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Introducing Clicker Training as a Cognitive Enrichment for Laboratory Mice

--Manuscript Draft--

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Abstract:	Establishing new refinement strategies in laboratory animal science is a central goal in fulfilling the requirements of Directive 2010/63/EU. Previous research determined a profound impact of gentle handling protocols on the well-being of laboratory mice. By introducing clicker training to the keeping of mice, not only do we create an amicable treatment of mice but we also enable them to experience a cognitive enrichment. Clicker training is a form of positive reinforcement training using a conditioned secondary reinforcer, the "click" sound of a clicker, which serves as a time bridge between the strengthened behavior and an upcoming reward. The effective implementation of the clicker training protocol with a cohort of 12 BALB/c inbred strain mice of each sex proved to be uncomplicated. The mice learned rather quickly when challenged with tasks of the clicker training protocol and almost all trained mice overcame the challenges they were given (100% of female mice and 83% of male mice). This study has identified that clicker training for mice strongly correlates with a reduced fear in the mice in human-mice interactions as shown by reduced anxiety-related behaviors (e.g., defecation, vocalization, and urination) and fewer depression-like behaviors (e.g., floating). By developing a reliable protocol that can be easily integrated into the daily routine of the keeping of laboratory mice, the mice's lifetime experience of welfare can be improved substantially.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a	

certain date, please indicate the date below and explain in your cover letter.	

Cover Letter

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Mainz, den 25.10.16

Dear Jaydev Uppon,

Please find attached a revised version of our manuscript JoVE55415 entitled “Introducing Clicker Training as a Cognitive Enrichment for Laboratory Mice” for publication as an video-article in JoVE. We greatly appreciate the careful and constructive revision. All comments have been addressed, with corresponding changes made directly to the manuscript where appropriate. Detailed responses to the editorial comments are included on the next pages.

We are looking forward to your response.

Sincerely,

Nadine Baumgart

Editorial comments:

•NOTE: Please download this version of the Microsoft word document (File name: 55415_R1_091416) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Scattered grammar and formatting issues should be corrected:

-Title – Is there some confusion as to the effect of clicker training? Please remove the question mark and also consider rephrasing the title as an investigation into the effect of clicker training.

-New Title: **Introducing Clicker Training as a Cognitive Enrichment for Laboratory Mice**

-Line 102: Also routinely performed laboratory procedures like general handling, restrain, blood or tissue sampling can cause stress responses."

➔ "Furthermore, routinely performed laboratory procedures like general handling, restraining, blood or tissue sampling can cause stress responses, which can be addressed by measuring different parameters such as stress hormones or behaviour"

-Line 153: "The reward must not be a risk for animal health." This is a bit awkward.

➔ Use food rewards, which are vacuum-packed food or animal feed that meets the requirements of food safety standards. This sentence makes "The reward must not be a risk for animal health." Unnecessary. The remarked sentence got deleted.

-Step 2.2.1: Second sentence should be "alternate" rather than "alter."

➔ Word is changed

-Step 2.3.2, 2.4.7, 2.4.10: Is "the access of the tunel" the right term here? Perhaps "entrance"?

➔ The term is changed to "end of the tunnel" throughout the entire document

-Step 2.3.6 has an extraneous close parentheses and is missing a period at the end of the sentence.

➔ Step 2.3.5 is deleted and 2.3.6 modified to make the step more clear

-Step 2.4.8: "Allows" should be "allow."

➔ Is modified to allow

-Figure legend for Fig. 7 has capitalized "Mouse" when it is not necessary to do so.

➔ Is now "mouse"

•Formatting

-Step 1.1 would make more sense presented at the end of the section rather than the beginning.

➔ Step 1.1 now is step 1.6 at the of the section

-Step 2.2.3: The punctuation/formatting here is a little confusing.

➔ The formation is changed by adding more sections

•Additional detail or clarification is needed:

-Step 2.4.10 says to present the reward as soon as the mouse shows "this behavior" appropriately, but as written it is not clear specifically to what "this behavior" refers.

➔ "This behaviour" is changed into "mouse starts continuously to reenter the tunnel"

-Step 2.6 needs to be rewritten so it is clearer what "the hand" refers to. The experimenter's other hand (the one not holding the target stick) one assumes?

➔ One explaining sentence is inserted as Step 2.6.1

➔ "the hand" is modified to "the experimenter's hand"

-Step 3.1.1: What kind of "grid"?

➔ As step 3.1.1 is no longer part of the protocol it is no further addressed

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

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➔ The bibliography style is changed from Nature to JOVE

•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version

➔ The manuscript has been edited by Elsevier professional copyediting service

•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors present an interesting, novel and valuable approach towards the stress free handling and cognitive enrichment of mice. The presented method and data will be of interest for the audience, I have

just a few comments that should be addressed by the authors before the manuscript can be recommended for publication:

Major Concerns:
1) Page 4, line 100: Are there any hints that mice are capable to develop "trusting relationships" with humans? Or is the goal of this approach just to decrease anxiety when being handled (a process of learning that a situation is not harmful)?

- ➔ The goal of this approach is to decrease anxiety when being handled and the hope would be that the mice experience this as a relationship of trust.

2) I see that it is an important proof of the training success to test for "welfare" parameters. I agree it is important to mention the tests you use (restraint, floating) and to show the resulting data. Nevertheless, I disagree that there is a need to describe the tests in this manuscript in detail. I assume the prospective reader of this article aims to refine his or her mouse handling/housing when applying the described training. Describing two stressful tests (restraint, floating) here may imply that there is a need to regularly and routinely test the welfare effects of the training.

