample size, the fact that unexposed control mice were not entirely naive, and the mice's advanced age, this study serves as a crucial starting point, demonstrating its potential contribution to the ongoing endeavour to enhance the welfare of research animals.

Introduction

Mice's ability to thrive in limited spaces, their short gestation periods and their dietary adaptability are just a few of the reasons why they make ideal models for a wide range of experiments. Naturally, they have become extensively employed across various scientific disciplines in order to expand our scientific knowledge (Bryda, 2013; Phifer-Rixey & Nachman, 2015). However, the use of mice is not without consequence regarding their welfare. In the current study, welfare is defined as the state in which an individual can actively adapt to its living conditions, thereby attaining a state perceived as positive by the individual. (Arndt *et al.,* 2022; Faculteit Diergeneeskunde, 2024; Ohl & Hellebrekers, 2009). However, mice are often subjected to situations in research settings to which they cannot actively adapt. For instance, mice are frequently forced into novel surroundings, causing them to experience anxiety attributed to their inherent neophobia (Misslin & Cigrang, 1986). Their neophobia also prompts them to exhibit avoidance reactions when introduced to unknown food sources (Griebel *et al*., 1993; Kronenberger & Médioni, 1985). Although familiarisation with the unknown food sources may occur, allowing them to overcome neophobia, it's important to note that stress-induced anorexia can persist due to stress unrelated to neophobia (Yamada *et al*., 2020). Despite the possibility of habituation to experimental settings, mice possess an innate fear of humans. Consequently, routine procedures such as handling and sampling can be stressful due to their invasive nature and mice's instinctual apprehension towards human interaction. Invasive procedures like intraperitoneal (IP), intramuscular (IM) and subcutaneous (SC) injections induce an elevation in heart rate in mice. Heart rate has been demonstrated to correspond with plasma corticosterone (pCORT) values following various restraint methods, with pCORT being a confirmed parameter for assessing acute stress (Korte, 2001). Therefore, the elevated heart rate in mice undergoing injections indicates an acute stress response to the injection procedure (Meijer *et al*., 2006).

Although stress is a crucial adaptive response that enhances animals' ability to manage aversive stimuli more effectively and to restore homeostasis (Johnson *et al*., 1992), chronic exposure to stress can lead to persistent behavioural and physiological changes in mice, including altered basal hormone levels, modifications in gene expressions, and increased manifestations of anxiety-related behaviours (Sterlemann *et al*., 2008). These changes could cause the mice's adaptive capacity to be surpassed, thereby compromising their welfare (Ohl & Hellebrekers, 2009). Furthermore, these changes could also potentially introduce confounding variability into the data obtained from animal studies (Balcombe *et al*., 2004; Gouveia *et al*., 2017). Failing to reduce stress in these animals can therefore raise scientific concerns, but also ethical ones. Many regulatory bodies mandate researchers to adhere to the principles of the 3Rs (Replacement, Reduction, and Refinement, as outlined by Russell & Burch (1992)) in designing and conducting animal experiments (Directive 2010/63/EU; Wet op de Dierproeven, 2023).

A method that has shown promise in reducing stress in animals and providing cognitive enrichment, thereby refining animal studies, is the use of training (Coleman *et al.,* 2008; Daugette *et al*., 2012; Leidinger *et al*., 2017; Swan *et al*., 2023). One such example is clicker training. Primarily applied in other species, clicker training has demonstrated stress-reducing effects, as observed in shelter cats (Verdino, 2021), in chickens (Mählis *et al.,* 2023), and in pigs (Jønholt et al., 2021). The advantage of clicker training over traditional training methods is that it enables precise timing of reinforcement. The click sound serves as an instantaneous marker, allowing animals to better understand and associate specific behaviours with rewards. In contrast, traditional training methods may be prone to delayed rewards or marking incorrect behaviours, possibly affecting animals' ability to learn new tasks (Browne, 2015). Therefore, clicker training allows for more clarity and precision, enhancing the effectiveness of training sessions.

Previous studies have explored the application of clicker training in mice, demonstrating its successful implementation (Dickmann *et al*., 2022; Leidinger *et al*., 2017). Using positive reinforcement, clicker training could help mice to establish a positive association with researchers. This, in turn, may have the potential to also reduce stress in mice during handling and other routine procedures (Leidinger *et al*., 2017). Van Eldik (2021) conducted a study where laboratory mice were clicker trained to undergo a subcutaneous (SC) injection using minimal physical restraint. The study also evaluated the impact of clicker training on mouse behaviour, particularly stress-related behaviours and contact seeking behaviour towards the researcher. These findings suggested that trained mice exhibited less stress-related behaviour and displayed more contact seeking behaviour compared to untrained, unhabituated mice, yet only non-significant trends for effects were found, and further research is needed to establish statistical significance and validate these preliminary observations. This may support the hypothesis that clicker training can minimise stress in laboratory mice, thereby improving their overall well-being. Furthermore, its alignment with the principles of refinement in laboratory animal research contributes to the goals of the 3Rs framework (Russell & Burch, 1992).

The application of clicker training in mice for intraperitoneal (IP) injections has not yet been explored. The primary objective of this study is therefore to investigate the potential of clicker training in reducing stress in mice undergoing IP injections. The emphasis lies in the development of a refined, practical and effective training protocol that holds promise for future applications in research studies. To do this, the study will build upon and modify the protocol developed by Van Eldik (2021). As Van Eldik (2021) trained mice for subcutaneous injections, the primary modification pertains to the injection method. The conventional technique for IP injection includes scruffing the mouse and pressing its tail to the palm of the hand. Subsequently, the mouse is positioned in dorsal recumbency with a slight downward tilt of the head (Miner *et al*., 1969). In order to refine this method, this study opts for a minimal-restraint injection method. Baek *et al* (2015) compared stress-related hormone concentrations (ACTH and corticosterone) in mice undergoing the conventional IP injection method with those in mice subjected to a method termed the 'novel technique'. This novel technique involved lifting the mouse's rear end by the base of the tail and by one hind leg. The mouse is not held in dorsal recumbency, but is allowed to remain upright and to grasp a wire rack with its front paws. The novel technique resulted in significantly lower stress-hormone concentrations compared to the conventional method.

Notably, there was no significant difference in hormone concentrations between mice undergoing the novel technique and control mice, which did not receive any stressors. These findings supported the study's expectations that the selected injection method would effectively minimise stress in the mice, validating it as a promising refinement technique. Drawing inspiration from this innovative approach, the objective of this study is to enhance and streamline the method by minimising restraint to the greatest extent. Consequently, the mice's rear ends will solely be lifted by the base of their tails, eliminating the practice of holding one of their hind legs. Additionally, to afford the mice greater freedom to interact with the researcher, injections will be administered on a training pad rather than on a wire rack.

To evaluate the effect of clicker training, mice will be assigned to three treatment groups. The training group will undergo clicker training to receive an IP injection with minimal restraint. The exposed control group will be familiarised with the researcher, the reward, and the research environment. However, they will not be familiar with the employed handling method for injection and will lack exposure to the sensation of the needle against their abdomen, as opposed to the trained mice. The unexposed control group will not be familiar to any of the aforementioned factors. The purpose of the two control groups is to explore whether exposure alone might also have a positive impact on mouse behaviour. After the training period, an IP injection will be given to all mice and several behaviours will be observed.

As the mice in the training group will undergo training to remain still during injection, the study will measure the duration each mouse spends standing still during injection. Given that an IP injection induces an acute stress response in mice (Meijer *et al.,* 2006), it is anticipated that this response will manifest as excessive movement during the injection, constituting a flight reaction (Eilam, 2005). In the context of this study, excessive movement is defined as any movement during injection that deviated from the the description of standing still (which will be described further in the article). Henceforth, such movement will be denoted as 'struggling', and the study will measure the duration each mouse spends struggling during injection, along with the total duration of the injection. Excessive movement can complicate the process of safely administering the injection, leading to multiple injection attempts and an extended total duration. However, an alternative reaction that mice might exhibit in response to the injection is freezing (Campos *et al.,* 2013; Walker *et al.,* 2003). In such instances, it may become easier to administer the injection, as these mice will not move.

Following the injection, the mice will be presented with a food reward, and a voluntary approach test will be conducted to observe their behaviour post-injection. Recognising once more the mice's neophobia, initial apprehension toward the reward and researcher is natural (Kronenberger & Médioni, 1985; Misslin & Cigrang, 1986). Continued reluctance to accept the reward may be due to stress-induced anorexia (Yamada *et al*., 2020). Therefore, it is crucial to observe and score the mice's interaction with the reward, and with the researcher they have been trained by or exposed to. Behaviours directed at the reward and the researcher will be categorised as contact seeking behaviours, specifically: accepting and eating the reward offered by the researcher, sniffing, touching, and nibbling the researcher's hand, as well as sitting on the researcher's hand. Sitting in close proximity of the researcher's hand without direct contact will also be considered contact seeking behaviour. Freezing and flinching will also be scored, both behaviours well-known to be related to fear (Campos *et al.,* 2013; Walker *et al.,* 2003). Grooming is a behaviour that can be challenging to interpret. While it may be linked to stress, serving as a displacement behaviour, it can also be routine maintenance behaviour (Mu *et al.,* 2020; Song *et al*., 2016). Stress-related

grooming may be distinguished from maintenance behaviour by grooming duration, and by changes in grooming patterns (Fernández-Teruel & Estanislau, 2016; Kalueff *et al.,* 2015).
In less aversive situations, grooming duration tends to be low, but increases as situations become more aversive. However, there appears to be a peak grooming duration at a certain level of aversiveness. When the aversiveness of the situation surpasses that level, the grooming duration decreases again. (Fernández-Teruel & Estanislau, 2016). Determining the exact level of aversiveness of the procedures in the current study and whether it surpasses the peak is difficult, making it hard to formulate expectations for this behaviour. Nonetheless, it will be scored to observe possible differences among the treatment groups.
Lastly, when confronted with a potentially dangerous situation, mice employ various behaviours as a means of risk assessment. These behaviours encompass performing stretch attends, rearing and peering over the edge of surfaces to look at the floor (Cole & Rodgers, 1994; Rodgers & Dalvi, 1997). While typically categorised as exploratory behaviours, they also serve as indicators of anxiety in mice, as evidenced by a study administering anxiolytic drugs. Mice subjected to these drugs exhibit a reduction in these risk assessment behaviours (Cole & Rodgers, 1994). Hence, stretch attends, rearing and looking over the edge of the injection table will be scored. Stretched walking, an extension of the stretch attend, will also be scored.

Trained mice are least likely to experience a stress response during injection, or react avoidant toward the researcher and the reward after injection. Therefore, is it expected that they will display shorter total injection durations, will stand still longer, and struggle less compared to mice from both control groups. Furthermore, it is also expected that they will exhibit more contact seeking behaviours, fewer fear-related behaviours, and fewer risk assessment behaviours compared to both control groups. Conversely, mice in the exposed control group are more likely to experience a stress response during injection than trained mice. While they have been familiarised, the injection procedure might startle them. This would lead them to stand still for shorter durations, struggle more, and have longer total injection durations compared to trained mice. It is also expected that they will demonstrate fewer contact seeking behaviours, more fear-related behaviours and more risk assessment behaviours than trained mice.
Mice in the unexposed control group are predisposed to heightened anxiety towards the unfamiliar research environment, the researcher and the reward (Misslin & Cigrang, 1986; Kronenberger & Médioni, 1985). The expectation is therefore that they will struggle the most, stand still the least, and have the longest total injection durations among all three treatment groups. However, in case of a freezing respons to the injection, it is expected that unexposed mice will be the most fearful and will therefore exhibit more freezing. This would lead to less struggling and shorter total injection durations, even though the mice are still undergoing stress. Unexposed control mice are also expected to exhibit fewer contact seeking behaviours, more fear-related behaviours and more risk assessment behaviours than mice from the other treatment groups.

Materials And Methods

Animals and housing

A total of 29 adult female mice were used in this study, comprising 5 C3H (C3H/HeOuJ) and 24 Balb/c (Balb/cAnNCrl). All were born on 24-08-2022. One of these mice had been designated as a sentinel prior to the study, and blood was collected twice during this study to assess the overall health of the mice as a group. The mice were distributed across six homecages, each containing four or five mice. The groups in which the mice were housed were established prior to this study and remained unchanged throughout the research period. Given that this is a pilot study designed to explore the potential of clicker training to refine the process of IP injections and reduce stress in mice undergoing the procedure, there was an absence of preceding data to estimate the standard deviation needed for a power analysis. Initially, conducting an a priori analysis based on the data collected in Van Eldik (2021) was considered. However, since that study involved training mice for a different procedure, it was deemed insufficiently comparable to the current study. Therefore, the decision was made to forego the a priori power analysis. Despite this, it was anticipated that the sample size of 29 mice would likely be insufficient, considering the sample sizes used in multiple studies in which mice were successfully trained for other procedures (Dickmann *et al*., 2022; Swan *et al*., 2023). As a result, the sample size will be divided into batches, with the current study serving as batch one. Subsequent studies will concentrate on training and analysing the remaining batches.

The experiment took place in the Central Laboratory Animal Research Facility (*Gemeenschappelijk Dierenlaboratorium, GDL)* at Utrecht University. The mice have previously been used as teaching animals in animal handling classes for students attending Utrecht University, and will continue to serve this purpose afterward. These classes involved various handling and restraining techniques, as well as SC injections and conventional IP injections. Each mouse has received approximately four to five injections in total prior to the start of this study. The utilisation of these animals for educational purposes and training was approved by the Dutch Central Authority for Scientific Procedures on Animals (CCD) and the Animal Ethics Committee of Utrecht University (licence: AVD10800202216046). Furthermore, the involvement of these animals adheres to Dutch laws (Wet op de Dierproeven) and complies with European regulations (Directive 2010/63/EU). Makrolon type IV-S cages were used (59 x 38 x 20 cm). The cages contained the following enrichment: two large orange tunnels, two cardboard houses, tissue paper and a small transparent tunnel. The latter was fastened to the lid and was used to transport the mice from their homecages to the training room and back (Gouveia & Hurst, 2013). The bedding consisted of wood chips. Figure 1 illustrates the homecage arrangement. The cages were cleaned every first Monday of each month. A 12:12 h light:dark cycle was used (lights on at 7 AM). Room temperature was around 22 degrees Celsius, with a humidity of approximately 65%. Mice had ad libitum access to food and water. The food consisted of pellets (Rat/Mouse maintenance, Ssniff Spezialdiäten GmbH, DE-50494 Soest).

