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TITLE:

Using Clicker Training and Social Observation to Teach Rats to Voluntarily Change Cages

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KEYWORDS:

Cage change, Clicker training, Positive reinforcement training, Refinement, Hygiene, Well-being

SUMMARY:

This protocol introduces a method of cage change for rats via clicker training. Rats learn the desired behavior not only by direct training but also by observational learning. The implementation of this fast and easy protocol might help to improve well-being and hygiene in rodent facilities.

ABSTRACT:

Cage cleaning is a routinely performed husbandry procedure and is known to induce stress in laboratory rats. As stress can have a negative impact on well-being and can affect the comparability and reproducibility of research results, the amount of stress experienced by laboratory animals should be minimized and avoided when possible. Further, the direct contact between the rat and animal caretaker during the cage change bears hygiene risks and therefore possibly negatively impacts the well-being of the rats and the quality of the research.

Our protocol aims to improve the routinely performed cage changing procedure. For this reason, we present a feasible protocol that enables rats to learn via clicker training and observation to voluntarily change to a clean cage. This training helps to reduce stress caused by the physical disturbance and handling associated with the cage changes and concurrently enables a reduction in direct contact between animal and animal caretaker after the training phase is completed.

The implementation of clicker training to rats is fast and easy. Rats are generally interested in the training and efficiently learn the desired behavior, which entails changing cages through a pipe. Even without training, the rats learn to perform the desired behavior by observation, as 80% of the observational learning group successfully changed cages when tested. The training further

helps to establish a relationship of trust between trainer and animal. As hygiene and well-being are both very important in animal experiments, this protocol might also help to improve high-quality research.

INTRODUCTION:

Routine procedures can cause stress in laboratory animals¹⁻⁴. It has been shown that cage changing does increase cardiovascular parameters and general activity in rats⁴⁻⁶. Such stress responses can at least be partially due to the physical disturbance and handling associated with cage changing procedures rather than the new unfamiliar environment^{2,4}. Of particular importance is the negative effect of stress on the well-being of the regarded animal⁷. Further, stress induces changes in behavior and in other body parameters, including the autonomic nervous system, the neuroendocrine system and the immune system. Therefore, stress is often referred to as a possible source of unexpected deviations across animal experiments and should be avoided as much as possible in high-quality animal research^{7,8}. One way to reduce stress in laboratory animals is by training. Training animals can generally be a very helpful tool for laboratory animal management and is, in fact, demanded by EU Directive 2010/63/EU⁹. Training can serve as a form of enrichment and helps to prepare animals for experiments; thus, training contributes to sustaining and enhancing well-being in laboratory settings¹⁰⁻¹². A possible training method is positive reinforcement training (PRT). PRT is a form of operant conditioning where a reward (*e.g.*, a food reward), is linked with a desired behavior¹³. This form of training is already commonly used in laboratory settings for nonhuman primates in order to reduce stress and to enhance well-being and has gained popularity in various other animal species^{10,13-16}. Training of voluntary movement is also frequently used for refining animal husbandry management¹⁷⁻¹⁹. PRT is not only a helpful tool for cooperation when working with animals; it likewise is generally beneficial for the animals' well-being, regardless of whether the trained behavior is directly used²⁰. The positive reinforcement training protocol described here aims to avoid any form of stress during routinely performed cage changes by giving the rat the opportunity to voluntarily participate in the procedure.

In addition to the possible stress for the animals, working processes in animal facilities always provide pitfalls for the maintenance of hygiene status, especially as the direct contact between laboratory animals and animal caretakers bears the risk of hygiene contamination. In animal facilities specializing in rodents, the transfer of the animals from dirty into clean cages provides a regular and high workload. This procedure usually includes direct contact between animals and humans and thus represents a hygienic risk factor due to the possible transfer of pathogens settled on the human skin²¹. Aside from transfer from other animal vectors, pathogenic and nonpathogenic organisms intrude animal facilities most frequently via humans^{22,23}. As reduced health is associated with reduced well-being, even subclinical infections can be a cause for nonreproducible experimental results, and this should clearly be avoided^{24,25}. On this account, training animals to voluntarily change cages further enables the conduction of the cage change after the training with hardly any direct contact between animal and animal caretaker, thereby reducing a potential hygienic risk while performing experimental procedures.

