Better welfare for mice through handling and training

Research Project Veterinary Medicine University Utrecht

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Table of contents

Abstract

The question to be answered with this study was whether it was possible to train mice to use less restraining during a subcutaneous injection, so that it could ultimately lead to less stress and better well-being for the mice, and a less negative association with the researcher. This was done with female C3H mice by means of clicker training (the bridging stimulus was a dog whistle and the reward peanut butter), the mice were trained through several steps / levels to stand to the right side of the platform and to allow an injection on a platform in a training cage. Attention was paid to the time it took for the mouse to first stand to the right, how much percentage of the time the mice stood to the right side of the platform during training, behaviors (which could be influenced by stress) and contact-seeking behaviors towards the researcher. The study ultimately found that there was more habituation than training, because only touch the hand of the researcher, eating of the reward, the number of times the mouse withdrew and the time it took to inject were significantly different. And there were no differences in time right, behaviors or many contact seeking behaviors with the researcher, which would be affected by training. Thus habituation has caused that an injection could be given with less restraining. This led to a stress reduction and with that an improvement in well-being, and also ensured a less negative association with the researcher.

Introduction

History C3H strain

In this study, laboratory mice of the inbred strain C3H (C3H / HeOuJ) are used. These mice descend from the species *Mus musculus* (in common language the house mouse), which consists of three main subspecies. The laboratory strains originated from several subspecies and the most from the subspecies *M. musculus domesticus* [1-3], but the exact percentage attributed to the subspecies *M. musculus domesticus* differs in a number of studies [4,5].
So that is in short the history of the C3H mouse and how this inbreeding strain originated.

Characteristics C3H strain

A number of characteristics of this strain C3H, and specifically C3H/HeOuJ mice, is that these mice are homozygous for the retinal degeneration 1 mutation (Pde6brd1), which causes the mice to be blind at weaning age [6]. So studies can not work with sight.
In a study by Southwick et al. [7], it is indicated that there is little aggression between individuals of this mouse strain [7]. In another study by Wahlsten et al. [8], it is stated that this also contributes to that the mice of this strain are easy to handle. This was reflected in the fact that the mice showed little evasive behavior during capture and the wrestling while holding was also low, but the squeak of the mice was average [8]. For research it is nice to know that mice are easy to handle, this can be of benefit in comparison to animals that are difficult to handle.

Laboratory use of mice

As is clear from above, the origin of the laboratory mice, including the inbred C3H strain, has a long history. But why are mice actually used as laboratory animals? Mice are used as laboratory animals because they can live and breed in a small area, have a small size and are omnivorous. They can also be handled regularly by humans and have short generation times of between 9-11 weeks. In the lab, mice can breed all year when kept under the right environmental conditions and provided with sufficient food [2]. So because of its properties, the mouse is easy to keep as a laboratory animal.
Besides being easy to house, mice are also used in pre-clinical trials and research because they can serve as a model for human diseases. Mice are popular for these studies because, like humans, they are mammals and genes from mice are for 95% similar to those of humans. Mice can also be used in genetic studies, because genes can easily be manipulated in mice to provide more information about the functioning of certain genes and this genetic modification may be another way of making a model for human disease [9].
Mice have been used as research animals for a century. As a result, a lot of resources and tools have been developed to work with mice in laboratory environments [10]. It is also often the case that the used mice come from one and the same inbred strain. This makes research even more reliable, because there are few genetic differences between the mice of one inbreeding strain, which are also referred to as identical multiples [11-13] (although some form of genetic variation always remains).
Not only are mice being used in all kinds of research, they are also often used in teaching, for example to train future researchers, caretakers or vet students how to handle these animals and to perform actions such as injections.

Stress

It is conceivable that a mouse as a laboratory animal can experience stress. When looking at what exactly stresses mice, there are many things that can cause stress in mice. Mice can become stressed from social stressors, such as isolation or the opposite, crowding, but also from a social defeat (for example, placing a dominant animal in an animal's home cage) [14]. But mice can also get stressed from many things in the environment/laboratory. These things include for instance bright light, noises and smells, but also handling and restraining by researchers because they exist in the social environment of the mice [15,16]. Handling is known to cause stress, because mice get an acute stress response, seen by increasing the heart rate and body temperature, through handling [17]. Repeated restraining can be used to cause chronic stress in rats, which can then lead to depression-like behavior in these animals [18]. Not only handling and restraining can cause stress, but also certain experimental procedures such as injections can cause an acute stress response in mice [19].

Welfare and reduction

When using animals as laboratory animals, the animal must always be taken into account and care must be taken to ensure that the welfare of an animal is not unnecessarily compromised. As described above, a mouse is likely to experience stress as a laboratory animal and from a welfare point of view this should be reduced. This is also included in the law, which states that it must be avoided to let an animal experience unnecessary pain and stress [20]. In a research by Neely et al. [15] it is also stated that reducing the amount of stressors can help to improve the welfare of animals, and here specifically that of mice [15].
It is most important to improve the welfare of laboratory animals, but a second reason why stress is not good in laboratory animals is because it can affect the results of studies [19,21]. It is given by research from Gouveia et al. [21] that the test results of behaviour tests are “better” if the mice have less stress through handling [21]. In this research mice had to distinguish between 2 urinary stimuli in successive experiments. Mice with less stress by the use of the tunnel handling method (described below) had much willingness to explore and investigate test stimuli. This was an advantage in this behaviour test, because it was recorded how long the animals were sniffing/examining the urinary stimulus. While mice picked up by the tail had little willingness [21]. So given that restraining and handling stressed mice, it can be tried to reduce this in the favor of the mouse's welfare and the results of the study.
This certainly also corresponds to the three Rs (replacement, reduction and refinement) that need to be checked for each animal experiment. Replacement stands for the replacement of animals by non-sentient alternatives. Reduction means minimizing the number of animals used. And Refinement indicates that animals used in experiments should experience as little pain and stress as possible [22]. The latter term therefore also includes handling, restraining and injection of laboratory animals, which causes stress and pain. So it would be good to do this in such a way that it causes less stress for the animals.

Non-aversion handling methods

Much research has already been done on reducing restrainment when picking up a mouse and thus looking at the form of handling to make this less stressful for mice. A distinction is made between (non-)aversive methods. A tunnel and a cup (using hands to form a cup) are two non-aversive handle methods, because mice show less anxiety and seek contact with the handler [23] (also after a subcutaneous injection) [24]. While mice handled with the standard

tail handling show aversive reactions towards the handler. Tail handling is thus regarded as an aversive method, and aversion could be significantly reduced with cup or tunnel handling[24]. Several additional studies claim that mice that are handled with non-aversive methods have less stress in comparison with mice handled by the standard tail handling [23-28]. A study by Gouveia et al. [23] showed that some mice interacted more with a home cage tunnel laying already in the cage, then with an external tunnel between cages, but for other mice there was no difference between these two tunnels [23]. In another research from Gouveia et al. [24] it was found that 2 seconds is enough to familiarise mice with the tunnel handling method. For cup handling also brief but more frequent handling than with a tunnel is needed to familiarise mice. If looking at tail handling strong aversion is shown by infrequent and brief handling [24]. So it becomes clear from these studies that tail handling is strongly related to aversive behavior, while familiarise mice with the tunnel handling method is done in no time, can be used as a non-aversive method and is a less stressful method to pick up mice [21].

Training to reduce restraining

Since restrainment is another form of handling that can cause stress, finding ways to reduce the need to fully restrain animals can be an additional way to reduce unnecessary stress during procedures. A potential way to achieve this, is via training. Animals, and thus also mice [29], can be trained to undergo certain actions, such as injections or blood collection, without the frequent use of restraining and thereby having less stress and a better well-being. For example, dogs can be trained to like injections and vaccinations instead of being afraid of them. This can be done with counterconditioning, conditioning is described below, in combination with desensitization. This is mainly done by offering a reward, or things like play and petting, and thereby bringing the animal into a positive state of well-being [30].

There are also many examples of chimpanzees trained by positive reinforcement to perform routine medical actions, such as injections [31-34]. The training focuses on teaching the animal to stick out the body part in question for an injection, so often they are trained to give an arm. These trained animals have been compared to fully restrained animals, with the latter group showing more aggression and fear [33]. That nonhuman primates can be trained to give an arm has been known for some time and has been used for a while [34].

Training has also been used in (other) zoo animals. There is research done to train zebras to be able to give an injection without having to use darts. These darts are not only responsible for physical damage, but also trigger a fight or flight response and create a negative bond between the keeper and the animal. While training the zebras ensured that an injection could be made without restraining or darts, which reduced stress and thus improved the welfare of these animals. So training can also be applied to zoo animals to improve their well-being [35]. So training is often used in animals, but little is known about it in mice. Below, the general principles of training will be described and how they will be applied in this research.

Conditioning

By means of conditioning can animals be trained to do things and in this way an animal can learn new things (for example with training by means of counterconditioning or with positive reinforcement, which are both described above). There are two forms of conditioning, classic conditioning and operant conditioning. The difference would be briefly explained below.

Classic conditioning is an unconscious learning method and established a relation between a stimulus and a response. A neutral stimulus is a stimulus that will not by itself lead to a response (for example, a neutral sound). On the other hand is an unconditioned stimulus a stimulus that would lead to an automatic response (for example, a food reward). In classic conditioning, a link/relation is created between the first neutral stimulus and the unconditioned stimulus (with its automatic response), which finally results in using only the neutral stimulus to gain the automatic response. After using this form of learning, the neutral stimulus becomes the conditioned stimulus that induces the automatic response [36,37].
Operant conditioning is a more conscious learning method. Operant behaviour is, defined by Skinner, behaviour that is controlled by means of consequences (of that behavior) in the form of a reward or a punishment, which are negative and positive reinforcers. Operant conditioning uses intermittent reinforcement and reinforcement schedules. A reinforcement schedule contains a reinforcement for an animal with a good described rule. For example, giving a reward, which is a positive reinforcer, for an animal after it exhibits the desired behaviour the researcher wanted the animal to learn. Reinforcement is often food for an animal [38].

Reinforcement

So in summary, classic conditioning is based on creating associations, while operant conditioning trains to show the desired behavior (or to reduce unwanted behavior). As mentioned above, animals can be trained with negative or positive punishment (negative punishment: remove a positive stimulus to reduce unwanted behavior, positive punishment: applying a negative stimulus to reduce unwanted behavior) or with negative or positive reinforcement (negative reinforcement: removing a (negative) stimulus to reinforce behavior, positive reinforcement: applying a stimulus to reinforce behavior). Several articles have been written about positive and negative reinforcements.
An article about dogs clearly shows that there is a difference in the use of positive and negative reinforcers. It appears that the use of negative reinforcers causes problem behavior and that the dogs listen better when using positive reinforcers. Due to the fact that negative reinforcers cause problem behavior and are therefore a welfare concern, it is recommended to use positive reinforcers [39].
And a review written by Ziv et. al [40] looked at 17 studies on the effects of different training methods in dogs. Here, too, a distinction is made between aversive methods and non-aversive methods. The aversive method includes negative reinforcement and positive punishment, which can endanger the mental and the physical health of dogs. So it is stated that positive reinforcement should be used when training with animals and that the aversive methods should be used as little as possible [40].
Positive reinforcement is also used with nonhuman primates. In fact, a study by Fischer et al. [41] mentions that positive reinforcement is used as a gold standard for training these animals for cognitive tasks such as working memory. This article also showed that this method is a powerful technique [41]. As discussed above, there are also many studies with nonhuman primates that use positive reinforcement to teach the animals to receive an injection without using restraining [31-34]. In contrast, among the nonhuman primates there is also an article that says that if time is a limiting factor, negative reinforcement can be a solution [42]. In this study by Wergård et al. [42], it appears that none of the animals could complete the task trained with positive reinforcement and 10 of the 12 animals trained with negative reinforcement could complete the task within 30 training sessions. There was also no difference in response to

the trainer between the two groups. So this article argues that negative reinforcement can be seen as an alternative training method, especially with limited time [42].
In both articles about dogs and about nonhuman primates, it is clear that the use of positive reinforcement is recommended, because it ensures a better welfare of the animal. Dogs and nonhuman primates are of course not the same as mice. But it never hurts to work with rewards. And perhaps also with the benefits of positive reinforcement instead of negative reinforcement or punishment and its drawbacks.

Clickertraining

Both conditioning and positive reinforcement are reflected in clicker training. Classic conditioning is used to link the clicker to the reward, while operant conditioning is used to teach the mice to show the desired behaviour that must be learned.
Clicker training is also a form of training using positive reinforcement by using a bridging stimulus, often the sound of a click (hence the name 'clickertraining'). This bridging stimulus stands between the behaviour and the reward. The bridging stimulus is a conditioned secondary reinforcer and has been proven that the bridging stimulus strengthens that specific desired behaviour. The bridging stimulus can be given directly after the desired behaviour has been done by the animal and thereafter the reward is given, so no time between the behaviour and giving the reinforcement is lost. Because the bridging stimulus can be precisely given at the moment the desired behaviour is carried out, the animal will know what to do and perform this behaviour more frequently.
Research [43] in which rats were taught by clicker training to change cages when cleaning cages shows that the use of clicker training reduces stress in the rats, because there was less need for direct contact with the researcher/handler and the rat during cage cleaning (normally stressful) once the training was complete. It is also stated that clicker training is easy and quick to learn for rats [43].
Research done by Leidinger et al. [16] shows also that mice learned very quickly (in a week with 5 minutes of practice every day) what the intention was of the clicker protocol and what the researchers were wanting from them. Most of the mice overcame the challenges, for instance following a target stick [16].
Doing clicker training with mice enabled them to have cognitive enrichment and this can enable mice to use these cognitive skills for solving problems and having control over their environment. This gives the mice an extra way to cope towards the surroundings, finally contributing towards their own well-being. The research also shows that during human-mice interactions, trained mice expressed fewer anxiety-related behaviours (for example, squeaking) than non-trained mice, indicating that training might help reduce fear towards humans. This can also increase the welfare of laboratory mice [16].
Mice tend to react curiously towards novel objects, so there is no need to use reinforcements to get the mice to investigate new things. This is useful when mice have to touch or stand on something during clicker training and make this maybe a little bit easier [44].

Current study

As mentioned in the beginning, handling, restraining and performing actions on laboratory animals is very stressful. So the question arises from the University Utrecht and specific from the department Animal in Science and Society whether a protocol can be set up to use less restraining with mice when handling and during the time a subcutaneous injection is given to

ensure that mice have less stress and better well-being. And to see whether training ensures that the animals have a less negative association with the researcher, even after injections, the behavior of the mice towards the researcher is also examined.
Also, if looked further, the result of this study can be used to show students what training does with animals, to reduce stress during the practicals and maybe even motivate students to use training in their studies.
To be able to recognize stress in the mice, it is necessary to know which behaviors can or can not be seen under the influence of stress. The well-known phenomenon of freezing is known to be performed by mice under stress [45,46]. The behaviors defecation [16,47], urination [16], vocalization [16], jumping [48], stretch attend [49] and escape attempt [50,51] can also increase due to the influence of stress [16,47-51]. The behaviors sniffing and grooming are a bit more complicated. Grooming starts by little stress and can be longer by moderate stress, but can be stopped by high levels of stress that elicit freezing [46,52]. So grooming can be seen as a displacement behavior due to stress. But otherwise is grooming just done by the mouse as maintenance behavior and can have nothing to do with stress [52]. Sniffing can also occur due to stress, but is also exploration behavior and therefore does not always have to be performed under stress [53,54]. The rear is a behavior that occurs less often under stress, but it is also exploratory behavior and can therefore also go in two directions [46,49,55]. Food is also consumed less by mice under stress, so not eating the reward would increase under the influence of stress [45]. In the text will be further referred to behaviors when it comes to these above behaviors upon which stress can have an influence.
Answering the research question is done in this research by creating three groups of mice, one group with naive mice (a naive control group with no exposure to the researcher or the training environment - from here on called the ‘naive’ group), one group of mice that is the control group (exposed to the researcher, the training environment and the reward, but without receiving any actual training - from here on called the ‘control’ group) and one group that is trained with clicker training to train the mice to accept a subcutaneous injection in the neck without having to be fully restrained (from here on called the ‘trained’ group). To get an indication whether this type of training can be a way to reduce stress for mice undergoing a subcutaneous injection, there is investigate the mice’s behavioural response to a subcutaneous injection after training, and compare it to the behavioural response of mice that had not received any training (both control groups). If training mice for injections can reduce stress for these animals used in teaching, this can maybe be extended even further to use this protocol for better well-being for other laboratory mice too.

Materials and methods

Animals

The mice used for this study were 30 female C3H mice (C3H / HeOuJ) born on July 15 in 2020. These animals were used at Utrecht University for teaching students how to handle, restrain and perform injections (subcutaneous and intraperitoneal) on these mice, so their use was for educational purposes. The animals were housed in makrolon type 3 cages with five mice in each cage (six cages in total). The cage had the following dimensions: 21 x 37 x 19 cm (l x b x h). Inside the cage, the mice had an orange large tube, a cardboard house, tissues and a small transparent tube hanging from the lid (used for the tunnel handling method). The bedding in the cage consisted of woodchips. The cages were changed once every two weeks. Figure 1 shows one of the home cages as an example.

Figure 1. The home cage of the mice from above.

The mice were housed in the Central Laboratory Animal Research Facility (GDL) of Utrecht University in a room that also houses mice from another strain (Balb/c) and Syrian hamsters (all used for teaching). In the common animal laboratory (GDL), the mice had a circadian rhythm of 12 hours light and 12 hours dark, the light turned on at 07:00. The temperature in the home rooms lied around 22 degrees Celcius and the humidity around 65%. The lid contained a food trough with pellets (Rat/Mouse maintenance, 10 mm, batch no 98268044, Ssniff Spezialdiäten GmbH, DE-59494 Soest) in it and a drinking bottle with the opening facing down, so the animals received water and feed ad libitum.

To mark the mice, they were all individually lifted using the tunnel method. Then they emerged from the back of the tube into the cage, where the tail could be restrained. Then each mouse within a cage could be individually marked on the base of their tail (using permanent marker). The marking was repeated once a week, as the markings would fade over time.

Mice were randomly assigned to their treatments using the excel function RAND (). Mice in the training treatment and the control treatment (exposed to the training cage, the experimenter and the rewards, but not trained) were mixed across cages, so the two

treatments were housed together (2-3 animals from each of the two treatments in one cage, a total of 5 animals per cage) in order to control for potential cage effects. Because naive animals (used only at the end of this study, during the final measurements) had to remain completely naive, they were housed in two separate cages and not mixed with the other treatments.
The order in which animals from the trained group were trained, and the order in which control animals were exposed to the training cage and the other stimuli was also randomized across days. This randomization was done with the same function (RAND ()) in excel.

Methods of habituation, linking and training

The word session is used instead of a day, because the days were not consecutive, but sometimes there was a weekend or a day in between.
All training, linking or habituation sessions were performed in a separate cage of the same size as the home cage and with woodchips as bedding (training cage). The sessions took place in a different room than where the mice were housed and this room was slightly colder, so a heating pad was placed under the training cage. Mice were transported to the training cage using the tunnel handling method involving the transparent plastic tubes (length: 11 cm, diameter: 7.5 cm) that were already present in the mice's home cages (hanging from the cage lid). In the training cage, a self-assembled platform was placed so that the mice were more easily accessible for the injections at the end of the experiment. Figure 2 and 3 show what the training cage looks like, with the platform laying on its side (during the initial habituation phases - see below) and in its final position standing (during the training phases).

The mice from the training group were trained to accept a subcutaneous injection (of saline solution) in the neck without being fully restrained, following the principles of clicker training (so using positive reinforcement) with a bridging stimulus. It was decided to choose a dog whistle as a bridging stimulus, which allows for the trainer to perform procedures (such as injections) with both hands while still being able to use the bridge signal. However, before training could start, the mice were habituated to the experimenter, the training environment, the bridging stimulus and the reward (peanut butter, which the mice already knew from before, as it had been used as a reward after some of the practical lessons for which the animals had been used). The procedures of habituation, linking the bridging stimulus to the reward, and the training protocol are described below.

