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Positive Reinforcement Training for Laboratory Mice

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Positive Reinforcement Training for Laboratory Mice

Träning av laboratoriemöss med positiv förstärkning

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SUMMARY

The mouse (*Mus musculus*) is the most commonly used mammal in biomedical research and can easily be restrained by both hand and by a device. Restraining animals causes stress that not only decreases the animal's wellbeing, but may also influence the research results. The first aim of this study was to train mice to voluntarily stand still using positive reinforcement training. Another benefit of teaching a mouse to stand still is that you can avoid anaesthesia for some procedures. The second aim of this study was to investigate if postnatal handling could also facilitate learning.

That mice can be trained using positive reinforcement training (PRT), has been shown in numerous experiments as well as on video-sharing websites such as YouTube, yet most training of laboratory mice is pretty basic; focusing on habituation by calm, gentle handling and patience. In PRT, animals are being reinforced for performing a desired behaviour and will not be forced to cooperate. A reinforcer could be anything the animal likes, such as food or access to a favourable environment. Giving the animals control over their situation and a chance to work for their food are both factors that are associated with increased wellbeing.

Postnatal handling is a brief and daily separation of the pup from the mother for the first two to three weeks of the pups' life. This has been shown to have several different effects on the animals; mice that have been handled are more explorative, less anxious and have a lower response to stress response than unhandled mice. The effects of postnatal handling are thought to be mediated by a changed maternal behaviour towards the pups after handling and/or a result of the handling itself. Several experiments with mice have shown that pups receiving increased maternal care had a reduced stress response. There are many factors influencing the effect of postnatal handling; e.g. gender, strain and the time of day when being handled.

Housing factors have been shown to affect the brain development of laboratory animals. Mice are known to develop stereotypies when raised in standard laboratory cages. Stereotypies are repetitive behaviours that can indicate a suboptimal environment. An enriched environment has been shown to improve learning and memory in mice.

Our experiment included six pregnant NMRI females and their offspring. The females were handled prior to parturition and were presented with different rewards until the pups were weaned. This was to evaluate which one was most popular and make sure the pups were used to the smell of the reward from an early age. Half of the litters were randomly selected to be handled prior to weaning. 24 of the pups were randomly assigned to be part of the training group with equal distribution between genders and handling/non-handling. All mice were always lifted in a cupped hand by the researcher and never by the tail.

Training was done using a device with a sensor that triggered a reward-signal when the mouse was in the correct position. The reward was delivered through a metal nipple in the device. To further evaluate the effects of handling two behavioural tests were performed; an elevated plus maze and a handling test. A skilled animal technician, who fixated the mice by hand and scored them as compliant, hesitant or unwilling, performed the blind handling test. The mice had no prior experience of this person.

The most popular reward in the study was the strawberry ice cream. After spending 14 times training in the device, nine out of 24 mice had learnt to take the reward from the metal pipe. Five out of six unhandled females had learnt this task and only two males had learned to do so. The device did not function as planned and the reward-signal was not used since the refraction time was too long. Instead

reward was given to the mice with increasing intervals from 0 seconds up to 3.5 seconds by the end of the experiment.

In the elevated plus maze, the only difference was (when pooling both genders) that the non-handled animals exhibited a significantly higher number of stretch attend postures when compared to the handled animals (20,0 ± 5,8 vs. 27,6 ± 6,4 ($P < 0{,}001$)). The handling test showed no difference between handled and non-handled animals. Trained animals (including handled and non-handled animals) were more compliant than control animals (54 ± 0,7 % vs. 21 ± 1,2 % ($P < 0{,}003$)). The control animals consisted of the trained animal's littermates who had not been included in the training and were only used for the handling test.

The results showed that only two males started to take the reward from the device. Interestingly enough both times when the males learned this there had not previously been any females or unknown males in the training area. This indicates that the learning of males may be inhibited by the smell of females or other unknown males. The non-handled females had higher success in learning to drink from the device than the handled females had (83 % vs. 33 %). This could mean that postnatal handling has a negative effect on learning in females, but further research is necessary to be able to clarify this.

The fact that only one parameter (the stretch attend postures) differed between the groups in the elevated plus maze, and that the results of the handling test showed that trained mice were more compliant than control mice, indicates that friendly and gentle handling post weaning could have major effects on mice. Training mice to stand reasonably still is possible although it requires a long habituation period. With an improved device, further training and research can be done to optimize the training protocol.

SAMMANFATTNING

Musen är det vanligaste däggdjuret som används i biomedicinsk forskning. Möss är lätta att fixera både med handen och med hjälp av annan utrustning. När djur fixeras blir de oftast stressade. Stress påverkar inte bara djurets välmående negativt, det kan även påverka forskningsresultaten. Det huvudsakliga målet med den här studien var att träna möss till att stå stilla med hjälp av positivt förstärkning. Två fördelar med att lära en mus att stå stilla är att man kan undvika anestesi under vissa procedurer samt undvika fixering. Det sekundära målet med studien var att se om postnatal hantering kunde främja inlärning, eftersom postnatal hantering av möss har visat sig kunna minska inlärningssvårigheter.

Att möss kan tränas med hjälp av positiv förstärkning har visats både i flertalet experiment och på video-delande websidor såsom YouTube. Trots det är träning av laboratoriemöss väldigt basal och bygger på habituering genom lugn, vänlig hantering och tålamod. När djur tränas med positiv förstärkning, förstärker man det önskade beteendet med en belöning, något som djuret tycker om. Genom att ge djur kontroll över sin situation och en chans att arbeta för sin mat kan djurets välmående öka.