Figure 1: The image on the left illustrates the homecage arrangement, including the enrichment materials. The small transparent tunnel fastened to the lid of the cage can be seen on the image on the right.

Prior to training (familiarisation)

All familiarisation and training sessions took place outside the home room in which the mice were housed, in another area of the research facility known as the 'training room'. The animals were transported between the rooms using a metal cage rack system on wheels. The training room was situated approximately 20 metres from the home room, and was selected due to its frequent use for animal handling classing involving the mice used in this study, meaning the mice were already familiar with the location.

Before starting training, all mice were familiarised with the researcher, and the mice's overall acceptance of the reward was evaluated. A homecage was taken from the rack and placed on a table. The cage was then opened and all cage enrichment was removed, after which the researcher put a gloved hand in the cage, holding an unstandardized amount of crushed Yoghurt Drops (ESVE Knaagdier Drops Yoghurt, ESVE Knaagdieren en Vogels) and flattening their hand on the cage's substrate. The Yoghurt Drops were selected as the reward based on their existing availability at the GDL, and the fact that the mice had been exposed to them before, which proved to be practical and time-efficient. Each cage was allotted five minutes for the mice to approach the hand and consume the reward, while the researcher observed their response to the researcher's presence and to the chosen reward. Although the mice had had some prior exposure to Yoghurt Drops during animal handling classes, their familiarity was not significant enough to automatically rule out a potential neophobic reaction to the reward. When the five minutes had ended, the researcher returned the enrichment items to the cage, closed the cage and put it back in the cage rack system. The same procedure was repeated for all remaining homecages. This process of familiarisation was executed on four separate days, all within a total span of six days. When the majority of the mice accepted the reward and seemed to have been familiarised to the researcher, the researcher moved on to the next step in the process, namely marking the mice. The mice were deemed familiarised when they ceased to exhibit the following behaviours: freezing, avoiding the researcher's hand, or defensively burying the researcher's hand (Walker *et al*., 2003; Bourin *et al*., 2007; De Boer & Koolhaas, 2003). The determination of whether the majority was achieved was made based on visual estimation.

In order to control for any potential order effects, the order in which cages were habituated was randomised for each day using Excel's RANDBETWEEN() function.

Marking and dividing the mice

The study comprised 29 mice, which were distributed among six different homecages. Specifically, five of these cages housed five mice each, and one cage contained four mice. To distinguish and identify each individual mouse, a permanent marker was used to apply a varying number of stripes on their tails. The number of stripes ranged from one to five, depending on the number of mice within their respective cages. To pick up the mice, a tunnel (which was part of the enrichment in the homecage) was used, as this has been found to cause less anxiety compared to tail handling (Gouveia & Hurst, 2013).

After marking, the mice were randomly divided into three groups: the training group (n=10), the exposed control group (n=10) and the unexposed control group (n=9). All randomization was carried out using Excel's RANDBETWEEN() function.

Training and exposure

On training and exposure days, all 29 mice were transported to a 'waiting room', adjacent to the training room. The door to this room could be closed and was selected to minimise the likelihood of the mice hearing the clicker being used in the training room, while they were waiting to be trained or exposed. The sequence of each cage and mouse for each training and exposure day was randomised using Excel's RANDBETWEEN() function, ensuring a different order each time. This controlled randomisation process aimed to provide every cage with the opportunity to be first as well as last in the sequence, thereby minimising potential order effects. Furthermore, measures were taken to prevent a pattern where mice belonging to a specific treatment group were consistently selected as the first or last to be removed from each cage. Instead, the order of mice from different treatment groups was carefully balanced.
Upon removal from the cage rack system, a cage was positioned on a table within the waiting room. All enrichment items, except for the wood chip bedding, were then removed from the homecage. A mouse belonging to the treatment or exposure group was subsequently taken out of the cage using the tunnel handling method. The enrichment-free cage was properly closed and the mouse was transported to the research area using the tunnel. The door of the waiting room remained closed during each mouse's session. When training and exposure had been completed for all mice within a homecage, the enrichment materials were returned to the homecage. Subsequently, the homecage was placed back into the cage rack system before the researcher proceeded to acquire the next homecage.

Both training and exposure (see below for more details) was done on a training pad (60 x 60 cm) (Absorin Comfort, Medeco, Brandpuntlaan Zuid 14, 2665 NZ Bleiswijk, The Netherlands). On a training and exposure day, the researcher did not change into new gloves or replace the training pad after each mouse. Instead, the same training pad and pair of gloves was used for all mice. Additionally, the same clicker (4011905228600, Trixie Amazon) was employed for all mice, on each training and exposure day. The set up used on training and exposure days is shown in Figure 2.

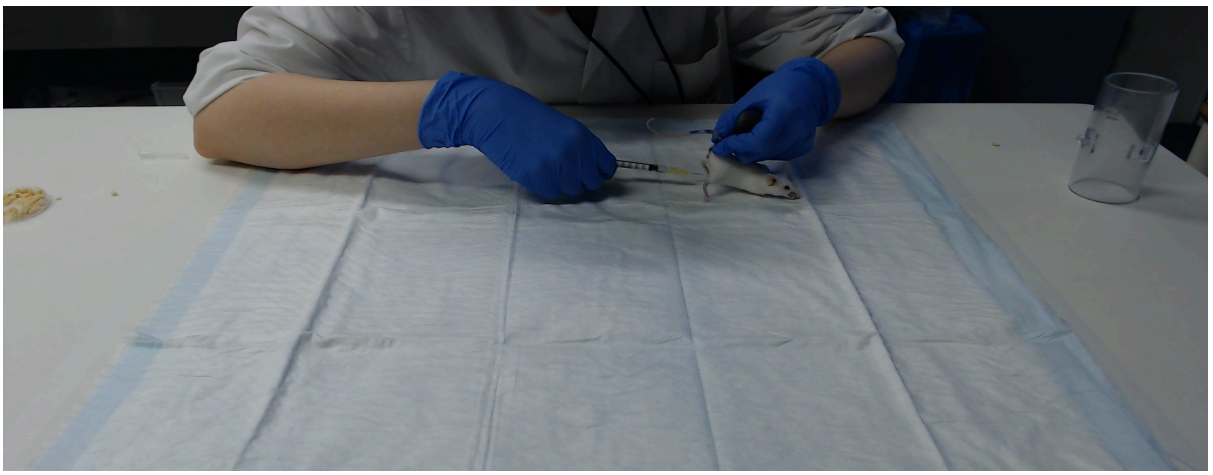

Figure 2: The setup used on training and exposure days, as well as on the injection day. The researcher is holding the clicker in the left hand. The reward is visible in a petri dish on the left side of the image, the tunnel in which the mice are transported is visible on the right side.

Different protocols were followed for the mice in the training group, the exposed control group, and the unexposed control group.

The protocol for mice in the training group was divided into six levels, as shown in Table 1.

Level	**Description**	**Criteria for completion**
Level 0 / Linking	In order to link the clicker with the reward, the clicker was utilised in rapid succession and the reward was presented after each clicker sound.	The first seven sessions were dedicated to establishing an association between the clicker sound and the reward. Subsequent to these sessions, it was anticipated that the linking process would persist as the mouse progressed through the levels.
1	The mouse was transported to the training pad by means of the tunnel. The researcher restrained the mouse by holding the base of the tail. The mouse was then subjected to the clicker sound while on the training pad. This sound was followed by a reward.	The level was considered completed when the mouse accepted the reward on the training pad.
2	The mouse was transported to the training pad by means of the tunnel. The researcher restrained the mouse by holding the base of the tail. The researcher used the clicker when the mouse sat still on the training pad. The mouse was then rewarded with a piece of Yoghurt Drop.	The level was considered completed when the mouse sat still on the training pad and accepted the reward.

3	The mouse was transported to the training pad by means of the tunnel. The researcher restrained the mouse by holding the base of the tail. The mouse's rear end was lifted by its tail. This was the position the mouse would later be held in during IP injection. The researcher used the clicker when the mouse stood still. The mouse was then rewarded with a piece of Yoghurt Drop.	The level was completed when the mouse stood still despite its rear end being lifted and accepted the reward.
4	The mouse was transported to the training pad by means of the tunnel. The researcher restrained the mouse by holding the base of the tail. The mouse's rear end was lifted by its tail. This was the position the mouse would later be held in during IP injection. A capped syringe was placed against the lower abdomen (the IP injection site). The researcher used the clicker when the mouse stood still. The mouse was then rewarded with a piece of Yoghurt Drop.	The level was completed when the mouse stood still despite the feeling of the capped syringe against its abdomen and accepted the reward.
5	The mouse was transported to the training pad by means of the tunnel. The researcher restrained the mouse by holding the base of the tail. The mouse's rear end was lifted by its tail. This was the position the mouse would later be held in during IP injection. An uncapped syringe was placed against the lower abdomen (the IP injection site) without piercing the skin. The researcher used the clicker when the mouse stood still. The mouse was then rewarded with a piece of Yoghurt Drop.	The level was completed when the mouse stood still despite the feeling of the uncapped syringe against its abdomen and accepted the reward.
6	The injection. The mouse was transported to the training pad by means of the tunnel. The researcher restrained the mouse by holding the base of the tail. The mouse's rear end was lifted by its tail. An IP injection was given. Further details about this level will be elaborated on in the paragraph titled "*The injection day"*.	-

Table 1: The training protocol, organised into six distinct levels, featuring detailed descriptions and the criteria the mice needed to meet to successfully progress through each level.

During training or exposure, a scoresheet was used to record details such as the number of completed sessions per mouse, dates, and the respective treatment group for each mouse. Furthermore, the achieved level of each trained mouse was noted, including whether it had completed the level during the training session (see Table A1 of the appendix). Each training session involved briefly revisiting prior levels to warm up the mice. Newly achieved levels were repeated until the criteria for completion were met, after which mice would advance to the next level, independently of the progress of the other mice. This individualised progression allowed mice to advance at their own pace, resulting in varying training levels among mice during a single session. Notably, the highest level a mouse had completed became its official level. Despite any setbacks during subsequent sessions, a mouse's level on the scoresheet did not regress. Even if it failed to meet the criteria of the officially reached level, the mouse retained this level until advancing to a higher one.

The protocol for the exposed control group mice remained the same for all sessions. This protocol was devised in order for these mice to obtain equal amounts of exposure to the reward, the researcher, the tunnel, the training room and pad, and the sound of the clicker as the mice in the training group. The exposed control group mice were transported to the training pad by means of the tunnel, where the researcher restrained them by holding the base of the tail. Unlike the training group, the exposed control group mice were not lifted by the rear end, as this is part of the training procedure. Although the mice were also exposed to the clicker, they did not undergo training with it. The clicker sound was presented at random intervals and was not immediately followed by a reward. This approach aimed to prevent the establishment of a direct association with the clicker. Despite the risk of unintentional training, the decision was made to offer the reward on the training pad instead of in the homecage. This aimed to prevent other mice in the homecage, including the unexposed control group, from accepting the reward. Offering the reward to the exposed control group mice in an entirely different location, such as on the lid of the home cage, would have differed too much from the treatment of the trained mice, complicating later (statistical) comparison.

The unexposed control group mice did not undergo any training, were not subjected to exposure involving the training pad or the clicker, and remained in the waiting room. Having been employed in university classes and included during the researcher's familiarisation with the mice, they were previously accustomed to being handled. While they were also familiar with receiving conventional IP injections, the restraining method implemented during this study was unknown to them. The only further interaction the mice had with the researcher occurred when renewing the tail markings. These markings faded quickly and required renewal every training and exposure day. This was done by placing the unexposed control group mice on the lid of the homecage using the tunnel. They were restrained by the base of the tail while the markings were renewed, then returned to their cage using the tunnel.

Another study aimed at training mice for SC injections ran in parallel to the present study, and was conducted with a sample size of 24 mice. Consequently, coordination was necessary for the shared use of the training room, as both studies planned their training and exposure days on the same day. All 53 mice were collectively transported to and from the waiting room, requiring them to wait until both studies completed their respective training and exposure days. The training and exposure days commenced at either ten AM or two PM, alternating with the parallel study. Each session, irrespective of the mice being in the treatment or exposed control group, had a standardised duration of three minutes. Following their session, the mice remained on the training pad slightly longer to allow the researcher to renew the fading tail markings. In total, each training and exposure day for the current study lasted approximately 2.5 to 3 hours, while the parallel study's day lasted approximately 2 to 2.5 hours. This meant that mice were outside the home room for 4.5 to 5.5 hours. Typically, three days per week were reserved for training and exposure. These were not scheduled consecutively to allow for one or two resting days in between.

At the start of the study, the number of sessions required to train the mice was unknown, as there was no prior experience with training mice for IP injections using the current study's protocols. While the researcher did anticipate the need for several linking sessions, an exact prediction was unavailable. The expectation was that, based on the mice's behaviour during familiarisation with the researcher, a consistent acceptance of the reward would be achieved after three or four linking sessions. However, this proved insufficient, with only three mice accepting the reward by session 4. Consequently, the decision was made to extend the linking phase, ultimately using seven sessions for linking. Due to time constraints, training commenced on session 8, with the expectation the other mice would gradually follow suit. Despite some mice consistently accepting the reward during the linking sessions, they did not progress through the levels. This was not due to incapability, but because training to sit still (level 2) did not start until session 8.

An additional decision arose concerning the total number of sessions required before concluding training and moving onto the injection. As some mice progressed slowly through the levels, or not at all, and with the increasing number of sessions, it became apparent that waiting for all mice to reach level 5 was not feasible. This was due to time constraints. The researcher determined that a minimum of 50% of the mice needed to reach and experience (but not necessarily completed) level 4. Level 4 was selected because the mice would have been trained to remain still while their rear end was lifted by the base of the tail, and would have at least experienced the sensation of the uncapped syringe against their abdomen. Session 18 marked the first session where a minimum of 50% of the mice had reached (but not necessarily completed) at least level 4. However, the supervisor was only available to oversee the injection one week later, allowing for five additional sessions for training. Eventually, two more mice reached level 4 or higher, raising the percentage to 70%.