Habituation

The mice that were used already know the use of the tunnel handling method. Because the mice used in this study were also used for practicals to teach students how to restrain the animals, they might have a negative association with hands and being handled by humans (as restraining is a stressful experience [15,16]). The intention of the habituation part of the protocol was therefore that they get used to the training cage and hands, and had a lowered stress response towards researchers and general handling. This was important, because stress might interfere with training, as mentioned in the introduction.

This was built up very slowly. In the first session, the mice were given the opportunity to sniff the hand of the researcher for 1 minute. Then they were transferred with the tunnel handling method to the training cage (all five mice within one cage were transferred consecutively) and back to the home cage. When they were back in the home cage they were offered a

little peanut butter (which would eventually also be used as a reward during the training sessions) for one minute.
In the second session, the mice were also moved back and forth using the tunnel handling method, but were marked for individual recognition (when transferred to the training cage) and given a minute before and after being transferred to lick peanut butter off the hand of the researcher in the home cage. Because some mice did not eat the peanut butter from the hand of the researcher, some small tufts of peanut butter were smeared on the wall of the home cage to see if all the mice would eat it, to make sure the peanut butter in itself was not an aversive stimulus.
The mice were introduced to the dog whistle (to be used as the bridging stimulus during training) on the third session. The whistle was blown twice in front of the home cages of the mice to assure that the mice would not be startled by the whistle (which would be shown as mice run away to hide or sit still, freezing). Furthermore, the same was done on this session as the session before (session two). During the last minute peanut butter was given on this third session, it was checked whether everyone eated it from the hand/fingers of the researcher.
The mice must also be habituated to the platform. This was done from the fourth session onwards. The mice were again transported to the training cage. There the platform was laying on one side to make sure the animals could reach the platform and explored it. Figure 2 shows the laying platform.

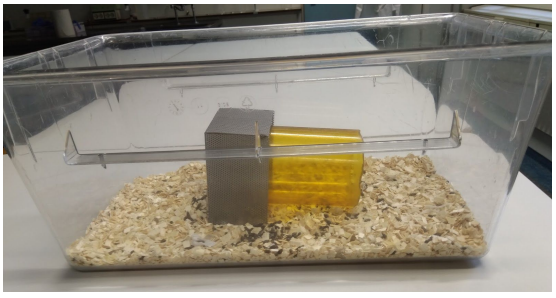


Figure 2. The platform lying on one side from the side.

They were given two minutes to get used to the platform. Then they got five minutes to eat peanut butter. Hereafter the platform was removed and the whistle was whistled twice to assure the mice would also not be startled by the whistle while in the training cage. Afterwards, the mice were returned to their home cage using the tunnel handling method where they were given the opportunity to eat peanut butter for one minute.
The procedure of session 4 was repeated once on session five.

Linking

In session six, the next part of this study was continued, where it was aimed to create a link for the mice between the bridging stimulus (hearing the dog whistle) and getting a reward (peanut butter). Habituation also continued during the linking and training sessions, as the linking and training was done in the training cage.

The mice were transported to the training cage, without the platform this time, by means of the tunnel method. Here, the group with trained mice got to hear the whistle and immediately afterwards offered the reward, which was peanut butter. While the control group just got a reward (also peanut butter) every half minute on the first linking session and every quarter of a minute on the second linking session, because the mice in the control group thus received

a reward about as often on average as the animals from the trained group (this was done to make sure that control mice and trained mice had a similar amount of exposure to the training environment, the peanut butter, and the experimenter, to control for these factors, but without further training the control mice). Two sessions were used to link the bridging stimulus (dog whistle) to the reward. On linking session 2 there were animals that waited after the sound of the whistle, waited for the reward and then walked on. It showed that they understood the association between the bridging stimulus and the reward, but there were also animals that did not have this or did not eat peanut butter. At that time it was decided to continue the training, because the linking would go even further there for the trained animals.

Training

Described here was the part of the protocol that involves the clicker training with the final result with each training session lasting 5 minutes. There were several levels to be taken in this section to end up giving the mice a subcutaneous injection without restraining. These levels are listed one by one below (see table 1). The level of each animal was noted and they were only trained at a higher level if all criteria of the lower level were met. So it could also be that the animals were at different levels.

The first three sessions of the training, the mice were transferred to the standing platform (figure 3 shows the standing platform) in the training cage using the tunnel handling method and were placed to the left. The group with trained mice got to hear the whistle and immediately afterwards offered the peanut butter. While the control group was offered the peanut butter every quarter of a minute and this remained that way throughout the whole training part. Then they were transported back to their home cage and given 2 minutes to eat peanut butter. This was also done every session after training in every home cage for all the mice. After this, the training continued, because 90% of the mice accepted the reward on the platform and understood the link between the whistle and receiving the reward (the animals were waiting after the sound of the whistle for the reward, ate the reward and then walked on). Table 1 shows that all mice were in level 1 at that moment (so only 10% did not meet the conditions of level 1, but was pulled up a level so that the mice could all be trained further from there).

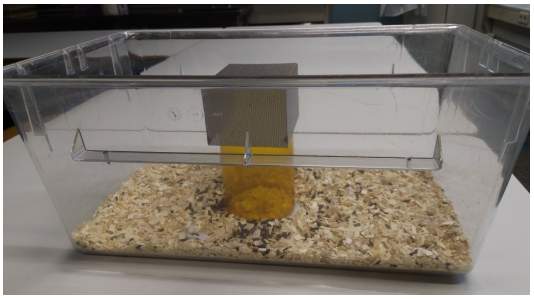


Figure 3. The platform standing from the side.

From here on (session 4) there was also worked with diluted peanut butter (tap water was added to make the peanut butter thinner and less sticky), because some mice sometimes took too much of the reward and subsequently showed choking symptoms. Diluting the peanut butter did not affect the mice’s eagerness to take the reward.

To got the mice to progress from level 1 to level 2 (see table 1), the animals had to stand on the right side of the platform (as the experimenter was right handed, injecting the mice was done from the right) and had to take the reward frequently (20/30 times) on that side. So the following levels were for the mice that took the reward often (around 20-30 times) on the right side of the platform and thus had reached level 2. Each subsequent level was passed if the conditions could be met, for example: level 4 was achieved if the researcher could stroke the mouse. And this went up to level 7, where the mouse could be injected. It was possible that each mouse was at a different level and therefore should receive a different treatment at the same time at this point in the training, because one mouse learned faster than the others.

Levels	***Description***	***Conditions***
Level 1	The mouse is standing on the platform and accepts the reward after hearing the whistle.	- Eating the reward
Level 2	The mouse is standing/facing to the right side of the platform and accepts the reward after hearing the whistle.	- Eating the reward frequently (20/30 times) - Standing/facing right
Level 3	The mouse stands to the right side of the platform and the researcher is able to hold his hand above the head of the mouse (the mouse does not turn away or walk back and remains standing to the right); the mouse accepts the reward after hearing the whistle.	- Eating reward - Standing/facing right - Hand above head - Standing still
Level 4	The mouse stands to the right side of the platform and the researcher is able to stroke the mouse, while the mouse is sitting still; the mouse accepts the reward after hearing the whistle	- Eating reward - Standing/facing right - Stroking - Standing still
Level 5	The mouse stands to the right side of the platform, accepts the reward after hearing the whistle and the researcher is able to grab a fold in the neck, while the mouse is eating the reward.	- Eating reward - Standing/facing right - Fold in neck - Standing still
Level 6	The mouse stands to the right side of the platform, accepts the reward after hearing the whistle and the researcher is able to grab a fold in the neck and hold a syringe (with cap on) against the fold of the neck.	- Standing/facing right - Fold in neck - Syringe against fold - Standing still
Level 7	The mouse stands to the right side of the platform, accepts the reward after hearing the whistle and the researcher is able to inject.	- Standing/facing right - Fold in neck - Injection - Standing still

Table 1. Different training levels and their description.

Injections (‘last measurement’)

After finishing and reaching the final level, the trained animals were injected, along with an equal number of control and naive animals for comparison. The animals were injected in the

fold of the neck with NaCl 0.9% using 1 ml syringes and 30 G needles. All animals except one were injected following the same procedure - by picking up the fold of the neck using index finger and thumb, while the animal was still sitting on the platform and without fully restraining the animal and picking it up. As is described, in only one animal (individual 45A03, which belonged to the control group) it was not possible to pick up a fold in the neck, because the animal kept withdrawing. So it was necessary to fully restrain this animal.
The course of this last measurement looked slightly different than a normal training session. During the last measurement, the animal was placed on the platform as usual. For the trained animals, the animal was injected when a syringe would be placed against the fold of the neck in the previous level, for the control animals an attempt was made to pick up a fold in the neck when they were standing on the right side of the platform and ate the reward and for the naive animals an attempt was made to pick up a fold in the neck if they were on the right side of the platform. After the injection was given, the examiner would place his hand on the platform for one minute. After that time, training was resumed for the trained animals to train them to stand on the right side of the platform and the naive and control animals were offered a reward every quarter minute. In total, this last measurement also took 5 minutes, just like a training session.

Measurements

To measure the effect of the clicker training, the time that it took for the trained mice to walk to the right side of the platform was tracked in each training session from session 6 onwards, using a stopwatch and a voice recorder. The time spent on the right side of the platform (measured during specific sessions, see table 2 for more details), the behaviors (such as ‘grooming’, ‘sniffing , see ethogram - these behaviours were only measured at the beginning of the training period - ‘first measurement’, and at the end, after the final injections - ‘last measurement’ - see table 2 for more details) and the contact seeking behaviors (see ethogram - these behaviours were only measured at the end, after the final injections - ‘last measurement’ - see table 2 for more details), were recorded using a webcam connected to a laptop (see figure 4). The behavior of the mice could then be scored afterwards, using Solomon Coder, version 19.08.02 as a scoring program [56]. See below for more details.

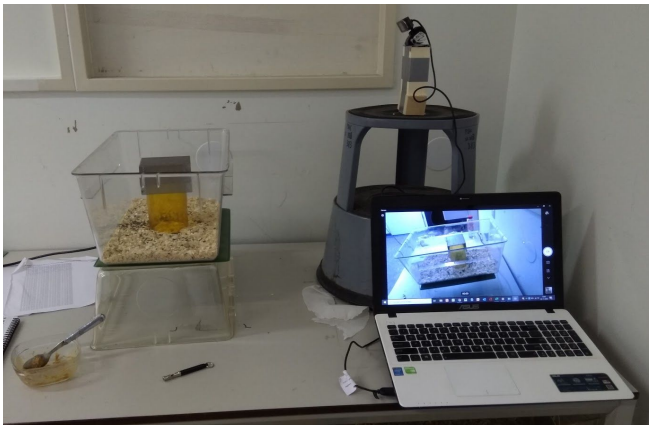


Figure 4. The setup used for filming in this study.

Parameter	***Session/measurement***	***How many animals were measured per treatment***

The time the mice take to stand directly to the right side of the platform	Session 6 Session 7 Session 8 - 19 Session 20 - 24 (depending on when animals reached level 7)	Trained animals: 10 Trained animals: 8 Trained animals: 10 Trained animals: 7
The time the mice stand to the right side of the platform	Session 7 Session 8 Session 9, 13, 16 Session 19 (‘last measurement’) Session 20 Session 24 (‘last measurement’ or last training session)	Trained animals: 8 Control animals: 7 Trained animals: 2 Controle animals: 3 Trained animals: 10 Control animals: 10 Trained animals: 3 (one not filmed) Control animals: 3 Naive animals: 3 Trained animals: 7 Control animals: 7 Trained animals: 7 Control animals: 7 Naive animals: 5
Detailed behavioral scoring, contact seeking excluded	Session 7 (‘first measurement’) Session 8 (‘first measurement’) Session 19 (‘last measurement’) Session 24 (‘last measurement’)	Trained animals: 8 Control animals: 7 Trained animals: 2 Controle animals: 3 Trained animals: 3 (one not filmed) Control animals: 3 Naive animals: 3 Trained animals: 5 Control animals: 5 Naive animals: 5
Contact seeking behaviors	Session 19 (‘last measurement’) Session 24 (‘last measurement’)	Trained animals: 3 (one not filmed) Control animals: 3 Naive animals: 3 Trained animals: 5 Control animals: 5 Naive animals: 5

Table 2. The number of animals per treatment that are measured for a parameter in each session or measurement.

There were a number of sub-questions that were important in this study to answer the research question. Repeating this question briefly: “Can mice be trained to give them an injection without being fully restrained, and whether this ultimately results in less stress and therefore better well-being for the mice, and in a less negative association towards the researcher ”. This will be broken down below in four different parameters, which were used to answer the research question and the results of the sub-questions will be listed one by one in the section Results.

The time it takes for mice to stand on the right side of the platform

Since the trained mice were trained to go to the right side of the platform, it was hypothesised that these mice would continue to move to the right side of the platform faster with each training session. Standing on the right side of the platform was counted when the mouse was standing with its head to the right side of the platform or was standing on the right part of the platform with its head to the right side of the platform, so if they were oriented to the right side of the platform.
The animals were always placed on the platform in the same way, by letting them come out of the tube on the left. As soon as they were out of the tube, it was recorded how long it took the trained mice to stand on the right side of the platform (using a voice recorder). The mice were recorded from session 6 onwards until the last measurement (when the animals received their injection, or simply until their last training session for the three trained animals that did not reach level 7 and therefore did not proceed to the injection phase).

The time the mice are on the right side of the platform

The same applies as above: since the trained mice were trained to go to the right side of the platform, there was hypothesised that these mice would stay on the right side of the platform for a longer amount of time per training session with each consecutive session. In addition, it was expected that over time they would learn to stay on the right side of the platform for longer than the control and naive animals.

The number of times the mice exhibited behaviors

Since the trained mice were trained to receive an injection in a manner in which full restraint would not be necessary, there was hypothesised that these mice would show less or more of the behaviors (as described in the introduction depending on the behavior) between the first and last measurement, and that they would show less or more of the behaviors during the last measurement than the control and naive animals.

The number of times / the time the mice seek contact with the researcher

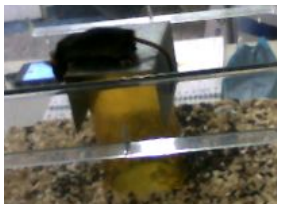
Since the trained mice were trained to receive an injection in a manner in which full restraint would not be necessary, there was hypothesised that these mice would seek more contact with the researcher in the minute after injection during the last measurement than the control and naive animals, as the experience would likely be less stressful for them and aversion towards the handler should be reduced, when compared to control or naive animals.

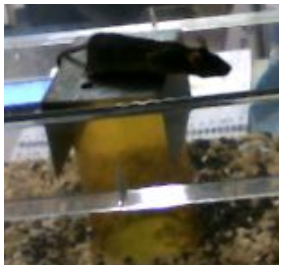
Scoring behaviors

The number of times the mice stood to the right side on the platform was peated with the program solomon, but also the time to the left side of the platform, the time when there was nothing to see (hand for the camera), the total time on the platform and the percentage of the total time on the right side of the platform. This was filmed every Tuesday and on the last measurement.
The number of times the mice exhibited behaviors was peated with the program solomon. The ethogram below (see table 3) shows which behaviors were counted during the first and last measurement (already described in the introduction).
The number of times / the time the mice sought contact with the researcher was peated with the program solomon. This was done by placing the investigator's hand on the platform for 60 seconds after giving the injection and allowing the interaction between investigator and mouse to be observed. The ethogram below (table 3) shows which behaviors were registered. It was also tracked how often the mice retracted and how long it took before the mice could be injected (could be seen if the researcher's hand wanted to start taking the mouse) before the injection was given, because picking up a fold was also done by the naive and control animals which were not trained for this in comparison to the trained animals. These seeking contact behaviors with the researcher were only peated during the last measurement and were compared between the three treatments (naive, control and trained animals).
Table 2 indicates exactly when what was measured.

Ethogram

Behavior categories	***Behavior***	***Expression of the behavior***
Detailed behavioral scoring, contact seeking excluded		
	Freezing	Sitting still, not eating the reward and almost no movement of limbs or head
	Grooming/hair coat	Washing with front legs

	Sniffing platform	Nose on platform
	Rear	Stand on the hind legs
Not visibly happened	Urinating	Pee
	Defecation	Defecate
Not happened	Vocalisation	Squeak
	No feed intake	Not assuming the reward, while it is being offered
Not happened	Jumping	Jump up
	Escape attempt	Lean over the edge of the platform and have its head down

	Stretch attend	Mouse makes itself very tall and has its head stretched out forward
Contact seeking with researcher		
	Sniffle at 0,5 cm of the hand	With nose
	Put a paw on the hand	With paw
	Touch the hand	With nose or body (not paw)
	Jump on the hand	With the whole body

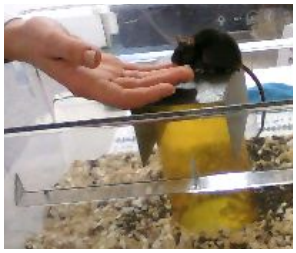
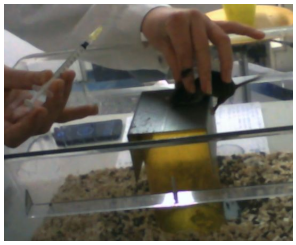
	Eating the reward	Eating the reward of the finger of the researcher's hand
	Pull back	Resisting to be injected and start pulling away
	Time before the mice can be injected	Starting from the moment the researcher's hand wants to start taking the mouse

Table 3. Ethogram of all stress and contact-seeking related behaviors.

Statistics

All statistical analyses done in this study were done using R-studio 4.0.3 [57]. Linear mixed models were done using the lme4 package [58]. Some of the graphs were made using the ggplot2 package of R-studio [59].

Prior to executing models, all data was checked to see whether the data was normally distributed by means of a histogram and Shapiro-Wilk Test. If this was not the case, a log transformation or sqrt transformation attempted to get the data distributed normally. And whether adjustments of data were needed is described with the results. If it was not possible to get the data distributed normally, then non-parametric statistics were used on this data. If it was possible to get the data distributed normally, statistical tests were continued and the model residuals were always checked for normality using QQ-plots and histograms. Which tests were used on the data is described below.

For the time it took for the mice to stand directly on the right side of the platform (the first parameter) a linear mixed model was used to analyse whether the behaviour of the mice changed across sessions. Where the time (in seconds) (the behaviour) was the dependent variable, the factor sessions was the independent variable and mouse ID was included as a random factor (to control for repeated measures). This parameter was only measured in trained animals.

For the time the mice were on the right side of the platform and for the behaviors 'sniffing the platform', 'defecating', 'escape attempt' and 'stretch attend', linear mixed models were used to analyse whether the behaviour of the mice changed over time (across sessions in the case of time spent on the right of the platform, first versus last measurement in the case of

the other behavioral parameters), whether the behaviour differed between trained animals and control animals, and whether the changes in behaviour across sessions or between measurements depended on the treatment that the mice had received. In the models, the behaviour (for example, percentage of time spent on the right side of the platform or defecating) was the dependent variable and treatment, session/measurement and their interaction (treatment*session/measurement) were independent variables. Mouse ID was again included as a random factor (to control for repeated measures). If the models showed no interaction, the model was run again without the interaction to look at the individual effects of session/measurement and treatments.
There was also made use of two Post hoc tests (linear mixed models) for the time the mice are on the right side of the platform to be able to look separately at the sessions per treatment (separate models for control and trained animals) and whether there was a line in it, because there was a trend for interaction in this parameter. This was done by creating different datasets in Rstudio to separate the control and trained animals, where the independent variable was the sessions and with the percentage time as dependent variable. The Bonferroni correction was also used here to correct the critical level of alfa (Bonferroni corrected p-value = $\frac{\alpha\,(original\ p\text{-}value)}{n\,(number\ of\ tests\ performed)} = \frac{0.05}{2} = 0.025$).
For the behaviors ‘freezing’, ‘grooming’ and ‘rear’ four non-parametric Wilcoxon tests were used, because the data was not distributed normally. For each of these behaviors a separate datasheet had been created. In which the difference in behavior between the first and last measurement was calculated (behavior last measurement - behavior first measurement) and named as behavior difference, which, as with the linear models above, was used to analyse whether the behaviour differed between measurements (the difference in behavior was tested against mu = 0), whether the changes in behaviour across sessions depended on the treatment that the mice had received (with the difference in behavior as the dependent variable and treatment as factor) and whether the behaviour within each session differed between the trained animals and the control animals was analysed using the data from each session separately (with the behavior as dependent variable, treatment as factor). The Bonferroni correction was also used here to correct the critical level of alfa (Bonferroni corrected p-value = $\frac{\alpha\,(original\ p\text{-}value)}{n\,(number\ of\ tests\ performed)} = \frac{0.05}{4} = 0.013$).