Postnatal hantering är en kortvarig och daglig separation av musungen från mamman under det första två till tre levnadsveckorna. Detta har visats sig ha många olika effekter: möss som har hanterats är mer undersökande, mindre oroliga och har ett lägre stress-svar vid stress än icke-hanterade möss. Effekterna av postnatal hantering tros bero på ett förändrat beteende hos modern efter hanteringen och/eller hanteringen i sig själv. Flera experiment har visat att musungar som får en ökad maternell omvårdnad har ett reducerat stress-svar. Effekten av hantering påverkas dock av många faktorer; t.ex. kön, stam och tiden på dygnet som musungarna hanteras.

Levnadsmiljön kan påverka djur på flera olika sätt. Exempelvis kan möss utveckla stereotypa beteenden när de föds upp i standardburar. Stereotypier är repetitiva beteenden som kan indikera att miljön är suboptimal. En berikad miljö däremot, har visat sig kunna påverka hjärnans utveckling och förbättra inlärning och minne hos möss.

I denna studie användes sex dräktiga NMRI-honor och deras avkommor. Innan födseln hanterades honorna och de fick tillgång till olika belöningar fram till avvänjning av ungarna. Den tidiga presentationen av belöningen gjordes för att utvärdera vilken som var mest populär så snart som möjligt och för att ungarna skulle vara vana vid lukten av belöningen från en tidig ålder. Hälften av kullarna valdes slumpmässigt ut att hanteras från födsel till avvänjning. 24 ungar valdes slumpmässigt ut att ingå i träningsgruppen med jämn fördelning av kön och hanterade/icke-hanterade möss. Alla möss lyftes alltid i en kupad hand av forskaren och aldrig i svansen.

Vid träning användes en specialbyggd apparat med en sensor som triggade en belöningssignal när musen var i rätt position. Belöningen levererades genom en metallnippel framför musens mun. För att vidare utvärdera effekterna av hanteringen utfördes två beteendetester, en upphöjd plus-maze och ett hanteringstest. Hanteringstestet var ett blindtest som bestod av en för mössen okänd person som fixerade dem med handen och karaktäriserade dem som medgörliga, tveksamma eller ovilliga.

Den populäraste belöningen i studien var jordgubbsmjukglass. Efter 14 besök i träningsapparaten hade nio av 24 möss lärt sig att ta belöningen ifrån nippeln. Fem av sex icke-hanterade honor och endast två av hanarna lärde sig detta. Träningsapparaten fungerade inte som planerat och belöningssignalen användes inte, då refraktionsperioden var för lång. Istället för en belönings-signal så ökades intervallen mellan belöningarna från 0 till 3,5 sekunder under försökets gång.

I den upphöjda plus-mazen var den enda skillnaden att de icke-hanterade djuren visade ett högre antal ”stretch attend postures” jämfört med hanterade djur (20,0 ± 5,8 vs. 27,6 ± 6,4 (P <0,001)). Hanteringstestet visade ingen skillnad mellan hanterade och icke hanterade djur. De tränade mössen (inkluderande både hanterade och icke hanterade djur) var mer medgörliga än kontroll djuren (54 ± 0,7 % vs. 21 ± 1,2 % (P <0,003)).. Kontroll djuren bestod av de kullsyskon som inte valts ut för att tränas och användes endast under hanteringstestet.

Resultaten visade att bara två hanar tog belöningen från apparaten. Båda två lärde sig detta efter ett besök där ingen hona eller okänd hane hade varit i träningsområdet innan. Detta tyder på att hanars inlärning störs av doften av honor och andra främmande hanar. Det var fler honor ur den icke-hanterade gruppen som lärde sig att ta belöning från apparaten än de hanterade honorna (83 % vs. 33

%). Detta kan betyda att postnatal hantering har en negativ effekt på inlärning, men vidare studier krävs för att kunna dra en konklusiv slutsats.

Att grupperna visade så få skillnader i beteenden i den upphöjda plus mazen samt att resultaten från hanteringstestet visade att tränade möss var mer lätthanterliga, indikerar att en vänlig och försiktig hantering efter avvänjning kan ha en stor effekt på möss. Att träna en mus att stå relativt stilla är möjligt men kräver en lång habitueringsperiod. Med en förbättrad träningsapparat kan vidare träning och forskning utföras för att utveckla ett träningsprotokoll för möss.

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INTRODUCTION

The mouse (*Mus musculus*) with over 1000 genetically defined inbred strains, a short life span, small size and low cost of maintenance makes it the most commonly used mammal in biomedical research.

The three R’s, reduction, refinement and replacement have been the foundation for laboratory animal science for over four decades. Reduction means that the number of animals being used should be kept as precise as possible so no more animals than necessary are included in the experiment. Replacement means to find different research models that do not include the use of animals. This study is a so-called refinement project, aiming at increasing the wellbeing of the animals being used for studies. (Kaliste, 2007)

To maximize the welfare of laboratory animals, it is important to be able to perform the necessary procedures with as little stress as possible (Laule, 2010). Restraining animals causes stress (Hedrich, 2004; Kaliste, 2007) and is not only a method used when collecting data, but it is also used as a model of psychiatric stress (Buynitsky & Mostofsky, 2009). Restraint stress can induce temporal weight loss, gastric ulcers, an increase of glucocorticoids, delay cutaneous healing and decrease memory acquisition (Hedrich, 2004; Buynitsky & Mostofsky, 2009). Berridge & Dunn, (1989) showed that restraint stress could reduce exploratory behavior.

The first aim of this study was to train mice to voluntarily stand still using positive reinforcement training (PRT). Two benefits of teaching a mouse to stand still is that you can avoid anesthesia for some procedures and use this as a possible alternative to restraining. To aid the training process, a device specifically constructed for this experiment was used. The device has a sensor paired with an automatic reward signal. Training a mouse to willingly stand still could make it possible to collect data from animals that are awake, while keeping stress to a minimum. Several studies (Bloomsmith, 1992; Reinhardt, 1997; Lambeth et al., 2006) have shown that the least stressed animals produce the most reliable research results. Avoiding unnecessary stress of the animal would not only increase its wellbeing but could also contribute to more accurate data being collected.