- ➔ The detailed description of the stress test is not anymore part of the protocol, as the concerns have a point.

In contrast, for establishing and describing such a training, as you do here, I would additionally expect to add more data that prove that the training is reducing anxiety and increases welfare. Did you assess any physiological data (endocrine, heart beat etc.)? Or other behavioural signs of good wellbeing (e.g. certain home cage behaviours)? I do not think you have to include this data now, but please highlight the need to further evaluate the effects of training in the future.

- ➔ No such data were obtained
- ➔ "In further studies supplementary information about the mice wellbeing, such as a whole behavioral battery, physiological data as corticosterone levels or the impact of training on brain development should be gained"

Minor Concerns:
1) The style of the manuscript should be improved by a native speaker.

- ➔ The manuscript has been edited by Elsevier professional copyediting service.

2) Please, change gender to sex in the whole manuscript. Gender is a socio-cultural definition, sex a biological.

➔ Gender is changed to sex in the whole manuscript

3) When talking about "cupping" in the description of the training, please cite Hurst et al.

➔ An additional sentence regarding cupping is inserted and Hurst et al. are cited

4) 2.2.2): remove the food? How?

➔ An additional sentence is inserted

5) Figure 10, 11: mean +/- SEM?

➔ The columns are expressed in % of all tested mice.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
This manuscript concisely describes a method for clicker training mice that results in ease of handling and reduced stress to the animals. The goals of the study were described adequately and the authors documented the technique through the figures. It is admirable that a study like this was undertaken since mice are typically not given any positive reinforcement training (PRT). While I am not certain that this technique will be used widely simply because of the shear numbers of mice used in research, I believe it provides a good example of how PRT can and should be used for mice to decrease stress and theoretically improve scientific data.

Major Concerns:
I have no major concerns about the paper.

Minor Concerns:
I have a few minor concerns.

While the authors acknowledge that the study has limitations because they only used inbred mice, I think they should also address the issue that there are many strains of mice with different behavioral characteristics that may or may not lend themselves to successful PRT.

➔ A sentence referring to this issue is inserted in line 444-446.

There are also some minor grammatical corrections that need to be made for example:

Line 65 should be 3Rs Principles (and throughout)

- ➔ 3R Priciple is not changed into 3Rs Principles
- ➔ „Bailoo, J. D., Reichlin, T. S., & Würbel, H. (2014). Refinement of experimental design and conduct in laboratory animal research. ILAR Journal / National Research Council, Institute of Laboratory Animal Resources, 55(3), 383–91. http://doi.org/10.1093/ilar/ilu037" use as well "3R Principle"

Line 103 - restraint, not restrain

- ➔ changed

Line 341 - Figure 10 not Figures

- ➔ changed

Line 342 - Figures 11 and 12 not Figure

- ➔ changed

Additional Comments to Authors:
No additional comments.

TITLE:

Introducing Clicker Training as a Cognitive Enrichment for Laboratory Mice

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KEYWORDS:
Enrichment; Positive reinforcement training; Clicker training; Welfare; Well-being; Refinement; 3R's; Mouse; BALB/c

SHORT ABSTRACT:
The development of new refinement strategies for laboratory mice is a challenging task that contributes towards fulfilling the 3R principle. This protocol introduces clicker training as a cognitive enrichment program for laboratory mice.

LONG ABSTRACT:
Establishing new refinement strategies in laboratory animal science is a central goal in fulfilling the requirements of Directive 2010/63/EU. Previous research determined a profound impact of gentle handling protocols on the well-being of laboratory mice. By introducing clicker training to the keeping of mice, not only do we promote the amicable treatment of mice, but we also enable them to experience cognitive enrichment. Clicker training is a form of positive reinforcement training using a conditioned secondary reinforcer, the "click" sound of a clicker, which serves as a time bridge between the strengthened behavior and an upcoming reward. The effective implementation of the clicker training protocol with a cohort of 12 BALB/c inbred mice of each sex proved to be uncomplicated. The mice learned rather quickly when challenged with tasks of the clicker training protocol, and almost all trained mice overcame the challenges they were given (100% of female mice and 83% of male mice). This study has identified that clicker training for mice strongly correlates with reduced fear in the mice during human-mice interactions, as shown by reduced anxiety-related behaviors (*e.g.,* defecation, vocalization, and urination) and fewer depression-like behaviors (*e.g.,* floating). By developing a reliable protocol that can be easily integrated into the daily routine of the keeping of laboratory mice, the lifetime experience of welfare in the mice can be improved substantially.

INTRODUCTION:
The development of new refinement strategies for laboratory mice is a challenging task that contributes to the fulfillment of the 3Rs (replacement, reduction, and refinement) of laboratory animal science[1]. Improvements in the field of refinement can further contribute to the well-being of the millions of animals that are used for experimental purposes. Therefore, intensive research is needed in the field. This is also a defined aim of Directive 2010/63 of the European Union. Directive 2010/63/EU points out that the lifetime experience of laboratory animals has to be enhanced and that "establishments shall set up

habituation and training programs suitable for the animals, the procedures and length of the project" (Article 3.7)[2].

Laboratory animals can experience many stressful situations while they are being kept and bred for experiments. Usually, the interaction between the laboratory mice and the responsible persons is rather limited. Therefore, a trusting relationship cannot develop. This can elicit increased anxiety and stress in reaction to handling, which is detrimental to the behavior and physiology, and therefore, the well-being, of the animals[3–6]. Furthermore, routinely performed laboratory procedures like general handling, restraint, and blood or tissue sampling can cause stress responses, which can be examined by measuring different parameters, such as stress hormones or behavior[7,8]. It has been shown that handling programs can efficiently decrease the anxiety toward the investigator in laboratory rodents[9,10,11]. Handling programs can therefore improve the animals' conditions and could contribute considerably to animal welfare[5].