During the last measurement, when animals received their injections, additional comparisons were made between the behavior of trained animals, control animals and naive animals. Here for the time the mice are on the right side of the platform, for the behaviors ‘sniffing the platform’, ‘defecating’, ‘escape attempt’ and ‘stretch attend’ and the contact seeking behaviors ‘sniffle at 0.5 cm of the hand’, ‘touch the hand’, ‘total time on hand researcher’, ‘total time seeking contact with researcher’ and finally the time it takes before injection a One Way ANOVA was used to analyse whether the behaviour differed between trained, control and naive animals. In the models, the behaviour (for example, percentage of time spent on the right side of the platform or defecating) was the dependent variable and treatment was the independent variable. If an effect of treatment emerged, Post hoc t-tests were used to identify between which treatments there was a significant difference (behavior as dependent variable and treatment as independent variable). The Bonferroni correction was also used here to correct the critical level of alfa (Bonferroni corrected p-value = $\frac{\alpha\,(original\ p\text{-}value)}{n\,(number\ of\ tests\ performed)} = \frac{0.05}{3} = 0.017$).
For the behaviors ‘freezing’, ‘grooming’ and ‘rear’ and for the contact seeking behaviors ‘jump on the hand’, ‘put paw on the hand’, ‘eating the reward’ and ‘pull back’ non-parametric

Kruskal–Wallis tests (data was not distributed normally) were used to analyse whether the behaviour differed between trained, control and naive animals. In the models, the behavior (for example, freezing or pull back) was the dependent variable and treatment was the independent variable. If an effect of treatment emerged, Post hoc Wilcoxon tests were used to identify between which treatments there was a significant difference (behavior as dependent variable and treatment as independent variable). The Bonferroni correction was also used here to correct the critical level of alfa (Bonferroni corrected p-value = $\frac{\alpha\,(original\ p\text{-}value)}{n\,(number\ of\ tests\ performed)} = \frac{0.05}{3} = 0.017$).

No statistics had been done on the behaviors ‘vocalisation’, ‘urinating’ and ‘jumping’, because these behaviors did not occur.
Also, no statistics on the behavior ‘no feed intake’ (see ethogram) had been done, because it could not be kept track of how often the reward was offered during the first and last measurement due to the time limit. As a result of which the outcome of this behavior would have little meaning.

Missing data / results

The first measurement was filmed one session (session 8 instead of 7) later for 5 animals (of which two animals belonged to the trained group and three to the control group) from one cage (2A14), because those animals had been caught and trained incorrectly (treated the trained animals from that group as control animals and the control animals were trained) in the previous session (session 7) and so it was decided to train them properly and then record them in the session afterwards (session 8). It has not been investigated whether this affected these animals because it was so early in the training.

During the last measurement, filming failed for one mouse (individual 35A03, see table 2), which belonged to the trained group. Therefore no data for that mouse is known from the last measurement.
There is also no data on defecation for some mice (individuals 15A03, 25A03, 45A03, 24, 34 and 54A) during the last measurement. These were simply forgotten to count with those animals.
Also, some animals did not reach the final level of training and therefore did not proceed to the last measurements. Because there were tested equal numbers of animals from each treatment group at the last measurement, this means some animals from the control group and the naive group were also excluded from the last measurement. From each of the three treatments two animals were not included (so a total of six animals).
There had also been one mouse (individual 42A18, which belonged to the trained group) where filming during session 24 did not go well and only the last 3 minutes were filmed. Only the percentage on the right side of the platform is included from this mouse, because this mouse did not reach the last measurement and only the last 3 minutes were scored for this animal (behavior and contact seeking behavior is therefore not peated by this individual).

Results

The results of the statistical tests are presented including transformations if necessary. The graphs are a representation of the raw data.

The time it takes for trained mice to move directly to the right side of the platform

A log transformation (Ln) was needed to get the data distributed normally.
A significant difference was found between sessions ($F_{(18, 145.08)} = 1.800$, $p = 0.030$). Namely, there was a significant difference between session 6 and 8 ($t_{(144.728)} = 2.589$, $p = 0.011$), 6 and 9 ($t_{(144.728)} = 2.797$, $p = 0.006$) and 6 and 13 ($t_{(144.728)} = 2.252$, $p = 0.026$). This can also be seen in figure 5, where there are peaks at session 8,9 and 13.

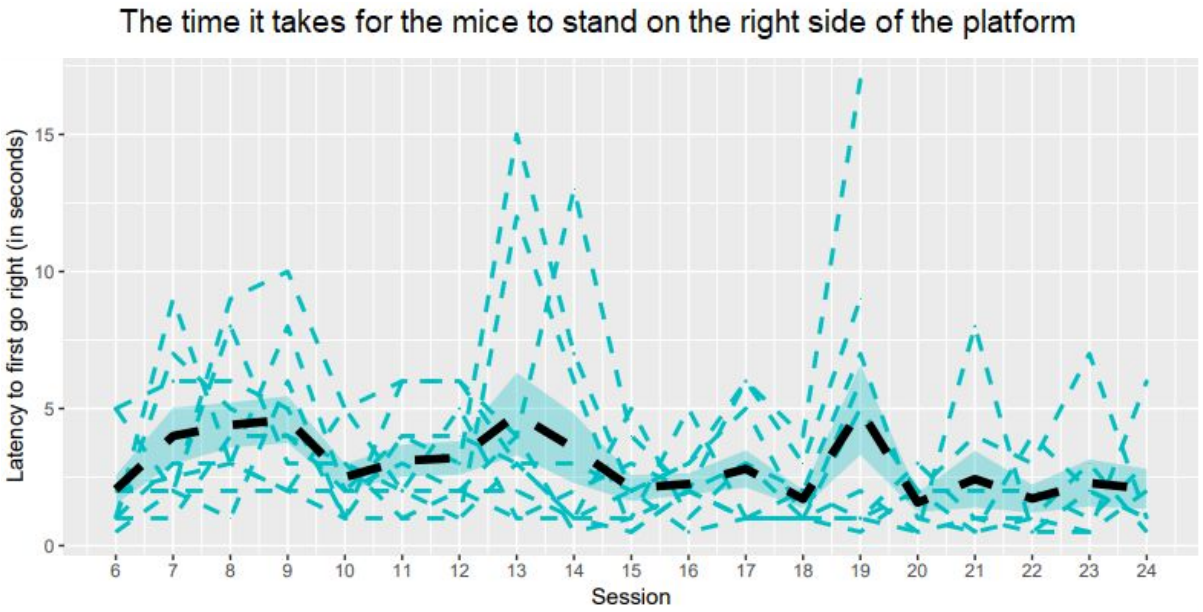


Figure 5. The time it takes for the trained mice to stand directly or for the first time to the right side of the platform every session. With the broken black dotted line the mean of the trained animals and the blue color around it the mean variations. And all loose blue dashed lines are trained individuals.

The percentage of total session time the mice stand on the right side of the platform

Differences between trained and control animals across sessions

The raw data is distributed normally, so no adjustment was needed. There was a trend for an interaction between session and treatment ($F_{(6,69.432)} = 2.107$, $p = 0.063$). With the Post hoc tests (the critical level of alpha in this case was 0.025 due to the bonferroni correction - see methods) a significant effect of session was found for the control animals ($F_{(6, 35.025)} = 3.196$, $p = 0.013$) as well as for the trained animals ($F_{(6, 34.434)} = 6.666$, $p < 0.001$). The control animals stayed around 50% of the time to the right side of the platform with an outlier in one session providing the significant effect. By the trained animals there were multiple sessions where the animals sat longer on the right side of the platform, but there was no upward trend.

Once the interaction was removed from the model, a significant main effect of both treatment: trained mice spent on average 14% of the total session time more on the right side of the platform than control mice ($F_{(1, 16.870)} = 38.517$, $p < 0.001$) and session ($F_{(6, 75.594)} = 6.757$, $p < 0.001$) was found. Namely, there was a significant difference between session 7 and 9 ($t_{(73.858)} = 4.712$, $p < 0.001$). All these effects, both with and without interaction, are shown in figure 6.

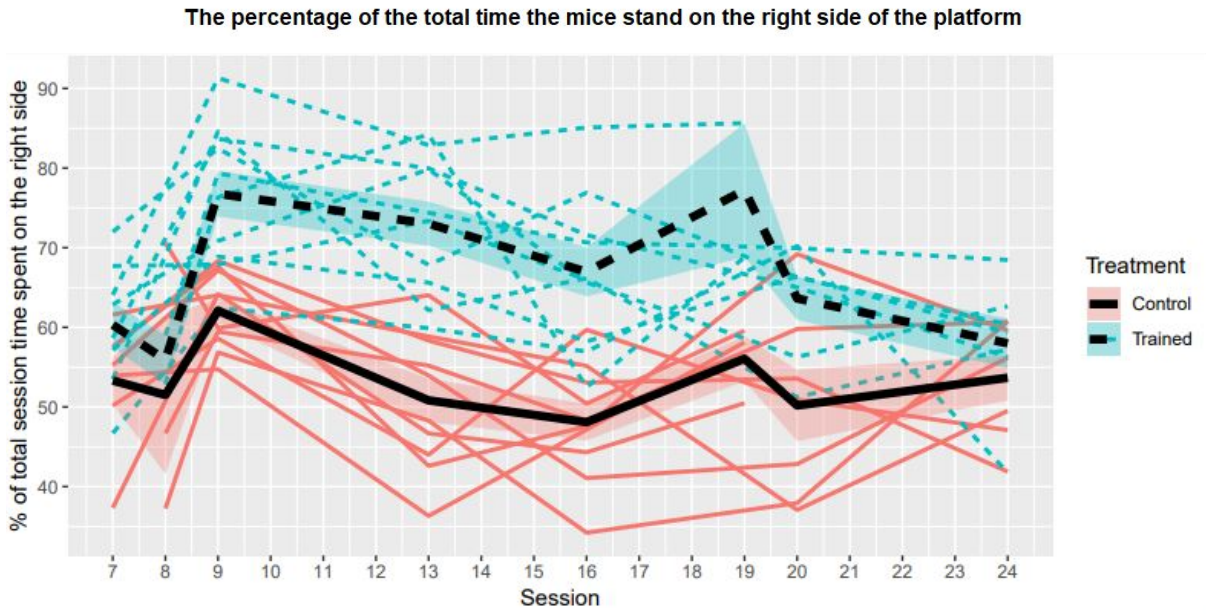


Figure 6. The percentage of the total time the mice stand on the right side of the platform in the five minutes of training between the two treatments over the sessions. With the broken black dotted line the mean of the trained animals and the blue color around it the mean variations. The same applies to the control animals, but with a solid line and the red color around it. And all loose red solid lines are control individuals and all loose blue dashed lines are trained individuals.

Differences between trained, control and naive animals during the last measurement

During the last measurement, where the animals received a subcutaneous injection in the neck, there was no significant difference found between the trained, control and naive animals in the percentage of time they spend on the right side of the platform ($F_{(2, 20)} = 1.478$, $p = 0.252$). This can also be seen in figure 7.

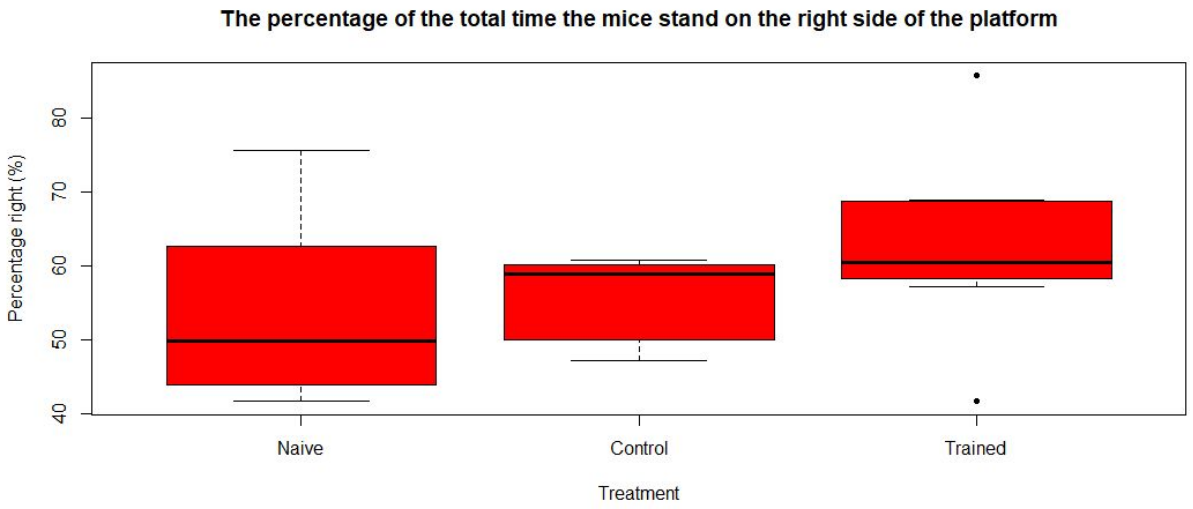


Figure 7. The percentage of the total time the mice stand on the right side of the platform in the five minutes of training between the three treatments during the last measurement.

The number of times the mice exhibited behaviors

Freezing; differences between trained and control animals, the first and the last measurement, and their interaction

There was no significant difference in the number of times the mice were freezing between the first and last measurement (Z = 39.5, p = 0.050, while the critical level of alpha in this case was 0.013 due to the bonferroni correction - see methods) and treatment had no effect on the change in freezing behaviour between the first and the last measurement either (Z = 31.5, p = 0.719). And there was also no significant difference of freezing between the two treatments (Z = 48, p = 0.871 and Z = 32, p = 0.673). This is also reflected in figure 8.

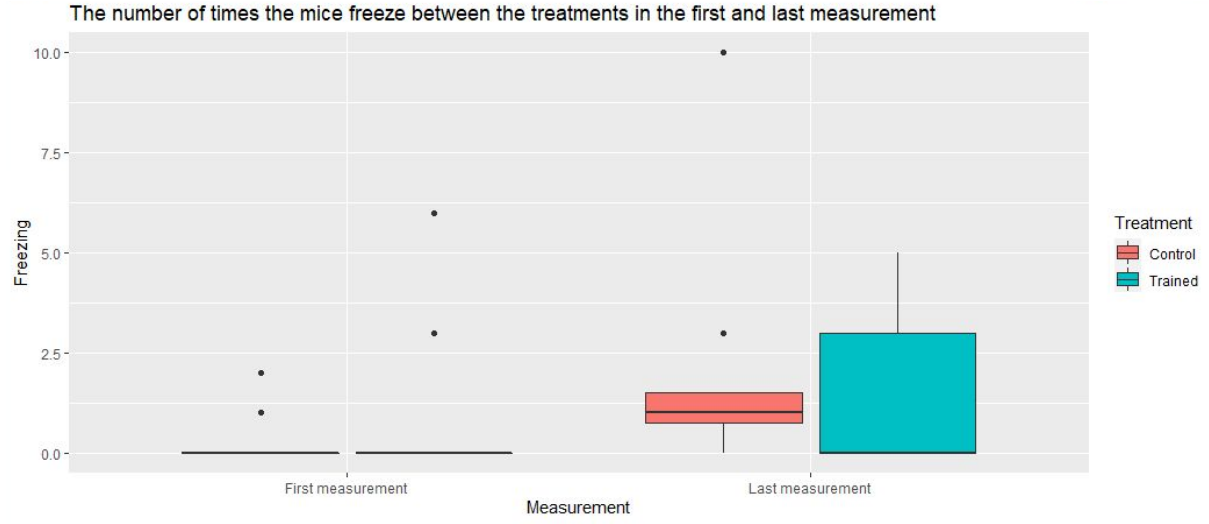


Figure 8. The number of times the mice freeze in the five minutes of training between the two treatments in the first and last measurement.

Grooming; differences between trained and control animals, the first and the last measurement, and their interaction

There was no significant difference in the number of times the mice were grooming between the first and last measurement (Z = 13.5, p = 0.048, while the critical level of alpha in this case was 0.013 due to the bonferroni correction - see methods) and treatment had no effect on the change in grooming behaviour between the first and the last measurement either (Z = 28.5, p = 1). And there was also no significant difference of grooming between the two treatments (Z = 53.5, p = 0.818 and Z = 31, p = 0.769). Figure 9 confirms this once again.

Figure 9. The number of times the mice groom in the five minutes of training between the two treatments in the first and last measurement.

Rear; differences between trained and control animals, the first and the last measurement, and their interaction

There was no significant difference in the number of times the mice performed the rear between the first and last measurement (Z = 11.5, p = 0.034, while the critical level of alpha in this case was 0.013 due to the bonferroni correction - see methods) and treatment had no effect on the change in rear behaviour between the first and the last measurement either (Z = 25, p = 0.771). And there was also no significant difference in performing the rear between the two treatments (Z = 66, p = 0.235 and Z = 29, p = 0.948). This is also shown in figure 10.

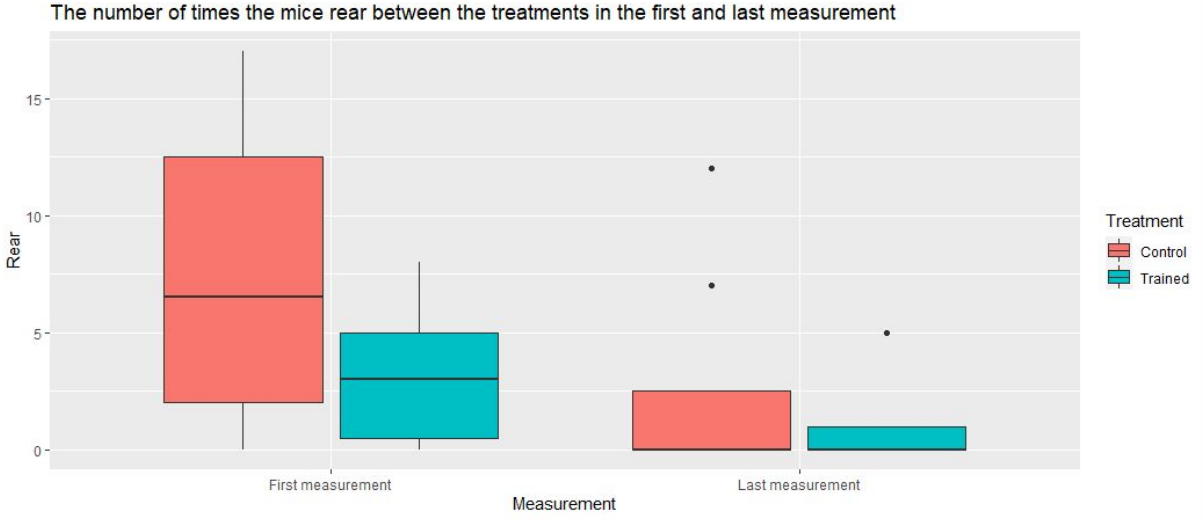


Figure 10. The number of times the mice rear in the five minutes of training between the two treatments in the first and last measurement.

Sniffing the platform; differences between trained and control animals, the first and the last measurement, and their interaction

For the number of times the mice sniffed the platform, normality of the data could be achieved using a log transformation (Ln(number of times sniffing the platform +1)). Subsequent tests showed that there was no interaction between treatment and measurement ($F_{(1, 31)} < 0.001$, $p = 0.991$). Overall the mice sniffed significantly more (on average 7.78 times more) during the last measurement compared to the first measurement ($F_{(1, 32)} = 12.270$, $p = 0.001$, see figure 11), but there was no significant differences between trained and control mice ($F_{(1, 32)} = 1.371$, $p = 0.250$).