Postnatal handling is a brief and daily separation of the pup from the mother for the first two to three weeks of the pups’ life (Würbel, 2001). Postnatal handling has been proven to reduce learning impairments (Zaharia et al., 1996). The second aim of this study was to investigate whether postnatal handling could facilitate learning/training of the mice after weaning.

LITERATURE REVIEW

Classical conditioning

A Russian physiologist named Ivan Pavlov described one of our best-known learning theories, classical conditioning. In his experiment he showed that if the sound of a metronome were immediately followed by meat powder, the sound alone would eventually elicit salivation in the dogs being tested. This happened through a process called classical conditioning (Manning & Dawkins, 1998; Barnard, 2003; Jensen, 2007)

The animal learns that an event is followed by another, for example a dog can learn that when its owner open a specific cabinet this will be followed by getting food. This leads to the anticipation of food when opening the cabinet and thus elicits salivation before the dog sees the food itself.

Pavlov termed the meat as an unconditional stimulus (UCS) and the salivation that followed to be an unconditional response (UCR) (Manning & Dawkins, 1998; Barnard, 2003; Jensen, 2007). An UCS is any stimulus that evokes a response without previous learning, i.e. the smell/sight of food or a painful stimulus (Barnard, 2003). When pairing a UCS to a neutral stimulus like a sound, the sound will eventually become a conditional stimulus (CS) and elicit a conditioned response (CR) (Manning & Dawkins, 1998; Barnard, 2003; Jensen, 2007).

Operant conditioning

Operant conditioning is learning by consequence (Skinner, 1981). The animal learns that a specific behavior is followed by an event, and depending on that event the behavior increases or decreases in likelihood. There are four basic consequences following behavior:

1. **Positive reinforcement** – the behavior **increases** in frequency by **adding** something pleasant when behavior is performed
2. **Negative reinforcement** – the behavior **increases** in frequency by **removing** something unpleasant when the behavior is performed
3. **Positive punishment** – the behavior **decreases** in frequency by **adding** something unpleasant when the behavior is performed
4. **Negative punishment** – the behavior **decreases** in frequency by **removing** something pleasant when the behavior is performed. (Skinner, 1991; Barnard, 2003; Jensen, 2007).

One example could be a pigeon pecking at a key and receiving food, making the pecking more likely to occur when the key is present (Manning & Dawkins, 1998; Barnard, 2003). Another example could be a dog that has jumped up on the kitchen counter to steal food and the owner scolds them, making jumping up on the counter less likely to occur (Jensen, 2007).

When teaching a behavior, it is not as clear a difference between classical and operant conditioning as one might think. A good example is the pigeon pecking at a key. It has been shown that if the pigeon is being rewarded with food for pecking at the key, the pigeon will treat the key as if it was food. It will peck with its bill open and eyes closed just as if it was seizing a food item. If the pigeon on the other hand is being rewarded with water its bill is almost closed and its eyes open as if it was drinking water (Manning & Dawkins, 1998; Barnard, 2003).

In order for the animal to learn a certain behavior it is important to have the right response paired with the right stimulus. For example teaching a rat to press a pedal using electric shocks will not work, but using electric shocks to teach it to jump up on a shelf will work. This is because jumping up and escaping from the pain caused by the electric shock is natural for the rat. Pressing the lever is a behavior used when searching for food and the association between the pain and searching for food is therefore hard to make. (Manning & Dawkins, 1998; Jensen, 2006)

There are limits to what an animal can learn. In order for the animal to learn something it must be a behavior that is natural for the animal to perform. As an example, a pigeon cannot be taught to operate a lever with its feet in order to get food, but it is easy to teach a hen the same behavior. This has to do with the fact that pigeons do not use their feet when searching for food, but a hen does. (Jensen, 2006)

Habituation

Habituation means that the animal learns through repetition that there is no significance to a stimulus and eventually stops reacting to it. It could be a new and scary sound and first the animal reacts by trying to hide. As time goes by and the animal hears the sound over and over it no longer tries to hide and then the sound has become habituated (Manning & Dawkins, 1998; Barnard, 2003; Jensen, 2007).

Training animals for procedures

Most of the efforts to train animals for procedures have focused on nonhuman primates and dogs. Dogs are a domestic species and training dogs for procedures have focused on maintaining a positive human-animal bond. Nonhuman primates are non-domesticated, as well as being very strong and can easily harm a care taker. Therefore, training has focused on providing safe access to animals during veterinary care, cleaning living areas, as well as reducing the need to separate individual animals from the rest of the pack (Laule, 2010).

That mice can be trained with positive reinforcement using operant conditioning has been shown in numerous experiments (Heyser et al., 2000; Bussey et al., 2001; Wenger et al., 2004; Brigman et al., 2005; Carp et al., 2006; Bartko et al., 2010) as well as on video-sharing websites such as YouTube (Search phrase: mouse training immediately showed over 14 different PRT videos, 2013), yet most training is pretty basic, focusing on habituation by calm, gentle handling and patience (Laule, 2010)

Positive reinforcement training (PRT)

In PRT animals are reinforced with something they like, in return for performing a desired behavior (Brown & Jenkins, 1968; Pryor, 1999; Barnard, 2003). This gives the animals the option of cooperating willingly instead of being forced to do so (Laule, 2010).