The goal of this study is to introduce positive reinforcement training for mice as a specific handling program. Positive reinforcement training is a form of operant conditioning that gives the investigator a means to shape animal behavior. When the animal performs a desired behavior, it is followed by a positive stimulus (here, a food reward). The intention is that the animal links the reward to the respective behavior. Clicker training is a form of positive reinforcement training using a conditioned secondary reinforcer, the "click" sound of a clicker, and has been proven to strengthen a specific behavior[12].

More specifically, the click sound serves as a "time bridge" between the behavior and the upcoming reward[13]. The trainer clicks precisely when the animal performs the desired behavior, without any time delay[14], and then presents the food reward. This strengthens the rewarded behavior, which will be performed with a higher frequency. Clicker training is widely used with companion animals and has made its way into laboratory animal science, where it has been successfully implemented with nonhuman primates[13,15,16]. As mice learn rather quickly when challenged with operant conditioning paradigms, the introduction of a second reinforcer should not overstrain their cognitive abilities[5,17–19].

By introducing clicker training to the keeping of mice, we enable mice to experience cognitive enrichment. The design of cognitive enrichment must enable the mice to use their cognitive skills to solve problems and to gain control over their environment[20,21]. Several studies with different species prove the positive impact of cognitive enrichment on the welfare of captive animals[22–24]. Enhancing the ability of the animals to successfully cope with environmental challenges contributes to their well-being[25,26].

In addition, if the animals experience low levels of stress during their lifetime, they are less prone to develop detrimental coping strategies when confronted with stressors that occur in biomedical research. Thus, the consistent implementation of cognitive enrichment can contribute to a homogenization of the subjects' phenotypes. This will contribute to the 3R principle of reduction, as it can reduce the number of subjects required to meet statistical requirements[27].

By developing a reliable protocol that can be easily integrated into the daily routine of keeping laboratory mice, we can substantially improve their lifetime experience of welfare.

PROTOCOL:

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

NOTE: The protocol includes five days of interventions (Monday to Friday), with breaks on the weekends (Saturday and Sunday). The protocol can be easily adapted to meet specific needs.

1. Determining a reward suitable as a second reinforcer

NOTE: Use food rewards, such as vacuum-packed food or animal feed that meets food safety standards. For example, different kinds of nuts, chocolate, gummy bears, or dried fruits are suitable.

1.1) Insert a small petri dish with different food rewards (pieces of 0.5 cm^3) into the home-cage (Figure 1).

1.2) After 10 min, check for leftovers.

1.3) If all rewards are eaten after 10 min, shorten the time span until a preference can be estimated.

1.4) On days 4 and 5 (*e.g.,* Thursday and Friday), add small amounts of the preferred ("winner") reward to the home-cage to establish an affectation of all mice in the cage to the reward.

1.5) If the mice are not habituated to mouse tunnels, add a tunnel to the cage (at least 2 days before beginning the training, *e.g.,* over the weekend).

1.6) Repeat steps 1.2-1.4 on 3 consecutive days (*e.g.,* Monday to Wednesday).

2. Clicker training

2.1) Preparations for each training session

NOTE: Every time a mouse needs to be lifted up in this protocol, lift it in a calm and gentle manner. Put a hand under the mouse to lift it up. During the first few times, the mice might be agitated, but the habituation process will be finished after several days. If this step creates difficulties, it is useful to refer to the protocol for "cupping on the open hand"[28].

2.1.1) Prepare a cage with bedding material.

2.1.2) Transfer the cage of the mouse being trained to a quiet place.

2.1.3) Prepare the reward and have a timer and the clicker/target stick combination ready.

NOTE: If the mice are habituated to handling and already have a relationship of trust with

the experimenter, the mice can be fed by hand. Otherwise, attach the reward to a stick or forceps in such a way that the mice can keep a distance from the experimenter.

2.1.4) Remove all objects from the home-cage, which now serves as the training area (*e.g.,* mouse houses, nest-building material, etc.).

2.1.5) Transfer the cage companions to a prepared cage.

2.2) General rules that apply for all training sessions

NOTE: The following general rules apply for all sessions and will not be mentioned again throughout the protocol.

2.2.1) Train each mouse for 5 min. Alternate between 30-s training and 15-s break periods.

2.2.2) Let the mouse gnaw on the reward no longer than a second. Then, take the stick (with the reward) out of the cage.

2.2.3) Execute the following pattern of rewarding:
1–10 performances of behavior: reward every time.
11–21 performance of behavior: reward every second time.
From 22 performance of behavior on: reward every third time.

2.2.4) After 4 min of training, the next performance of the desired behavior is reinforced by a jackpot reward. Allow the mouse to gnaw at the food reward three times longer than before.

2.3) Training session Day 1: “Linking the secondary reinforcer with the food reward”

2.3.1) Set the timer to 5 min, press start, and add the mouse tunnel to the home-cage.

NOTE: Take advantage of the innate thigmotaxis of mcie and place the tunnel next to a wall (Figure 2). For the first few sessions, this will enhance the probability of the mouse entering the tunnel.

2.3.2) Wait until the mouse inspects the tunnel. As soon as the mouse enters the tunnel, click and present the reward at the end of the tunnel.

2.3.3) Let the mouse feed on the reward while sitting in the tunnel.

2.3.4) Click continuously for 15 s while the mouse is sitting in the tunnel.