Our protocol for training rats to voluntarily change cages can be a helpful tool for laboratory rat

management, as it links the training as a form of cognitive enrichment with performance of routine work. Accordingly, our protocol is an animal-friendly procedure that helps to increase the well-being of rats in laboratory animal facilities. As the cage change after the training phase can be performed with hardly any direct contact between animal and animal caretaker, it might additionally help to maintain hygiene at a high level and therefore further improve animal research.

PROTOCOL:

The handling of the rats and the experimental procedures were conducted in accordance with European, national, and institutional guidelines for animal care.

1. Acclimatization and Habituation

Note: If rats were not transported, the acclimatization and habituation time can be reduced. For noninvasive identification, color the tail with skin-friendly marker.

1.1. Week 1: Acclimatization

Note: Two sessions should be performed during the first week, one on the day of the arrival and a second one 2 days after arrival.

1.1.1. On the day of arrival of the rats:

1.1.1.1. Transfer the rats in pairs of two to their new home cage. Transfer some of the old nesting and bedding material as well as some of their familiar food pellets to facilitate the habituation to the new environment.

1.1.1.2. Avoid lifting the rat by its tail. If possible, put one hand under the belly or around the chest and the other hand supporting the hind legs of the rats to lift them (**Figure 1**)²⁶.

1.1.2. Two days after the day of arrival:

1.1.3. Open the home cage of the rats and put a hand in the cage. Let the rats sniff and explore the hand. If the rats are trying to get out of the cage, hinder them by gently pressing them back in the cage. Continue with this procedure for the next four minutes.

1.1.4. If the rats are afraid and do not start exploring their environment, repeat the procedure once per day until the rats start to explore. In this case, a habituation to the clicker might be necessary.

Note: Rats explore new things with all of their senses, so after some time, they will recognize the same experimenter as being familiar. It might happen that the rat does oral explorations of the hands, but it should not be in an invasive manner. If this situation occurs, the hand must be slowly

taken away to ensure that the gloves are not destroyed.

1.1.5. Add small pieces of the intended food reward to the home cage.

Note: The food reward must be in accordance with the requirements for laboratory animal nutrition. In this protocol, white chocolate is used (held at the end of a syringe), but other food, such as dry fruits, fresh fruits or cereals, is possible. If the animal facility hygiene requirements demand special conditions, there are various suppliers for treats for laboratory animals. The healthiest option is preferable, but a mix of high fat and high sugar may give the animals a greater motivation to participate.

1.2. Week 2: Habituation to the scientist and the food reward

1.2.1. Continue with the habituation to the hand and the food reward started in the previous week. Limit the interaction time to two minutes per day.

1.2.2. In cases where the rat is very anxious, habituation to the clicker might be necessary.

1.2.2.1. To evaluate this behavior, click once every five seconds after the session on day 1. If the rat reacts with a fear-related behavior, the clicker must be habituated.

1.2.2.2. For habituation to the clicker, click five times while the rat consumes the reward that is placed in the cage after the session. The habituation to the clicker is completed as soon as the rat no longer shows any fear-related behavior.

1.3. Week 3: Habituation to intense handling

1.3.1. On days one to three, start with lifting or stroking the rat for several seconds and slowly extend the time (max. two minutes per rat). Stop if the rat shows any aversive signs such as freezing, dodging or hiding.

1.3.2. On days four and five, end the handling in tickling, as described in Cloutier *et al.* (2012).²⁷

Note: Indicators for a successful habituation are, for example, the rat taking the food reward if it is offered by hand or the rats getting close to the hand, exploring it, or touching it.

2. Clicker Training

Note: A training session takes four minutes and consists of intervals of 30 seconds of training followed by 15 seconds of break. Remove any added material during the pause. The observer rat is free to do what it wants, and it is allowed to participate and to consume the reward, but the focus is solely on the other rat. All rats were tickled on day one and day five. For the training success, it is crucial that the experimenter pays attention to the animals' behavior and is acting in a calm manner. The training strongly depends on the experimenter's abilities of timing and

power of observation. No verbal feedback occurs in the training, and verbal communication with the animals may just occur outside the sessions. An indicator for the learning of the desired behavior is the repetition of the behavior.

2.1. Preparation

2.1.1. Prepare the required material, such as the reward, the clicker/target stick combination, a timer, a clean cage, the metal tube, and any other specific training material.

2.1.2. Transfer the home cage to a quiet place.

2.1.3. Remove all items from the cage. Only the two rats (trained and observer) and the bedding material should remain in the cage.