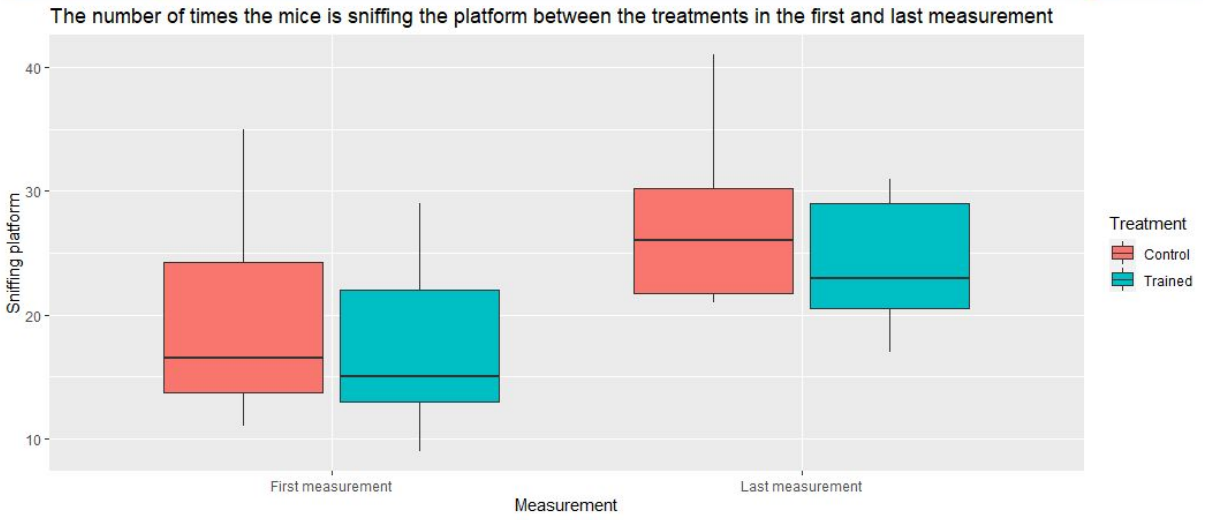


Figure 11. The number of times the mice is sniffing the platform in the five minutes of training between the two treatments in the first and last measurement.

Defecation; differences between trained and control animals, the first and the last measurement, and their interaction

For the number of times the mice defecated, normality of the data could be achieved using a log transformation (Ln(number of times defecation +1)).
Subsequent analysis indicated that there was no interaction between treatment and measurement ($F_{(1, 9.022)} = 2.306$, $p = 0.163$). Overall, mice defecated more (on average 0.95 times more) during the last measurement compared to the first measurement ($F_{(1, 9.638)} = 7.791$, $p = 0.020$) and trained mice defecated more (on average 1.06 times more) than control mice ($F_{(1, 9.732)} = 6.472$, $p = 0.030$). Which can both be seen in figure 12.

Figure 12. The number of times the mice defecate in the five minutes of training between the two treatments in the first and last measurement.

Escape attempt; differences between trained and control animals, the first and the last measurement, and their interaction

The escape attempt data is distributed normally, so no adjustment was needed.
There was no interaction between treatment and measurement ($F_{(1, 16.569)} = 0.097$, $p = 0.760$). Overall, mice were trying to escape less (on average 11.85 times less) during the last measurement compared to the first measurement ($F_{(1, 17.560)} = 13.491$, $p = 0.002$) and trained mice were trying to escape less (on average 10.76 times less) than control mice ($F_{(1, 17.161)} = 9.487$, $p = 0.007$). Which can both be seen in figure 13.

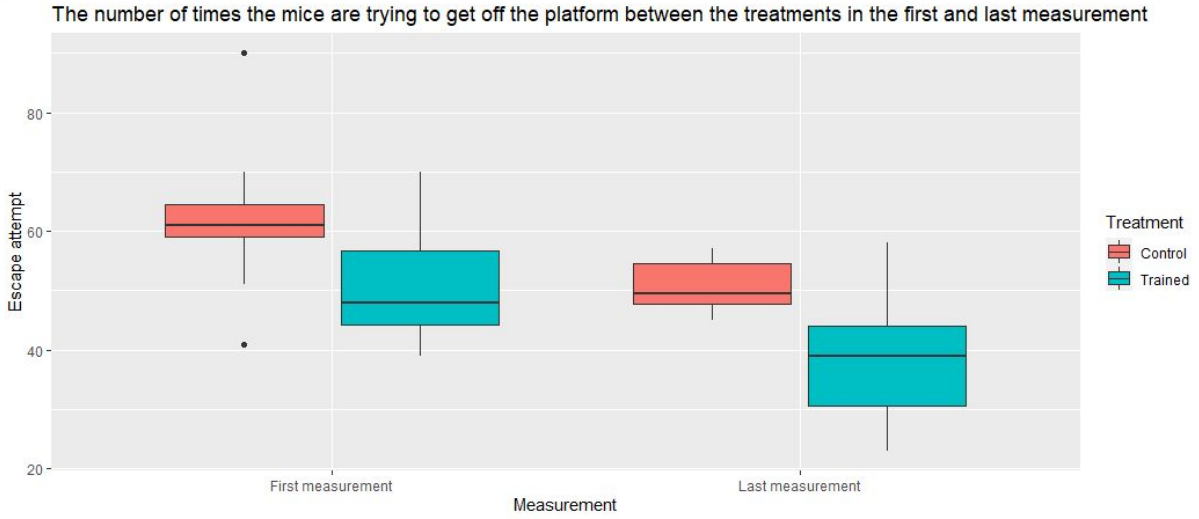


Figure 13. The number of times the mice are trying to get off the platform (escape attempt) in the five minutes of training between the two treatments in the first and last measurement.

Stretch attend; differences between trained and control animals, the first and the last measurement, and their interaction

The stretch attend data is distributed normally, so no adjustment was needed.
There was no interaction between treatment and measurement ($F_{(1, 31)} = 0.003$, $p = 0.955$).
There was no significant difference between the first and the last measurement ($F_{(1, 17.155)} =$

2.464, p = 0.135) and there was no significant difference between treatment, trained and control mice ($F_{(1, 16.355)}$ = 0.363, p = 0.555). Which is confirmed in figure 14.

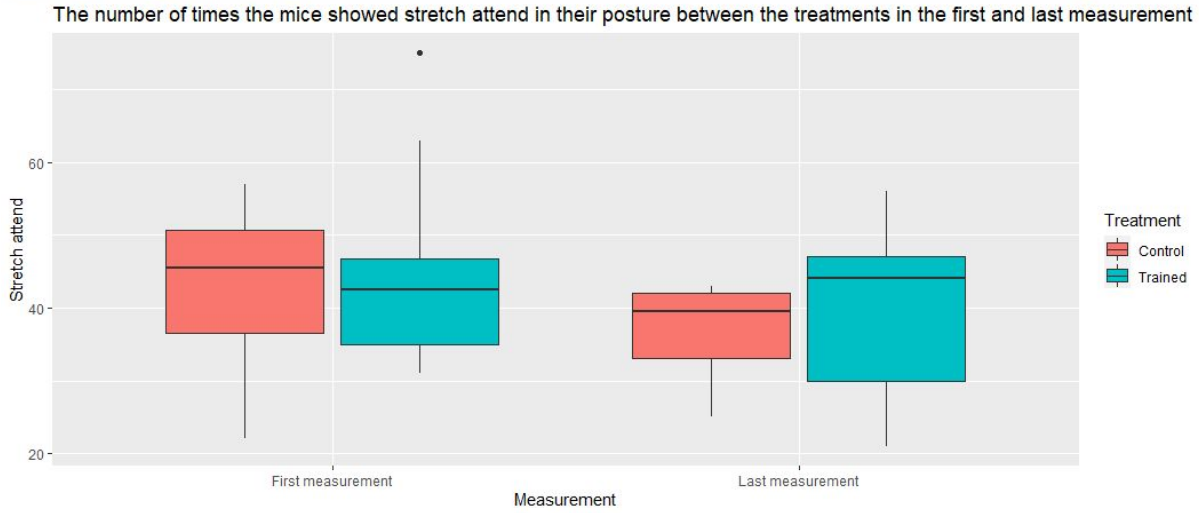


Figure 14. The number of times the mice showed stretch attend in the five minutes of training between the two treatments in the first and last measurement.

Freezing; differences between trained, control and naive animals during the last measurement

There was no significant difference found in the number of times the mice were freezing between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)}$ = 0.865, p = 0.649, while the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods). This is also reflected in figure 15.

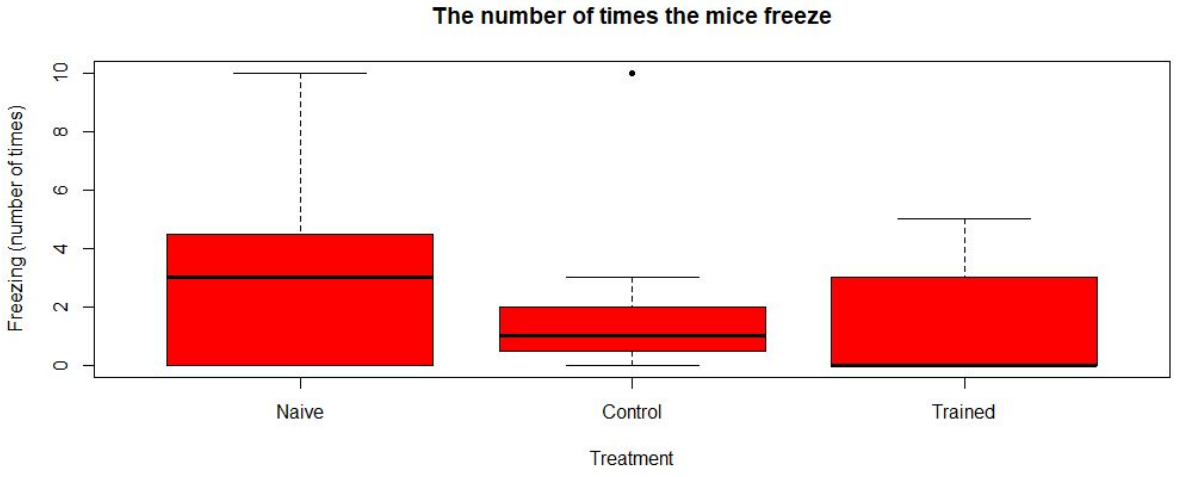


Figure 15. The number of times the mice freeze in the five minutes of training between the three treatments during the last measurement.

Grooming; differences between trained, control and naive animals during the last measurement

There was no significant difference found in the number of times the mice were grooming between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)}$ = 0.546, p = 0.761, while the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods). This is also reflected in figure 16.

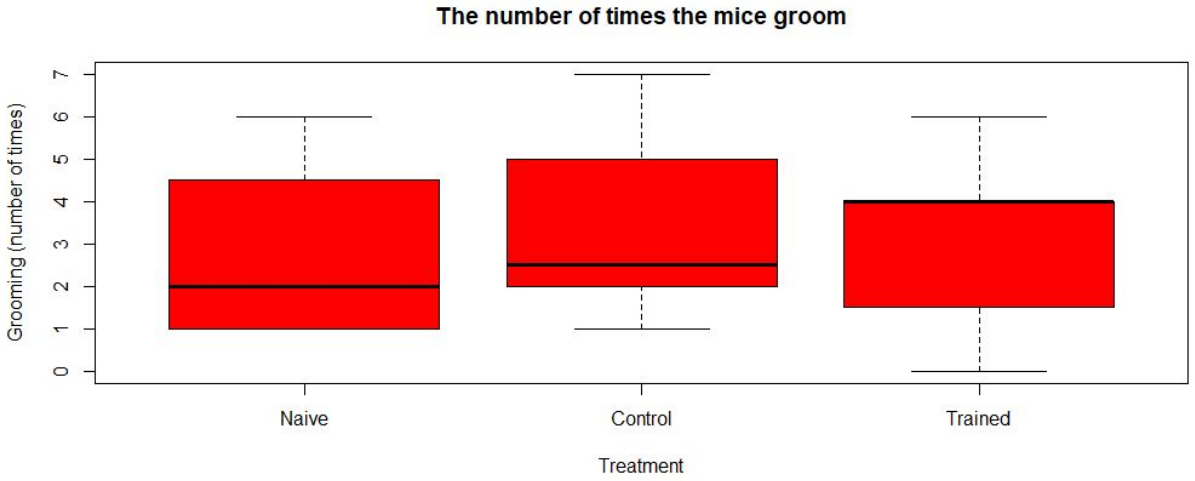


Figure 16. The number of times the mice groom in the five minutes of training between the three treatments during the last measurement.

Rear; differences between trained, control and naive animals during the last measurement

There was no significant difference found in the number of times the mice rear between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)}$ = 0.119, p = 0.942, while the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods). This is also reflected in figure 17.

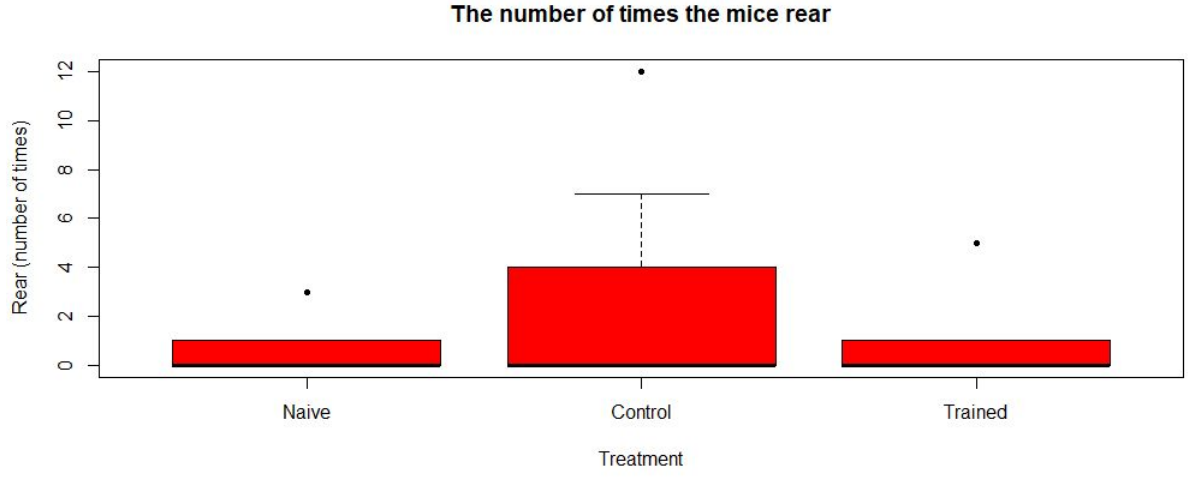


Figure 17. The number of times the mice rear in the five minutes of training between the three treatments during the last measurement.

Sniffing the platform; differences between trained, control and naive animals during the last measurement

For the number of times the mice sniffed the platform, normality of the data could be achieved using a log transformation (Ln(number of times sniffing the platform +1)).
There was no significant difference found in the number of times the mice sniffled the platform between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)}$ = 2.207, p = 0.136). This can also be seen in figure 18.

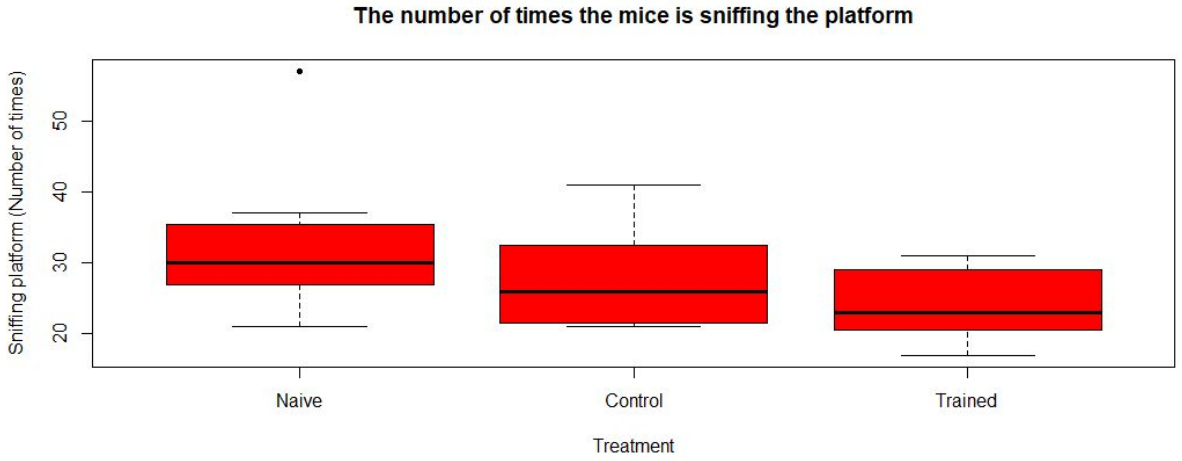


Figure 18. The number of times the mice is sniffing the platform in the five minutes of training between the three treatments during the last measurement.

Defecation; differences between trained, control and naive animals during the last measurement

For the number of times the mice defecated, normality of the data could be achieved using a log transformation (Ln(number of times defecation +1)).

There was no significant difference found in the number of times the mice defecated between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 14)} = 0.276$, $p = 0.763$). This can also be seen in figure 19.

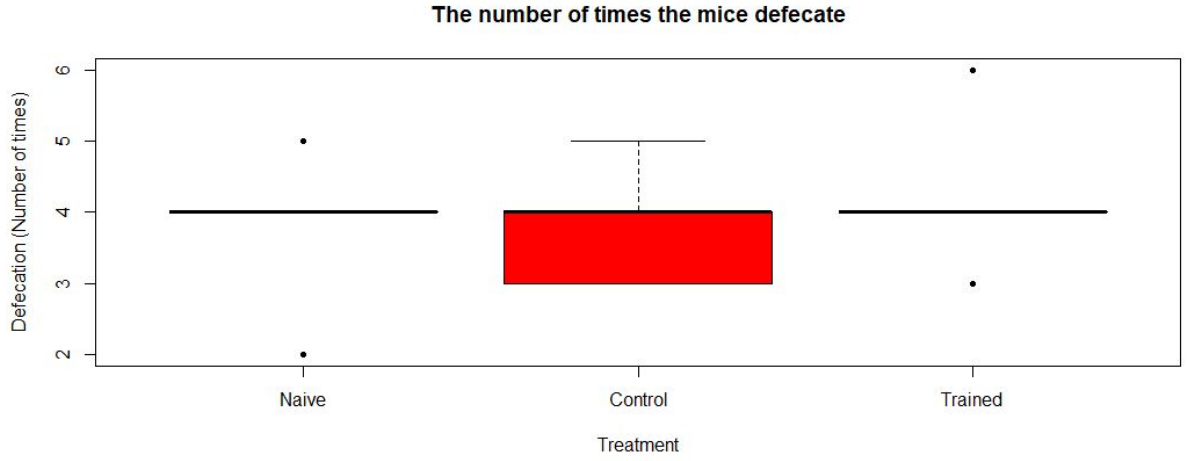


Figure 19. The number of times the mice defecate in the five minutes of training between the three treatments during the last measurement.

Escape attempt; differences between trained, control and naive animals during the last measurement

The escape attempt data is distributed normally, so no adjustment was needed.

There was a trend found in the number of times the mice were trying to escape the platform between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 3{,}256$, $p = 0.060$). But with the post hoc tests (the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods), there were no significant differences between naive and trained mice ($t_{(12.207)} = 2.087$, $p = 0.058$), naive and

control mice ($t_{(7.842)} = 0.594$, $p = 0.569$) and control and trained mice ($t_{(7.437)} = 2.594$, $p = 0.034$). This is made visible in figure 20.