2.3.5) As soon as the mouse leaves the tunnel, go back to step 2.3.2.

2.4) Training session Days 2-5: “Running through a tunnel”

2.4.1) Set the timer to 5 min, press start, and add the mouse tunnel to the home-cage.

2.4.2) As soon as the mouse enters the tunnel, click and present the reward at the end of

the tunnel.

2.4.3) As long as the mouse is in the tunnel, click and immediately present the reward in the same manner.

2.4.4) Repeat this for the next 30 s, and then pause for 15 s to take the tunnel out of the cage.

2.4.5) Add the tunnel again and click immediately when the mouse reenters the tunnel.

2.4.6) Present the reward at the end of the tunnel.

2.4.7) Allow the mouse to gnaw for up to 1 s.

2.4.8) Take away the reward.

2.4.9) As soon as the mouse starts re-entering the tunnel by itself, present the reward in front of the end of the tunnel (Figure 3).

2.5) Training session week 2: “Following a target stick”

2.5.1) Set the timer to 5 min, press start, and place the globe at the end of the target stick in the cage.

2.5.2) As soon as the mouse shows interest in the globe, click and present the reward next to the globe (Figures 4 and 5).

2.5.3) Reward the mouse only after it touches the globe with its nose.

2.5.4) When the mouse has linked the performed action to the reward, change the position of the globe during the training session (Figure 6).

2.5.5) Wait for the mouse to meet this last challenge.

2.5.6) Place the globe in the cage.

2.5.7) Shortly before the mouse touches the globe, slowly and carefully change the position of the globe by 1 cm.

2.5.8) Click and reward if the mouse has crossed the distance and touches the globe.

2.5.9) Repeat this procedure until the mouse follows reliably.

2.5.10) Slowly extend the distance the mouse has to cross.

2.6) Training session week 3: “Following the target stick to the experimenter’s hand”

2.6.1) Place one hand in the training cage while holding the clicker/target stick combination and the reward with the other hand.

2.6.2) Set the timer to 5 min, press start, and place the globe at the end of the target stick next to the hand.

2.6.3) As soon as the mouse shows interest in the globe, click and present the reward next to the globe.

2.6.4) As soon as the mouse has met this challenge, move the globe several steps closer to the hand.

2.6.5) Place the globe on the palm of the hand. Click and reward while the mouse is sitting on the palm of the hand (Figure 7).

REPRESENTATIVE RESULTS:

The first and also one of the most important steps was the determination of an appropriate food reward. Therefore, the mice were offered different kinds of nuts, a sugar solution, marmalade, and different kinds of chocolate in a petri dish (Figure 1). In our experience, the mice showed an obvious preference for white chocolate. Hence, we used white chocolate for all further training processes.

The actual training was implemented with a cohort of 12 BALB/c inbred mice of each sex. All mice were highly interested in the training. For the evaluation of training success, we checked for the proper performance of the desired behavior: “following the target stick” (Figure 4). The vast majority of the trained mice—all female mice and 83% of the male mice—followed the target stick (Figure 8). Female mice displayed a higher motivation for training in general and performed the respective behavior with a higher frequency throughout the training sessions. After completing 4 days of training, female mice followed the target stick with a mean of 64 times per 5 min, whereas male mice displayed this behavior only 50 times per 5 min. In 5 min, the task “following to the palm of the hand” was performed on average 55 times by female mice and 35 times by male mice (Figure 9).

To check whether the training had a positive effect on the well-being of the trained mice, we evaluated the tolerance to manipulations after completing the training. Therefore, we analyzed anxiety-related behaviors while the mice were singlehandedly restrained (*i.e.,* grasping the scruff of the neck and the base of the tail with one hand) for 15 s. Spontaneous urination, defecation and vocalization were recorded. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as a control group. Trained mice displayed a significantly lower frequency of anxiety related behaviors than untrained mice. (Figure 10). Similar results were obtained when recording the vocalization linked to handling while performing the Morris Water Maze Test (Figures 11 and 12). The trained mice squeaked significantly less than the untrained mice (Figure 11) and the total number of squeaks per squeaking mouse was significantly reduced. To further evaluate this issue, we analyzed the floating behavior during the Morris Water Maze Test. Floating behavior during the Morris Water Maze Test is described as periods of time when the mice are not

swimming, but are merely floating on the surface. This depression-related floating behavior turned out to be significantly reduced in the trained group (Figure 12).

FIGURE LEGENDS:

Figure 1. Presentation of possible rewards. Six positions for the presentation of rewards are marked in a petri dish.

Figure 2. Position of mouse tunnel. To enhance the probability of the mouse entering the tunnel, the tunnel is placed next to a cage wall.

Figure 3. Presenting the reward. The appropriate position for presenting the reward outside the end of the tunnel is shown.

Figure 4. Training success. A trained mouse follows the target stick while being trained.

Figure 5. Rewarding. A mouse is rewarded next to the target stick during the training session.

Figure 6. Alternating target stick positions in the second week of training. The positions of the training stick are indicated in this schematic drawing.

Figure 7. Training success. A mouse has followed the target stick onto the hand of the trainer.

Figure 8. Success of training protocol. A cohort of 12 BALB/c inbred strain mice of each sex was trained. By the end of training session week 2: "Following a target stick," all female mice and 83% of the male mice successfully overcame the challenge.