2.2. Training day 1: Establishing a connection between the primary and secondary reinforcer

2.2.1. Place a short tunnel in the home cage, preferably one the rat is familiar with.

2.2.2. Wait until the rat sits in the tunnel and then feed the rat with the reward.

Note: If the rat is not feeding under the circumstances of the training situation, the habituation phase must be extended or adjusted.

2.2.3. As soon as the rat shows interest in the reward and is about to start nibbling on the reward, click and then let the rat feed on the reward. To ensure that the pairing of the two reinforces is successful, repeat clicking and then rewarding for 15 seconds. Do this at least twice during this first training session.

2.2.4. If the rat did participate, check for signs of the establishment of a connection between the primary and the secondary reinforcer. The main criterion for this classification is the estimation of the food reward, which is shown by the rat by sniffing, looking around and at the hand that presents the food reward or lifting to its hind limbs in order to look for the reward (**Figure 2**). If this occurs, move on with the next part; otherwise, repeat "Training day 1"

2.3. Training days 2 to 5: Passing through a short tunnel

Note: A training session consists of alternating phases of 30 seconds of training and 15 seconds of break for four minutes.

2.3.1. Place a short tunnel of the same material as that of the first used/original tunnel that will link the two cages in the home cage.

2.3.2. Start the timer and immediately begin with the first 30 seconds of training.

2.3.2.1. The first time the rat enters the tunnel, present the reward at the end of the tunnel. As soon as the rat enters the tunnel, click and let the rat eat the reward.

2.3.2.2. Take away the food reward and wait until the rat shows interest for the tunnel once more. Click as soon as the rat enters the tunnel and present the reward at the end of the tunnel (Figure 3).

2.3.2.3. After 30 seconds of training, add a 15 second break (remove the tunnel).

2.3.3. Repeat the 30 seconds of training with a subsequent 15 second break for four minutes.

2.3.4. If the rat shows the desired behavior more than four times a minute, stop clicking and just reward with the primary reinforcer.

2.3.5. Stop after four minutes.

2.4. Training week 2: Touching a target stick

2.4.1. Start the timer and immediately begin with the first 30 seconds of training. Add a break every 15 seconds and then go on with the training.

2.4.2. Place the globe of the target stick near the rat.

2.4.3. As soon as the rat shows interest in the globe, click and reward the rat. Repeat this several times.

2.4.4. If the rat shows interest in the globe more than four times a minute, the next behavior to reinforce is "touching the globe with the nose".

2.4.5. If the rat repeats that behavior for more than four times a minute, stop clicking and just reward with the primary reinforcer.

2.4.6. Stop after four minutes.

2.5. Training week 3 (five days) and week 4 (three days): Changing the cage through a tunnel

Note: Here, only passing through the tunnel towards the clean cage is reinforced. The training in week 4 has been shortened to three training days. This time is already sufficient for training success and gives the opportunity for one additional day for evaluation or data recording for further experiments.

2.5.1. Build up a connection between the home cage and a new clean cage by a tunnel by inserting a longer version of the tunnel already familiar to the rat.

265 2.5.2. On day one, perform no training, and just let the rats inspect the tunnel. There is a good
266 chance that they will even transfer to it on their own.

267
268 2.5.3. On day two, give the rat two minutes to transfer through the tunnel on its own from the
269 dirty to the clean side. If the rat freely transfers through the tunnel, skip to step 2.5.8.

270
271 2.5.4. Otherwise, start the first 30 second training session (alternate between 30 seconds of
272 training and a 15 second break) for four minutes.

273
274 2.5.5. Click and reward the rat as soon as it shows interest in the tunnel.

275
276 2.5.6. Go on to click and reward the entrance into the tunnel.

277
278 2.5.7. Go on to click and reward if the rat goes deeper in the tunnel.

279
280 2.5.8. Continue in a similar manner until the rat runs through the whole tunnel. As soon as the
281 rat starts peeking out at the end of the tunnel into the clean cage, present the globe of the target
282 stick, click, and reward for touching the globe.

283
284 2.5.9. At last, guide the rat to leave the tunnel. For this process, use the globe of the target stick.
285 Present the globe in front of the tunnel and click and reward the rat as soon as it steps in the new
286 cage and is touching the globe.