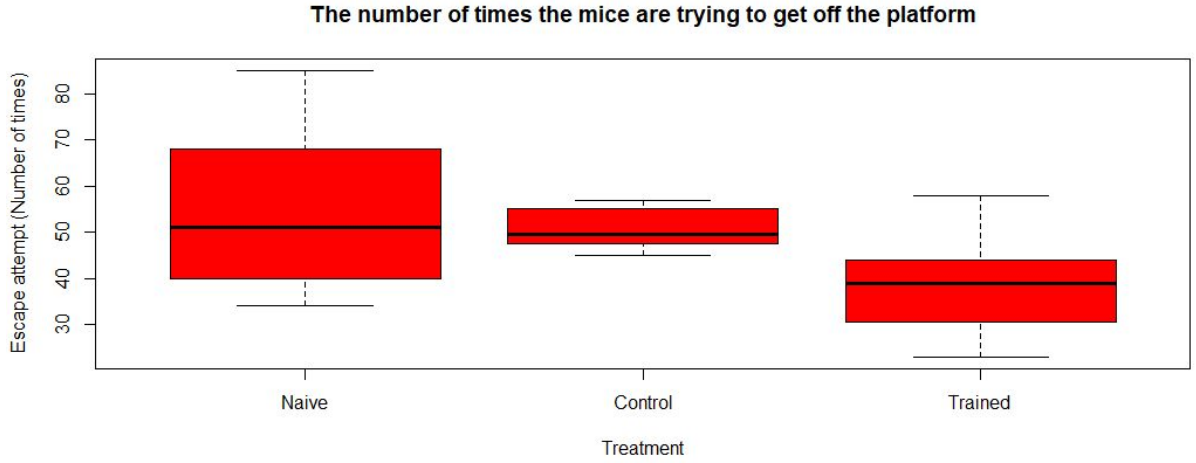


Figure 20. The number of times the mice are trying to get off the platform (escape attempt) in the five minutes of training between the three treatments during the last measurement.

Stretch attend; differences between trained, control and naive animals during the last measurement

The stretch attend data is distributed normally, so no adjustment was needed.
There was no significant difference found in the number of times the mice showed stretch attend between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 0.260$, $p = 0.773$). This can also be seen in figure 21.

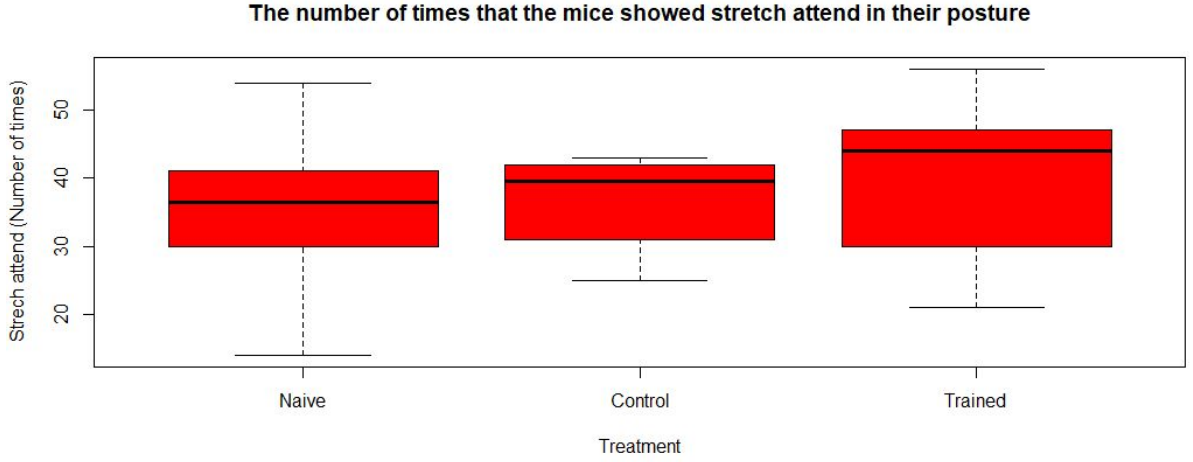


Figure 21. The number of times the mice showed stretch attend in the five minutes of training between the three treatments during the last measurement.

The number of times / the time the mice seek contact with the researcher

Sniffle at 0.5 cm of the hand; differences between trained, control and naive animals during the last measurement

For the number of times the mice sniffled at 0.5 cm of the hand of the researcher, normality of the data could be achieved using a log transformation (Ln(number of times sniffle at 0.5 cm of the hand +1)).

There was no significant difference found in the number of times the mice sniffled at 0.5 cm of the hand of the researcher between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 0.160$, $p = 0.853$). This can also be seen in figure 22.

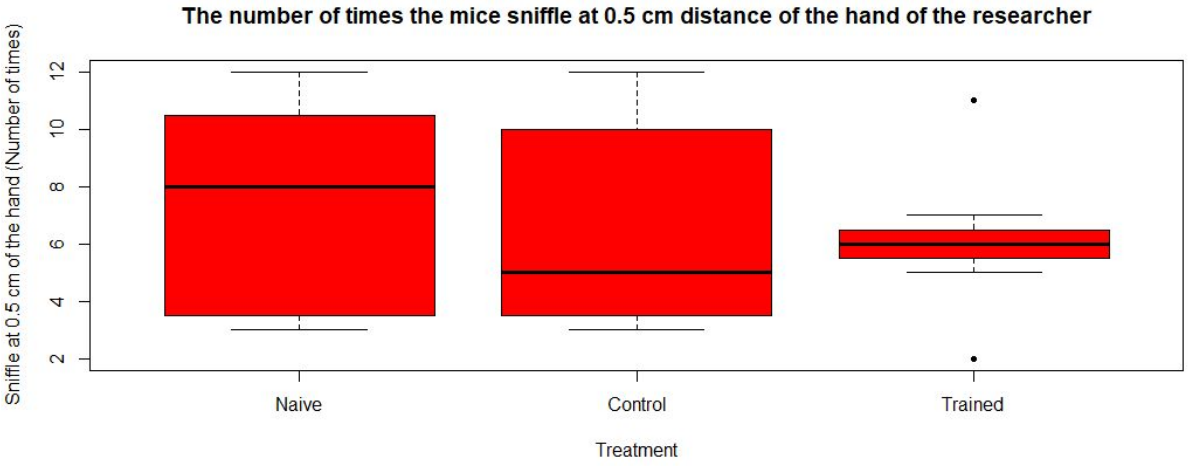


Figure 22. The number of times the mice sniffle at 0.5 cm distance of the hand of the researcher in one minute after injection between the three treatments during the last measurement.

Put a paw on the hand; differences between trained, control and naive animals during the last measurement

There was no significant difference found in the number of times the mice put a paw on the hand of the researcher between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)} = 1.047$, $p = 0.592$). This is also reflected in figure 23.

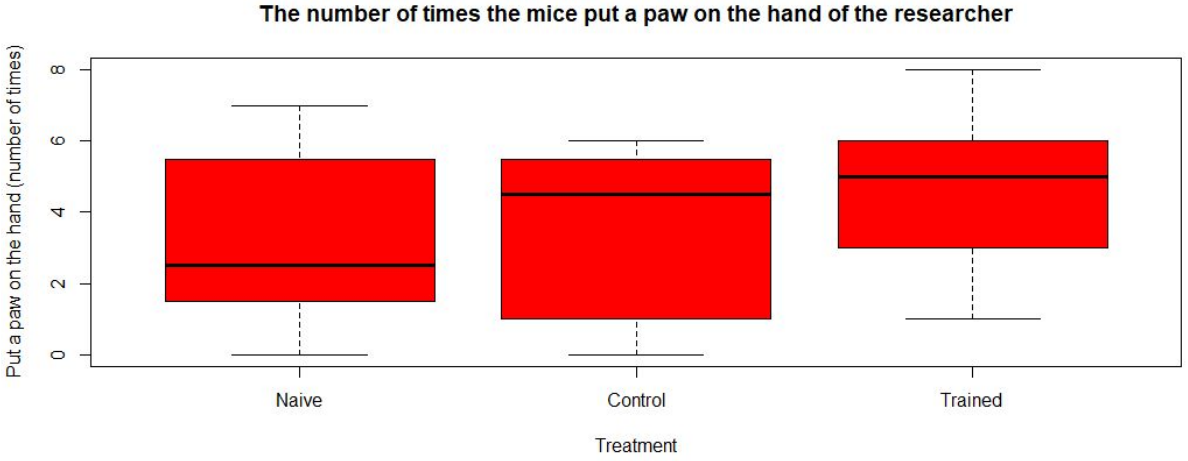


Figure 23. The number of times the mice put a paw on the hand of the researcher in one minute after injection between the three treatments during the last measurement.

Touch the hand; differences between trained, control and naive animals during the last measurement

For the number of times the mice touched the hand of the researcher, normality of the data could be achieved using a sqrt transformation (sqrt(number of times touch the hand +1)). There was a significant difference found in the number of times the mice touched the hand of the researcher between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 6.032$, $p = 0.009$). Post hoc tests (the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods) showed that overall, the trained mice touched the hand of the researcher more (on average 4.70 times more) compared to the naive mice during the last measurement ($t_{(11.825)} = -3.198$, $p = 0.008$), which is seen in figure 24. But there was no significant difference found between naive and control mice ($t_{(11.365)} = -2.045$, $p = 0.065$) and control and trained mice ($t_{(12.553)} = -1.643$, $p = 0.125$).

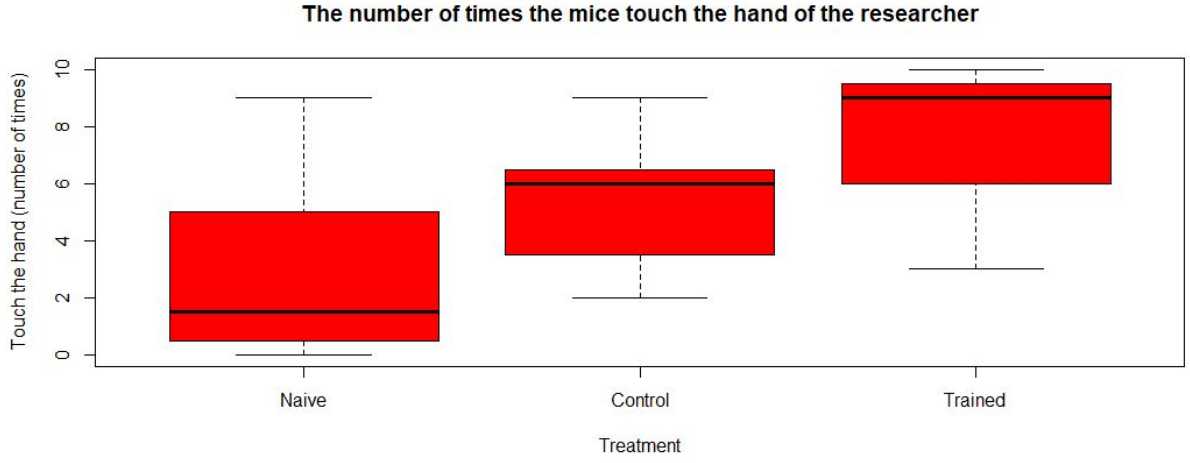


Figure 24. The number of times the mice touch the hand of the researcher in one minute after injection between the three treatments during the last measurement.

Jump on the hand; differences between trained, control and naive animals during the last measurement

There was no significant difference found in the number of times the mice jumped on the hand of the researcher between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)} = 2.477$, $p = 0.290$). This is also reflected in figure 25.

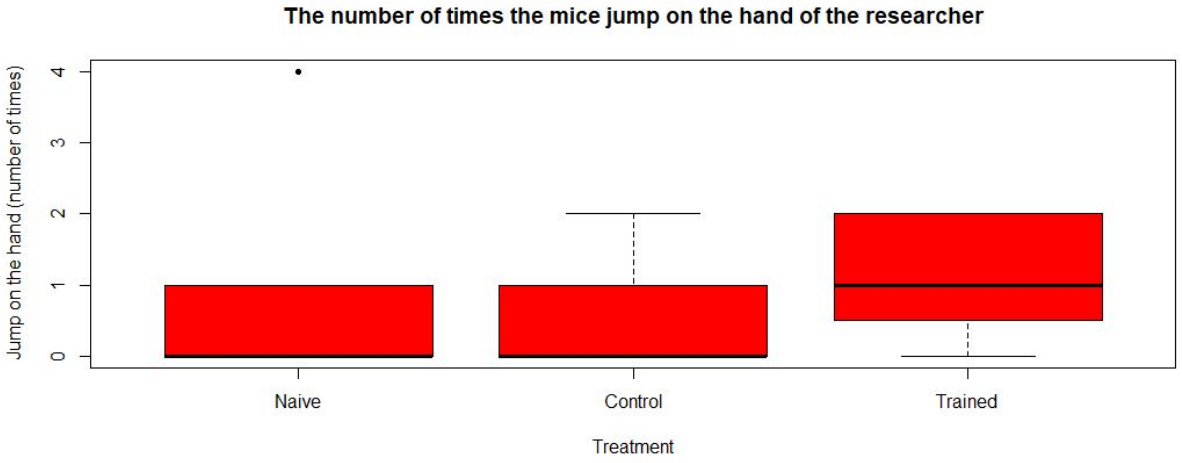


Figure 25. The number of times the mice jump on the hand of the researcher in one minute after injection between the three treatments during the last measurement.

Eating the reward; differences between trained, control and naive animals during the last measurement

There was a significant difference found in the number of times the mice were eating the reward between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)} = 10.81$, $p = 0.004$). Post hoc tests (the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods) showed that overall, the control mice were eating the reward more (on average 1.25 times more) compared to the naive mice during the last measurement ($Z = 12$, $p = 0.013$) and also the trained mice were eating the reward more (on average 2.43 times more) compared to the naive mice during the last measurement ($Z = 4$, $p = 0.002$), which both are also seen in figure 26. But there was no significant difference found between control and trained mice ($Z = 17.5$, $p = 0.236$).

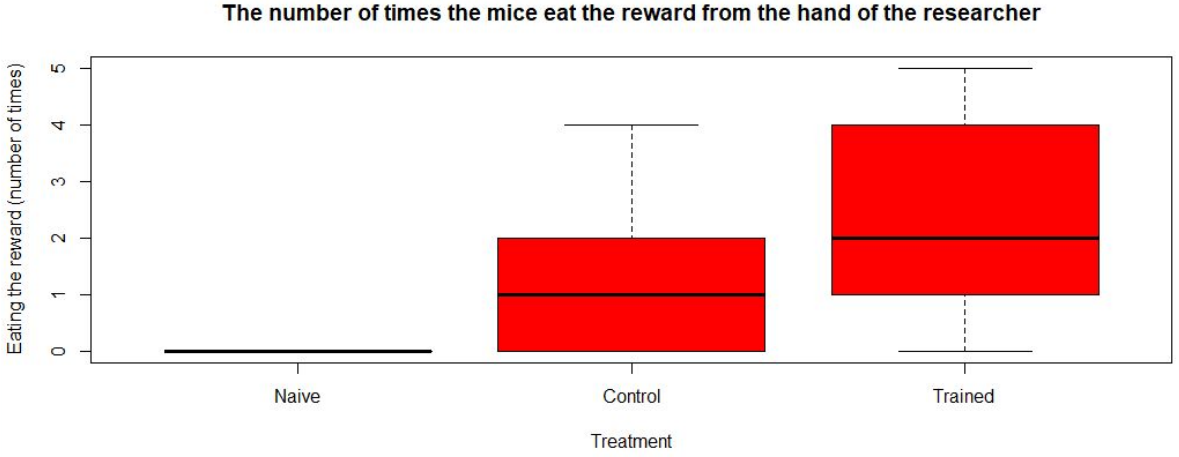


Figure 26. The number of times the mice eat the reward from the hand of the researcher in one minute after injection between the three treatments during the last measurement.

Total time the mice are on the hand; differences between trained, control and naive animals during the last measurement

For the total time the mice were on the hand of the researcher, normality of the data could be achieved using a sqrt transformation (sqrt(total time the mice are on the hand +1)).

There was no significant difference found in the total time the mice were on the hand of the researcher between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 0.097$, $p = 0.908$). This is also shown in figure 27.

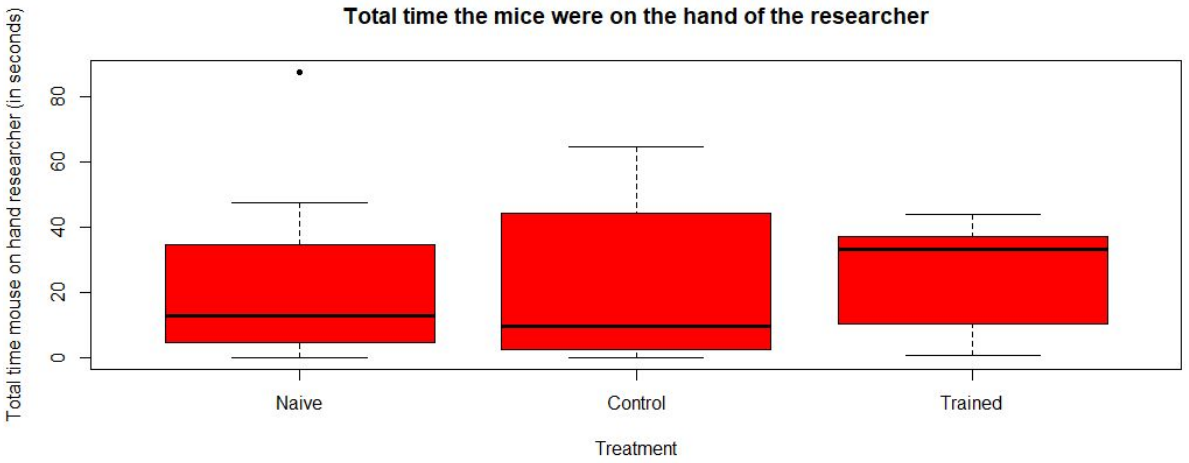


Figure 27. The total time the mice were on the hand of the researcher in one minute after injection between the three treatments during the last measurement.

Total time the mice seek contact; differences between trained, control and naive animals during the last measurement

For the total time the mice seek contact with the researcher, normality of the data could be achieved using a sqrt transformation (sqrt(total time the mice seek contact +1)).
There was no significant difference found in the total time the mice sought contact with the researcher between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 1.473$, $p = 0.253$). This is also shown in figure 28.

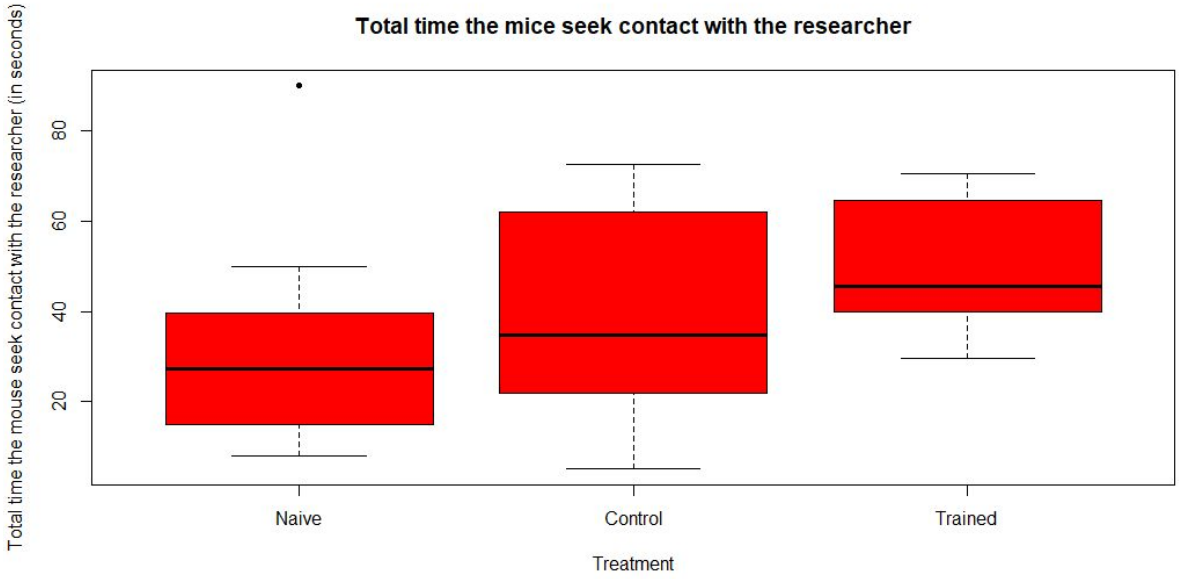


Figure 28. The total time the mice seek contact with the researcher in one minute after injection between the three treatments during the last measurement.