Figure 9. Repetitions of respective behavior after one week of training. A cohort of 12 BALB/c inbred mice of each sex was trained. On the last day of training session week 2: "Following a target stick" and 3: "Following the target stick to the hand", the repetitions of the respective behavior were counted during a 5-min training session. In both weeks, female mice displayed the strengthened behavior with a significantly higher frequency than male mice ("Target stick": 63.92 ± 3.72, $p = 0.0300$; "On hand": 54.92 ± 4.01, $p = 0.0069$). The frequency of the strengthened behavior did not significantly vary in female mice from week two to week three. However, male mice showed a significant decrease in repetitions from week two to week three ("Target stick": 50.42 ± 4.48; "On hand": 35.92 ± 5; $p = 0.0408$). A Mann-Whitney U-test was performed. The results of the data were expressed as the mean ± S.E.M.

Figure 10. Displayed behavior triggered by scruff holding. A cohort of 12 BALB/c inbred mice of each sex was trained following the clicker training protocol. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as a control group. The mice were singlehandedly restrained (*i.e.,* grasping the scruff of the neck and the base of the tail with one hand) for 15 s. Spontaneous urination, defecation, and vocalization were recorded. There was a profound difference between the displayed behavior of the trained and the control group. Trained mice displayed all behaviors with a significantly lower frequency than

untrained mice. (“Urination”: $p < 0.001$; “Defecation”: $p < 0.001$; “Vocalization”: $p < 0.001$). A Mann-Whitney U-test was performed. The columns are expressed as the percent of all tested mice.

Figure 11. Displayed behaviors during the Morris Water Maze Test. A cohort of 12 BALB/c inbred mice of each sex was trained following the clicker training protocol. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as a control group. A Morris Water Maze Test was performed with all mice after the third week of training. Floating behavior and vocalization linked to handling were recorded. Floating behavior occurred significantly less in trained mice ($p < 0.001$). Further, there was a profound difference in the vocalization of the trained and the control group. Trained mice squeaked significantly less when handled than mice of the untrained control group ($p < 0.001$). A Mann-Whitney U-test was performed.

Figure 12. Prevalence of squeaks per mouse. A cohort of 12 BALB/c inbred mice of each sex was trained following the clicker training protocol. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as control group. A Morris Water Maze Test was performed with all mice after the third week of training. The vocalization linked to the handling of the mice was recorded. Untrained mice squeaked significantly more often than trained mice. Untrained mice displayed vocalization 1.167 ± 0.7 times and untrained mice 4.071 ± 0.83 times. An unpaired t-test with Welch's correction was performed. The results of the data were expressed as the mean ± S.E.M.

Figure 13. Further applications. A mouse is following a target stick to cross a bridge from one cage to another.

DISCUSSION:

The effective implementation of the clicker training protocol with a cohort of 12 BALB/c inbred mice of each sex proved to be uncomplicated. Previous studies have confirmed the effectiveness of clicker training with several species, and we extended this to mice. As mice are the lowest developed mammals among laboratory animals, their abilities are often underestimated. Therefore, the most surprising aspect of the data is that training success could be achieved with almost all mice.

One critical step within this protocol is the timing of the second reinforcer, which is very important to establishing a connection between the displayed behavior and the reinforcement. As mice are very agitated, it is slightly difficult to mark an exact performance while they are scampering around. The more experienced the trainer is, the faster training success can be achieved. We observed that the mice learned quite quickly, even with unexperienced trainers. Even little mistakes could be compensated for in the course of the clicker training protocol. Common sources of error included reinforcing a wrong behavior and a lack of interest in the food reward after clicking.

An uncontrollable factor is the individual character of each mouse. Although this study was performed with inbred mice, born and raised under the same conditions, they develop a variety of different characters. Due to previous research and our own empirical data, we know that this includes different tastes, different expressions of appetite, and different

exploration behavior[29,30]. The first critical step was to find an appropriate food reward. The mice were offered different kinds of nuts, a sugar solution, marmalade, and different kinds of chocolate. For the mice in this study, white chocolate proved to be the most appropriate reward. The success of this protocol depends on the interest of the mice in the training. If anything else arouses their interest more, the training reaches its limits. The more equipment that was added to the cage to fulfill a training session, the less the mice participated in the training. The elimination of all attention-grabbing items in the surrounding is essential (*e.g.,* leaving even a small amount of bedding material in the cage to prevent the mice from burrowing). Sometimes the mice gain more interest in the training after a habituating to the new environment for several minutes.

Gentle handling protocols have already been determined to benefit the wellbeing of mice when treated amicably[9–11]. The results of the present study indicate that a closer human-animal relationship contributes to mouse tolerance to treatments. Under manipulation, trained animals displayed fewer anxiety-related behaviors (*i.e.,* urination, defecation, and vocalization)[31]. In the Morris Water Maze Test, the untrained but gently handled mice expressed a significantly higher prevalence of floating behavior. Trained mice displayed less of this depression-related behavior[32]. It could be argued that the positive results were due to increased well-being in the trained mice.

A limitation of this study is that only one strain of inbred mice was studied. Due to high behavioral differences between different strains of mice, additional inbred strains, as well as outbred strains, should be investigated[29]. In further studies, supplementary information about mouse wellbeing, such as a whole behavioral battery, physiological data (*e.g.,* corticosterone levels), or the impact of training on brain development, should be gathered.

Further applications of clicker training could assist in improving health and hygiene conditions in animal facilities. By applying the clicker training protocol, we succeeded in teaching mice to follow a target stick and to cross a bridge from one cage to another (Figure 13). If this technique is perfected, it makes manual cage-changing unnecessary. In addition, mice could possibly be trained to voluntarily walk onto measurement equipment (*e.g.,* scale or behavioral areas). This could provide reliable protection against anthropozoonotic disease transmission, as direct contact with the animals will no longer be necessary.