Ultimately, each training and exposed control mouse underwent a total of 23 training or 23 exposure sessions, respectively. The progression through levels for each training group mouse is detailed in Table A2 of the appendix, including their acceptance of the reward. Table A3 of the appendix outlines whether the exposed control group mice accepted the reward. A timeline of the study is illustrated in Figure 3.

By the 14th session, it became evident that three mice in the training group consistently declined to accept the reward (X27Y1, X20Y4 and X12Y5). To investigate the possibility of an aversion to Yoghurt Drops, these mice were presented with the reward in their respective homecages, where they did accept it. This suggests that the rejection of the reward was likely due to apprehension about accepting it on the training pad. In case the Yoghurt Drops were not a sufficient incentive for the mice to overcome their apprehension, peanut butter was also offered to assess their response, but they rejected this as well. Attempts to train them were continued with the Yoghurt Drops, as this was more practical. However, these three mice failed to respond to the training protocol due to their inconsistent acceptance of the reward. They remained at level 0 or 1 throughout all 23 sessions and were consequently deemed untrained. As they were not considered representative for the mice in the training group, they were later excluded from analysis.

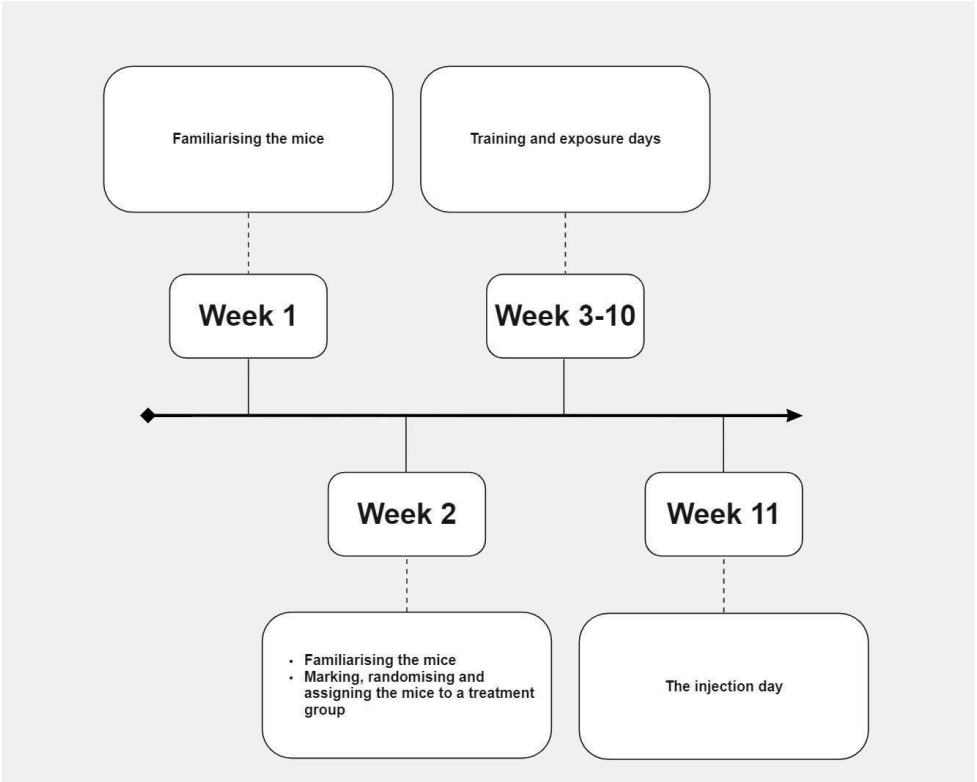

Figure 3: Timeline of the study.

The injection day

In order to evaluate the effect of training, exposure, and non-exposure on mouse behaviour surrounding an IP injection using minimal restraint, different tests were performed. This entailed administering an IP injection to all 29 mice, using the same method the mice in the training group were trained to undergo. The injection was recorded so that assessment of each mouse's behaviours during and after the injection could take place at a later moment.

Prior to the injection day, the research area was set up. A webcam was positioned to record each mouse's behaviour on the training pad. Yoghurt Drops were crushed and were once again used as the reward. Standardisation of the reward quantity was not implemented, given that the previous training and exposure sessions had revealed variability in the amount each mouse accepted. This rendered standardisation impractical. The set up is depicted in Figure 4.

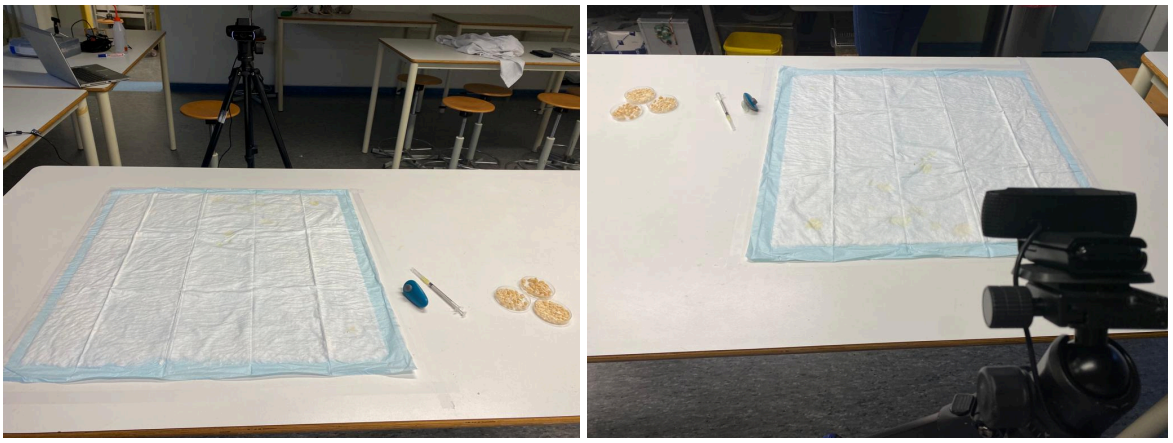

Figure 4: Set up of the research area on the injection day.

In the waiting room, an additional webcam was installed to be able to capture the mice's behaviour within the homecage both before and after injection. The set up is depicted in Figure 5.

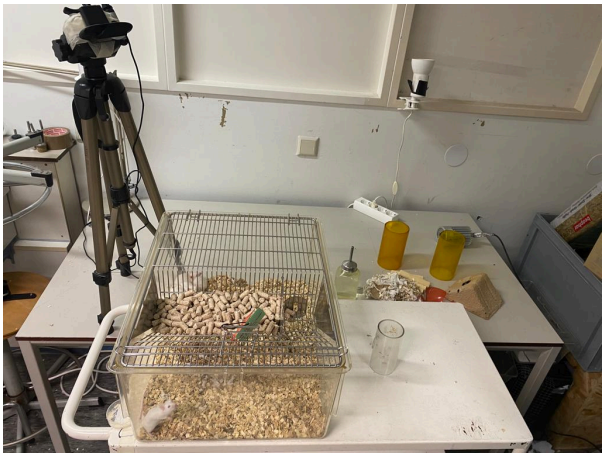

Figure 5: Set up of the waiting room on the injection day.

To obscure the treatment group from the researcher, the homecage numbers were altered, and all mice were randomly assigned new tail markings using a permanent marker. This was done by an independent blinding consultant. It is important to note that while the blinding process aimed to maintain objectivity and prevent bias, complete blinding was not achievable. Some mice had very notable characteristics, such as a high Body Condition Score, and remained identifiable to the researcher.

On the injection day, all 29 mice were once again transported to the waiting room. The sequence in which each homecage and each mouse was used, was randomised by the supervisor of this study using Excel's RAND() function. The manner of randomisation was controlled, akin to the randomisation procedure for training and exposure days.
The first homecage was placed upon the table in the waiting room, and after removing the enrichment, the homecage lid was placed beside it. First, a voluntary approach test was conducted within the homecage. This was done by placing a gloved hand flat on the substrate, palm up, at the centre of the cage. The researcher minimised hand movement and did not force interaction with the mice. The duration of this test was five minutes. Due to time constraints, this initial voluntary approach test in the homecage will not be further discussed here.
Subsequently, the researcher changed into fresh gloves. This was done to avoid any variation in glove scent between the first and last mouse of the cage, thereby reducing potential order effects.
The first mouse was then transferred to the training pad in the training room using the tunnel. There, the researcher held the mouse by the base of its tail and lifted its rear end. This was the same position the training group mice had been trained to accept. An IP injection was administered in the lower left abdominal cavity, positioned cranially to the second-to-last nipple. The researcher used the clicker at the moment the needle penetrated the skin. The researcher aspirated first to ensure the bladder, bowels or blood vessels had not been punctured. If this was not the case, 0.1 ml NaCl was injected using a 30G needle. Following this, the needle was withdrawn and the researcher gently returned the mouse's rear end to the training pad, releasing the tail. The bladder was punctured on a few occasions. In such instances, the researcher refrained from injecting the NaCl, withdrew the needle, and opted not to reinject the mouse. This decision was made considering that the mouse did undergo the handling method used for the IP injection, and experienced the needle piercing the skin. Subsequent follow-ups later on the injection day and the day after indicated that the mice did not appear to be adversely affected by the bladder being punctured.
For the mice of the first homecage, the researcher initially had one minute for injection. If a mouse displayed non-cooperation by moving excessively, the supervisor would intervene. This involved restraining the mouse by a skinfold on its neck or back while the researcher lifted its rear end by the tail to administer the IP injection. However, it became evident that a one-minute cut-off point was too lengthy. Some mice of the first homecage exhibited significant resistance within the initial 30 seconds, making a safe injection impossible without assistance. Recognising this, the researcher was granted 3 attempts to inject the mice of the remaining five homecages. Each attempt involved lifting the mouse's rear end and positioning the needle to its abdomen. If the mouse was not injected by the third attempt, the supervisor would step in and assist with the injection. In the end, the supervisor provided assistance with the injection for a total of seven mice. Among them, five belonged to the exposed control group, one to the unexposed control group, and one to the training group.

After injection, the researcher would present Yoghurt Drop pieces to the mouse as a reward. This reward acceptance test lasted for one minute. The timer started when the first Yoghurt Drop piece was offered to the mouse.
Upon completion of the one-minute rewarding period, a voluntary approach test was conducted on the training pad. The researcher positioned their right hand flat on the training pad, palm up, in the bottom right corner. Mice were free to move around and explore the training pad and the researcher's hand. The researcher minimised hand movement and did not force interaction with the mice. The voluntary approach test on the training pad lasted two minutes, with the timer starting the moment the researcher's hand made contact with the training pad. At the conclusion of the two minutes, the mouse was returned to the homecage. In case a mouse went out of frame during the test, the researcher would manually return it to the centre of the training pad. The timer would continue during this action. The criteria for defining when a mouse was considered to be out of frame are specified in the ethogram (Table 3, 'out of frame').
The process on the training pad was repeated for each mouse within a homecage. To minimise order effects, fresh gloves and a new training pad were introduced after each mouse. Once the last mouse completed the voluntary approach test on the training pad, it was returned to the homecage. A 10-minute waiting period was implemented before repeating the voluntary approach test in the homecage in the same manner as previously described, once again lasting for five minutes. Again, due to time constraints, the results of this voluntary approach test within the homecage is to be published elsewhere.
Subsequently, the enrichment was placed back into the homecage, and the homecage was returned to the cage rack system.
All procedures were then repeated for each subsequent homecage. Table 2 gives an overview of the different tests performed, including their order and duration.

Test	Duration
Voluntary approach test within the homecage (all the mice within the homecage)	5 minutes
Intraperitoneal injection on the training pad (one mouse at a time)	3 attempts*
Testing reward acceptance (one mouse at a time)	1 minute
Voluntary approach test on the training pad (one mouse at a time)	2 minutes
Voluntary approach test within the homecage (all mice within the homecage)	5 minutes

Table 2: The stages the injection day consisted of, in the correct order, and their duration.
**Except for the first homecage, where 1 minute was taken as the cut-off point, after which the supervisor would assist during injection.*

BORIS, the ethogram and the final dataset

To assess the mice's behaviour recorded during injection, the reward acceptance test and the voluntary approach test on the training pad, an ethogram was created (Table 3). The foundation of this ethogram was established before the injection day but was later expanded upon using insights gathered from the videos. The behaviours were categorised into distinct groups, namely the behaviour during injection, contact seeking behaviour towards the researcher (including accepting the reward), risk assessment behaviour, fear- and/or stress-related behaviour, and 'other behaviours'. Behaviours during injection comprised 'standing still' and 'struggling'. Additionally, the ethogram included the total duration of injection. Contact seeking behaviours encompassed 'accepting the reward/eating', 'sniffing the researcher', 'touching the researcher', 'nibbling the researcher', 'sitting on the researcher', and 'sitting next to the researcher'. Risk assessment behaviour included 'stretch attend', 'stretched walk', 'rear', and 'looking over the edge of the table'. Fear- and/or stress-related behaviour included 'freezing', 'flinching', and 'grooming'. Other behaviours encompassed 'sitting elsewhere on the training pad' and 'out of frame'. All behaviours were scored during specific stages of the injection day, and detailed descriptions of each behaviour and the respective stages can be found in the ethogram (Table 3).

This ethogram was subsequently incorporated into BORIS (Behavioral Observation Research Interactive Software), the software employed by the researcher for scoring the observed behaviours (Friard & Gamba, 2016). Each behaviour was categorised as either a state event or a point event. A state event indicates a behaviour with a duration (in seconds), whereas a point event lacks a clear duration. It is therefore easier to count a point event behaviour's frequency of occurence. Flinching was classified as a point event, while the other behaviours were considered state events. To score the duration of the state event behaviours, specific start and stop moments were chosen for each behaviour to ensure standardisation across all mice. These start and stop moments are detailed in the expanded version of the ethogram in Table A4 of the appendix. Particular emphasis was placed on scoring the behaviour during the voluntary approach test on the training pad for a precise duration of two minutes.

Upon the initial scoring of each video, four videos were randomly selected to undergo a secondary scoring to assess the intra-observer reliability, employing Cohen's Kappa. The average reliability yielded a Cohen's Kappa value of 0.84 (84%).

In addition to the scored duration of each behaviour, documentation included whether the supervisor provided assistance during injection. Once each mouse had been scored and the researcher was informed of the treatment assignments, the treatment group and the achieved level for each mouse in the training group was also noted. Furthermore, the order in which the mice were brought to the training pad on the injection day was noted to analyse possible order effects. The dataset containing all this information can be found in appendix X.