Negative reinforcement

Traditionally most training of captive animals has consisted of negative reinforcement training. In negative reinforcement training the animal performs behaviors in order to escape something unpleasant. There are quite a few negative reinforcers in a laboratory; most of them are used for restraining for example thick gloves and crowding boards. Sometimes these restraining procedures is accompanied with loud voices and physical threats (Laule, 2010). Negative reinforcement works but the anxiety the animal feels when being forced to cooperate decreases their welfare (Pryor, 1999), and as mentioned before, increased stress may compromise research results

Benefits of positive reinforcement training

Hiby *et al.*, 2004 found that dogs trained exclusively using reward-based methods were more obedient and showed less problem behaviors than dogs trained with negative methods (including punishment) or a combination of positive and negative methods.

From a welfare perspective, PRT may offer the animals more control over their situation and a chance to work for their food (Institute for Laboratory Animal Research (U.S.) Staff, 2005), both factors that are associated with increased wellbeing (Lambeth *et al.*, 2006). PRT also provides increased possibilities in husbandry and veterinary care, making it easier to collect urine, semen and blood samples (Institute for Laboratory Animal Research (U.S.) Staff, 2005). PRT has also been shown to reduce stereotypic and abnormal behaviors (Laule, 1993; Bloomsmith *et al.*, 2007; Heidenreich, 2007; Baker *et al.*, 2009; Coleman & Maier, 2010).

Training methods

To train an animal using PRT you must first establish a functioning reinforcer. This could be food, toys or access to a favored environment. Anything the animal finds reinforcing and that is feasible in your experimental conditions. In operant conditioning it is common to use a metal noisemaker, called a clicker, as a signal of correct behavior. The animal is taught that the sound of the clicker (CS) is followed by a reward (UCS) using classical conditioning. (Pryor, 1999; Jensen, 2007; Laule, 2010)

Factors that could affect the outcome of a study on learning

Rodents undergo a number of important developmental processes concerning neurology and endocrinology during the first two weeks of life. These processes are mainly controlled by genes but environmental factors may also play an important role on the course of development (Ladd *et al.*, 2000; Matthews, 2002). This means that factors such as husbandry routines and handling practices can affect the development of animals, which in turn can have impact on the research results.

Prenatal maternal handling

If pregnant mice are stressed during the late prenatal period it can change the development of the brain in the offspring (Sternberg & Ridgway, 2003), one known stressor is handling (as reviewed by Braastad, 1998).

Handling of C57BL/6J dams resulted in an increased litter size, decreased length of gestation and decreased weight of the pups (Werboff *et al.*, 1968). When comparing the offspring of rats that were non-handled or handled during pregnancy, the prenatally handled rats were less emotional than controls when handled. The prenatally handled females also had a lower corticosterone response during the test (Ader & Plaut, 1968). Another study showed that prenatal handling decreased the serotonin levels in the offspring (Plaut *et al.*, 1972).

The effects of prenatal handling have also been studied in farmed blue foxes, where handling has been shown to be an important stressor. In two studies the prenatally handled cubs were more reactive in new environments (Braastad *et al.*, 1998) and/or had an increased adrenocortical function than the unmanipulated cubs (Braastad *et al.*, 1998; Osadchuk *et al.*, 2001).

Postnatal handling

Postnatal handling is a brief and daily separation of the pup from the mother for the first two to three weeks of the pups' life (Würbel, 2001) which has been shown to have several different effects on the animals. Mice that have been handled are more explorative, less anxious and has a lower corticosterone response to stress than unhandled mice (D'Amato et al., 1998; Moles et al., 2004; Parfitt et al., 2004). The behavioural and endocrine effects of handling persist throughout the animal's life (Meaney et al., 1988).

BALB/CByJ mice exhibit learning impairments in the Morris water-maze and hyper secretion of corticosterone. Zaharia *et al.* (1996) showed that postnatal handling in this mouse strain reduced the learning impairments and inhibited stress-induced disturbances during the Morris water-maze task.

There are two theories of what causes the effects of postnatal handling:

1. The effects of postnatal handling are mediated by changes in maternal behavior; that is the treatment of the pups affects the mothers' behavior, which in turn, affects the pups' development
2. The effects are a result of the handling itself (as reviewed by Denenberg, 1999)

Zaharia *et al.* (1996) also showed that cross-fostering BALB/CByJ mice with C57BL/6ByJ dams had the same effect as the postnatal handling, showing that maternal factors could influence the development of mice. Mice that were postnatally handled in combination with receiving new bedding showed less anxiety compared to mice that were handled but did not receive new bedding. The mothers of the litters receiving new bedding spent more time in nursing posture and in the nest than control mothers. These differences were also observed far apart from the daily manipulation, indicating a change in mothering style. (D’Amato & Cabib, 1987; D’Amato et al., 1992; Cabib et al., 1993).

D’Amato et al. (1998) repeated their earlier experiment with postnatal handling combined with receiving new bedding, but this time treated the mother with an anxiolytic drug. This showed that in the litters of mothers receiving the anxiolytic drug the effects of postnatal handling was prevented. The drug did not directly affect the pups, but the dams had decreased maternal behavior following separation from the pups. Several experiments with mice reared by a rat prone to maternal behavior and/or their mother has shown that pups receiving more maternal care had a reduced stress response and decreased anxiety behaviors on the elevated plus maze (Rosenberg et al., 1970; as reviewed by Denenberg, 1999). Taken together, these results support the importance of the mother-infant interactions on the effects of postnatal handling.

Several different factors can affect the outcome of studies on postnatal handling. Parfitt et al., 2007 observed that the stress response in C57BL/6 male mice is influenced by the time of day the pups are being handled. Pups handled during the last three hours of the light phase showed less stress response compared to pups handled during the first hours of the light phase. There are also findings that suggest there is a strain- and sex-related effect of handling (Anisman et al., 1998; Millstein & Holmes, 2007). Effects of handling have been reported to be evident primarily in males (Meaney et al., 1994), which is why we found it interesting to include both males and females were in this study. Exposing the mother to a stressful environment during separation can also induce long-term and short-term changes in offspring (Moles et al., 2004). Handling effects seem to be multifactorial and these factors mentioned above are some of the reasons why it is difficult to clearly assess the effects of handling.