Positive reinforcement training is highly recommended for captive animals, as it contributes to their mental health[33]. If mice experience a high level of wellbeing, they display few individually different coping strategies. Clicker training, therefore, makes a noteworthy contribution to the second R, reduction, as previous work shows that a reduction in the variability of mice phenotypes leads to a reduced number of animals necessary to meet statistical requirements[27]. This study has demonstrated that clicker training in mice strongly correlates with a reduced fear of human-mice interactions and matches results observed in earlier studies with different species[34]. Clicker training has further potential, as it could be considered cognitive enrichment. The broad implementation of positive reinforcement training in laboratory animal facilities could make a valuable contribution to the 3R principle, as it refines the keeping and biomedical research of mice.

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DISCLOSURES:

The authors have nothing to disclose.

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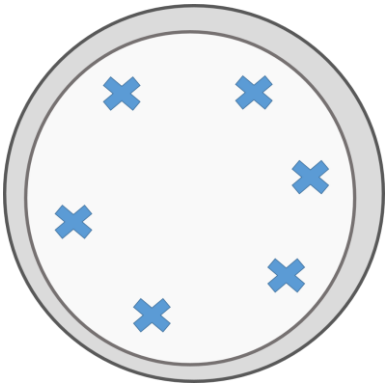


Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

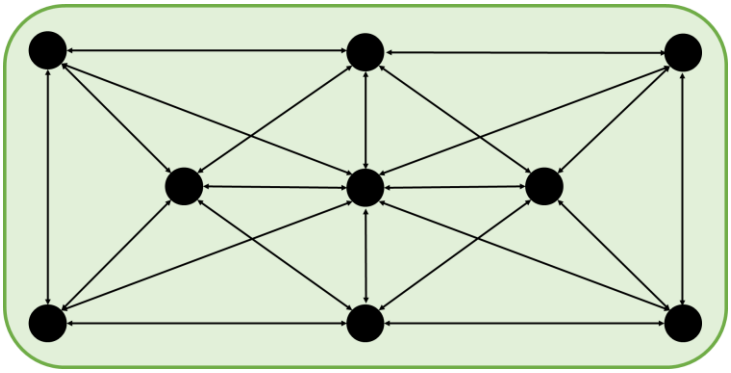
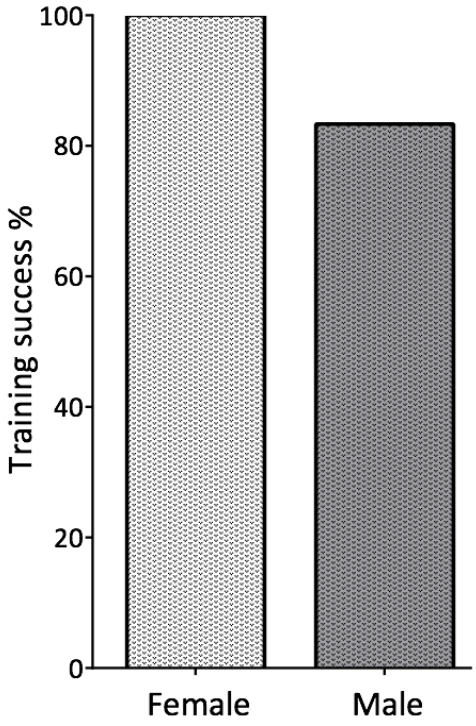


Figure 6

Figure 7



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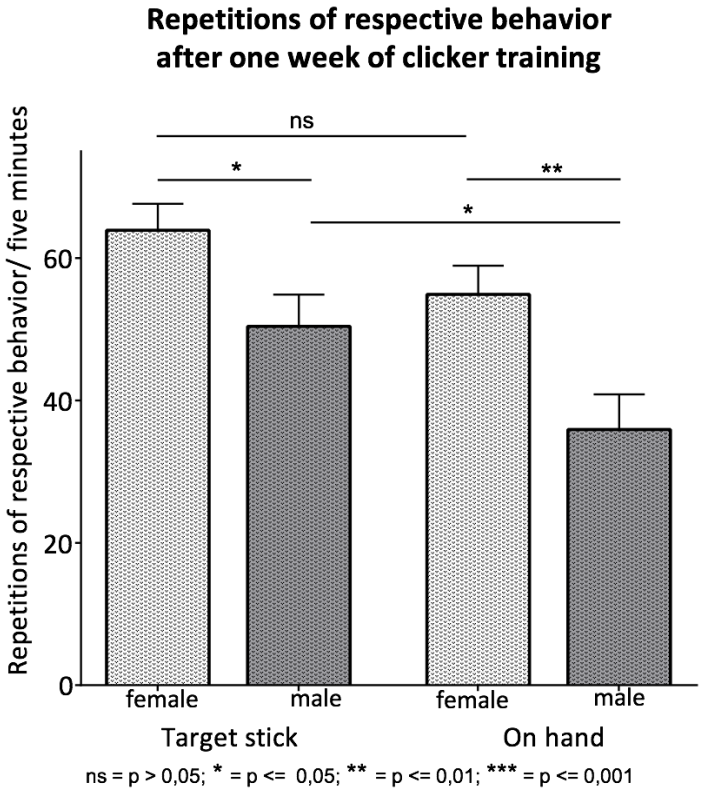