287
288 2.5.10. Transfer the rat back to the “dirty” home cage. Put one hand under the belly or around
289 the chest and the other hand supporting the hind legs of the rats to lift them (**Figure 1**). Present
290 the globe of the target stick in the center of the clean cage. Click and reward as the rat runs
291 through the tunnel and is touching the target stick (**Figure 4**).

292
293 2.5.11. If the rat repeats the behavior for more than four times a minute, stop clicking and just
294 reward with the primary reinforcer.

295
296 2.5.12. Stop after four minutes.

297 **REPRESENTATIVE RESULTS:**

298
299 The training was conducted on a cohort of ten female Lister hooded (LD) rats. Ten untrained but
300 gently handled female LD rats served as a control group. Gentle handling means that the rats
301 were only lifted by their body and not lifted at the base of the tail. To evaluate the learning by
302 observation, we added one further group of 10 female LD rats, which were not trained but were
303 cage-mates of the trained rats and were able to observe the training. All rats completed the
304 acclimatization and habituation to handling. The training comprised moving to a new cage
305 through a metal pipe (**Figure 4**). Demonstration of the desired behavior was rewarded (**Figure 4**).
306 Every training session was evaluated, and the training success was analyzed in three categories:
307 participation, no participation and explorative behavior. The trained rats were generally
308 interested in the training but also showed a high motivation to explore the new environment.

Especially during the first training days, the explorative behavior was very prominent (**Figure 5**). After only three days of training, a majority of the rats displayed the desired behavior and changed the cage via the metal pipe (**Figure 5**). After completing five days of training, all trained rats demonstrated the behavior (**Figure 5**). The training was continued for four weeks, with five days of training each. After four weeks of watching the “training status” of the observational training group, these rats were evaluated. For this evaluation, we counted the number of times the rats ran through the tunnel into the clean cage during one training session. Overall, 80% of the rats learned the desired behavior by watching (**Figure 6**).

We further examined whether the training had a positive effect on the trained rats. Therefore, we evaluated fear-related behaviors of the rat during restraint after four weeks of training. Urination, defecation and vocalization were recorded, and an aversive score was calculated (no fear-related behavior = 0, one fear-related behavior = 1, two fear-related behaviors = 2, three fear-related behaviors = 3). The untrained group showed a notable increase in fear-related behavior scores (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1: Gentle lifting of the rat. One hand is placed under the belly or around the chest, and the other hand supports the hind legs of the rat.

Figure 2: Sniffing and Rearing. The rat shows interest in the food reward by sniffing, looking around, or lifting its hind limbs.

Figure 3: Short tunnel. Rewarding a rat after passing through a short tunnel. The observer rat shows interest in the procedure.

Figure 4: Rewarding after cage change. A rat is rewarded after moving into a clean cage through a metal pipe.

Figure 5: Days until learning. A group of ten female Lister hooded rats were trained to change cage via a metal pipe by the use of clicker training. The training was evaluated in three categories: participation, no participation and explorative behavior (nd: not detected: none of the rats showed the respective behavior). All rats acquired the trained behavior after five days of training.

Figure 6: Observational learning. A group of ten female Lister hooded rats that were cage mates of trained rats did not undergo training themselves but were allowed to observe the training. After four weeks of observation, the rats were evaluated for voluntary cage changing behavior. Overall, 80% of the observational learning group changed cages via the metal pipe.

Figure 7: Fear-related behavior score. After four weeks of training, all rats across the 3 groups (trained, observational learning, untrained control group) were restrained, and vocalizations, defecation and urination were recorded and subsequently used to calculate a fear-related behavior score for each rat (no fear-related behavior = 0, one fear-related behavior = 1, two fear-related behaviors = 2, three fear-related behaviors = 3). The fear-related behaviors are depicted

as the mean \pm SEM. For statistical analysis, a one-way ANOVA with subsequent Tukey's multiple comparisons test was performed. The untrained group showed an increased fear-related behavior score compared to that of the trained and observational learning groups.