There is currently more literature on the effects of handling in rats than in mice (Meaney, 2001). However, rats have been shown to have similar responses as mice to postnatal handling. In addition, there are studies showing that rats enhance learning and cognition in response to postnatal handling (Meaney et al., 1988, 1991; Oitzl et al., 2000; Tang, 2001).

Environmental enrichment

Barren housing of laboratory animals has been shown to impose constraints on their brain development as well as causing stereotypic behaviors (Rosenzweig & Bennett, 1996; van Praag et al., 2000; Gross et al., 2011). Mice are also known to develop stereotypies when raised in standard laboratory cages (Latham & Mason, 2010; Tilly et al., 2010; Gross et al., 2012; Leger et al., 2012). Stereotypies are repetitive behaviors that once fully developed, persist even under conditions where they would normally not be acquired (Mason, 1991; Garner, 2005). Two distinct forms of stereotypic behaviors in mice are bar gnawing and jumping. Both behaviors are the result of the animals trying to escape from their cages. These behaviors can account for up to 50 % of their daily activity (Würbel et al., 1996; Garner, 2005). With a few exceptions, environments that causes stereotypies are generally sub-optimal and plays a role in assessing welfare in animals (Mason & Latham, 2004; Mason et al., 2007). An enriched environment induces several changes in the brains of rats and mice, including increased numbers of neurons, synapses and dendritic branches, resulting in improved learning and memory (Rosenzweig & Bennett, 1996; Kempermann et al., 1997; van Praag et al., 2000; Lambert et al., 2005).

MATERIAL AND METHODS

Animals & Housing

Six pregnant mice (*Mus musculus*, NMRI) were purchased from Charles River (Sulzberg, Germany) and delivered via Nova-SCB (Sollentuna, Sweden). The mice were 12 +/- 2 days pregnant upon arrival. The mice were housed in Makrolon type III (38 cm × 22 cm × 15 cm) with food and water ad libitum. The cages were enriched with hay, a PVC pipe and paper tissues as nesting material (SCA, Belgium). The litters were kept intact and the number of pups per dam was 12.5 ± 3.5. The pups were weaned at 21 days of age and 24 of them were randomly assigned to be part of the training group. The training group consisted of 12 females and 12 males equally divided between the handled and non-handled pups. Pups that were visually much smaller than their littermates were excluded. The littermates not being used for training (control mice) were used in a handling test to evaluate any effects of training. For an overview of the groups see table 1.

The mice were housed in Makrolon type III cages (38 cm × 22 cm × 15 cm) with a density of maximum five per cage. The cages were enriched with autoclaved hay and a PVC pipe (Ø six cm) in the trained animals' cages (Figure 1) and autoclaved hay and a play tray made of wood in the control animals' cages (Figure 2). The mice were given water ad libitum at all times. An adult mouse eats approximately five grams of standard pellets (R3, Lantmännen, Kimstad, Sweden) each day (Maslov, 2013). During training the mice were fed approximately 90% of their recommended intake: 13.4 - 13.7 grams per cage of three. The temperature was kept at 22.8±0.4°C and the humidity at 42 ± 2,1%.

Figure 1. *Housing of trained animals.*

Figure 2. *Housing of control animals.*

Table 1. *Overview of the groups in the experiment*

Trained mice	Control mice
Handled males (THM)	Handled males (CHM)
Non-handled males (TNHM)	Non-handled males (CNHM)
Handled females (THF)	Handled females (CHF)
Non-handled females (TNHF)	Non-handled females (CNHF)

Handling

Prenatal maternal handling

The pregnant mice were handled for five minutes on a rotating schedule twice a day for five days until the first litter was born. During handling the mothers were picked up with a cupped hand, were gently stroked and allowed to move freely on the researchers arms. In this study the pregnant mice were handled in the late phase of pregnancy to minimize their stress during the postnatal handling (Kaliste, 2007).

Postnatal handling

After parturition, half of the litters were randomly selected to be handled (H) from the day of birth. During handling the pups were removed from their home cage and placed in an artificial nest with nesting material. Gloves were worn at all times during the first two weeks and the dams of the H litters were removed from the cage before handling of the pups, to keep the dams' stress to a minimum (Hedrich, 2004). Non-handled (NH) litters were also separated from their mother but were left undisturbed. Handling and separation occurred twice a day for five days a week on a rotating schedule. Both H and NH litters was weighed once a week in connection to cage cleaning.

Choosing a reward

The rewards tested in this study were banana drink (Danone, Poland), chocolate milk (Arla Foods, Stockholm, Sweden) and two flavors of soft ice cream (Arla Foods, Stockholm, Sweden) vanilla and strawberry. Liquid rewards were chosen because of the reward-delivery system.

The pregnant mice had access to the rewards from the second day of arrival and until the pups were 20-21 days of age. The rewards were placed in the cage once a day in small glass bowls (IKEA, Uppsala, Sweden). This meant that the pups were used to the smells of the rewards from an early age. To decide which reward was the most favored, the number of approaches and times the adult mouse drank was recorded for the first two minutes upon presentation (see table 2). Every reward was compared to each of the other rewards twice. The most popular reward was the reward from which the mouse drank the most, if a tie were to occur the number of approaches decided which reward the mouse preferred. Each reward was able to be most favored up to 50 times during testing.

Table 2. *Definition of behaviors recorded during reward testing*

Behaviour	Description
Approaching	Touching the bowl with the head or leaning over the edge of the bowl
Drinking	Tongue touching the reward or continuing for >30 second, only pausing briefly for grooming and without moving hind paws

Training area

The training area consisted of a plastic box (55x35x28cm, Kis, Italy). The box was wiped with water after each session. Fecal boli and urine was wiped off after each mouse. The training device was placed against the wall in the middle of the long side of the box. This was to make it the most desirable place to be.