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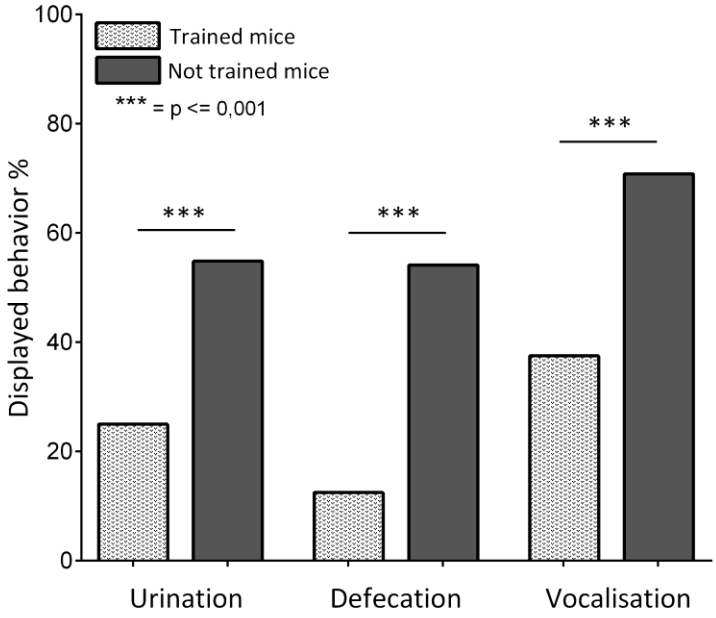

Figure 10

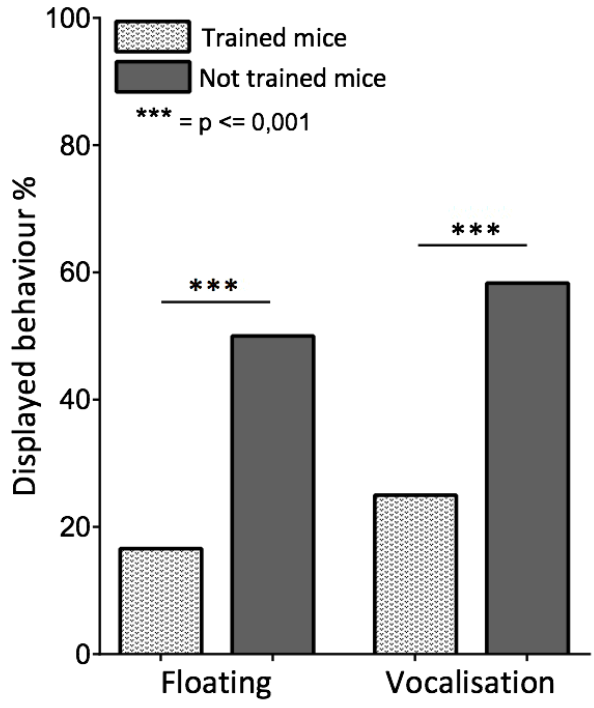

Figure 11

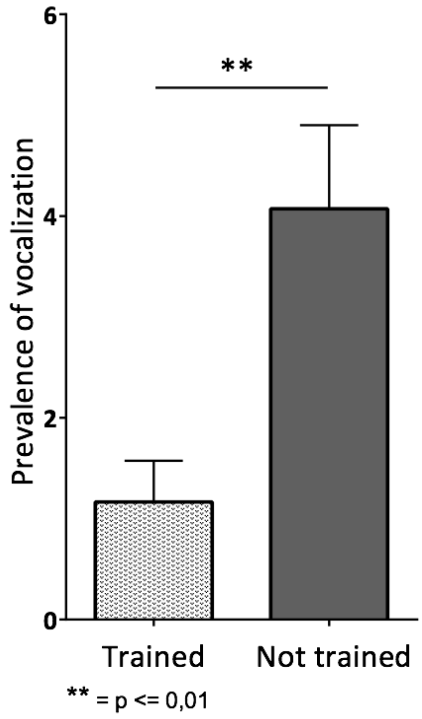

Figure 12

Figure 13

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Table of Materials editorial review.xlsx

Name of the Material/Equipment	Company
Lid for open housing	Tecniplast
SealSAfe Plus top for open housing	Tecniplast
Type II long, filter top cages	Tecniplast
Aspen bedding material	Lab & Vet Service GmbH
Red polycarbonate Mouse House	Tecniplast
Tissue papers	Tork, SCA Hygiene Products GmbH
Food - ssniff M-H Extrudat	ssniff
Target Stick with Clicker	Trixie
PVC Tube (Tunnels)	Thyssen Krupp
White Chocolate/ white chocolate cream	
Forceps	FineScienceTools
Prism Version 6.0 for Windows	GraphPad Software

Catalog Number

GM500LID117
GM500400SU
GM500PFSPC
H0234-300
ACRE011
290179
V1126-000

2282
RTPVCU04003005

e.g. 11150-10

Comments/ Descriptions

Environmental enrichment
Environmental enrichment
Environmental enrichment
ad libitum

Company doesn't matter, preferable organic quality
Or any other tool to fixate chocolate

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Editorial comments:

•NOTE: Please download this version of the Microsoft word document (File name: 55415_R1_091416) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Scattered grammar and formatting issues should be corrected:

-Title – Is there some confusion as to the effect of clicker training? Please remove the question mark and also consider rephrasing the title as an investigation into the effect of clicker training.

-New Title: Introducing Clicker Training as a Cognitive Enrichment for Laboratory Mice

-Line 102: Also routinely performed laboratory procedures like general handling, restrain, blood or tissue sampling can cause stress responses."

➔ “Furthermore, routinely performed laboratory procedures like general handling, restraining, blood or tissue sampling can cause stress responses, which can be addressed by measuring different parameters such as stress hormones or behaviour”

-Line 153: "The reward must not be a risk for animal health." This is a bit awkward.