Pull back; differences between trained, control and naive animals during the last measurement

There was a significant difference found in the number of times the mice pulled back between the three treatments (naive, control and trained animals) during the last measurement ($\chi^2_{(2)} = 7.711$, $p = 0.021$). Post hoc tests (the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods) showed that overall, the trained animals pull back less (on average 1.96 times less) compared to the control animals ($Z = 48.5$, $p = 0.015$), which is also seen in figure 29. But there was no significant difference found between the naive and control mice ($Z = 40.5$, $p = 0.396$) and between the naive and trained mice ($Z = 47$, $p = 0.024$).

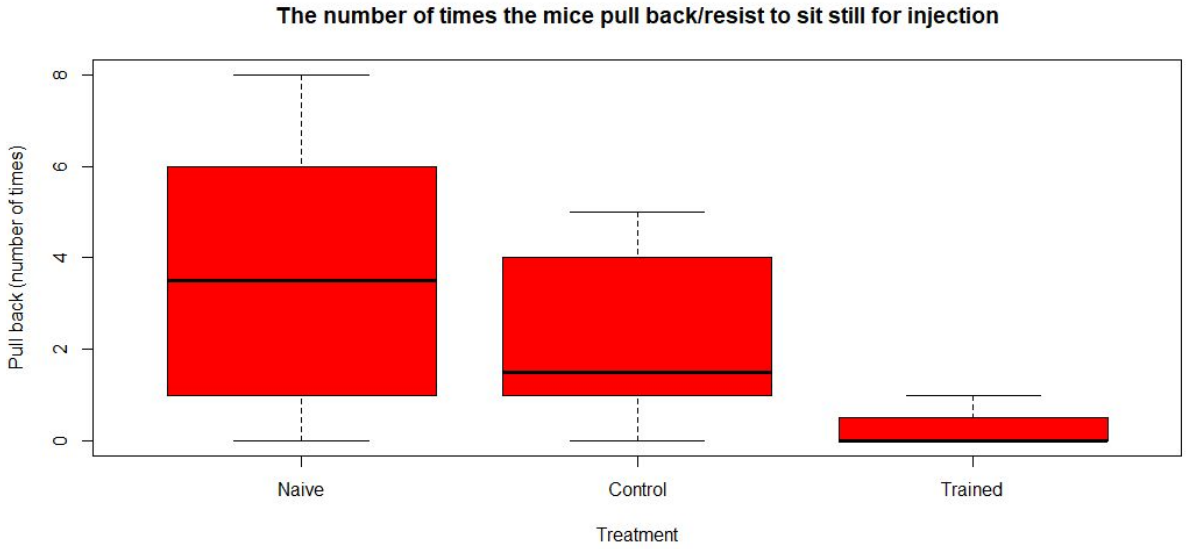


Figure 29. The number of times the mice pull back or resist to sit still before injection between the three treatments during the last measurement.

Time it takes for the mice to be injected; differences between trained, control and naive animals during the last measurement

For the time it takes for the mice to be injected, normality of the data could be achieved using a log transformation (Ln(time it takes for the mice to be injected +1)).
There was a significant difference found in the time it takes for the mice to be injected between the three treatments (naive, control and trained animals) during the last measurement ($F_{(2, 20)} = 4.480$, $p = 0.025$). Post hoc tests (the critical level of alpha in this case was 0.017 due to the bonferroni correction - see methods) showed that overall, the trained animals need less time (on average 29.2 secondes less) to be injected compared to naive animals ($t_{(12.869)} = 3.140$, $p = 0.008$), which is also shown in figure 30. But there were no significant differences between naive and control animals ($t_{(13.857)} = 1.281$, $p = 0.221$) and between control and trained mice ($t_{(13)} = 1.712$, $p = 0.111$).

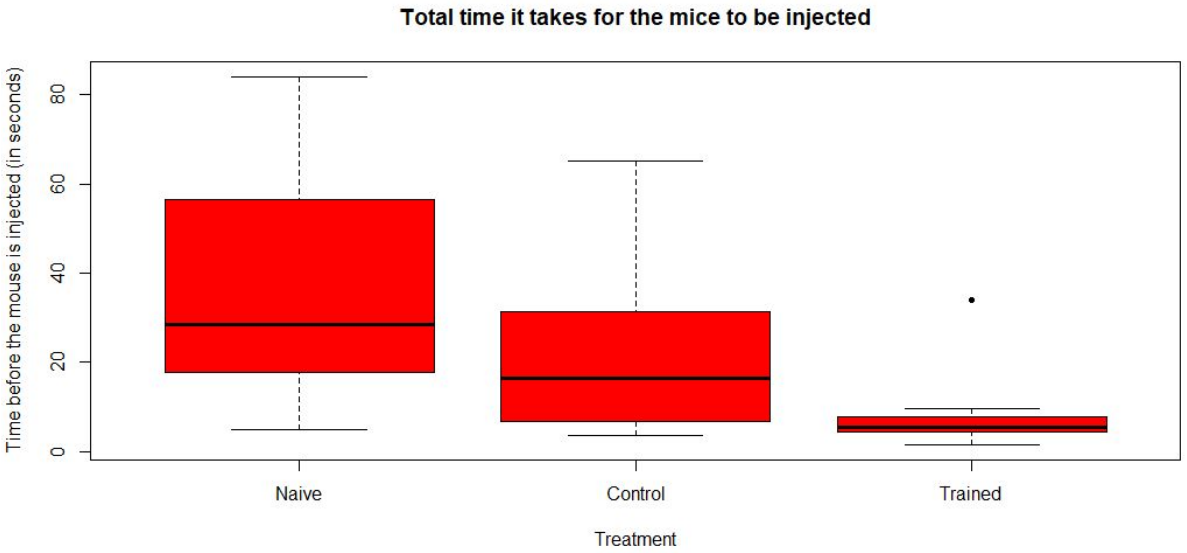


Figure 30. The total time it takes for the mice to be injected between the three treatments during the last measurement.

The data sheets will be enclosed as an attachment, so that they can be viewed.

Discussion

Results

This study was conducted to investigate whether mice could be trained to receive an injection without the use of restraining, which could ultimately reduce stress and improve the welfare of laboratory mice, and could also ensure a less negative association toward the researcher. As described above, this research question was answered by means of four parameters. Here the results will be discussed.

Training to go to the right side of the platform

By means of clicker training the mice were trained to stand to the right side of the platform in the training cage, to stay still while the researcher took the fold of the neck, and placed a subcutaneous injection in the fold of the neck.

The time it takes for the mice to stand directly to the right side of the platform

The first parameter was the time it took for the trained mice to go directly to the right side of the platform (from session 6 to session 19/24) and this could say something about the effect of the clicker training: if the training was successful, the mice would be expected to move to the right side of the platform faster with each consecutive training session [16].

As described in the results, there was a significant difference between sessions in the time it took the trained mice to go to the right side of the platform. However, this change in the latency for the animals to go to the right side of the platform did not follow the expected direction: differences were only found between session 6 and session 8,9 and 13, where the animals spent more time reaching the right side of the platform on the latter sessions. And also when looked closer at the data, every independent session the data went back and forth independently and without a downwards trend across the sessions, while that was expected in advance, should the training be successful. It is therefore unlikely that the differences between sessions were a result of training. The fact that there was no trend in the data did not follow the expected pattern shows that the clicker training had likely not been effective in teaching mice to orient directly towards the right side of the platform. This makes it clear that the mice were not trained for the time at which they move to the right side of the platform. The reason why the mice were not trained to stand directly to the right side of the platform may be because the mice had just been put on the platform and they were stressed, so the first time could always take longer because they had to get used to it again. Or because the mice were blind, so they may not have realized what was the left side of the platform and what was the right side of the platform. Another reason could be that the noise of the bridging stimulus (a dog whistle, which made a loud noise) was also heard in the room where the mice were housed, so that the association that was made in the training disappeared / extincted, because no reward was followed there [60]. This last reason can be solved in the future by providing a soundproof room. These are all hypothetical explanations however, and the real answer is not known.
It is unclear what exactly caused the variation in responses between the different sessions, but something that may have played a role was the changing of the cages. For example, on days after a cage change, animals might have been more stressed at the start of a training session [61] and therefore performed less well [21]. It had not been checked on which days the

cages of the animals were changed and this is something that could be done in the future to explain or rule out such differences.
So overall it is clear that in this study, clicker training has had no effect on how quickly mice moved to the right side of the platform across sessions.

Percentage of time the mice are on the right side of the platform

The second parameter measured was the percentage of the total session time the mice stood on the right side of the platform, as this could also say something about the effect of the clicker training: if the training was successful, trained mice would be expected to stand to the right side of the platform for a longer time with each consecutive training session (session 7/8, session 9, session 13, session 16, session 19, session 20, session 24), and eventually spent more time on the right side of the platform than untrained control animals[16].
There was no interaction between the sessions and the treatment, meaning that the development of how long mice spent on the right side of the platform was not affected by treatment (training versus control), but there were overall significant differences between sessions and treatments.
Trained mice on average spent more time on the right side of the platform than control mice. The overall effect of session was caused by the fact that, during session 9, mice suddenly stood much more to the right side of the platform than on session 7, while in other sessions, this difference was not there anymore. But when looking at the data, there was a lot of variation in time spent on the right side of the platform with every session, ant there was no clear upward trend for this behavior across the sessions for trained animals, while here too it was expected that training should cause the mice to show more of the desired behavior (to stand to the right side of the platform) over time. The variation in behavior across sessions might have been caused by different factors. For example, offering the reward often varied in duration, and the amount of peanut butter offered was not standardized, affecting the time it would take the mice to eat the reward. Mice likely spent more or less time on a spot, depending on how long a reward was offered, or how much of it was offered – even when not eating the reward, investigating the hand of the experimenter might cause mice to stay at a certain spot for the time the reward was offered. It had also been noted that the mice were more often on the side where the researcher himself stood. And there were differences in accepting the reward, because one cage with animals (2A18) accepted much less reward than the other cages from the beginning and therefore stood less to the right side of the platform, while animals that ate the peanut butter well stood much to the right side of the platform. In addition, as with the latency to go to the right side of the platform, on days after cages had been changed, the behavior of the mice might have differed, and hearing the noise of the bridging stimulus outside of the training cage (while standing in the adjacent room before or after training) might have caused the sound of the whistle to not be clearly linked to the reward for the animals. As there was no upward trend in standing on the right side of the platform, it is questionable whether the mice were aware that they had to stand on the right side of the platform, again indicating that the training protocol used in this study might not have been effective for that part.

Finally, a statistical test was also done to see how much percentage of the time the mice were on the right side of the platform between the three treatment groups (naive, control and trained mice) during the last measurement (session 19/24). It was previously expected that the trained mice would stand on the right side of the platform the longest, while the naive and control animals would stand on the right side of the platform about 50% of the time [16].

There was no significant difference found between the treatments, while this would be expected because the trained animals were trained with the clicker training to stand on the right side of the platform. One reason why this happened less during the last measurement may be because the mice then received an injection and during one minute after the injection the hand of the researcher was laying on the platform. So the last measurement looked very different from the other sessions. This change may have caused stress to the mice / to some individuals and can caused some animals to perform less well [62]. To avoid this in the future, there can be chosen to compare the session before the last measurement between the treatments.
Another reason may also have been that the five mice that had their last measurement in session 24 were less well trained / habituated than the three mice that had already their last measurement in session 19. These individual differences in response to the clicker training could be due to differences in intelligence [63,64]. So a subdivision had been made here into better trained animals (three) and less trained animals (five), the latter being in the majority and therefore fewer differences may be visible between the naive, control and trained animals.
Here too it has become clear that no effect of clicker training has been found between the treatments.

So overall there was no effect of training on both standing immediately to the right side of the platform and the percentage of the time that the mice stood on the right side of the platform. The next section discussed the behaviors and the seek contact behaviors in more detail and can perhaps show whether there has been an effect.

Training to experience less stress and seek more contact with the researcher

In this study, the aim was not only to teach the mice to stand to the right side of the platform and to undergo an injection, but also to investigate whether training can help reduce stress and seek contact with the researcher in response to an injection, in case mice were trained to undergo the injection without fully restraining them. Various behaviors have been scored to assess the stress response of the mice and the contact seeking of the mice towards the researcher, the results of which will be discussed below.

The number of times the mice exhibited behaviors

First, each behavior was examined to see whether there was an interaction between the first (session 7/8) and last measurement (session 19/24) and the two treatments (control and trained mice), after which the overall effect of treatment and session was assessed.
It was previously expected that the behaviors would be lessened or would be multiplied (depending on the behavior – see introduction) by training [29], so over the time (between the first and last measurement) and also between the treatments (trained mice versus control mice).

Changes in the behaviors measured in this study over time (the first versus the last measurement) were not affected by treatment (trained versus control animals).
For the behaviors ‘freezing’, ‘grooming’, ‘rearing’ and ‘stretch attend’ there were also no significant differences between the first and last measurement (all treatment groups taken together). This might have partly been caused by the fact that the injection might have

induced some distress in all animals, making a comparison to the first measurement (where no injection was given, but where the introduction to training might have caused some distress) difficult. Here, too, the session before the last measurement could be looked at in the future, but then it was no longer taken into account whether the training, even after the injection, influences the behaviors to be lower or higher than in untrained animals, as described in the literature above [29].
Another reason with the behaviors could be that there was not enough data or that a choice was ultimately made for the control animals to pick up a fold in the neck and only restrain the mice completely when necessary (in only one animal (individual 45A03) the case where it really was not possible to pick up a fold in the neck). As a result, the difference between a non-aversive method (fold in neck) and an aversive method (fully restraining) had not been very clear and it may therefore also be that the data of the behaviors show minimal differences between control and trained animals.
There was also no difference between the control and trained animals for the behaviors 'freezing', 'grooming', 'rearing', 'sniffing the platform' and 'stretch attend'. One reason could be that there has not really been an effect of training (mentioned above), given that no effect on standing on the right side of the platform had occurred. In that case, habituation of the animals to the training environment probably affected their behavior, and this counted for both the trained mice as well as the control mice (because control mice were also exposed to the training environment, the researcher and the rewards – they were simply not specifically trained towards an injection without restraint). So that may have ensured that there was no difference between the control and trained mice either.
Specific to the behavior of 'grooming', there could be no differences because, as described in the introduction, this behavior is a maintenance behavior and a behavior that can be influenced by stress (occur by moderate levels or stop by high levels of stress). That makes grooming so complicated and in this case it was difficult to say anything about it. In order to be able to distinguish these two forms of grooming in the future, attention can be paid to the frequency of grooming, the total time of the number of bouts, disorganized grooming pattern and incomplete bouts, which all should increase due to stressors [65].
For the behavior 'sniffing the platform' was a significant difference found between the first and the last measurement, with the behavior occurring more frequently during the last measurement than during the first measurement. As described in the introduction, this behavior can be performed either under stress or as an exploratory behavior. It is possible that there was more sniffing the platform due to stress during the last measurement because an injection was given then, which causes stress. On the other hand, it may also be the case that the mice had less stress as a result of the training and therefore started sniffing the platform more during the last measurement versus the first measurement due to exploration[53,54]. So this behavior can go both ways, making it difficult to say anything about it.
For the number of times the mice defecated there was also a significant difference between the first and last measurement, with the behavior occurring more often during the last measurement, probably because during the last measurement an injection was given. This caused stress and made the mice defecate more. For the behavior 'defecating' was also a significant difference between the control and trained mice, this showed that the trained animals defecated more than control animals. A reason for this can be that the mice do not like noises [15] and they were stressed every time the bridging stimulus was used (whistle, which had a loud noise) [16,47]. The control animals did not hear the sound of the bridging stimulus and were only offered a reward every quarter minute, so they were not affected by this stressor. It was checked in advance whether the mice were startled by the dog whistle

by looking at their reaction (freezing or running away), then nothing was found. But it may still be the case that the mice did not like the sound.
Only the behavior 'escape attempt' was following the hypothesis. During the last measurement, the number of times that this behavior occurred was a lot lower than during the first measurement. Moreover trained animals showed fewer escape attempts than control animals. Again, these effects were probably due to habituation instead of training, as written above, because the training had no effect on the time the mice were on the right side of the platform and the other behaviors. Habituation had ensured that the mice had become accustomed to the environment and the procedure over time, and the trained mice may also be more habituated than the control mice by going through the different levels of the protocol.

Secondly, each behavior was examined during the last measurement (session 19/24), to see whether there was a difference between the three treatments (naive, control and trained animals). It was previously expected that the behaviors would be lessened or multiplied (depending on the behavior – see introduction) by training [29], so that the trained mice showed the least or the most behavior and the naive mice the opposite, whereby the control mice were somewhere in between the two treatments (naive and trained animals).
However, there was no evidence that training affected the behaviors measured in this study. None of the behaviors showed significant difference between the treatments (only a trend was found for the number of escape attempts, but Post hoc tests were not able to reveal any differences between treatments). Potential explanations might be (as already described above): a lack of data due to low sample sizes (n = 7 trained mice, n = 8 control mice and n = 8 naive mice for the last measurement, only for the behavior defecation it was n = 6 trained mice, n = 6 control mice and n = 5 naive mice for the last measurement) or that the last measurement was different from the other sessions by giving an injection, which caused the mice stress.
Another reason may be that a choice was ultimately made for the naive and control animals to pick up a fold in the neck and only restrain the mice completely when necessary (in only one animal (individual 45A03) the case where it really was not possible to pick up a fold in the neck). As a result, the difference between a non-aversive method (fold in neck) and an aversive method (fully restraining) had not been very clear and it may therefore also be that the data of the behaviors showed minimal differences between naive, control and trained animals.

Finally, though urination almost never occurred and was therefore not statistically analyzed, it was striking that during the last measurement two naive animals urinated while none of the control or trained animals did, which also indicated a stress reduction in these latter animals[16].

The number of times / the time the mice seek contact with the researcher

How long and how often the mice sought contact with the researcher after the final injection was measured to say something about the effect of clicker training and the way of handling and restraining on the reaction of the mice towards the researcher.
To do this, each contact seeking behavior was examined to see whether there was a difference between the three treatments (naive, control and trained animals) during the last measurement (session 19/24). It was previously expected that the contact seeking behaviors would be increased by training, so that the trained mice showed the most contact seeking

behavior and the naive mice the least, whereby the control mice were somewhere in between these two treatments (naive and trained animals) [29].

For ‘touch the hand’ was a significant difference found, but only between the naive and trained mice, whereby the trained mice touched the hand more often than the naive mice. So here it was clear that the trained mice sought more contact with the researcher, even after injection, which was in line with the literature. The control animals needed not differ significantly from the naive and trained animals if they were exactly in between, which would also correspond to what was predicted.
Another behavior with a significant difference was eating the reward – trained mice and control mice ate the peanut butter significantly more often than the naïve animals (they never ate the reward). This might have been caused by the fact that, though the naive mice had eaten peanut butter previously (after some of the practical lessons the animals were used for), they had not been exposed to it as often and as much as the control and trained animals. They were possibly less used to the reward than the trained and control animals. Another reason can be that the naive mice still experienced more stress than the control and trained mice and therefore consumed the reward assumed not at all. There was no difference in eating the reward between the control and trained mice, probably because they were both equally used to being offered the reward.
There was also a significant difference found between treatments for the number of times the mice resisted and the time it took for the mice to be injected, but only between naive and trained mice, whereby the trained mice resisted the injection procedure less, required fewer attempts to inject and could therefore be injected faster than naive mice. This was also expected, because the study had trained these mice to stand still and accept the injection, while the naive mice were not used to anything and were startled by the attempt to pick up the fold of the neck. This also said that the control animals were in between these groups as expected. They were better habituated than naive animals, but not trained, so fell right in between the two other treatments.
Taken together the results showed that there were differences between trained mice and naive mice in their response to the injection, but not much difference between the control and trained mice, further confirming that habituation (which control animals also underwent for some parts – although not for the injection procedure) played a large role in these responses, in addition to training for the injection procedure itself.