Training device

The device was constructed from a PVC pipe with an optic sensor attached. It rested on two plastic feet and contained a smaller plastic test-tube (head holder) which function was to stop the mice from getting out from the front of the PVC pipe. Under the sensor, a small metal pipe for presenting the reward (figure 3 and 4), was connected to a long plastic tube and a pipette (10μl per click), which was

manually controlled from the outside of the cage. A small speaker (Salcon 80W, China) was also connected to the device emitting a short sound that was to function as a reward marker. When the sensor was activated a short sound was triggered. For the sound to be triggered the mouse had to block the sensor, and for it to be triggered again, the mouse had to unblock and then re-block the sensor. A delay could be set for which the mouse had to block the sensor and stay there for a chosen number of seconds. After blocking the sensor the device had to be manually reset by pressing a button.

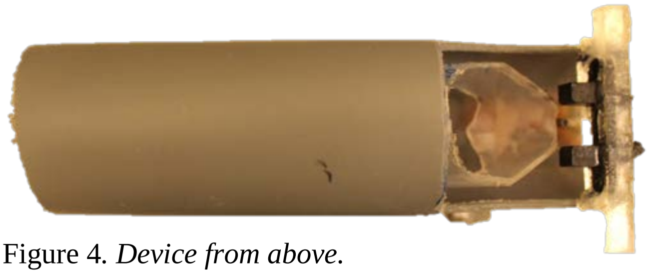

Figure 4. *Device from above.*

Training protocol

The training consisted of three phases: habituation, building value and increasing criteria. The mice spent a total of 14 times in the training area during this experiment. All mice spent one minute in the training area twice a day, every weekday. Exceptions were on days of behavioral testing and two days after the training started on which no training was done. During the first and last day of training there was only one training session. For an overview see table 3.

Habituation was considered to be done once the mice started to take the reward from the metal nipple. They then spent the next visit on building value for staying in the device by constantly receiving the reward when their head was in the correct position. After that the criteria was increased so that the mouse had to stay a chosen amount of time before receiving the reward (for criteria see table 4). When the session ended the mice that were still in the pipe, were gently tapped by a finger from behind making it leave the pipe. Mice were always lifted in a cupped hand and never by the tail. All mice were individually marked with a pen (Faber-Castell, Germany) around the base of the tail and the markings were filled in again after the training sessions when necessary.

Table 3. *Overview of training sessions*

	Monday	Tuesday	Wednesday	Thursday	Friday
First week	T	T		T	T

Second week	B	T	T	B	B
Third week	T	T			

T = days of training, B = days of behavioural testing and blank = no manipulation of the mice

Table 4 *Criteria for receiving reward (seconds)*

1
2
2,5
3
3,5

Elevated Plus maze (EPM)

At 44-46 days of age, the mice were tested on an Elevated Plus Maze. The EPM was elevated to approximately 50 cm above the ground and the arms' dimensions were 40 x 10 cm (see figure 5). The two closed arms had sidewalls that were 40cm high with open ends. The mice were placed in the central zone of the maze (10 x 10 cm) facing one of the open arms. The testing time was five minutes. The test was performed in the animal room at normal light and it was videotaped (Logitech webcam C920, China) from above.

The tapes were analyzed for latency to the first visit to an open arm and the number of transits between open and closed arms. During testing the frequency of stretch attend posture (SAP), rearing and the number of fecal boli were recorded (see table 5). The maze was cleaned with soap water after each mouse.

Table 5. *Definition of behaviours in the EPM*

Behaviour	Description
Stretched attend posture	The animal's body is stretched forward and then retracted to the original position without moving forward
Rearing	Standing on hind legs with both forearms in the air or against the wall
Arm entry	Entering the arm with 3 out of 4 paws

Figure 5. *Elevated plus maze with mouse.*

Handling test

At age of six weeks the mice were tested in a handling situation. The person performing the test was a skilled animal technician who was unfamiliar to the mice. For the test person to be unbiased the cage labels were removed and treatments of the different mice were not disclosed until the test was finished. The test was videotaped (Logitech webcam C920, China) from the side. The test person first put her hand in the cage for 30 seconds. The closest third of the cage was defined as “hand zone”. The number of visits to this zone was recorded. After that, the test person caught a mouse and restrained it. The test person then scored the mouse as compliant, hesitant or unwilling to be fixated (see table 6). Mice in this test included the control mice and the mice being trained.

Table 6. *Definition of behaviours during the handling test*

Behaviour	Description
Visit in hand zone	Entering with 3 out of 4 paws
Compliant	Accepting restraining without attempting to get free. Minor movements, e.g. of hind legs were accepted
Hesitant	Moderate movement of leg and body while being restrained
Unwilling	Moves constantly and does not relax

Statistical analysis

The data from the elevated plus maze was analyzed using Two Way Analysis of Variance (ANOVA), with gender as factor one and handling as factor two. For analysis of the number of mice who succeeded in taking the reward in the training arena a Two Way ANOVA was used with gender as factor one and handling as factor two. For testing the effects of gender, handling and training on compliance in the handling test, a Three Way ANOVA was used with gender as factor one, handling as factor two and training as factor three. The scores from the tests were set as unwilling=1, hesitant=2 and compliant=3. The Holm-Sidak method was used for all pairwise multiple comparisons. All Statistical calculations were performed using SigmaPlot 12.0 for Windows. Results were considered significant at $P<0.05$.

RESULTS

Choosing a reward

The results showed a strong preference to the soft ice creams, with the strawberry flavor most favored (see figure 6).