➔ Use food rewards, which are vacuum-packed food or animal feed that meets the requirements of food safety standards. This sentence makes "The reward must not be a risk for animal health." Unnecessary. The remarked sentence got deleted.

-Step 2.2.1: Second sentence should be "alternate" rather than "alter."

➔ Word is changed

-Step 2.3.2, 2.4.7, 2.4.10: Is "the access of the tunel" the right term here? Perhaps "entrance"?

➔ The term is changed to “end of the tunnel” throughout the entire document

-Step 2.3.6 has an extraneous close parentheses and is missing a period at the end of the sentence.

➔ Step 2.3.5 is deleted and 2.3.6 modified to make the step more clear

-Step 2.4.8: "Allows" should be "allow."

➔ Is modified to allow

-Figure legend for Fig. 7 has capitalized "Mouse" when it is not necessary to do so.

➔ Is now “mouse”

•Formatting

-Step 1.1 would make more sense presented at the end of the section rather than the beginning.

➔ Step 1.1 now is step 1.6 at the of the section

-Step 2.2.3: The punctuation/formatting here is a little confusing.

➔ The formation is changed by adding more sections

•Additional detail or clarification is needed:

-Step 2.4.10 says to present the reward as soon as the mouse shows "this behavior" appropriately, but as written it is not clear specifically to what "this behavior" refers.

➔ “This behaviour” is changed into “mouse starts continuously to reenter the tunnel”

-Step 2.6 needs to be rewritten so it is clearer what "the hand" refers to. The experimenter's other hand (the one not holding the target stick) one assumes?

➔ One explaining sentence is inserted as Step 2.6.1

➔ “the hand” is modified to “the experimenter’s hand”

-Step 3.1.1: What kind of "grid"?

➔ As step 3.1.1 is no longer part of the protocol it is no further addressed

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➔ The bibliography style is changed from Nature to JOVE

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•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors present an interesting, novel and valuable approach towards the stress free handling and cognitive enrichment of mice. The presented method and data will be of interest for the audience, I have just a few comments that should be addressed by the authors before the manuscript can be recommended for publication:

Major Concerns:
1) Page 4, line 100: Are there any hints that mice are capable to develop "trusting relationships" with humans? Or is the goal of this approach just to decrease anxiety when being handled (a process of learning that a situation is not harmful)?

➔ The goal of this approach is to decrease anxiety when being handled and the hope would be that the mice experience this as a relationship of trust.

2) I see that it is an important proof of the training success to test for "welfare" parameters. I agree it is important to mention the tests you use (restraint, floating) and to show the resulting data. Nevertheless, I disagree that there is a need to describe the tests in this manuscript in detail. I assume the prospective reader of this article aims to refine his or her mouse handling/housing when applying the described training. Describing two stressful tests (restraint, floating) here may imply that there is a need to regularly and routinely test the welfare effects of the training.

- ➔ The detailed description of the stress test is not anymore part of the protocol, as the concerns have a point.

In contrast, for establishing and describing such a training, as you do here, I would additionally expect to add more data that prove that the training is reducing anxiety and increases welfare. Did you assess any physiological data (endocrine, heart beat etc.)? Or other behavioural signs of good wellbeing (e.g. certain home cage behaviours)? I do not think you have to include this data now, but please highlight the need to further evaluate the effects of training in the future.

- ➔ No such data were obtained
- ➔ "In further studies supplementary information about the mice wellbeing, such as a whole behavioral battery, physiological data as corticosterone levels or the impact of training on brain development should be gained"

Minor Concerns:
1) The style of the manuscript should be improved by a native speaker.

- ➔ The manuscript has been edited by Elsevier professional copyediting service.

2) Please, change gender to sex in the whole manuscript. Gender is a socio-cultural definition, sex a biological.

- ➔ Gender is changed to sex in the whole manuscript

3) When talking about "cupping" in the description of the training, please cite Hurst et al.

- ➔ An additional sentence regarding cupping is inserted and Hurst et al. are cited

4) 2.2.2): remove the food? How?

- ➔ An additional sentence is inserted

5) Figure 10, 11: mean +/- SEM?

➔ The columns are expressed in % of all tested mice.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
This manuscript concisely describes a method for clicker training mice that results in ease of handling and reduced stress to the animals. The goals of the study were described adequately and the authors documented the technique through the figures. It is admirable that a study like this was undertaken since mice are typically not given any positive reinforcement training (PRT). While I am not certain that this technique will be used widely simply because of the shear numbers of mice used in research, I believe it provides a good example of how PRT can and should be used for mice to decrease stress and theoretically improve scientific data.

Major Concerns:
I have no major concerns about the paper.

Minor Concerns:
I have a few minor concerns.

While the authors acknowledge that the study has limitations because they only used inbred mice, I think they should also address the issue that there are many strains of mice with different behavioral characteristics that may or may not lend themselves to successful PRT.

➔ A sentence referring to this issue is inserted in line 444-446.

There are also some minor grammatical corrections that need to be made for example:

Line 65 should be 3Rs Principles (and throughout)

➔ 3R Priciple is not changed into 3Rs Principles
➔ „Bailoo, J. D., Reichlin, T. S., & Würbel, H. (2014). Refinement of experimental design and conduct in laboratory animal research. ILAR Journal / National Research Council, Institute of Laboratory Animal Resources, 55(3), 383–91. http://doi.org/10.1093/ilar/ilu037" use as well "3R Principle"

Line 103 - restraint, not restrain

➔ changed

Line 341 - Figure 10 not Figures

➔ changed

Line 342 - Figures 11 and 12 not Figure

➔ changed

Additional Comments to Authors:
No additional comments.