DISCUSSION:

The protocol described above is a useful extended application of our previously described clicker training protocol for laboratory mice¹⁰. The implementation requires only minutes per day over a total of seven weeks, including acclimatization, habituation and clicker training. For feasibility, this protocol was limited to week days with sessions of approximately ten minutes per pair of rats. The protocol can appear to be time-consuming to establish in large rat colonies, but if the animals are designated for long-term experiments or as breeders, it can help to improve the process of cage changing in the long term. In addition, a reduced disturbance while performing experiments can improve the quality of the results. Further, all time spans noted in this protocol are recommendations and can be amended to fit specific requirements any laboratory. Time spans can be shortened if the animals learn the tasks quickly or if the rats have already habituated. Generally, the rats are interested in the training and require only a few repetitions to acquire the desired behavior "cage change through a pipe". Even without training, rats learned to perform the desired behavior by observation, as 80% of the observational learning group successfully engaged in volunteer cage-changing when tested. Here, we present a method for trained cage changing because it is a frequently performed routine procedure in animal husbandry and is known to cause stress in animals. This stress is at least partially due to the physical disturbance during the cage change^{2,4}. Training gives the rats an option to voluntarily cooperate in performing this husbandry procedure. Having a choice in engaging in a task is suggested to have beneficial effects on the animals and therefore might help to reduce stress²⁸. Training is also beneficial in others ways, as it serves as a form of cognitive enrichment. The positive effect on the rats became clearly visible by a reduction in fear-related behavior upon being restrained. Not only did direct training appear to be beneficial for later handling of these rats, but observational learning reduced the fear related behavior score as well. Our rats were kept in standard environments, enriched with a PVC-tunnel and tissue paper. In addition to the training, all rats across the three groups were subjected to a tickling assay. Accordingly, we did not detect any deficits in well-being in any of the groups, as there were no differences in sucrose preference or burrowing behavior between the three groups (data not shown). Nevertheless, the training appeared to be beneficial for the rats, as they showed reduced fear-related behavior while being restrained.

On the one hand, training the rats might lead to a nonstressful interaction between humans and animals, from which the animals clearly benefit. On the other hand, however, contact between humans and animals always bears a risk of hygiene contamination. In particular, contact between the animals and uncovered skin of the experimenter or animal caretaker subjects results in a higher infection rate in rats²⁴. Therefore, depending on the hygiene level, the animals should be trained carefully, and protective clothing should be worn by trainers with necessary adaptations made to the protocol depending upon the requirement of the facility. For example, the metal pipe can be omitted if sufficiently cleaning the pipe is not feasible or too time-consuming for everyday work. We used the pipe because it allows an easy measurement of training success and

also prevents rats from spontaneously alternating between cages. However, it is also possible to train rats to change cages by moving the cage walls from the old to the new cage. This process might even speed up the procedure if cage changing occurs frequently because rats are very curious and explorative. In selecting the pipe and the reward with respect to hygiene, ensure that both are easy to clean, disinfect, or autoclave. The protocol can be adjusted to a range of research settings. For instance, bridges can be utilized, which allow the rats to change cages without physical risk or disturbance of wounds after surgical interventions. In the long term, this protocol allows less direct contact between the trained animals and the animal keeper. Thus, the animals are exposed to a lower germ load. Further, because of the successful observational learning of the rats, not all rats will require training. This observational learning might be particularly helpful in breeding cages, as the offspring may be able to learn the behavior directly from the mother. Reducing the direct but not overall contact between rats and animal caretakers can help to reduce the risk of contamination while simultaneously establishing a relationship of trust through the training. Hygienic level is of great importance because intercurrent infections occurring during breeding and experimental phases can significantly limit the scientific significance of animal experiments; thus, this protocol may help to further improve well-being and science²⁹.

For successful application of this protocol, several points should be taken into consideration. An acclimatization period for the rats must be maintained if they have been transported prior to training. The length of the acclimatization period should be adapted to the scientific research question. Previous studies have demonstrated that a time span of three days may be sufficient for certain experiments^{30,31}, whereas another study demonstrated that plasma glucocorticoids remain increased over two weeks³². In the present study, we choose an acclimatization period of 14 days after transport from the commercial breeder to the animal facility. Every rat must be monitored for any signs of discomfort prior to the training. If the experimenter is not familiar with rats, tools such as the rat grimace scale can be used for reference³³. Without these considerations, the training success rate might be reduced. Rats can be inherently very anxious and may require additional habituation phases. Attempting to train a fearful animal will yield limited success. The less stressed and fearful the animals are in the beginning of the experiment, the faster the training will be completed.

The end of the habituation phase is the optimal time point to record baseline data for further experiments. If invasive methods are necessary for the experiment, the protocol should be paused. An invasive method denotes any method that is equal or above mild severity set out in Annex VIII “Severity classification of procedures” of Directive 2010/63/EU⁹.