The importance of habituation

As already mentioned, the clicker training was not successful to train the animals to stand to the right side of the platform and stayed there to receive an injection, so habituation of the animals to the training environment and procedure had probably affected their behavior. This was consistent with the data of the behaviors in which there were often no differences between the control and trained mice due to this habituation, while there were sometimes differences between the naive mice (not habituated) compared to the control and trained animals (both habituated).
However, although the clicker training might not have been completely effective, the habituation that resulted from the training procedures by breaking up the injection procedure eventually did result in the mice undergoing an injection without having to be fully restrained and in a quick and easy manner. Afterwards, the ‘habituated’ animals (the trained animals and the control animals) showed more contact seeking behavior towards the researcher than

naive animals, indicating that the naive animals were likely avoiding the researcher after the injection procedure, while habituated animals did not. This might indicate that simply habituating the mice properly might reduce the need for restraining and might therefore lead to stress reduction and thus an improvement in well-being.

Limitations

One of the limitations of this research was the small sample size. A trade-off had been made in advance between the number of animals used and the time it took to train them. Due to the small number of animals used and the large variation in the data, it was sometimes difficult to be able to say something about the measured values. In order to be able to say more about the effect of clicker training and performed behaviors, it might be helpful to use more animals. So this study can now be seen more as a pilot in which there was explored some of the possibilities of training mice, but the study in itself could not say much about the effects (except the mentioned advantages of habituation).

Peanut butter was used as a reward in the study. This had to be diluted to prevent choking symptoms in the mice. This however was not standardized, which could have been done by mixing the exact same amount of peanut butter and water every day, so that the dilution is always the same.
Also the amount of peanut butter that was offered as a reward each time was not standardized, which meant that the amount of peanut butter the mice received varied per reward moment. As mentioned, larger rewards will take longer to eat and influence the amount of time spent in the location where the mice received the reward (the right side of the platform for trained mice, random locations on the platform for control mice). This could be avoided by standardizing the amount of reward offered, by weighing the reward and offering an exact amount of peanut butter each time. A standardized time in which mice were allowed to eat the reward of, for example, 2 seconds could also be used for this to keep this the same for every mouse.

Sometimes when the reward was offered, the mouse was more concerned with examining the researcher's hand than eating the reward. Again, it is good to have a standardized time of, for example, 2 seconds in which the reward is offered. If no attention is paid to the reward within this time, the reward will be removed again. This way, a mouse can be prevented from standing on one side for longer during follow-up research
It had also been noted that the mice moved more to the side where the researcher was standing. So this can be prevented in the future by just standing in the middle and only if the reward is given, for example, give the reward on the side where the mouse is currently standing for the control mice. This can also be standardized by placing a dot on the floor where the researcher should stand.

There was made use of a platform, which at the top consisted of a bent square made of iron with small holes in it. When the mice were stressed, it could sometimes happen that they got stuck in the holes with their nails on the side. The researcher then had to help release them. This has of course been stressful and perhaps painful. The iron was also quite cold, which may not have been very pleasant for the mice. During the training it was also visible that the mice continued to find the platform frightening (performing behaviors and not eating the reward on the platform, while the reward was eaten by everyone in the home cage). All

those things may also have affected the training, so it would be better to solve these things in future research. It could be possible to solve these three things in one time by means of a vetbed (described by RISE (Research Institutes of Sweden) in their video [29]) on the platform (a soft piece of fabric). This vetbed could already be used during the habituation, making it familiar to the mice. As a result, the mice are no longer stuck with their nails in the holes, the iron no longer feels cold and the mice may be less stressed because the vetbed already looks familiar to them and they have a nice association with it [29].

It was also noted that in training, the mice sometimes never accept the reward again after 2.5 / 3 minutes. It seemed like the concentration arc was no longer than that, because they did very well before that time and became much busier and more restless after this time (so probably not saturated which could otherwise be a reason). This can be addressed in follow-up research by having a training session lasting only 2.5 minutes or 5 minutes where 30 seconds of training is alternated with a 15 second break [16].

As is described above, the clicker training had not really an effect on the time it took the mice to go directly to the right side of the platform and on the time the mice stood on the right side of the platform. This may be because the association may not have been complete / correct in the beginning between the reward and the bridging stimulus (dog whistle), so that if there had been a stronger association in the beginning, the mice could be trained more. Other reasons could be caused by the environment or the trainer. But also because the reinforcer was too weak, so a solution is to make it stronger and therefore look for things that the mice like even more and want to work harder for. It was also possible that the speed of the reinforcement is too low, so the number of times a reward could be given because the desired behavior was performed. This may be because the task was too difficult. The aim is to try to click 10-15 times per minute. So if the behavior is too difficult, it should still be broken down further into smaller steps during the training [66].
If the behavior occurs more often than 10x after each other, it can also help to reward only when the mouse shows the behavior twice in a row. And also the use of a super reward at the end of the training can improve clicker training [16]. These things could help to actually train the animal the next time instead of habituating it.

Furthermore, it was difficult to count the number of times the mouse was defecating. So this was solved to count the number of poops that were on the platform. This may not have always been correct because it was also necessary to keep track of how often the mouse accepted the reward, so sometimes it was difficult to remember both. And another reason was because the mouse could also be at the edge of the platform, so that the poo fell right on the ground instead of first on the platform. A solution for this can be to use a different camera set-up in the future, because the mice were filmed with a set-up with one camera. With this camera set-up it was difficult to see if the mice were defecating, but also the behavior the mice performed on the left side or the behavior if the investigator's hand was in front of it. So there could be made use of a setup where there are 2 cameras (one on the right and one on the left or one on the right and one camera from above).

Recommendations future research

Based on the limitation, which is described above, here a brief description of the recommendations for further research is described.

- The use a greater number of animals
- Standardization of the peanut butter dilution, how much peanut butter offered and the time to consumed the peanut butter
- The use of a vetbed on top of the platform [29]
- Standardization of how long the reward is offered to the mice each time a reward is given
- Standardizing the training setup even more: for example by positioning the experimenter on exactly the same location in relation to the training cage
- The use of a different camera set-up to make more detailed behavioural observations possible (e.g. score defecation)
- Shorten the training time or insert breaks [16]
- Use a stronger reinforcement and divide the behaviors into even smaller steps [66]
- In case of frequent occurrence of the behavior, reward only when showing the behavior twice and give a super reward at the end of the training [16]

Conclusion

There was no clear effect of the clicker training, which was aimed at training the mice to stand to the right side of the platform and stay there to undergo a subcutaneous injection in the neck. There were also little/no differences between the control and trained animals for the behaviors and the contact seeking behaviors, which might indicate that the trained animals are more habituated to the procedure instead of actually trained. As a result of this habituation, the trained animals touched the hand of the researcher more often after the injection, ate the reward more often, pulled back less during the injection procedure, took less time to inject and did not urinate during the last measurement compared to naive animals. Thus, mice from the trained group allowed an injection without restraining, likely due to habituation to the procedure. So simply habituating the mice might reduce the need for restraining and might therefore lead to stress reduction and thus an improvement in well-being. And also ensures a less negative association with the researcher. It could also not be ruled out with this study that training does not work in mice, but by taking the above recommendations into account, hopefully, in a follow-up study, the training protocol used in this study can be further refined and optimized, so that the additional effects of training on ease of handling and injecting, and subsequently on indicators of stress can be investigated.

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Appendix 1

The time it takes for the mice to stand directly to the right side of the platform

Session	Date	Time (seconds)	Mouse	Group	Individual	Treatment	Level
6	23-11-2020	2	4	2A 18	42A 18	2	1
6	23-11-2020	0.5	1	2A 18	12A 18	2	1
6	23-11-2020	1	2	2A 18	22A 18	2	1
6	23-11-2020	1	3	5A 14	35A 14	2	1
6	23-11-2020	5	2	5A 14	25A 14	2	1
6	23-11-2020	1	4	5A 14	45A 14	2	1
6	23-11-2020	2	5	2A 14	52A 14	2	1
6	23-11-2020	2	1	2A 14	12A 14	2	1
6	23-11-2020	5	3	5A 03	35A 03	2	1
6	23-11-2020	1	1	5A 03	15A 03	2	1
7	24-11-2020	2	4	2A 18	42A 18	2	1
7	24-11-2020	2	1	2A 18	12A 18	2	1
7	24-11-2020	3	2	2A 18	22A 18	2	1
7	24-11-2020	6	3	5A 03	35A 03	2	1
7	24-11-2020	7	1	5A 03	15A 03	2	1
7	24-11-2020		5	2A 14	52A 14	2	1
7	24-11-2020		1	2A 14	12A 14	2	1
7	24-11-2020	9	3	5A 14	35A 14	2	1
7	24-11-2020	2	2	5A 14	25A 14	2	1
7	24-11-2020	1	4	5A 14	45A 14	2	1
8	26-11-2020	3	3	5A 14	35A 14	2	1
8	26-11-2020	8	2	5A 14	25A 14	2	1
8	26-11-2020	4	4	5A 14	45A 14	2	1
8	26-11-2020	9	4	2A 18	42A 18	2	1
8	26-11-2020	1	1	2A 18	12A 18	2	1
8	26-11-2020	3	2	2A 18	22A 18	2	1
8	26-11-2020	2	5	2A 14	52A 14	2	1
8	26-11-2020	3	1	2A 14	12A 14	2	1
8	26-11-2020	6	3	5A 03	35A 03	2	1
8	26-11-2020	5	1	5A 03	15A 03	2	3
9	1-12-2020	2	3	5A 14	35A 14	2	1
9	1-12-2020	3	2	5A 14	25A 14	2	3
9	1-12-2020	4	4	5A 14	45A 14	2	3
9	1-12-2020	5	3	5A 03	35A 03	2	3
9	1-12-2020	4	1	5A 03	15A 03	2	4
9	1-12-2020	2	5	2A 14	52A 14	2	3
9	1-12-2020	2	1	2A 14	12A 14	2	3
9	1-12-2020	10	4	2A 18	42A 18	2	1
9	1-12-2020	6	1	2A 18	12A 18	2	3
9	1-12-2020	8	2	2A 18	22A 18	2	1
10	2-12-2020	1	3	5A 14	35A 14	2	2
10	2-12-2020	3	2	5A 14	25A 14	2	4
10	2-12-2020	3	4	5A 14	45A 14	2	3
10	2-12-2020	5	5	2A 14	52A 14	2	4
10	2-12-2020	2	1	2A 14	12A 14	2	3

10	2-12-2020	1	3	5A 03	35A 03	2	4
10	2-12-2020	2	1	5A 03	15A 03	2	4
10	2-12-2020	5	4	2A 18	42A 18	2	1
10	2-12-2020	1	1	2A 18	12A 18	2	3
10	2-12-2020	2	2	2A 18	22A 18	2	1
11	3-12-2020	6	4	2A 18	42A 18	2	1
11	3-12-2020	1	1	2A 18	12A 18	2	3
11	3-12-2020	2	2	2A 18	22A 18	2	2
11	3-12-2020	4	3	5A 03	35A 03	2	4
11	3-12-2020	3	1	5A 03	15A 03	2	4
11	3-12-2020	6	3	5A 14	35A 14	2	3
11	3-12-2020	2	2	5A 14	25A 14	2	4
11	3-12-2020	1	4	5A 14	45A 14	2	3
11	3-12-2020	2	5	2A 14	52A 14	2	4
11	3-12-2020	4	1	2A 14	12A 14	2	4
12	4-12-2020	3	3	5A 03	35A 03	2	4
12	4-12-2020	2	1	5A 03	15A 03	2	4
12	4-12-2020	1	5	2A 14	52A 14	2	4
12	4-12-2020	4	1	2A 14	12A 14	2	4
12	4-12-2020	6	3	5A 14	35A 14	2	2
12	4-12-2020	2	2	5A 14	25A 14	2	4
12	4-12-2020	1	4	5A 14	45A 14	2	3
12	4-12-2020	6	4	2A 18	42A 18	2	1
12	4-12-2020	2	1	2A 18	12A 18	2	3
12	4-12-2020	5	2	2A 18	22A 18	2	2
13	8-12-2020	3	4	2A 18	42A 18	2	1
13	8-12-2020	1	1	2A 18	12A 18	2	3
13	8-12-2020	1	2	2A 18	22A 18	2	3
13	8-12-2020	15	3	5A 03	35A 03	2	4
13	8-12-2020	4	1	5A 03	15A 03	2	4
13	8-12-2020	12	5	2A 14	52A 14	2	4
13	8-12-2020	3	1	2A 14	12A 14	2	3
13	8-12-2020	4	3	5A 14	35A 14	2	2
13	8-12-2020	2	2	5A 14	25A 14	2	3
13	8-12-2020	3	4	5A 14	45A 14	2	3
14	10-12-2020	6	5	2A 14	52A 14	2	4
14	10-12-2020	1	1	2A 14	12A 14	2	4
14	10-12-2020	7	3	5A 03	35A 03	2	4
14	10-12-2020	13	1	5A 03	15A 03	2	4
14	10-12-2020	0.5	3	5A 14	35A 14	2	3
14	10-12-2020	1	2	5A 14	25A 14	2	3
14	10-12-2020	1	4	5A 14	45A 14	2	3
14	10-12-2020	3	4	2A 18	42A 18	2	1
14	10-12-2020	2	1	2A 18	12A 18	2	3
14	10-12-2020	1	2	2A 18	22A 18	2	3
15	11-12-2020	2	4	2A 18	42A 18	2	1
15	11-12-2020	3	1	2A 18	12A 18	2	3
15	11-12-2020	1	2	2A 18	22A 18	2	3

15	11-12-2020	0.5	5	2A 14	52A 14	2	4
15	11-12-2020	5	1	2A 14	12A 14	2	4
15	11-12-2020	2	3	5A 03	35A 03	2	4
15	11-12-2020	4	1	5A 03	15A 03	2	4
15	11-12-2020	1	3	5A 14	35A 14	2	3
15	11-12-2020	2	2	5A 14	25A 14	2	3
15	11-12-2020	0.5	4	5A 14	45A 14	2	3
16	15-12-2020	2	5	2A 14	52A 14	2	4
16	15-12-2020	1	1	2A 14	12A 14	2	3
16	15-12-2020	3	3	5A 03	35A 03	2	5
16	15-12-2020	2	1	5A 03	15A 03	2	5
16	15-12-2020	3	4	2A 18	42A 18	2	1
16	15-12-2020	2	1	2A 18	12A 18	2	3
16	15-12-2020	2	2	2A 18	22A 18	2	3
16	15-12-2020	5	3	5A 14	35A 14	2	3
16	15-12-2020	0.5	2	5A 14	25A 14	2	3
16	15-12-2020	2	4	5A 14	45A 14	2	3
17	16-12-2020	6	3	5A 03	35A 03	2	5
17	16-12-2020	6	1	5A 03	15A 03	2	5
17	16-12-2020	1	3	5A 14	35A 14	2	3
17	16-12-2020	1	2	5A 14	25A 14	2	3
17	16-12-2020	1	4	5A 14	45A 14	2	3
17	16-12-2020	5	4	2A 18	42A 18	2	1
17	16-12-2020	1	1	2A 18	12A 18	2	3
17	16-12-2020	3	2	2A 18	22A 18	2	3
17	16-12-2020	1	5	2A 14	52A 14	2	5
17	16-12-2020	3	1	2A 14	12A 14	2	3
18	17-12-2020	1	3	5A 14	35A 14	2	3
18	17-12-2020	1	2	5A 14	25A 14	2	3
18	17-12-2020	1	4	5A 14	45A 14	2	3
18	17-12-2020	4	3	5A 03	35A 03	2	6
18	17-12-2020	3	1	5A 03	15A 03	2	6
18	17-12-2020	1	5	2A 14	52A 14	2	6
18	17-12-2020	1	1	2A 14	12A 14	2	3
18	17-12-2020	1	4	2A 18	42A 18	2	1
18	17-12-2020	2	1	2A 18	12A 18	2	3
18	17-12-2020	2	2	2A 18	22A 18	2	3
19	18-12-2020	1	3	5A 14	35A 14	2	3
19	18-12-2020	1	2	5A 14	25A 14	2	3
19	18-12-2020	0.5	4	5A 14	45A 14	2	3
19	18-12-2020	5	4	2A 18	42A 18	2	1
19	18-12-2020	7	1	2A 18	12A 18	2	4
19	18-12-2020	1	2	2A 18	22A 18	2	3
19	18-12-2020	17	3	5A 03	35A 03	2	7
19	18-12-2020	9	1	5A 03	15A 03	2	7
19	18-12-2020	6	5	2A 14	52A 14	2	7
19	18-12-2020	2	1	2A 14	12A 14	2	4
20	5-1-2021		3	5A 03	35A 03	2	7

20	5-1-2021		1	5A 03	15A 03	2	7
20	5-1-2021	2	3	5A 14	35A 14	2	4
20	5-1-2021	3	2	5A 14	25A 14	2	4
20	5-1-2021	2	4	5A 14	45A 14	2	4
20	5-1-2021	2	4	2A 18	42A 18	2	1
20	5-1-2021	1	1	2A 18	12A 18	2	4
20	5-1-2021	0.5	2	2A 18	22A 18	2	3
20	5-1-2021		5	2A 14	52A 14	2	7
20	5-1-2021	0.5	1	2A 14	12A 14	2	4
21	6-1-2021		3	5A 03	35A 03	2	7
21	6-1-2021		1	5A 03	15A 03	2	7
21	6-1-2021	4	4	2A 18	42A 18	2	1
21	6-1-2021	0.5	1	2A 18	12A 18	2	4
21	6-1-2021	8	2	2A 18	22A 18	2	3
21	6-1-2021		5	2A 14	52A 14	2	7
21	6-1-2021	1	1	2A 14	12A 14	2	4
21	6-1-2021	2	3	5A 14	35A 14	2	4
21	6-1-2021	1	2	5A 14	25A 14	2	4
21	6-1-2021	0.5	4	5A 14	45A 14	2	4
22	7-1-2021		3	5A 03	35A 03	2	7
22	7-1-2021		1	5A 03	15A 03	2	7
22	7-1-2021	2	3	5A 14	35A 14	2	4
22	7-1-2021	0.5	2	5A 14	25A 14	2	4
22	7-1-2021	4	4	5A 14	45A 14	2	4
22	7-1-2021	3	4	2A 18	42A 18	2	1
22	7-1-2021	1	1	2A 18	12A 18	2	4
22	7-1-2021	0.5	2	2A 18	22A 18	2	4
22	7-1-2021		5	2A 14	52A 14	2	7
22	7-1-2021	1	1	2A 14	12A 14	2	4
23	8-1-2021	7	4	2A 18	42A 18	2	1
23	8-1-2021	3	1	2A 18	12A 18	2	5
23	8-1-2021	2	2	2A 18	22A 18	2	1
23	8-1-2021		5	2A 14	52A 14	2	7
23	8-1-2021	0.5	1	2A 14	12A 14	2	5
23	8-1-2021		3	5A 03	35A 03	2	7
23	8-1-2021		1	5A 03	15A 03	2	7
23	8-1-2021	1	3	5A 14	35A 14	2	5
23	8-1-2021	0.5	2	5A 14	25A 14	2	5
23	8-1-2021	2	4	5A 14	45A 14	2	5
24	12-1-2021		3	5A 03	35A 03	2	7
24	12-1-2021		1	5A 03	15A 03	2	7
24	12-1-2021	1	4	2A 18	42A 18	2	1
24	12-1-2021	0.5	1	2A 18	12A 18	2	7
24	12-1-2021	1	2	2A 18	22A 18	2	3
24	12-1-2021	3	3	5A 14	35A 14	2	7
24	12-1-2021	6	2	5A 14	25A 14	2	7
24	12-1-2021	1	4	5A 14	45A 14	2	7
24	12-1-2021		5	2A 14	52A 14	2	7