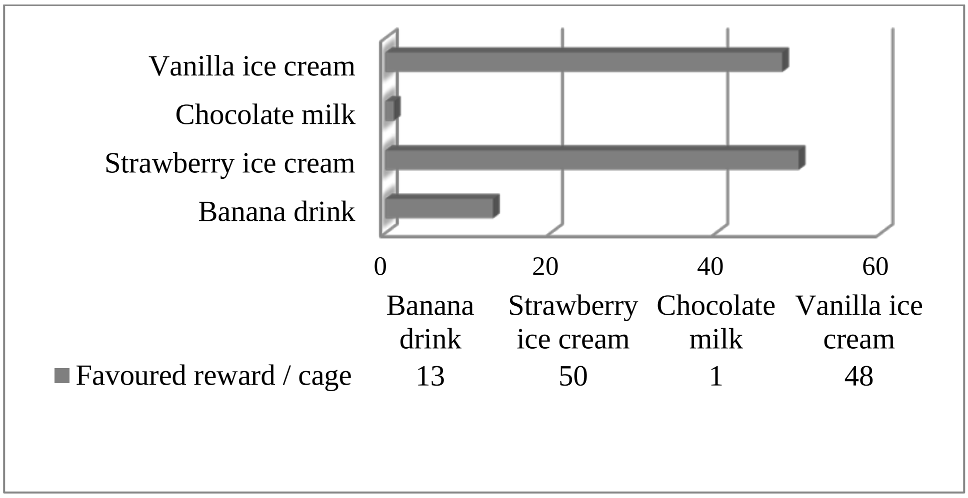

Figure 6. *Reward testing*

Training results

When the training was done nine out of 24 mice had taken the reward from the device. After the three day break there was no reversed learning. At that time one mouse had taken the reward from the device and had had a building-value session. After the four-day break one mouse did no longer drink from the device. That mouse had taken the reward for the first time during the session before the break. There were two males that had taken the reward from the device, one from the handled and one from the non-handled group. Of the handled females two mice took the reward from the device and in the non-handled group five took the reward from the device (see table 7). Females were significantly more successful than males in taking the reward (P=0.007)

Table 7. *Percentage of mice that have taken the reward from the device after 14 visits*

Group (n=6)	Percentage
HM	17%
NHM	17%
HF	33%
NHF	83%

HM= handled males, NHM= non-handled males, HF= handled females, NHF= non-handled females

Elevated Plus Maze

In the elevated plus maze, the only factor that had effect on the behaviour was handling. The non-handled mice showed a significantly higher number of stretch attend postures compared to the handled mice (28 ± 6,3 vs. 20 ± 5,8, P <0,001). No other differences between handled and non-handled mice could be detected in any of the parameters measured. Neither were any differences between males and females found.

Handling test

The trained animals were significantly more compliant than the control animals (54 ± 0,7 % vs. 21 ± 1,2 % scored compliant (=score 3), average scores were 2.38 ± 0,16 vs. 1,69 ± 0,14 (P = 0,003)). No effects of handling or gender on the compliancy of the animals could be detected. The results showed no difference between all of the compared groups in the number of visits to the hand zone or compliance to handling. Results from the analyses are presented as means ± standard error of the mean.

DISCUSSION

Evaluation of the training device

Shape, size and placement

The PVC pipe was large enough for the mice to sit up straight and comfortable while contemplating the situation. Mice being highly susceptible to predators are likely to show fear in unfamiliar environments, if there is not a place they can seek shelter. The device's placement in the arena and the choice of a narrow tube for the device were successful features. The mice quickly went into the pipe and did not prefer to stay in any particular corner of the training area. However, urine and fecal boli were often deposited in the PVC pipe with little access to properly clean it. Mice that had received reinforcement in the pipe could sometimes be hard to persuade to leave. The head holder was not small enough to keep the mice from crawling through it. The head holder's length required the mice to stand on top of it with their front legs in order to be in a comfortable position while training. The metal pipe had a good placement.

Reward-delivery system

The reward delivery system was not adapted to the reward and had only previously been tested on water. When using the pipette the reward did not stay in place on top of the metal pipe resulting in pooling of the reward under the head holder. Mice were on numerous occasions drawn to this puddle of strawberry ice cream; especially mice, which had learned to drink from the metal pipe. Sometimes the mice were distracted by the puddle, which disrupted the training sessions. The small plastic tube connecting the pipette and metal pipe would often fall off of the pipette interrupting the training.

Sensor-sound system

When the mice blocked the sensor a sound was emitted from the device. The sound did not seem to disturb the mouse. After the mice licked the reward from the metal pipe they first continued licking with increased intensity (interpreted as frustration). Once they stopped licking the metal pipe they immediately popped their heads up and sometimes tried again. The device required a much longer refraction period than the mice were willing to wait. Therefor the training continued by increasing the interval for which reward was delivered. The mouse with the most training sessions managed to stay in position while receiving reward with approximately 3.5 seconds in between rewards. The intense licking had seized although the mouse still licked at the metal pipe between rewards. The placement of the reward seems efficient for staying in the pipe. If the aim is to make the mouse stay still with its head this is not an optimal placement of reinforcement since the behavior being reinforced is the licking on the pipe.

Suggestions of improvements on the device

Mice differ in size depending on the strain, litter, gender and age, making it hard to make a head holder that fits all. Even though they could easily get out of the PVC pipe from the front it did not seem to be the preferred choice once the mice had learnt to drink the reward. Perhaps the openness of the pipe encouraged mice to spend time close to the sensors or perhaps it delayed learning by making it easier to climb away from it. Being able to open the pipe from the side would make it easier to clean and fetch mice not willing to leave. One solution for easy cleaning could also be to make it without a separate head holder since most urine and ice cream ended up under it.