For the training session, a training plan can be a helpful tool to ensure that the protocol will be consistently conducted in the desired manner. Such planning is especially important when different experimenters or handlers are involved.

Taken together, this very easy, application-orientated protocol may not only reduce stress during husbandry procedures and research settings but also possibly have beneficial effects on animal hygiene. Therefore, cage change training in rats can facilitate high-quality research and the well-being of the research animals.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

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Figure 3

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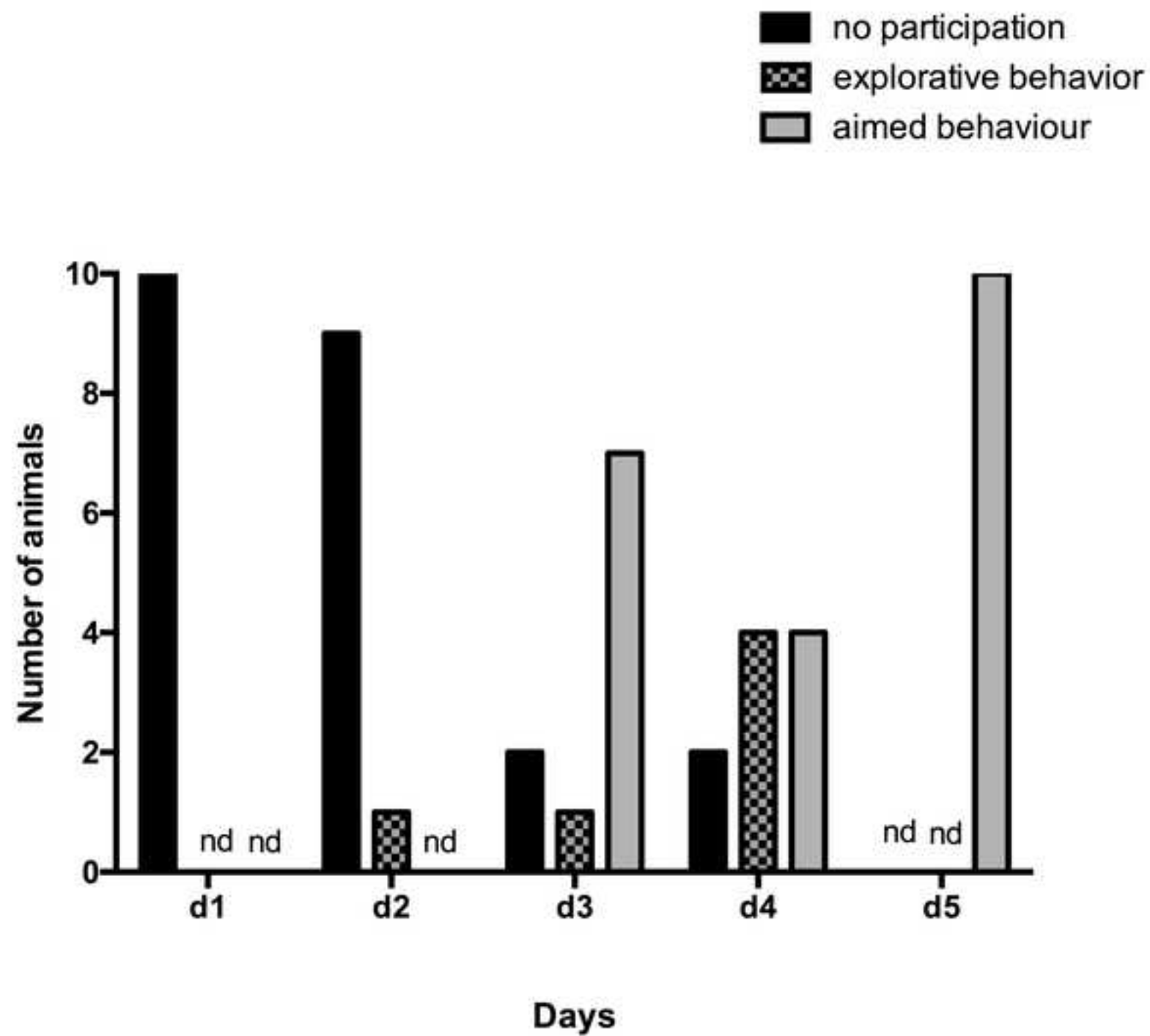