Upon completion of scoring, it became evident that certain behaviours were observed in fewer than 50% of all mice. ‘Freezing’ and ‘flinching’ were not exhibited by any of the mice. Additionally, ‘nibbling the researcher’ was observed in only one mouse, ‘sitting on the researcher’ and ‘looking over the edge of the table’ were observed in eight mice, and ‘rear’ in nine mice. ‘Grooming' was observed in 13 mice, constituting 44.8% of all mice. This resulted in insufficient data for these behaviours, leading to their exclusion from analysis. During the injection, all behaviours that deviated from the description of ‘standing still’ were scored as ‘struggling’. Given that mice were trained to stand still during injection, the decision was made to analyse only ‘standing still’ and the total injection duration, considering them sufficiently representative of the training’s impact on mouse behaviour during injection. Consequently, ‘struggling' was excluded. While ‘stretched walk’ was exhibited by nearly all mice, it proved challenging to differentiate from regular walking during scoring, resulting in its exclusion. In contrast, ‘stretch attend’, was displayed by all mice and easily recognisable. ‘Sitting elsewhere on the training pad’ and ‘out of frame’ were not indicative of contact seeking, fear- and/or stress-related, or risk assessment behaviour and were only added to the ethogram to ensure comprehensive scoring without subsequent analysis. Ultimately, eight out of the 20 behaviours outlined in the ethogram were included in analysis, marked in blue in Table 3. The behaviours include ‘standing still’, 'total duration of injection’, ‘accepting the reward/eating’, ‘sniffing the researcher’, ‘touching the researcher’, ‘sitting next to the researcher’, and ‘stretch attend’.

The total duration of injection was measured in seconds. Due to variations in this duration, ‘standing still’ was converted into a percentage of the total duration of injection. Additionally, as some mice went out of frame during the voluntary approach test (ranging from 0 to 22.86 seconds), the researcher was unable to score their behaviour during this period. Each behaviour observed during the voluntary approach test was therefore converted into a percentage of the total duration the mice were observable. The durations of ‘accepting the reward/eating’ and ‘total duration of injection’ were maintained in seconds for analysis. Despite the reward being offered for only one minute, certain mice continued eating beyond this duration. Consequently, they were engaged in eating during the two-minutes interval designated for the voluntary approach test on the training pad, rendering the calculation of a percentage impractical. The behaviours associated with the voluntary approach test were simultaneously scored to ensure an accurate representation of their duration and prevent any misrepresentation.

Category of behaviour	Behaviour	Description of the behaviour
During the injection		
Behaviour during injection	Standing still (state event)	The mouse stands still during injection: it does not move its hind paws, or turns its head/bends its body at a 90-degree angle. It is permitted to move its front paws within this restricted range and to observe its surroundings.
	Struggling (state event)	Any movement that deviates from the description of standing still.
	Total injection duration (state event)	The total duration of injection.
Testing reward acceptance		
Contact seeking behaviour	Accepting the reward/eating (state event)	The mouse accepts the reward offered by the researcher and eats it.
The voluntary approach test		
Contact seeking behaviour	Sniffing the researcher (state event)	The mouse sniffs the researcher's hand or arm.
	Touching the researcher (state event)	The mouse touches the researcher's hand or arm with one to three paws, the top of its head or its body. The tail is excluded from this.
	Nibbling the researcher (state event)	The mouse nibbles at the researcher's hand without there being a reward present.
	Sitting on the researcher (state event)	The mouse sits on the researcher's hand or arm with all four paws.
	Sitting next to the researcher (state event)	The mouse sits next to the researcher's hand within a distance of half a body length. The mouse does not touch the researcher.
Risk assessment behaviour	Stretch attend (state event)	The mouse elongates its body, potentially reaching forward with its forepaws. It does not move its hind paws.
	Stretched walk (state event)	The mouse walks forward with its body elongated, lifting its hind paws in an exaggerated fashion.
	Rear (state event)	The mouse sits back on its hind paws, lifting both forepaws off the training pad.
	Looking over the edge of the table (state event)	The mouse looks over the edge of the table, usually while performing a stretch attend.
Fear- and/or stress- related behaviour	Freezing (state event)	The mouse exhibits no movement apart from respiration.
	Flinching (point event)	The mouse makes a sudden, jerky movement.
	Grooming (state event)	The mouse sits back on its hind paws and cleans its snout/head with its front paws.

Other behaviours	Sitting elsewhere on the training pad (state event)	The mouse sits somewhere on the training pad, at least half a body length away from the researcher.
	Out of frame (state event)	Out of frame encompasses multiple situations in which mouse behaviour was rendered unobservable due to lack of visibility: - The mouse leaves the training pad with a distance exceeding one body length. - The mouse remains on the training pad, but is in the webcam's blind spot. - The mouse ascends the researcher's arm and exits the frame entirely, including its tail. - The mouse burrows beneath the training pad.

Table 3: The ethogram containing the stages of the injection day, the various categories of behaviours, and the behaviours that belong to them. The ethogram served as a reference for scoring the mice's behaviours.

Statistics

Statistical analyses were performed using R 4.3.2. (R Core Team 2023).
General linear mixed models were fitted for 'percentage standing still', 'total duration of injection', 'accepting the reward/eating', 'percentage sniffing the researcher', 'percentage touching the researcher', 'percentage sitting next to the researcher', and 'percentage stretch attend'. This was calculated using the lmer function from the lme4 package in R, with the lmertest package utilised to extract F values and p values (Bates *et al*., 2015; Kuznetsova *et al.,* 2017). The residuals of the model were visually evaluated through histograms and Q-Q plots. Additionally, the Shapiro-Wilk test was utilised to confirm the normal distribution of the residuals. To establish a normal distribution of model residuals, the behaviour 'percentage sitting next to the researcher'' underwent a natural log-transformation. No transformation was required for the other behaviours. Initially, models incorporated the treatment group, the order of the mice on the injection day, and their interaction as fixed effects. Employing a stepwise approach, all non-significant fixed effects and interactions were eliminated ($p > 0.05$), with the exception of the most crucial one, which is the treatment group. Additionally, the model incorporated the homecage as a random effect. This model was employed for all behaviours. In case of significant main effects, the emmeans function from the emmeans-package was employed for post-hoc testing (Lenth, 2023). The Tukey method was employed for p-value adjustment.

Results

Standing still (percentage of total duration of injection)

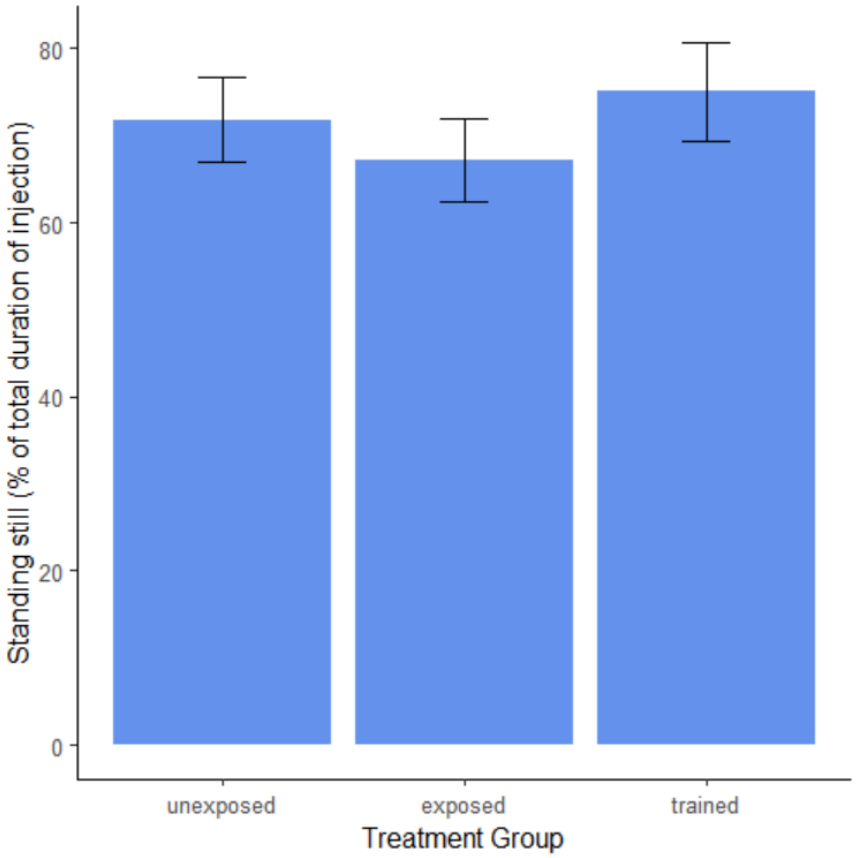

Figure 6: The duration of 'standing still' across all treatment groups, represented as a percentage of the total duration of injection (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).

No significant difference for 'standing still' was found between the training group (M = 75.07, SE = 5.64), the exposed control group (M = 67.15, SE = 4.71), and the unexposed control group (M = 71.75, SE = 4.87) ($F_{(2,19.429)}$ = 0.6089; p = 0.554).

Total duration of injection (seconds)

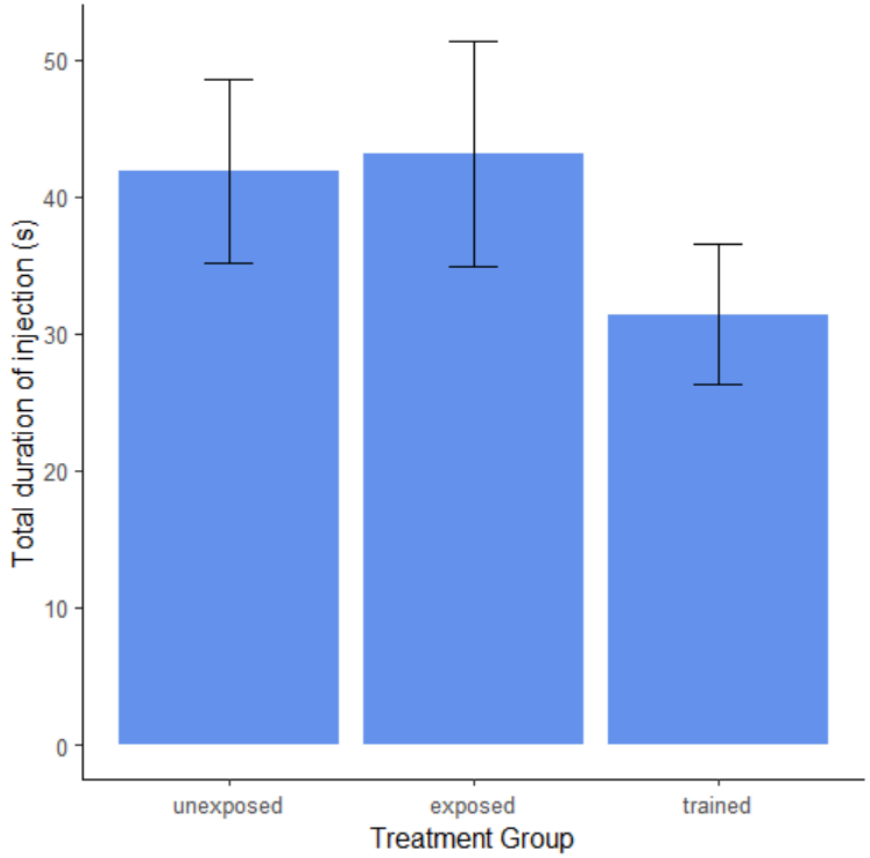

Figure 7: The total duration of injection across all treatment groups, represented in seconds (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).

No significant difference for the total duration of injection was found between the training group (M = 31.42, SE = 5.08), the exposed control group (M = 43.18, SE = 8.20), and the unexposed control group (M = 41.88, SE = 6.75) ($F_{(2,19.367)}$ = 0.8717; p = 0.434).

Accepting the reward/eating (seconds)

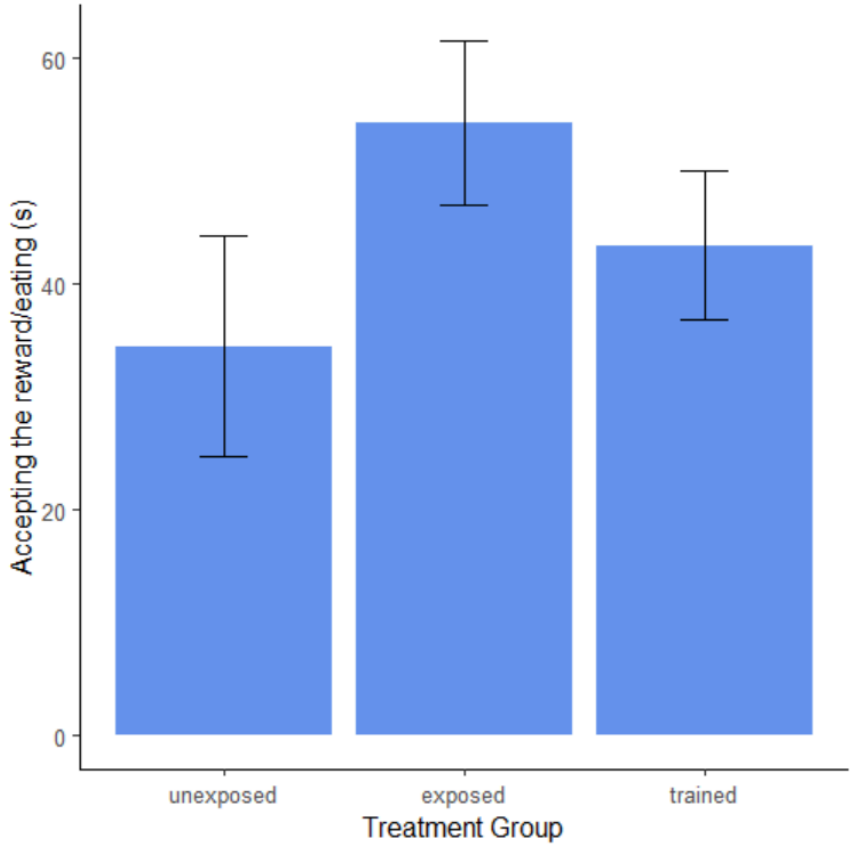

Figure 8: The duration of 'accepting the reward/eating' across all treatment groups, represented in seconds (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).