Either the reward or the metal pipe must be adjusted so the reward does not fall off creating a pool of ice cream under it. The reward tube cannot fall off during training since then the mice's behavior of blocking the sensor will start to be extinguished during that time. Skinner found out that in general, delays of more than eight seconds between a behavior and receiving the reward greatly slowed learning (Manning & Dawkins, 1998). On occasion it would take more than eight seconds to repair the reward system and it is reasonable to conclude that this slowed down the mice's learning.

The device needs a much shorter refraction period to be able to use it in training. It is also unwanted that the mouse must unblock and the re-block the sensor in order to get the next reward. If it cannot be managed automatically perhaps a light diode could help the trainer to indicate the mouse is in the right position and the trainer can trigger the reward-signal manually. Menzel and Erber (1978) researched how bees learn which type of flower produces nectar. They had artificial flowers that changed color with a sugar reward inside of them. In order to associate a certain color with the reward the color had to be presented three seconds before and 0,5 seconds after the bees got the reward. This is quite a narrow span to form an association between color and reward. I have not found the necessary span for mice to form association in the literature, and while mice not being bees one could assume the reward need to be presented within a short timeframe. On the other hand rats can learn to avoid certain food that makes them feel sick, even if they do not feel ill for hours after consuming it (Manning & Dawkins, 1998). More research is necessary to establish an efficient training protocol for mice.

Behavioral testing

When the mice in the present study were tested in the EPM, there was not a clear dissimilarity between the different groups (genders, handled and non-handled). This could either be due to effects of the training or the prenatal handling that all mice tested had received. The different housing of the trained and control animals could also have influenced their behavior. In the handling test the trained animals (including handled and non-handled animals) were more compliant than the control animals (littermates which had not been trained). This could indicate that our gentle and friendly handling of training group after weaning had an effect on the animals. In Hurst & West's (2010) study they showed that mice had less anxiety during handling and behavioral testing when lifted in a cupped hand or tube than when being lifted by the tail. Perhaps friendly handling could also have an effect on the behavior of mice on its own and this would explain the difference between the trained animals and the control animals.

Restricted diet

In this study the mice were fed approximately 90 % of their recommended daily intake of food. The estimate of a mouse eating roughly five grams per day was highly generalized as nutritional requirements depend on many factors. For most of the time there was still some pellets left in the cage when training started indicating that the animals in fact were fed ad libitum. When given the choice between working for food or eating food that is continuously available, animals prefer to work for their food. This phenomenon is called contra-freeloading (Osborne, 1977; Inglis, 1997). Contrafreeloading has been shown in mice (as reviewed by Inglis, 1997), rats (Hothersall *et al.*, 1973; Morgan, 1974; Carlson & Riccio, 1976), birds (Duncan & Hughes, 1972; Bilbrey *et al.*, 1973; Inglis & Ferguson, 1986), pigs (de Jonge *et al.*, 2008), primates (Menzel, 1991; Reinhardt, 1994) and humans (Tarte, 1981). Since most PRT with other animals do not require fasting and rewards are given as additional treats (Laule, 2010) this could indicate that it is also unnecessary when training mice.

Training procedures

Our results showed that only two males, one from the handled and one from the non-handled group, started to drink the reward from the device. The two males seemed more restless than the females while receiving reward and frequently stopped to walk around the training area. Interestingly enough both times when the males learned this there had not previously been any females or unknown males in the training area. Mice have many social olfactory cues, one is urinary odors. One study has shown that when transferring sawdust, containing urine during cage cleaning, aggression significantly increased (Van Loo *et al.*, 2000). In the wild, male mice form territories and will defend his territory from unknown males (Kaliste, 2007). Male mice will mark their territory with urine to communicate with other males and to recognize one another. Female odor cues have been shown to have a number of behavioral effects on males, including aggressiveness (Kavaliers, 2001). The males would probably have performed better in this test, if the area was free of odors from females and/or unknown males.

After a four-day break one mouse did not take the reward the following training session. This mouse had on the previous session taken the reward for the first time. On the second training session after the four-day break the mouse resumed to drink from the device. After a three-day break a mouse that had received reinforcement during two sessions in the device before the pause, did not stop drinking from it. Four days may have been too long and the mouse forgot how to drink from the device or pausing after only one training session made the mouse forget what behavior was reinforced last time. Maybe pausing caused the mouse to be uneasy with the environment of the training area making investigating a priority over food. The mice that had received reinforcement for more than two sessions did not seem affected by these breaks. How pausing from the training affected those who had not yet learnt to drink from the device is uncertain but it might have slowed their learning. In rats, the length of memory retention depend on the intensity of training when learning a task, where spaced training is generally more effective than massed training (Scharf *et al.*, 2002; Commins *et al.*, 2003). It is difficult to know for how long a mouse will remember a learnt task since the protocol for acquisition differs between experiments.

In our study there was a trend of the non-handled females having higher success in learning to drink from the device. There are findings of females being affected differently by handling than males (Anisman et al., 1998; Millstein & Holmes, 2007). In rats it has been reported that postnatally handled females have a higher hormonal stress response than unhandled females (Papaioannou *et al.*, 2002; Park *et al.*, 2003). This could be an explanation of the results in this experiment, but further research is necessary to be able to clarify this.

The facility manager and the animal technicians reported that the mice used in this study were more active during the daytime when the staff went into the room than mice in the rest of the facility. In our study we trained the animals during the light phase since other mice that could not reverse their daylight cycle were in the same room. This could explain their active behavior towards the staff as they were used to activities during the daytime.

In conclusion training mice to stand reasonably still is possible although requiring a long habituation period. With an improved device further training and research can be done. More research is necessary to acquire a training protocol for mice. Males might be disturbed while learning if the scent of females and/or unknown males is present. Gentle and friendly handling after weaning could affect stress and anxiety in mice, so much that the effects of postnatal handling are abolished.

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