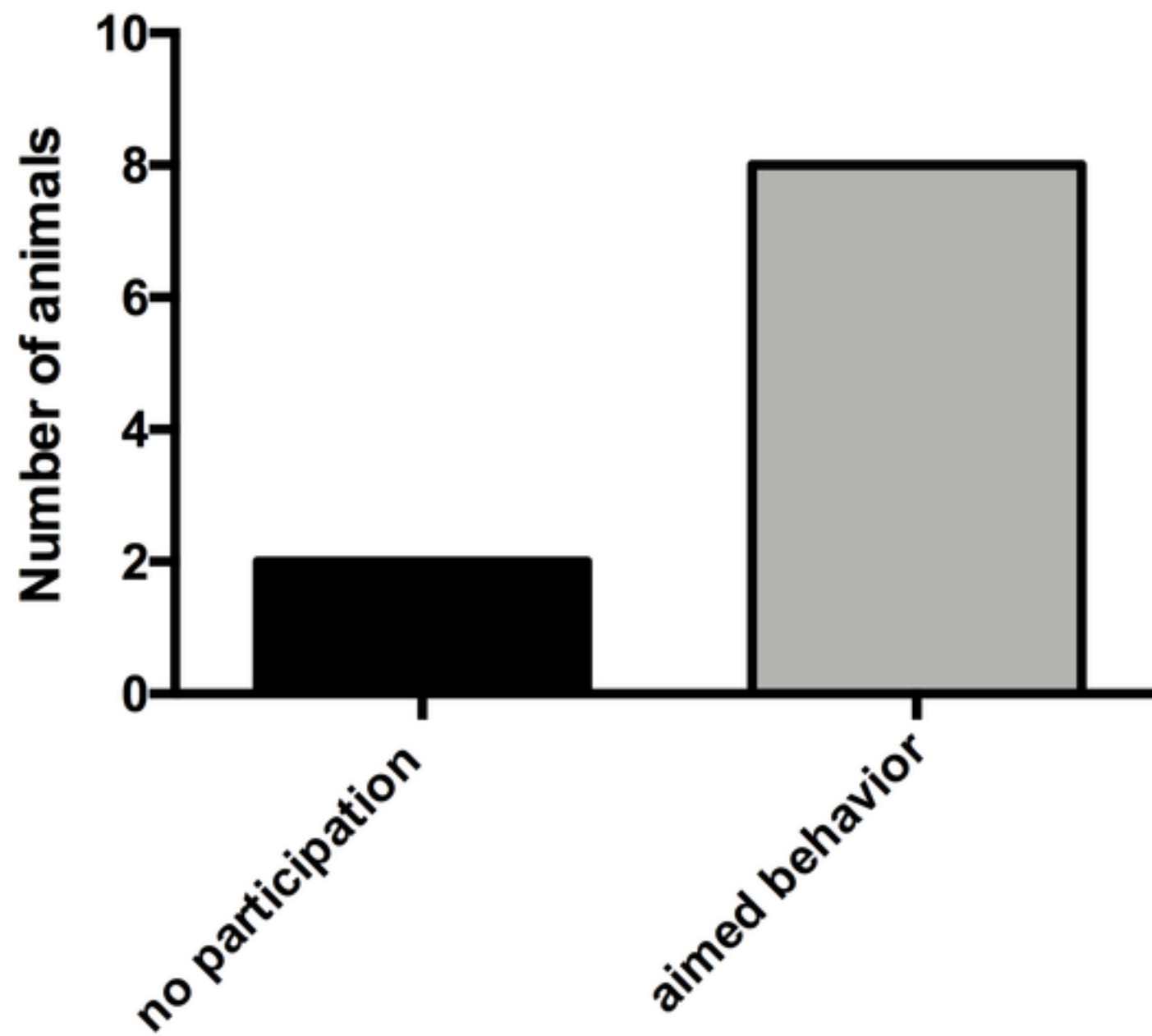
Figure 4

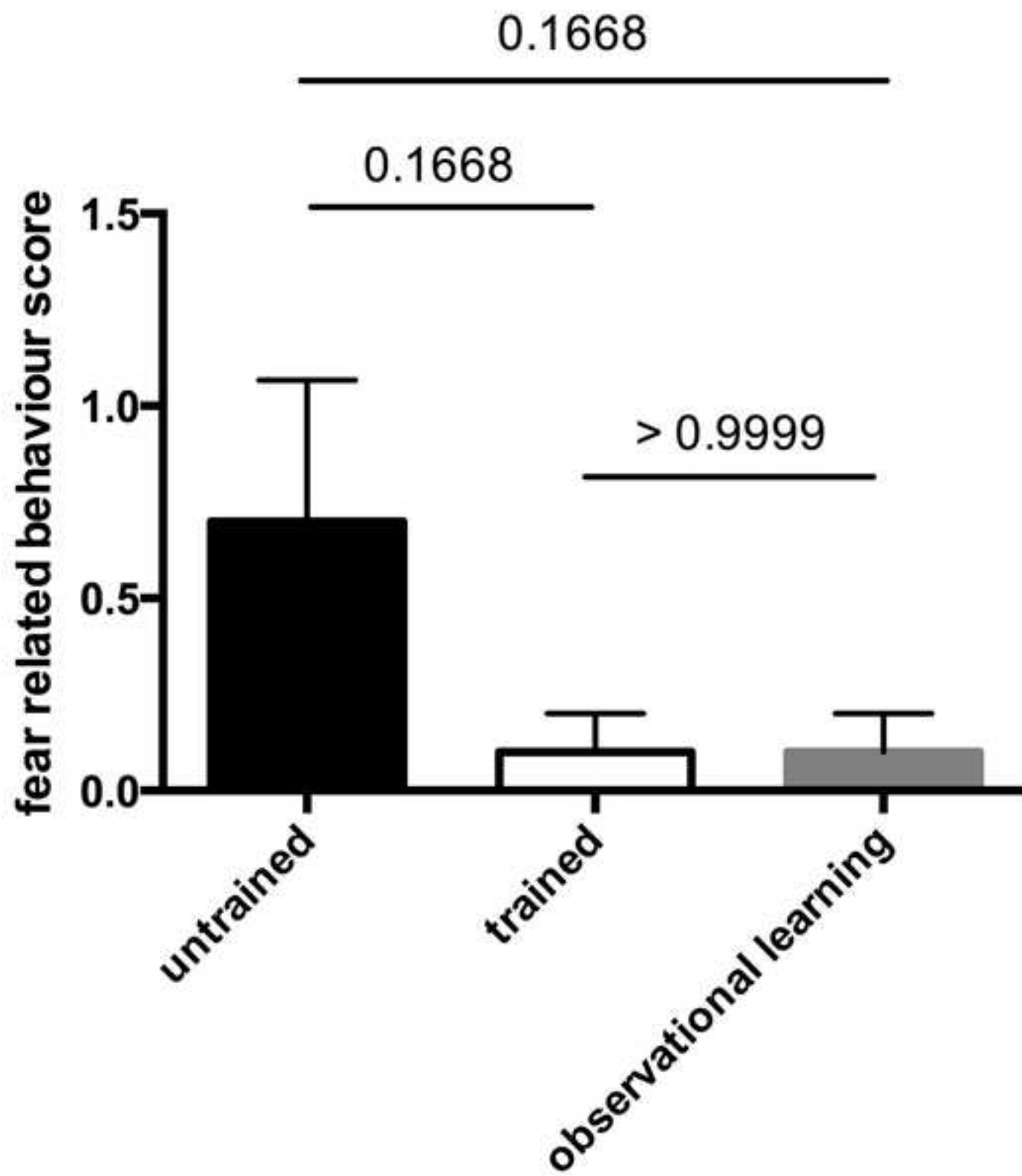
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Figure 5







Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Target Stick with Clicker	Trixie	2282	
Metal Pipe (Alu Flexrohr nw 100)	Rotheigner		available in construction marktes
White Chocolate/ white chocolate cream			Company doesn't matter, preferable organic quality
Prism Version 6.0 for Mac	GraphPad Software		

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Best regards,
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Editorial comments:

1. The manuscript is hard to understand in places (see e.g. notes in manuscript); please proofread, ideally by a fluent English speaker.
The manuscript underwent professional proofreading.
2. Some steps of the protocol are still unclear; see notes in manuscript.
All the notes in the manuscript were addressed to make the protocol better understandable.
3. Figure 3/4: Are this intended to show feeding? You mention that you feed rats white chocolate in the protocol, but this doesn't look like that.
The chocolate was added to the top of the syringe. This was just for holding, one can also use forceps. We also added this to the figure legend.