No significant difference for 'accepting the reward/eating' was found between the training group (M = 43.40, SE = 6.60), the exposed control group (M = 54.27, SE = 7.29), and the unexposed control group (M = 34.45, SE = 9.76) ($F_{(2,19.241)}$ = 1.8361; p = 0.1863).

Sniffing the researcher (percentage of duration mice were observable)

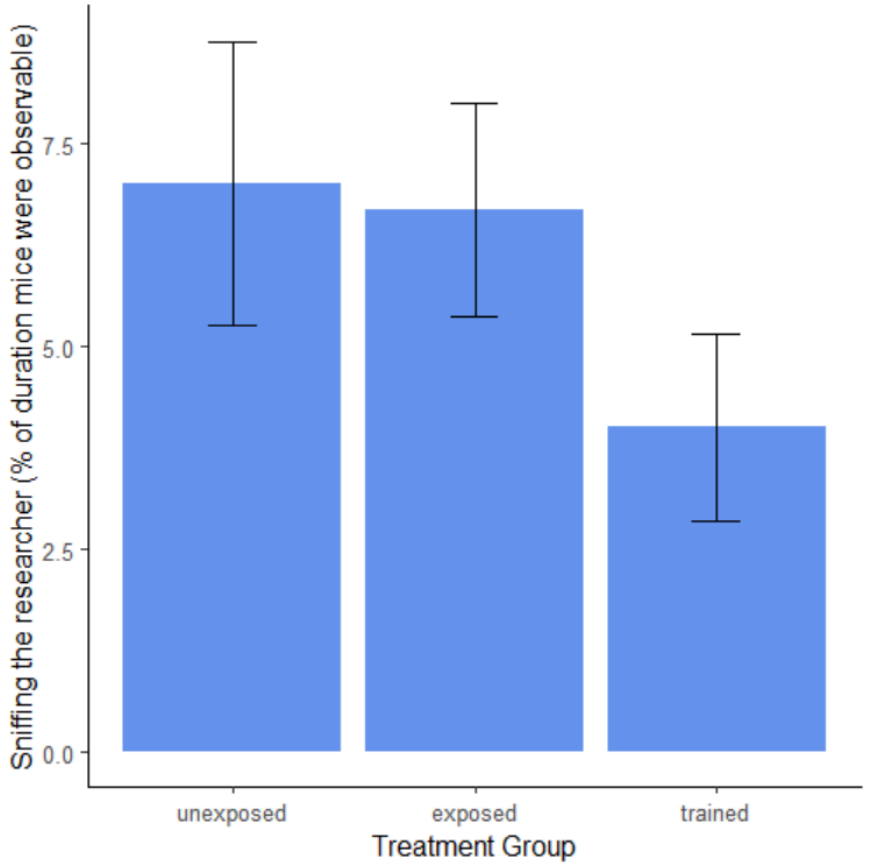

Figure 9: The duration of 'sniffing the researcher' across all treatment groups, represented as a percentage of the total duration of the total duration mice were observable by the researcher during the voluntary approach test (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).

No significant difference for 'sniffing the researcher' was found between the training group (M = 4.00, SE = 1.16), the exposed control group (M = 6.69, SE = 1.33), and the unexposed control group (M = 7.01, SE = 1.75) ($F_{(2,18.751)}$ = 1.6467; p = 0.2194).

Touching the researcher (percentage of total duration mice were observable)

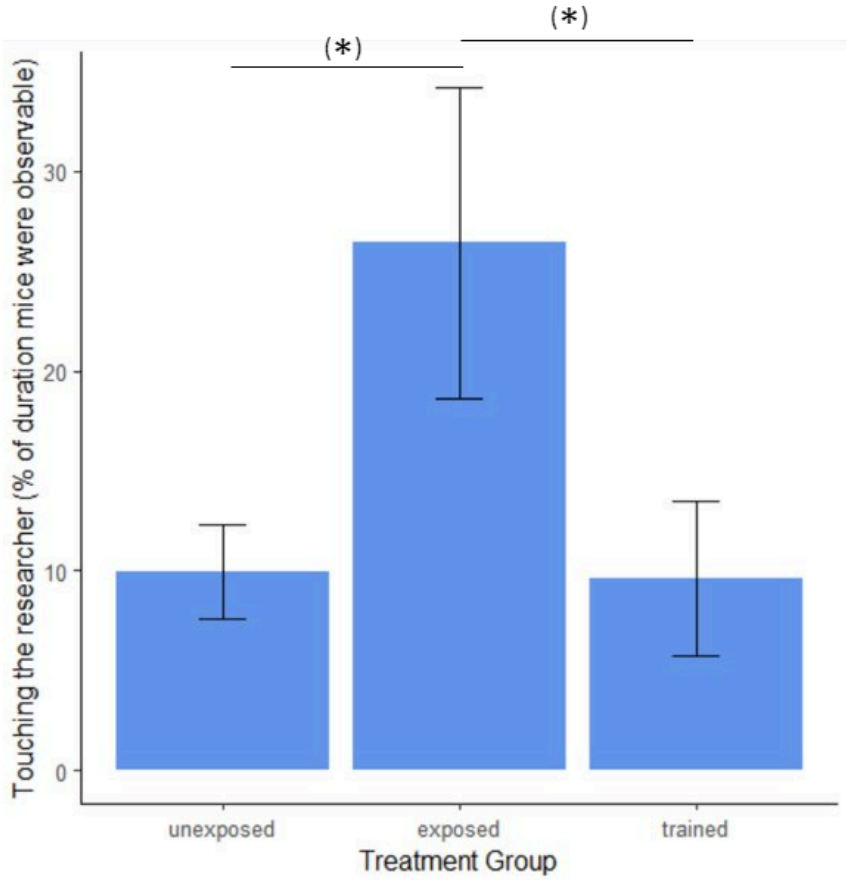

Figure 10: The duration of 'touching the researcher' across all treatment groups, represented as a percentage of the total duration of the total duration mice were observable by the researcher during the voluntary approach test (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).
(*) indicates a trend for a difference between groups (0.05<p<0.1).

A significant overall difference was found for 'touching the researcher' between the three treatment groups, as determined by the general linear mixed model ($F_{(2,19.197)}$ = 4.2034; p = 0.03062). Subsequently, a post hoc test was conducted. However, no statistically significant differences were found between the unexposed control group and the exposed control group (p = 0.0720), between the unexposed control group and the training group (p = 0.9721), or between the exposed control group and the training group (p = 0.0578). While these results were not significant, there appeared to be discernible trends. Specifically, the mice in the exposed control group (M = 26.43, SE = 7.80) appeared to touch the researcher more than the mice in the unexposed control group (M = 9.96, SE = 2.34). Additionally, the mice in the exposed control group (M = 26.43, SE = 7.80) appeared to touch the researcher more than the mice in the training group (M = 9.59, SE = 3.89).

Sitting next to the researcher (percentage of total duration mice were observable)

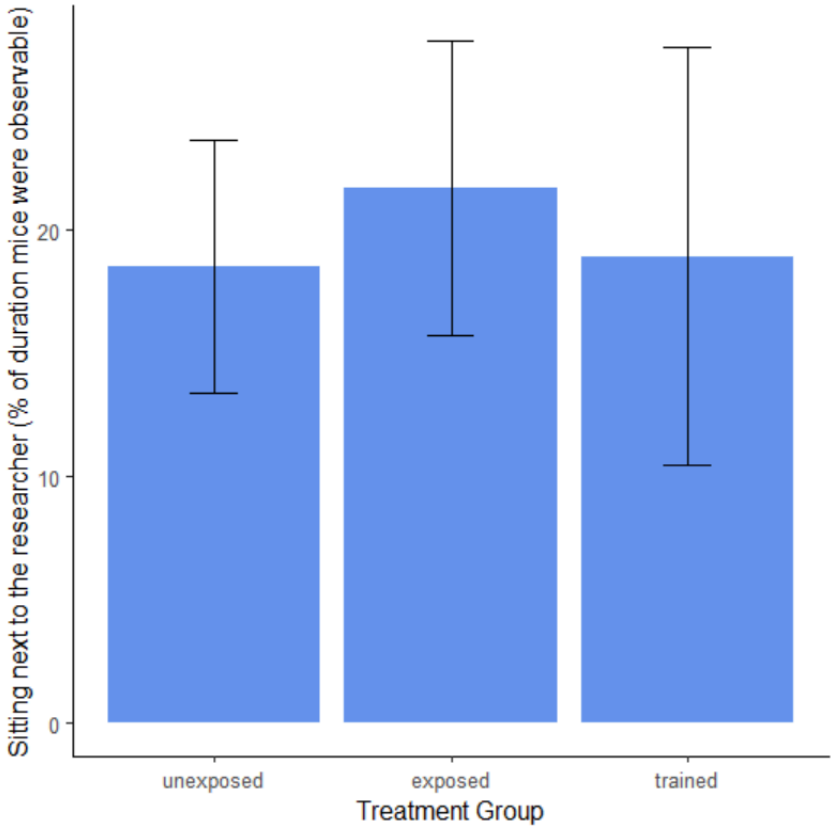

Figure 11: The duration of 'sitting next to the researcher' across all treatment groups, represented as a percentage of the total duration of the total duration mice were observable by the researcher during the voluntary approach test (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).

No significant difference was found for 'sitting next to the researcher' between the training group (M = 18.94, SE = 8.47), the exposed control group (M = 21.71, SE = 5.99), and the unexposed control group (M = 18.52, SE = 5.13) ($F_{(2,18.749)}$ = 0.7163; p = 0.5015).

Stretch attend (percentage of total duration mice were observable)

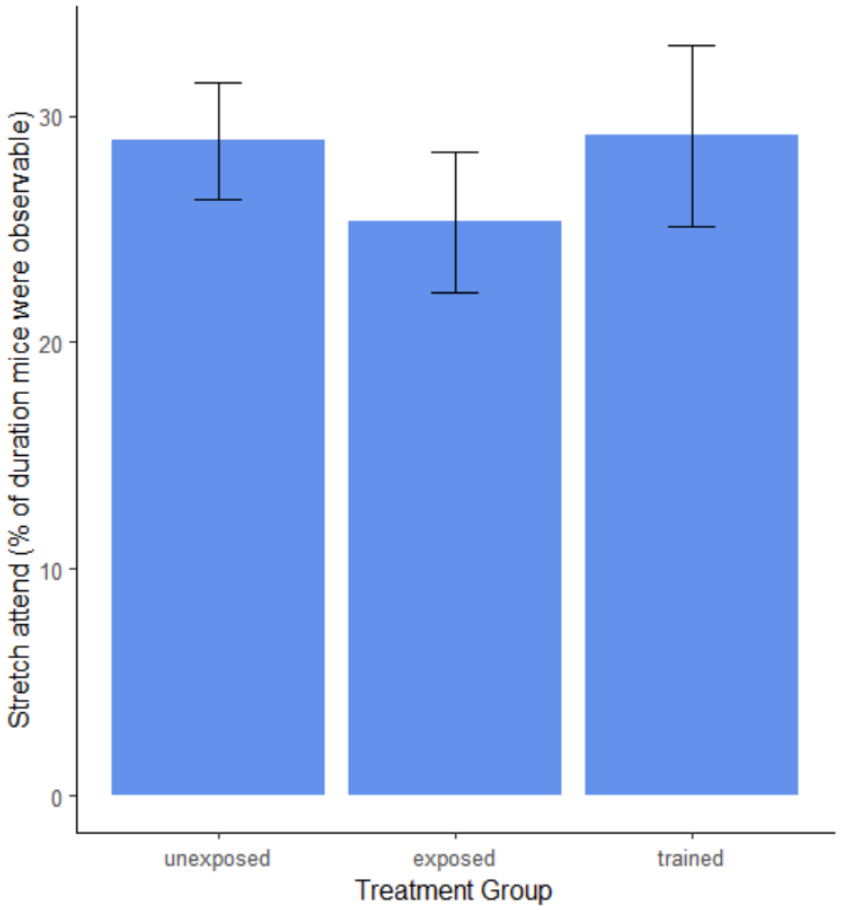

Figure 12: The duration of 'stretch attend' across all treatment groups, represented as a percentage of the total duration of the total duration mice were observable by the researcher during the voluntary approach test (mean ± 1 SE) (training group mice: n=7; exposed control group mice: n=10; unexposed control group mice: n=9).

No significant difference was found for 'stretch attend' between the training group (M = 29.11, SE = 4.02), the exposed control group (M = 25.31, SE = 3.12), and the unexposed control group (M = 28.90, SE = 2.58) ($F_{(2,19.867)}$ = 0.4988; p = 0.6146).

Discussion

This study sought to investigate the potential of clicker training as a refinement method for reducing stress in mice undergoing a minimal-restraint intraperitoneal injection. The effect of clicker training was assessed by observing specific behaviours exhibited by mice during and after injection, measuring their duration, and comparing them with two control groups. The exposed control group underwent familiarisation with the reward, the researcher, and environment, but not with the injection handling method. Conversely, the unexposed control group had minimal exposure to these factors. As mice were trained to remain still during injection, the duration of the behaviour 'standing still' was observed and analysed. Additionally, the total duration of the injection was analysed. After injection, a reward acceptance test and a voluntary approach test were conducted, during which the durations of various contact seeking behaviours were measured. These included 'accepting the reward/eating', 'sniffing the researcher', 'touching the researcher', and 'sitting next to the researcher'. The duration of the risk assessment behaviour 'stretch attend' was also measured during the voluntary approach test.

Despite the invasive nature of intraperitoneal injections, known to cause an acute stress response in mice (Meijer *et al*., 2006), the expectation was that trained mice would experience reduced susceptibility to this stress response due to the desensitising effect of the training protocol and familiarisation with the handling method (Clay *et al*., 2009; Laule *et al*., 2003). Exposure alone was anticipated to have a less pronounced desensitising effect on mice than the training protocol (Clay *et al*., 2009). Trained mice were also expected to experience minimal neophobia and reduced stress-induced anorexia (Griebel *et al*., 1993; Kronenberger & Médioni, 1985; Misslin & Cigrang, 1986; Yamada *et al*., 2020). Furthermore, they were expected to associate the handling procedure and the researcher with the positive experience of receiving a reward (Coleman *et al*., 2008; Jønholt *et al*., 2021; Leidinger *et al*., 2017; Mählis *et al*., 2023). Lastly, it was expected that trained mice would no longer perceive the situation as potentially dangerous, resulting in a reduction of anxiety-driven behaviours (Cole & Rodgers, 1994).