24	12-1-2021	2	1	2A 14	12A 14	2	7

Appendix 2

The time the mice are on the right side of the platform

Time (weeks)	Session	Date	Last measurement	Time right (seconds)	Time left (seconds)	Time nothing to see (seconds)	Time total (seconds)	Percentage right (%)	Mouse	Group	Individual	Treatment	Level
First measurement (pre-measurement) 26-10-2020 (week 1)	8	26-10-2020	0	180.5	125.5	0	306	58.99	1	2A14	12A14	2	1
First measurement (pre-measurement) 26-10-2020 (week 1)	8	26-10-2020	0	160	138.5	4.5	303	52.81	5	2A14	52A14	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	161.5	131	9.5	302	53.48	1	2A18	12A18	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	189.5	111.5	0.5	301.5	62.85	2	2A18	22A18	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	179.5	125	1.5	306	58.66	4	2A18	42A18	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	192.5	108	0	300.5	64.06	1	5A03	15A03	2	3
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	207.5	98.5	0.5	306.5	67.7	3	5A03	35A03	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	184	138	0	322	57.14	2	5A14	25A14	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	142	161	1	304	46.71	3	5A14	35A14	2	1
First measurement (pre-measurement) 24-10-2020 (week 1)	7	24-10-2020	0	218.5	85	0	303.5	71.99	4	5A14	45A14	2	1
Second measurement 01-12-2020 (week 2)	9	1-12-2020	0	244	63.5	0	307.5	79.35	1	2A14	12A14	2	3
2	9	1-12-2020	0	232.5	72	0	304.5	76.35	5	2A14	52A14	2	3
2	9	1-12-2020	0	255	45	1.5	301.5	84.58	1	2A18	12A18	2	3
2	9	1-12-2020	0	214	88	0	302	70.86	2	2A18	22A18	2	1
2	9	1-12-2020	0	253.5	49.5	0	303	83.66	4	2A18	42A18	2	1
2	9	1-12-2020	0	278.5	25.5	1	305	91.31	1	5A03	15A03	2	4
2	9	1-12-2020	0	211.5	99.5	0.5	311.05	67.9	3	5A03	35A03	2	3
2	9	1-12-2020	0	207	93.5	0	300.5	68.89	2	5A14	25A14	2	3
2	9	1-12-2020	0	188.5	113.5	0	302	62.42	3	5A14	35A14	2	1
2	9	1-12-2020	0	251.5	53	0.5	305	82.46	4	5A14	45A14	2	3
3	13	8-12-2020	0	228	78.5	0	306.5	74.39	1	2A14	12A14	2	3
3	13	8-12-2020	0	256	48	0	304	84.21	5	2A14	52A14	2	4
3	13	8-12-2020	0	192	117	0	309	62.14	1	2A18	12A18	2	3
3	13	8-12-2020	0	243.5	61.5	0	305	79.84	2	2A18	22A18	2	3
3	13	8-12-2020	0	241.5	60.5	0	302	79.97	4	2A18	42A18	2	1
3	13	8-12-2020	0	244.5	50.5	0	295	82.88	1	5A03	15A03	2	4
3	13	8-12-2020	0	224.5	81.5	0	306	73.37	3	5A03	35A03	2	4
3	13	8-12-2020	0	198.5	104	0	302.5	65.62	2	5A14	25A14	2	3
3	13	8-12-2020	0	180	120.5	0	300.5	59.9	3	5A14	35A14	2	2
3	13	8-12-2020	0	202.5	96	0	298.5	67.84	4	5A14	45A14	2	3
4	16	15-12-2020	0	215.5	89.5	0	305	70.66	1	2A14	12A14	2	3
4	16	15-12-2020	0	164.5	128.5	12.5	313.5	52.47	5	2A14	52A14	2	4
4	16	15-12-2020	0	196.5	100.5	0	297	66.16	1	2A18	12A18	2	3
4	16	15-12-2020	0	193	101	0	294	65.65	2	2A18	22A18	2	3
4	16	15-12-2020	0	215.5	85.5	0	301	71.59	4	2A18	42A18	2	1
4	16	15-12-2020	0	262.5	41	5	308.5	85.09	1	5A03	15A03	2	5
4	16	15-12-2020	0	201.5	103	1	305.5	65.96	3	5A03	35A03	2	5
4	16	15-12-2020	0	175.5	126	0	301.5	58.21	2	5A14	25A14	2	3
4	16	15-12-2020	0	170	128.5	0	298.5	56.95	3	5A14	35A14	2	3
4	16	15-12-2020	0	228	68.5	0	296.5	76.9	4	5A14	45A14	2	3
5 (05-01-2021)	20	5-1-2021	0	213	91.5	0	304.5	69.95	1	2A14	12A14	2	4
Last measurement (end measurement) 18-12-2020	19	18-12-2020	1	208.5	94.5	0	303	68.81	5	2A14	52A14	2	7
5 (05-01-2021)	20	5-1-2021	0	155.5	148	0	303.5	51.24	1	2A18	12A18	2	4
5 (05-01-2021)	20	5-1-2021	0	170.5	132.5	0	303	56.27	2	2A18	22A18	2	3
5 (05-01-2021)	20	5-1-2021	0	199	107	0	306	65.03	4	2A18	42A18	2	1
Last measurement (end measurement) 18-12-2020	19	18-12-2020	1	259.5	38	5.5	303	85.64	1	5A03	15A03	2	7
Last measurement (end measurement) 18-12-2020	19	18-12-2020	1						3	5A03	35A03	2	7
5 (05-01-2021)	20	5-1-2021	0	200.5	101.5	0	302	66.39	2	5A14	25A14	2	4
5 (05-01-2021)	20	5-1-2021	0	214.5	91	0	305.5	70.21	3	5A14	35A14	2	4
5 (05-01-2021)	20	5-1-2021	0	201.5	102.5	0	304	66.28	4	5A14	45A14	2	4
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	210.5	97	0	307.5	68.46	1	2A14	12A14	2	7
6	24	12-1-2021	0						5	2A14	52A14	2	7
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	174.5	130.5	0	305	57.21	1	2A18	12A18	2	7
6 (12-01-2021)	24	12-1-2021	0	189.5	113	0	302.5	62.64	2	2A18	22A18	2	3
6 (12-01-2021)	24	12-1-2021	0	90	70	0	160	56.25	4	2A18	42A18	2	1
6	24	12-1-2021	0						1	5A03	15A03	2	7
6	24	12-1-2021	0						3	5A03	35A03	2	7
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	180	123.5	0	303.5	59.31	2	5A14	25A14	2	7
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	126.5	176.5	0	303	41.75	3	5A14	35A14	2	7
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	185	121.5	0	306.5	60.36	4	5A14	45A14	2	7
First measurement (pre-measurement) 26-10-2020 (week 1)	8	26-10-2020	0	114	192	0	306	37.25	2	2A14	22A14	1	0

First measurement (pre-measurement) 26-10-2020 (week 1)		8	26-10-2020	0	144.5	165	0	309.5	46.69	3	2A14	32A14	1	0
First measurement (pre-measurement) 26-10-2020 (week 1)		8	26-10-2020	0	212.5	88.5	0	301	70.6	4	2A14	42A14	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	169	137	0	306	55.23	3	2A18	32A18	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	188.5	116	1.5	306	61.6	5	2A18	52A18	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	114	191.5	0	305.5	37.32	2	5A03	25A03	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	164.5	140.5	0	305	53.93	4	5A03	45A03	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	176.5	131	0	307.5	57.4	5	5A03	55A03	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	180.5	125	0	313.5	57.58	1	5A14	15A14	1	0
First measurement (pre-measurement) 24-10-2020 (week 1)		7	24-10-2020	0	150	149	0	299	50.17	5	5A14	55A14	1	0
Second measurement 01-12-2020 (week 2)		9	1-12-2020	0	174	132	0	306	56.86	2	2A14	22A14	1	0
	2	9	1-12-2020	0	179	122	0	301	59.47	3	2A14	32A14	1	0
	2	9	1-12-2020	0	189	126.5	0	315.5	59.9	4	2A14	42A14	1	0
	2	9	1-12-2020	0	212.5	104	0	316.5	67.14	3	2A18	32A18	1	0
	2	9	1-12-2020	0	198.5	102.5	9	310	64.03	5	2A18	52A18	1	0
	2	9	1-12-2020	0	199	111	0	310	64.19	2	5A03	25A03	1	0
	2	9	1-12-2020	0	170.5	140.5	0.5	311.5	54.74	4	5A03	45A03	1	0
	2	9	1-12-2020	0	209.5	97	0	306.5	68.35	5	5A03	55A03	1	0
	2	9	1-12-2020	0	211	101	0	312	67.63	1	5A14	15A14	1	0
	2	9	1-12-2020	0	179	126.5	0	305.5	58.59	5	5A14	55A14	1	0
	3	13	8-12-2020	0	147.5	152.5	5.5	305.5	48.28	2	2A14	22A14	1	0
	3	13	8-12-2020	0	170	138	0	308	55.19	3	2A14	32A14	1	0
	3	13	8-12-2020	0	202	113.5	0	315.5	64.03	4	2A14	42A14	1	0
	3	13	8-12-2020	0	170.5	145.5	0	316	53.96	3	2A18	32A18	1	0
	3	13	8-12-2020	0	179.5	125.5	0	305	58.85	5	2A18	52A18	1	0
	3	13	8-12-2020	0	140.5	160.5	0	301	46.68	2	5A03	25A03	1	0
	3	13	8-12-2020	0	109.5	192	0	301.5	36.32	4	5A03	45A03	1	0
	3	13	8-12-2020	0	178.5	127.5	0	306	58.33	5	5A03	55A03	1	0
	3	13	8-12-2020	0	131	176.5	0	307.5	42.6	1	5A14	15A14	1	0
	3	13	8-12-2020	0	136	173	0	309	44.01	5	5A14	55A14	1	0
	4	16	15-12-2020	0	104.5	201	0	305.5	34.21	2	2A14	22A14	1	0
	4	16	15-12-2020	0	147	157.5	0	304.5	48.28	3	2A14	32A14	1	0
	4	16	15-12-2020	0	153.5	151	0	304.5	50.41	4	2A14	42A14	1	0
	4	16	15-12-2020	0	124	178	0	302	41.06	3	2A18	32A18	1	0
	4	16	15-12-2020	0	171	139	0	310	55.16	5	2A18	52A18	1	0
	4	16	15-12-2020	0	132.5	166.5	0	299	44.31	2	5A03	25A03	1	0
	4	16	15-12-2020	0	145.5	163	0	308.5	47.16	4	5A03	45A03	1	0
	4	16	15-12-2020	0	160	141.5	0	301.5	53.07	5	5A03	55A03	1	0
	4	16	15-12-2020	0	142	156.5	0	298.5	47.57	1	5A14	15A14	1	0
	4	16	15-12-2020	0	181.5	122.5	0	304	59.7	5	5A14	55A14	1	0
5 (05-01-2021)		20	5-1-2021	0	110	180	0	290	37.93	2	2A14	22A14	1	0
5 (05-01-2021)		20	5-1-2021	0	180	121	0	301	59.8	3	2A14	32A14	1	0
Last measurement (end measurement) 18-12-2020		19	18-12-2020	1	183.5	123.5	1	308	59.58	4	2A14	42A14	1	0
5 (05-01-2021)		20	5-1-2021	0	130	173.5	0	303.5	42.83	3	2A18	32A18	1	0
5 (05-01-2021)		20	5-1-2021	0	114	193.5	0	307.5	37.07	5	2A18	52A18	1	0
Last measurement (end measurement) 18-12-2020		19	18-12-2020	1	156.5	153.5	0	310	50.48	2	5A03	25A03	1	0
Last measurement (end measurement) 18-12-2020		19	18-12-2020	1	179	127.5	1.5	308	58.12	4	5A03	45A03	1	0
5 (05-01-2021)		20	5-1-2021	0	157.5	136.5	0	294	53.57	5	5A03	55A03	1	0
5 (05-01-2021)		20	5-1-2021	0	200	89	0	289	69.2	1	5A14	15A14	1	0
5 (05-01-2021)		20	5-1-2021	0	153	147.5	0	300.5	50.92	5	5A14	55A14	1	0
Last measurement (end measurement) 12-01-2021		24	12-1-2021	1	188	121.5	0	309.5	60.74	2	2A14	22A14	1	0
Last measurement (end measurement) 12-01-2021		24	12-1-2021	1	185	120	0	305	60.66	3	2A14	32A14	1	0
	6	24	12-1-2021	0						4	2A14	42A14	1	0
6 (12-01-2021)		24	12-1-2021	0	172.5	134.5	0	307	56.19	3	2A18	32A18	1	0
Last measurement (end measurement) 12-01-2021		24	12-1-2021	1	154	157	0	311	49.52	5	2A18	52A18	1	0
	6	24	12-1-2021	0						2	5A03	25A03	1	0
	6	24	12-1-2021	0						4	5A03	45A03	1	0
6 (12-01-2021)		24	12-1-2021	0	122.5	170	0	292.5	41.88	5	5A03	55A03	1	0
Last measurement (end measurement) 12-01-2021		24	12-1-2021	1	181.5	123	0	304.5	59.61	1	5A14	15A14	1	0
Last measurement (end measurement) 12-01-2021		24	12-1-2021	1	145.5	163.5	0	309	47.09	5	5A14	55A14	1	0
Last measurement (end measurement) 18-12-2020		19	18-12-2020	1	142	165	1.5	308.5	46.03	1	4	14	0	0
Last measurement (end measurement) 18-12-2020		19	18-12-2020	1	174.5	128.5	0	303	57.59	2	4	24	0	0
Last measurement (end measurement) 18-12-2020		19	18-12-2020	1	163	144.5	0	307.5	53.01	3	4	34	0	0

Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	210.5	100	0	310.5	67.79	1	4A	14A	0	0
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	236.5	76.5	0	313	75.56	2	4A	24A	0	0
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	143	163.5	0	306.5	46.66	3	4A	34A	0	0
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	130.5	182.5	0	313	41.69	4	4A	44A	0	0
Last measurement (end measurement) 12-01-2021	24	12-1-2021	1	128.5	180	0	308.5	41.65	5	4A	54A	0	0

Appendix 3

The number of times / the time the mice exhibit behavior or seek contact with the researcher

Session	Date	Last measurement	Freezing	Grooming	Sniffing platform	Rear	Urinating	Defecation	Vocalisation	No feed intake	Jumping	Escape attempt	Strech attend	Sniffle at 0.5 cm	Paw on the hand	Touch the hand	Jump on the hand	Eating the reward	Totale time on hand researcher	Totale time seek contact with researcher	Pull back	Time it takes for the mouse to be injected	Individual	Treatment	Level
7	24-10-2020	0	0	6	13	8	0	2	0	13	0	70	75										12A18	2	1
7	24-10-2020	0	0	6	24	0	0	2	0	24	0	46	63										22A18	2	1
7	24-10-2020	0	0	4	13	8	0	3	0	15	0	51	44										32A18	1	0
7	24-10-2020	0	6	3	23	0	0	3	0	19	0	44	31										42A18	2	1
7	24-10-2020	0	0	7	12	2	0	2	0	20	0	63	46										52A18	1	0
7	24-10-2020	0	0	4	13	2	0	6	0	10	0	45	38										15A03	2	1
7	24-10-2020	0	1	1	11	0	0	2	0	15	0	62	34										25A03	1	0
7	24-10-2020	0	0	3	9	3	0	6	0	10	0	39	44										35A03	2	1
7	24-10-2020	0	0	3	35	17	0	1	0	1	0	65	45										45A03	1	0
7	24-10-2020	0	0	2	17	5	0	3	0	1	0	59	57										55A03	1	0
7	24-10-2020	0	0	5	16	16	0	2	0	0	0	70	52										15A14	1	0
7	24-10-2020	0	0	6	11	5	0	5	0	9	0	68	34										25A14	2	1
7	24-10-2020	0	0	5	13	5	0	3	0	18	0	58	41										35A14	2	1
7	24-10-2020	0	0	6	17	7	0	5	0	14	0	50	47										45A14	2	1
7	24-10-2020	0	0	7	25	2	0	2	0	20	0	59	55										55A14	1	0
8	26-10-2020	0	3	1	19	0	0	2	0	0	0	44	33										12A14	2	1
8	26-10-2020	0	0	3	16	14	0	3	0	0	0	90	47										22A14	1	0
8	26-10-2020	0	2	4	22	8	0	3	0	13	0	60	22										32A14	1	0
8	26-10-2020	0	0	7	29	0	0	2	0	19	0	41	28										42A14	1	0
8	26-10-2020	0	0	1	29	3	0	4	0	5	0	53	46										52A14	2	1
24	12-1-2021	1	5	4	31	0	0	4	0	8	0	48	26	6	3	6	0	5	4	45	1	5.5	12A18	2	7
																							22A18	2	3
																							32A18	1	0
																							42A18	2	1
24	12-1-2021	1	1	3	28	12	0	3	0	12	0	47	43	8	1	4	0	2	8.5	25	2	25.5	52A18	1	0
19	18-12-2020	1	0	0	23	1	0		0	1	0	29	56	2	7	10	2	5	34.5	65	0	9.5	15A03	2	7
19	18-12-2020	1	1	2	37	0	0		0	5	0	54	27	3	4	6	0	2	4	27	4	24	25A03	1	0
19	18-12-2020	1																					35A03	2	7
19	18-12-2020	1	0	2	26	0	0		0	5	0	48	35	4	5	7	1	0	64.5	72.5	5	65	45A03	1	0
																							55A03	1	0
24	12-1-2021	1	1	2	22	0	0	4	0	3	0	49	42	5	6	3	2	0	61.5	68	0	3.5	15A14	1	0
24	12-1-2021	1	4	6	17	0	0	4	0	3	0	23	21	11	1	10	0	3	0.5	29.5	0	3.5	25A14	2	7
24	12-1-2021	1	0	4	21	5	0	6	0	10	0	39	46	7	5	3	2	2	44	64	1	6	35A14	2	7
24	12-1-2021	1	2	2	29	0	0	4	0	11	0	32	34	6	3	6	1	0	16.5	34.5	0	34	45A14	2	7
24	12-1-2021	1	1	7	21	0	0	3	0	15	0	56	42	12	1	6	0	1	1	18.5	1	8.5	55A14	1	0
24	12-1-2021	1	0	4	20	1	0	4	0	6	0	58	48	5	8	9	2	1	33	45.5	0	1.5	12A14	2	7
24	12-1-2021	1	0	1	26	7	0	5	0	0	0	57	40	3	5	6	1	4	27	56	1	9	22A14	1	0
24	12-1-2021	1	10	5	41	1	0	4	0	14	0	50	25	5	0	2	0	0	0	5	1	5	32A14	1	0
19	18-12-2020	1	3	5	21	0	0	4	0	15	0	45	39	12	6	9	0	1	11	42.5	4	37	42A14	1	0
19	18-12-2020	1	0	1	29	0	0	3	0	8	0	40	44	6	5	9	1	1	40	70.5	0	5	52A14	2	7
19	18-12-2020	1	10	1	37	0	0	2	0	19	0	44	14	4	0	2	0	0	0	8	4	24.5	14	0	0
19	18-12-2020	1	3	1	21	0	0		0	19	0	34	44	6	3	9	0	0	8	26	6	32.5	24	0	0
19	18-12-2020	1	0	2	57	1	0		0	17	0	73	38	11	1	6	0	0	3	15.5	3	16.5	34	0	0
24	12-1-2021	1	4	2	34	0	1	4	0	14	0	36	29	3	7	0	4	0	87.5	90	2	19	14A	0	0
24	12-1-2021	1	0	1	25	1	1	5	0	14	0	63	54	3	2	1	1	0	47.5	50	0	5	24A	0	0
24	12-1-2021	1	0	4	31	3	0	4	0	10	0	85	35	10	5	1	1	0	21.5	28.5	6	78	34A	0	0
24	12-1-2021	1	3	5	29	0	0	4	0	10	0	52	31	10	2	0	0	0	6.5	14.5	8	84	44A	0	0
24	12-1-2021	1	5	6	29	0	0		0	16	0	50	38	12	6	4	0	0	17.5	29	0	35	54A	0	0