These expectations formed the hypothesis that trained mice would stand still the longest during injection, have the shortest total injection durations, and exhibit increased contact seeking and reduced risk assessment behaviour compared to both control groups. However, the analysis yielded no statistically significant difference between the three treatment groups for 'standing still', the total duration of injection, 'accepting the reward/eating', 'sniffing the researcher', 'sitting next to the researcher', and 'stretch attend'. This suggests that training did not noticeably influence the duration of these behaviours. The results also indicate that exposure alone had no discernible impact on the duration of these behaviours.

Interestingly, a statistically significant overall difference was found among the three treatment groups concerning ‘touching the researcher’. While post hoc testing failed to reveal statistically significant differences between these groups, a discernible trend emerged, indicating that mice in the exposed control group tended to touch the researcher more compared to mice in the other treatment groups. These observations imply that a potential influence of exposure does exist on this specific behaviour of mice following the minimal-restraint IP injection. This raises the question whether exposure alone might sufficiently desensitise mice, leading to reduced aversion towards the researcher. Such a possibility contradicts the study’s initial expectations that exposure would have a less desensitising effect compared to training (Clay *et al*., 2009). Further research is needed to validate these trends and potentially establish statistically significant differences between treatment groups. Should exposure alone be proven more effective, it challenges the necessity of clicker training as a refinement method, favouring exposure as a more practical and time-efficient refinement approach for future studies to consider.

Interpreting behaviour alone as a measurement of stress or anxiety in mice can be challenging due to the complex nature of their responses, and it is possible that certain behaviours may have been misinterpreted. For instance, prior to the study, the expectation was that the mice would react to the injection either by moving excessively or by freezing out of fear (Campos *et al.,* 2013; Eilam, 2005; Walker *et al.,* 2003). It is conceivable that a portion of the unexposed control mice indeed responded by freezing, which the researcher misinterpreted as standing still during injection. This could have influenced the outcomes. To enhance the accuracy of assessing if the mice experienced stress during the procedure, behavioural scoring could be combined with the measurement of stress-related hormone concentrations in the blood (Baek *et al.,* 2015) or faeces (as a non-invasive alternative; Rowland & Toth, 2019; Touma *et al*., 2004).

The lack of impact from both training and exposure on the durations of most behaviours, contrary to initial expectations, may be attributed to several limitations encountered in this study, one of which is the sample size. With access to only 29 mice, three of which were excluded from analysis, the training group ended up comprising only seven mice instead of the intended 10. Although no a priori power analysis was conducted, considering the sample sizes in other studies where mice were successfully trained for different procedures (Dickmann *et al*., 2022; Swan *et al*., 2023), it is likely that the sample size in the current study was too small, potentially contributing to the absence of statistical significance observed in the results. As the mice in this study were designated as batch one, it is expected that subsequent studies will build upon the protocol established here by incorporating larger sample sizes into subsequent batches to enhance the reliability of the results. The data collected in the current study can serve as a valuable foundation for these future studies to conduct their power analysis.

Moreover, seven out of the 29 mice had to be fixated by scruffing for the injection, as they exhibited excessive movement, posing a safety concern for the minimal-restraint method. Due to the low frequency of this occurence, whether the mice were scruffed or not was not included in the statistical analysis. Although it is conceivable that scruffing might have influenced the mice's behaviour after injection, existing studies indicate that it does not appear to have a significant impact on anxiety in mice (Gouveia & Hurst, 2019; Gjendal *et al.,* 2018). It is intriguing that five out of the seven mice belonged to the exposed control group, with only one mouse from the unexposed control group and one from the training group. The reason behind this pattern remains unclear. Future studies with larger sample sizes may involve a greater number of mice needing fixation, allowing for a more accurate analysis of its potential effects. This could also help determine whether the tendency for exposed control mice to require more frequent fixation persists.

Additionally, the mice in the unexposed control group were not entirely naive subjects. All mice, including the unexposed control mice, had previously served as teaching animals for students at Utrecht University. While the mice were unfamiliar with the minimal-restraint method employed in this study, they had substantial prior exposure to human interactions, had already undergone multiple (four to five) IP injections prior to the study, and they were familiar with the training room, as the animal handling classes took place there. Moreover, they had been included during the familiarisation with the researcher before training, establishing a certain level of familiarity. This previous experience may have caused the mice to respond with less apprehension towards the reward, researcher, and environment (Griebel *et al*., 1993; Misslin & Cigrang, 1986; Kronenberger & Médioni, 1985), potentially explaining the narrowing of the expected gap between the unexposed control mice and the other two control groups.

Another limitation and plausible explanation for the absence of statistically significant results could be the relatively advanced age of the mice in this study. At the onset of training, they were approximately 11 months old, and by the injection day, they were around 13 months old. Studies have indicated that aged mice (19-22 months old) may exhibit a decline in several functions compared to young mice (3-5 months old), encompassing cognitive functions such as learning and memory, as well as motor functions. The impact of age on these functions can vary among individual mice, with some aged mice performing these functions as effectively as younger mice (Forster *et al.,* 1996; Matzel *et al*., 2008). This variability in cognitive function with age is echoed in research on dogs. This research also suggests that ageing can lead to changes in behavioural patterns. Specifically, young dogs tend to engage more with humans compared to their aged counterparts. While older dogs with intact cognitive functions still interact with humans, there is a noticeable decline compared to the young dogs. Dogs with age-affected cognitive functions generally exhibit the least interaction with humans (Siwak *et al*., 2001). Despite inherent differences in behavioural responses to stimuli between dogs and mice, it remains possible that age could have influenced the mice's behaviour in the current study. The variation in cognitive function may have influenced the training outcomes, as reflected in the fact that not all mice reached the final training level. In younger mice, this variation could be narrower, potentially resulting in more consistent training outcomes. However, it's essential to note that these older mice were the only accessible subjects for this study. Future studies with younger mice could shed further light on how age influences training outcomes and mouse behaviour.

To refine the IP injection procedure, this study opted for a minimal-restraint injection. This decision was inspired by a study by Baek *et al* (2015), in which IP injections were performed by lifting the mice's rear ends by the tail and one hind paw. This method showed a significant reduction in stress-hormone concentrations compared to the conventional method (as described in Miner *et al* (1969)). To minimise restraint even further, the rear ends of the mice in the current study were only lifted by the base of the tail. Additionally, the mice in Baek *et al* (2015) were injected near a wire rack, allowing them to hold onto it with their front paws, whereas in the present study, injections were administered on a training pad to allow the mice to roam more freely during the voluntary approach test. While a number of training group mice responded positively to this minimal-restraint injection method and remained still during training sessions, it became evident, especially on the injection day, that this approach had limitations. Holding the mice only by the base of the trail allowed for too much freedom for the rest of the body. Many mice exhibited excessive movement during injection, including twisting their bodies even when the needle was already inserted into their abdomen. This behaviour poses potential dangers and raises concerns about inflicting pain on the mice. This undermines the study's intended goals of refinement (Russell & Burch, 1992). Consequently, modifications to the method are necessary before it can be implemented in further research. These modifications would be designed to find a balance between ensuring the mice's safety and maintaining the minimal-restraint approach during IP injections. For instance, a potential modification might still involve lifting the rear end by the base of the tail, while simultaneously placing the pinky finger of the same hand on the back of the mouse's neck and gently applying pressure. This method aims to restrict movement without causing discomfort to the mouse. Further research would be required to determine the stress-reducing efficacy of such a restraining method in comparison to the method employed by Baek *et al.* (2015).

The choice to incorporate clicker training in the current study was influenced by its successful application in several animal studies, as evidenced by Verdino (2021) and Mählis *et al.* (2023), demonstrating its stress-reducing effects. Additionally, its efficacy in mice was also established by Leidinger *et al*. (2017). However, it's important to note that these studies did not directly compare clicker training with traditional training methods. Studies that have made this comparison have shown that the use of a clicker as a secondary reinforcer does not lead to superior performance regarding newly learned behaviours compared to traditional training using a food reward as the primary reinforcer. In fact, clicker training was even associated with negative effects on certain learned behaviours, with traditionally trained animals exhibiting superior performance (Dorey *et al*., 2020; Gilchrist *et al*., 2021). In the current study, the majority of mice did not display distinct anticipatory behaviour, such as directing their attention towards the forthcoming award (Makowska & Weary, 2016). This raises uncertainty about whether an association was established between the clicker sound and the reward. Consequently, it is unclear whether the mice successfully underwent clicker training or were traditionally trained instead. While anticipatory behaviour was not specifically measured in the current study, future studies should consider its inclusion to confirm whether clicker training has taken place. Given these considerations, it's prudent to reconsider the necessity of incorporating a clicker. Traditional training methods may be equally or even more effective and offer the advantage of simplicity and efficiency, as linking sessions are not needed. Simplifying the training procedure may not only streamline the protocol but also reduce the risk of inducing additional stress, particularly from unfamiliar stimuli such as the clicker sound.

Due to time constraints, a total of 23 training sessions were completed before proceeding to the injection, even though not all trained mice (30%) had reached the final level. In the absence of established scientific guidelines, the necessary number of linking and training sessions was uncertain, but the researcher did not anticipate needing as many as were ultimately required. Initially, it was expected that mice would consistently accept the reward after just three or four linking sessions, based on their behaviour during familiarisation prior to training. However, linking was eventually extended to seven sessions as only three mice accepted the reward by session four. Another contributing factor to the relatively high number of sessions could be the researcher's limited training experience. During the initial sessions, this inexperience may have led to variability in the training procedures, particularly concerning the timing of clicker use. This variability might have conveyed unclear or inconsistent signals to the animals regarding the desired behaviours. As there are no recorded videos of the training sessions, verifying this variability is not possible, but it is plausible that it may have affected the efficacy and pace of the training process. A researcher with more training experience could likely streamline the training procedure significantly and enhance the protocol's practicality and applicability for use in subsequent studies.

Another element that might have influenced the pace of the training process is the specific obstacles encountered by mice as they advanced through the levels. Notably, the most substantial challenge revolved around the consistent acceptance of the reward, particularly evident in levels 0 and 1. Additionally, level 3, which introduced lifting their rear end by the tail base, posed another notable hurdle. Most mice spent extended periods on these levels compared to the other levels. However, once they did meet the completion criteria for both levels, they progressed swiftly to level 5 (refer to Table A2 of the appendix for more information). The prolonged stay at level 0 or 1 might be attributed to the initial experiences of neophobia, which gradually diminished as the mice became more familiar with the reward and the training process (Griebel *et al*., 1993; Misslin & Cigrang, 1986; Kronenberger & Médioni, 1985). Three trained mice consistently refused to accept the reward on the training pad, halting their progression through the levels. Notably, these mice demonstrated a more consistent acceptance of the reward within their homecages, suggesting that their aversion was specifically related to accepting it on the training pad. This reluctance persisted even after 23 sessions on the training pad, a duration well beyond what had been necessary for the other mice not only to accept the reward but also to have completed (almost) the entire training protocol. While this behaviour is most likely attributed to stress-induced anorexia triggered by the training room and training pad (Yamada *et al*., 2020), it is also noteworthy that individual temperament differences among the mice became apparent during training. Such temperament variations have been shown to influence food acceptance, which, in turn, influences the success of clicker training (Coleman *et al*., 2005; Leidinger *et al*., 2017). For instance, training group mice from homecage 26 displayed notably bolder behaviour compared to those from other homecages. They not only consistently accepted the reward from the first linking session but also reached and completed the final level ahead of others (see Table A2 and A3 of the appendix). It could be advantageous for future studies to assess and record each mouse's temperament by evaluating their interaction with the food reward before initiating training experiments (Coleman *et al*., 2005; Palmer *et al*., 2022), allowing for a more accurate prediction of individual mouse responses during training sessions. A possible explanation for the challenge posed by level 3 is that, despite the minimal-restraint IP injection method being less stressful than the conventional IP injection method (Baek *et al.,* 2015), the mice were still partly lifted by the tail. It is well-established that mice perceive

tail handling as aversive, even if it's brief (Davies *et al*., 2022; Gouveia & Hurst, 2013; Gouveia & Hurst, 2019). Hence, partial lifting by the tail likely induced some aversion, making this level particularly stressful. As training progressed, it likely desensitised the mice (Clay *et al.,* 2009; Laule *et al*., 2003), facilitating the completion of subsequent levels.

The challenges encountered by the mice during training sessions were evidenced in their occasional failure to meet the criteria of the highest level they had officially reached. However, these setbacks were not explicitly reported in the scoresheet, leading to uncertainty regarding their frequency. The overall impact of these setbacks on the training process also remains unclear, as a detailed analysis was not possible. Future studies could address this by documenting setbacks and adjusting the mice's levels based on their performance.

While scoring the behaviours using BORIS, the employed software (Friard & Gamba, 2016), several factors were identified that could introduce nuances into the interpretation of the results. Due to time constraints, only the total durations of each behaviour were measured. However, considering latency, which reveals when mice initiated these behaviours, also provides valuable insights. For instance, not all mice accepted the reward immediately after injection; some began eating midway through the designated minute, but continued eating beyond its duration. Consequently, these mice may have exhibited the same total eating duration as those accepting the reward immediately, potentially masking the impact of the stress caused by the injection. This latency in accepting and eating the reward could introduce a potential misrepresentation of the data for this behaviour. Future studies should consider incorporating latency in their data to ensure more precise and comprehensive results. Furthermore, when mice climbed up the researcher's arm and onto their shoulder, they were essentially displaying 'sitting on the researcher'. However, due to impaired visibility, they were categorised as 'out of frame'. Returning these mice to the training pad disrupted this behaviour unnecessarily and led to some mice becoming disoriented, which was reflected in reduced movement upon being returned. Adjusting the camera angle to include the researcher would resolve visibility issues and allow for accurate scoring of this behaviour as 'sitting on the researcher', thereby eliminating the need to return the mouse to the training pad.

While not formally measured or analysed, a subjective difference in mice behaviour between pre- and post-study was noticeable. Following the study, the mice appeared to possess a less anxious demeanour during animal handling classes. They displayed less apprehension towards the students and increased contact-seeking behaviour compared to their pre-study behaviour. This offers optimism that a repetition of this research, with adjustment to address the aforementioned limitations, could support these observations through statistically significant results.

In conclusion, this study gives rise to careful consideration regarding the future application of clicker training as a refinement method for scientific experiments involving laboratory mice. Despite the initial expectation of clicker training's potential to reduce stress during intraperitoneal injections with minimal restraint, the study did not produce significant results supporting this hypothesis. Notably, exposure to the researcher, the reward, and the research environment resulted in a statistical trend that suggested potential effectiveness, at least in terms of fostering increased contact-seeking behaviour towards the researcher. While exposure alone might have a positive impact on reducing aversion towards the researcher, the lack of conclusive evidence of clicker training's efficacy could also be attributed to several study limitations. However, this study does serve as a starting point, highlighting the necessity for adjustments in the methodology to overcome the limitations in future studies, ultimately enhancing the reliability and validity of results. These adjustments may contribute to the ongoing pursuit of advancing the welfare of research animals.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Esther Langen, for her indispensable guidance and unwavering support throughout this research endeavour. Under her mentorship, I have delved deeper into the intricacies of clicker training and stress reduction in laboratory mice. Additionally, her expertise has expanded my understanding of refinement strategies for laboratory animals on a broader scale. The privilege of completing my master's thesis at the Central Laboratory Animal Research Facility at Utrecht University is truly appreciated. I am thankful for the opportunity, and I acknowledge that this research holds the potential to contribute to the ongoing pursuit of enhancing the welfare of research animals.

References

Arndt, S. S., Goerlich, V. C., & Van Der Staay, F. J. (2022). A dynamic concept of animal welfare: the role of appetitive and adverse internal and external factors and the animal's ability to adapt to them. Frontiers in animal science, 3. https://doi.org/10.3389/fanim.2022.908513

Baek, J. M., Kwak, S. C., Kim, J., Ahn, S., Jun, H. Y., Yoon, K., Lee, M. S., & Oh, J. (2015b). Evaluation of a novel technique for intraperitoneal injections in mice. Lab Animal, 44(11), 440–444. https://doi.org/10.1038/laban.880

Balcombe, J., Barnard, N. D., & Sandusky, C. B. (2004). Laboratory routines cause animal stress. PubMed, 43(6), 42–51. https://pubmed.ncbi.nlm.nih.gov/15669134

Bates, D. M., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting Linear Mixed-Effects models using lme4. Journal of Statistical Software, 67(1). https://doi.org/10.18637/jss.v067.i01

Bourin, M., Petit-Demoulière, B., Dhonnchadha, B. N., & Hascoët, M. (2007). Animal models of anxiety in mice. Fundamental & Clinical Pharmacology, 21(6), 567–574. https://doi.org/10.1111/j.1472-8206.2007.00526.x

Browne, C. M. (2015). The Effects of Delayed Positive Reinforcement on Learning in Dogs (Thesis, Doctor of Philosophy (PhD)). University of Waikato, Hamilton, New Zealand. Retrieved from https://hdl.handle.net/10289/9808

Bryda, E. C. (2013). The Mighty Mouse: the impact of rodents on advances in biomedical research. Missouri Medicine, 110(3), 207–211. https://europepmc.org/articles/PMC3987984

Campos, A. C., Fogaça, M. V., Aguiar, D. C., & Guimarães, F. S. (2013). Animal models of anxiety disorders and stress. Revista Brasileira de Psiquiatria, 35(suppl 2), S101–S111. https://doi.org/10.1590/1516-4446-2013-1139

Clay, A. W., Bloomsmith, M. A., Marr, M. J., & Maple, T. L. (2008). Habituation and desensitization as methods for reducing fearful behavior in singly housed rhesus macaques. American Journal of Primatology, 71(1), 30–39. https://doi.org/10.1002/ajp.20622

Cole, J., & Rodgers, R. (1994). Ethological evaluation of the effects of acute and chronic buspirone treatment in the murine elevated Plus-maze test: comparison with haloperidol. Psychopharmacology, 114(2), 288–296. https://doi.org/10.1007/bf02244851

Coleman, K., Pranger, L., Maier, A., Lambeth, S. P., Perlman, J. E., Thiele, E., & Schapiro, S. J. (2008). Training rhesus macaques for venipuncture using positive reinforcement techniques: a comparison with chimpanzees. PubMed. https://pubmed.ncbi.nlm.nih.gov/18210997

Coleman, K., Tully, L. A., & McMillan, J. L. (2005). Temperament correlates with training success in adult rhesus macaques. American Journal of Primatology, 65(1), 63–71. https://doi.org/10.1002/ajp.20097

Daugette, K. F., Hoppes, S., Tizard, I. R., & Brightsmith, D. J. (2012). Positive reinforcement training facilitates the voluntary participation of laboratory macaws with veterinary procedures. Journal of Avian Medicine and Surgery, 26(4), 248–254. https://doi.org/10.1647/2011-056

Davies, J. R., Purawijaya, D. A., Bartlett, J. M., & Robinson, E. (2022). Impact of refinements to handling and restraint methods in mice. Animals, 12(17), 2173. https://doi.org/10.3390/ani12172173

De Boer, S. F., & Koolhaas, J. M. (2003). Defensive burying in rodents: Ethology, Neurobiology and Psychopharmacology. European Journal of Pharmacology, 463(1–3), 145–161. https://doi.org/10.1016/s0014-2999(03)01278-0

Dickmann, J., Gonzalez-Uarquin, F., Reichel, S., Pichl, D., Radyushkin, K., Baumgart, J., & Baumgart, N. (2022). Clicker training mice for improved compliance in the catwalk Test. Animals, 12(24), 3545. https://doi.org/10.3390/ani12243545

Directive 2010/63/EU. *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.* http://data.europa.eu/eli/dir/2010/63/oj

Dorey, N. R., Blandina, A., & Udell, M. A. R. (2020). Clicker training does not enhance learning in mixed-breed shelter puppies (*Canis familiaris*). Journal of Veterinary Behavior, 39, 57–63. https://doi.org/10.1016/j.jveb.2020.07.005

Eilam, D. (2005). Die hard: A blend of freezing and fleeing as a dynamic defense—implications for the control of defensive behavior. Neuroscience & Biobehavioral Reviews, 29(8), 1181–1191. https://doi.org/10.1016/j.neubiorev.2005.03.027

Faculteit Diergeneeskunde: Dierenwelzijn. Consulted through Dierenwelzijn - Faculteit Diergeneeskunde - Universiteit Utrecht in January 2024.

Fernández-Teruel, A., & Estanislau, C. (2016). Meanings of self-grooming depend on an inverted U-shaped function with aversiveness. Nature Reviews Neuroscience, 17(9), 591. https://doi.org/10.1038/nrn.2016.102

Forster, M. J., Dubey, A., Dawson, K. M., Stutts, W. A., Lal, H., & Sohal, R. S. (1996). Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proceedings of the National Academy of Sciences of the United States of America, 93(10), 4765–4769. https://doi.org/10.1073/pnas.93.10.4765

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods in Ecology and Evolution, 7(11), 1325–1330. https://doi.org/10.1111/2041-210x.12584

Gilchrist, R. J., Gunter, L. M., Anderson, S. F., & Wynne, C. D. L. (2021). The click is not the trick: the efficacy of clickers and other reinforcement methods in training naïve dogs to perform new tasks. PeerJ, 9, e10881. https://doi.org/10.7717/peerj.10881

Gjendal, K., Franco, N. H., Ottesen, J. L., Sørensen, D. B., & Olsson, I. A. S. (2018). Eye, body or tail? Thermography as a measure of stress in mice. Physiology & Behavior, 196, 135–143. https://doi.org/10.1016/j.physbeh.2018.08.022

Gouveia, K., & Hurst, J. L. (2013). Reducing Mouse Anxiety during Handling: Effect of Experience with Handling Tunnels. PLOS ONE, 8(6), e66401. https://doi.org/10.1371/journal.pone.0066401

Gouveia, K., & Hurst, J. L. (2017). Optimising reliability of mouse performance in behavioural testing: the major role of non-aversive handling. Scientific Reports, 7(1). https://doi.org/10.1038/srep44999

Gouveia, K., & Hurst, J. L. (2019). Improving the practicality of using non-aversive handling methods to reduce background stress and anxiety in laboratory mice. Scientific Reports, 9(1). https://doi.org/10.1038/s41598-019-56860-7

Griebel, G., Belzung, C., Misslin, R., Vogel, E. (1993). The free-exploratory paradigm: an effective method for measuring neophobic behaviour in mice and testing potential neophobia-reducing drugs. *Behavioural Pharmacology*, 4(6), 637-644. https://pubmed.ncbi.nlm.nih.gov/11224232/

Johnson, E. O., Kamilaris, T. C., Chrousos, G. P., & Gold, P. W. (1992). Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. Neuroscience & Biobehavioral Reviews, 16(2), 115–130. https://doi.org/10.1016/s0149-7634(05)80175-7

Jønholt, L., Bundgaard, C. J., Carlsen, M., & Sørensen, D. B. (2021). A case study on the behavioural effect of positive reinforcement training in a novel task participation test in Göttingen Mini pigs. Animals, 11(6), 1610. https://doi.org/10.3390/ani11061610

Korte, S. M. (2001). Corticosteroids in relation to fear, anxiety and psychopathology. Neuroscience & Biobehavioral Reviews, 25(2), 117–142. https://doi.org/10.1016/s0149-7634(01)00002-1

Kronenberger, J., & Médioni, J. (1985). Food neophobia in wild and laboratory mice (Mus musculus domesticus). Behavioural Processes, 11(1), 53–59. https://doi.org/10.1016/0376-6357(85)90102-0

Kalueff, A. V., Stewart, A., Song, C., Berridge, K., Graybiel, A. M., & Fentress, J. C. (2015). Neurobiology of rodent Self-grooming and its value for translational neuroscience. Nature Reviews Neuroscience, 17(1), 45–59. https://doi.org/10.1038/nrn.2015.8

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). LMERTest Package: Tests in Linear Mixed Effects models. Journal of Statistical Software, 82(13). https://doi.org/10.18637/jss.v082.i13

Laule, G., Bloomsmith, M. A., & Schapiro, S. J. (2003). The use of positive reinforcement training techniques to enhance the care, management, and welfare of primates in the laboratory. Journal of Applied Animal Welfare Science, 6(3), 163–173. https://doi.org/10.1207/s15327604jaws0603_02

Leidinger, C. S., Herrmann, F. J., Thöne-Reineke, C., Baumgart, N., & Baumgart, J. (2017). Introducing Clicker Training as a Cognitive Enrichment for Laboratory Mice. Journal of Visualized Experiments, 121. https://doi.org/10.3791/55415

Lenth, R. (2023). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.8.9. https://CRAN.R-project.org/package=emmeans

Mählis, G., Kleine, A., Lüschow, D., Bartel, A., Wiegard, M., & Thoene-Reineke, C. (2023). Clicker training as an applied refinement measure in chickens. Animals, 13(24), 3836. https://doi.org/10.3390/ani13243836

Makowska, I. J., & Weary, D. M. (2016). Differences in anticipatory behaviour between rats (Rattus norvegicus) housed in Standard versus Semi-Naturalistic laboratory environments. PLOS ONE, 11(1), e0147595. https://doi.org/10.1371/journal.pone.0147595

Matzel, L. D., Grossman, H. C., Light, K., Townsend, D. A., & Kolata, S. (2008). Age-related declines in general cognitive abilities of Balb/C mice are associated with disparities in working memory, body weight, and general activity. *Learning & Memory*, *15*(10), 733–746. https://doi.org/10.1101/lm.954808

Meijer, M. K., Spruijt, B., Van Zutphen, L., & Baumans, V. (2006). Effect of restraint and injection methods on heart rate and body temperature in mice. *Laboratory Animals*, *40*(4), 382–391. https://doi.org/10.1258/002367706778476370

Miner, N., Koehler, J. A., & Greenaway, L. (1969). Intraperitoneal injection of mice. *Applied microbiology*, *17*(2), 250–251. https://doi.org/10.1128/am.17.2.250-251.1969

Misslin, R., & Cigrang, M. (1986). Does neophobia necessarily imply fear or anxiety? Behavioural Processes, 12(1), 45–50. https://doi.org/10.1016/0376-6357(86)90069-0

Mu, M., Geng, H., Rong, K., Peng, R., Shu-Ting, W., Geng, L., Qian, Z. M., Yung, W., & Yao, K. (2020). A limbic circuitry involved in emotional stress-induced grooming. Nature Communications, 11(1). https://doi.org/10.1038/s41467-020-16203-x

Ohl, F., & Hellebrekers, L. (2009). “Dierenwelzijn” - de diergeneeskundige positie. Tijdschrift Voor Diergeneeskunde, 134(18), 754–755. https://dspace.library.uu.nl/bitstream/1874/202892/1/20090915%20Tijdschrift%20voor%20Diergeneeskunde.pdf

Palmer, S., Oppler, S. H., & Graham, M. L. (2022). Behavioral Management as a Coping Strategy for Managing Stressors in Primates: The Influence of Temperament and Species. Biology, 11(3), 423. https://doi.org/10.3390/biology11030423

Phifer-Rixey, M., & Nachman, M. W. (2015). Insights into mammalian biology from the wild house mouse Mus musculus. eLife, 4. https://doi.org/10.7554/elife.05959

R Core Team (2023). _R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria.https://www.R-project.org/.

Rodgers, R., & Dalvi, A. (1997). Anxiety, Defence and the Elevated Plus-maze. Neuroscience and Biobehavioral Reviews, 21(6), 801–810. https://doi.org/10.1016/s0149-7634(96)00058-9

Rowland, N. E., & Toth, L. A. (2019). Analytic and interpretational pitfalls to measuring fecal corticosterone metabolites in laboratory rats and mice. Comparative Medicine, 69(5), 337–349. https://doi.org/10.30802/aalas-cm-18-000119

Russell, W. M. S., & Burch, R. L. (1992). The Principles of Humane Experimental Technique. Hyperion Books.

Siwak, C. T., Tapp, P. D., & Milgram, N. W. (2001). Effect of age and level of cognitive function on spontaneous and exploratory behaviors in the beagle dog. *Learning & Memory*, *8*(6), 317–325. https://doi.org/10.1101/lm.41701

Song, C., Berridge, K., & Kalueff, A. V. (2016). “Stressing” rodent self-grooming for neuroscience research. Nature Reviews Neuroscience, 17(9), 591. https://doi.org/10.1038/nrn.2016.103

Sterlemann, V., Ganea, K., Liebl, C., Harbich, D., Alam, S., Holsboer, F., Müller, M. B., & Schmidt, M. V. (2008). Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: Implications for Stress-related Disorders. Hormones and Behavior, 53(2), 386–394. https://doi.org/10.1016/j.yhbeh.2007.11.001

Swan, J., Boyer, S., Westlund, K., Bengtsson, C., Nordahl, G., & Törnqvist, E. (2023). Decreased levels of discomfort in repeatedly handled mice during experimental procedures, assessed by facial expressions. Frontiers in Behavioral Neuroscience, 17. https://doi.org/10.3389/fnbeh.2023.1109886

Touma, C., Palme, R., & Sachser, N. (2004). Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Hormones and Behavior, 45(1), 10–22. https://doi.org/10.1016/j.yhbeh.2003.07.002

Van Eldik, K. C. (2021). Better welfare for mice through handling and training. Studentthesis. Referenced via https://studenttheses.uu.nl/handle/20.500.12932/39104.

Verdino, S. (2021) The Effects of Clicker Training on Stress Levels in Communally Housed Shelter Cats. Biological Sciences, 3-8.

Walker, D., Toufexis, D., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. European Journal of Pharmacology, 463(1–3), 199–216. https://doi.org/10.1016/s0014-2999(03)01282-2

Wet op de Dierproeven (WoD). Article 1d, paragraph 3. Consulted through https://wetten.overheid.nl/BWBR0003081/2021-07-01 in June 2023.

Yamada, C., Iizuka, S., Nahata, M., Hattori, T., & Takeda, H. (2020). Vulnerability to psychological stress-induced anorexia in female mice depends on blockade of ghrelin signal in nucleus tractus solitarius. British Journal of Pharmacology, 177(20), 4666–4682. https://doi.org/10.1111/bph.15219

Appendix

Session	Date	Group	Individual/ Mouse	Level	Level completed?	Notes
		Treatment	X26Y4			
		Treatment	X26Y5			
		Treatment	X27Y1			
		Treatment	X27Y2			
		Treatment	X22Y1			
		Treatment	X22Y3			
		Treatment	X21Y2			
		Treatment	X20Y4			
		Treatment	X12Y4			
		Treatment	X12Y5			
		Exposure	X26Y1			
		Exposure	X26Y2			
		Exposure	X27Y3			
		Exposure	X22Y2			
		Exposure	X22Y4			
		Exposure	X21Y4			
		Exposure	X21Y5			
		Exposure	X20Y3			
		Exposure	X12Y1			
		Exposure	X12Y2			

Table A1 : Scoresheet template used to track each mouse's progress.
X = homecage number
Y = individual mouse number (pertaining to the amount of stripes on their tails)

	X26Y4	X26Y5	X27Y1	X27Y2	X22Y1	X22Y3	X21Y2	X20Y4	X12Y4	X12Y5
Session 1	0	0	0	0	0	0	0	0	0	0
Session 2	0	0	0	0	0	0	0	0	0	0
Session 3	0	0	0	0	0	0	0	0	0	0
Session 4	0	0	0	0	0	0	0	0	0	0
Session 5	0	0	0	0	0	0	0	0	0	0
Session 6	0	0	0	0	0	0	0	0	0	0
Session 7	0	0	0	0	0	0	0	0	0	0
Session 8	1	1	0	0	1	1	1	0	0	0
Session 9	2	2	0	1	1	2	1	0	0	0
Session 10	2	2	0	1	1	2	1	0	1	1
Session 11	2	2	0	2	1	2	1	0	1	1
Session 12	3	2	0	2	1	2	2	0	1	1
Session 13	4	2	0	2	1	2	2	0	1	1
Session 14	5	2	0	2	1	2	2	0	1	1
Session 15	5	2	1	2	1	2	2	0	1	1
Session 16	5	3	1	2	1	2	2	0	1	1
Session 17	5	4	1	2	1	2	3	0	1	1
Session 18	5	5	1	3	1	3	4	0	2	1
Session 19	5	5	1	4	2	3	4	0	2	1
Session 20	5	5	1	4	3	3	4	0	3	1
Session 21	5	5	1	4	4	3	4	0	3	1

Session 22	5	5	1	4	4	4	4	0	4	1
Session 23	5	5	1	4	4	4	5	0	4	1

Table A2 : Progression of each mouse in the training group across 23 sessions. The numbers within each cell represent the highest level completed by each mouse during a session. The cell is highlighted blue if the mouse accepted the reward from the researcher's hand at least twice during that session. Sessions 1-7 served as linking sessions.
X = homecage number
Y = individual mouse number (pertaining to the amount of stripes on their tails)

	X26Y1	X26Y2	X27Y3	X22Y2	X22Y4	X21Y4	X21Y5	X20Y3	X12Y1	X12Y2
Session 1										
Session 2										
Session 3										
Session 4										
Session 5										
Session 6										
Session 7										
Session 8										
Session 9										
Session 10										
Session 11										
Session 12										
Session 13										
Session 14										
Session 15										
Session 16										
Session 17										
Session 18										
Session 19										
Session 20										
Session 21										

Session 22										
Session 23										

Table A3 : Progression of each mouse in the exposed control group across 23 sessions regarding reward acceptance. The cell is highlighted blue if the mouse accepted the reward from the researcher's hand at least twice during that session.
X = homecage number
Y = individual mouse number (pertaining to the amount of stripes on their tails)

Category of behaviour	Behaviour	Description of the behaviour	Start (duration)	Stop (duration)
During the injection				
Behaviour during injection	Standing still (state event)	The mouse stands still during injection: it does not move its hind paws, or turns its head or bends its body at a 90-degree angle. It is permitted to move its front paws within this restricted range and to observe its surroundings.	The time is considered to begin from the first frame the mouse stands still.	The time is considered to stop from the first frame the mouse turns its head or bends its body at a 90-degree angle or moves either of its hind paws.
	Struggling (state event)	Any movement that deviates from the description of standing still.	The time is considered to begin from the first frame the mouse moves either of its hind paws, or turns its head or bends its body at a 90-degree angle. Additionally, if the mouse moves its hind paws and then proceeds to walk, this is also considered struggling.	The time is considered to stop from the first frame the mouse ceases to move its hind paws, or repositions its head or body so it's no longer turned at a 90-degree angle. The mouse must also have ceased walking.
	Total injection duration (state event)	The total duration of injection.	The time is considered to begin from the first frame the researcher makes contact with the base of the mouse's tail. The researcher needs to be prepared to administer the injection, holding the syringe in advance. If the researcher initiates contact with the base of the mouse's tail before obtaining the syringe, the time only starts once the syringe is picked up and positioned appropriately for injection.	The time is considered to stop from the first frame the researcher releases the mouse's tail after the injection, regardless of whether the supervisor provided assistance during the injection.
Testing reward acceptance				
Contact seeking behaviour	Accepting the reward/eating (state event)	The mouse accepts the reward offered by the researcher and eats it.	The time is considered to begin from the frame in which the mouse opens its mouth to the widest extent to accept the reward from the researcher's hand. If the mouse resumes chewing after a previous cessation, without accepting an additional reward from the researcher's hand, the time begins from the frame in which the mouse reopens its mouth.	The time is considered to stop from the frame in which the mouse closes its mouth after chewing and refrains from an immediate resumption of chewing. In instances where the mouse turns away from the camera, obscuring visibility, it becomes challenging to determine whether it continues eating. If the mouse is actively chewing before turning away, and sustains this behaviour upon becoming visible again, the time remains uninterrupted.

				However, if the mouse has halted chewing upon reappearing to the camera, the time is stopped from the frame the mouse closes its mouth for the last time before turning away from the camera.
The voluntary approach test				
Contact seeking behaviour	Sniffing the researcher (state event)	The mouse sniffs the researcher's hand.	The time is considered to begin from the frame in which the mouse's nose makes contact with the researcher's hand or arm.	The time is considered to stop from the frame in which the mouse's nose no longer makes contact with the researcher's hand or arm.
	Touching the researcher (state event)	The mouse touches the researcher's hand or arm with one to three paws, the top of its head or their body. The tail is excluded.	The time is considered to begin from the frame the mouse's paw makes contact with the researcher's arm or hand. The term 'touching the researcher' encompasses situations in which the mouse has up to three paws in contact with the researcher. If the mouse is sitting next to the researcher's hand and there is no discernable space between the mouse's body and the researcher's hand, it is deemed as touching.	The time is considered to stop from the frame when the mouse's paw is no longer in contact with the researcher's hand. In instances where multiple paws (up to three) are in contact with the researcher, the time stops from the frame the last paw stops touching the researcher. If the mouse initially has three paws on the researcher and introduces the fourth paw, the behaviour transitions from touching to sitting on the researcher. The time is then stopped from the frame when the fourth paw makes contact with the researcher's hand or arm.
	Nibbling the researcher (state event)	The mouse nibbles at the researcher's hand without there being a reward present.	The time is considered to begin from the frame the mouse opens its mouth to the widest extent before nibbling or biting the researcher's hand or arm.	The time is considered to stop from the frame where the mouse's mouth no longer makes contact with the researcher's hand or arm.
	Sitting on the researcher (state event)	The mouse sits on the researcher's hand or arm with all four paws.	The time is considered to begin from the frame the mouse's fourth paw makes contact with the researcher's hand or arm, provided that the mouse already has three paws in contact.	The time is considered to stop from the frame when one of the four paws no longer makes contact with the researcher and touches the training pad.

	Sitting next to the researcher (state event)	The mouse sits next to the researcher's hand within a distance of half a body length. The mouse does not touch the researcher.	The time is considered to start from the frame when all four of the mouse's paws are touching the training pad. The mouse must be within a distance of half a body length from the researcher. Furthermore, the mouse is permitted to move its front paws and shift sideways with its upper body.	The time is considered to stop from the frame the mouse displays any of the other behaviours outlined in this ethogram, such as sniffing the researcher, touching the researcher, or performing a stretch attend. The time also stops when the mouse lifts either or both of its hind legs.
Risk assessment behaviour	Stretch attend (state event)	The mouse elongates its body, potentially reaching forward with its forepaws. It does not move its hind paws.	The time is considered to begin from the frame the mouse leans forward to elongate its body. If this movement is accompanied by the advancement of its front paws, the time starts from the frame when the first front paw is lifted off the training pad.	The time is considered to stop from the frame the mouse leans back to return to its original position. If the mouse has advanced one of its front paws to achieve the stretch attend, the time stops when the mouse lifts the first front paw off the training pad to retract it. This behaviour transitions into either sitting on the mat or sitting by the researcher, depending on the mouse's location on the pad. The time also stops if the mouse lifts either of its hind paws. If the mouse lifts one of its hind paws and continues to walk, the stretched attend concludes and transitions to a stretched walk from the frame when the mouse lifts the hind paw off the training pad.
	Stretched walk (state event)	The mouse walks forward with its body elongated, lifting its hind paws in an exaggerated fashion.	The time is considered to begin from the frame the mouse lifts its hind paw off the training pad, immediately after executing a stretched attend.	The time is considered to stop from the frame both hind paws touch the training pad and the mouse subsequently ceases walking.
	Rear (state event)	The mouse sits back on its hind paws, lifting both forepaws off the training pad.	The time is considered to begin from the frame when both of the mouse's front paws no longer make contact with the training pad.	The time is considered to stop from the frame when one of the mouse's front paws makes contact with the training pad again.
	Looking over the edge of the table (state event)	The mouse looks over the edge of the table, usually while performing a stretch attend.	The time is considered to begin from the frame the mouse points its snout downward, with its head leaning over the edge of the table.	The time is considered to stop from the frame the mouse lifts its head to align it with its body again.
Fear- and/or stress- related behaviour	Freezing (state event)	The mouse exhibits no movement apart from respiration.	NA	NA
	Flinching (point event)	The mouse makes a sudden, jerky movement.	NA	NA

	Grooming (state event)	The mouse sits back on its hind paws and cleans its snout with its front paws.	The time is considered to start from the frame where both of the mouse's front paws no longer make contact with the training pad and are one frame away from touching the mouse's snout.	The time is considered to stop from the frame where both of the mouse's front paws no longer touch the mouse's snout and are in the process of returning to the training pad.
Other behaviours	Sitting elsewhere on the training pad (state event)	The mouse sits somewhere on the training pad, at least half a body length away from the researcher.	The time is considered to start from the frame where all four of the mouse's paws are touching the training pad. The mouse must be at least half a body length away from the researcher. Furthermore, the mouse is permitted to move its front paws and shift sideways with its upper body.	The time is considered to begin from the frame the mouse displays any of the other behaviours outlined in this ethogram, such performing a stretch attend. The time also stops when the mouse lifts either or both of its hind legs.
	Out of frame (state event)	Out of frame encompasses multiple situations in which mouse behaviour was rendered unobservable due to lack of visibility: - The mouse leaves the training pad with a distance exceeding one body length. - The mouse remains on the training pad, but is in the webcam's blind spot. - The mouse ascends the researcher's arm and exits the frame entirely, including its tail. - The mouse burrows beneath the training pad.	- The time is considered to begin from the frame the mouse is at a distance greater than one body length from the training pad. - If the mouse is on the training pad, but in a blindspot, the time starts from the frame its behaviour is no longer visible. - If the mouse is on the researcher's arm, the time starts from the frame in which the tail of the mouse is no longer visible. - If the mouse burrows under the training pad, the time starts from the frame in which its behaviour is no longer visible.	- The time is considered to stop from the frame in which, after assistance from the researcher, all four of the mouse's paws make contact with the training pad. - If the mouse remains on the training pad, but in a blind spot, time stops from the frame the mouse is observable again and scoring of its behaviour can be resumed. - If the mouse is on the researcher and out of frame, the time stops when, assisted by either the researcher or a third person, all four of the mouse's paws make contact with the training pad. - If the mouse has burrowed under the training pad, the time starts from the frame the mouse is observable again and scoring of its behaviour can be resumed.

Table A4 The expanded ethogram containing 'start' and 'stop' moments. The researcher used these in BORIS to determine the specific video frames corresponding to the initiation and conclusion of the duration of each behaviour. This standardised the scoring process across all mice in the study. Certain behaviours were incorporated into the ethogram; however, they were not observed in any of the mice. Consequently, no specific start and stop frames have been determined for these behaviours, and this is indicated in the ethogram as 'NA' (not applicable). No duration was counted for 'flinching', only the frequency at which it occurred.