Aus dem Institut für Tierschutz, Tierverhalten und Versuchstierkunde
des Fachbereichs Veterinärmedizin der Freien Universität Berlin

Refinement strategies in breeding and keeping of laboratory mice

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der Freien Universität Berlin

vorgelegt von
Charlotte Sophie Leidinger
Tierärztin aus Homburg

Berlin 2017
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Erster Gutachter:	Univ.-Prof. Dr. Christa Thöne-Reineke
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Dritter Gutachter:	PD Dr. Bettina Bert

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Dedicated to

The Foundling Fox

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LIST OF ABBREVIATIONS

ACTH	**A**drenoc**c**orticot**t**ropic **H**ormone
APOPO	**A**nti-**P**ersoonsmijnen **O**ntmijnende **P**roduct **O**ntwikkeling: "Anti-Personnel Landmines Removal Product Development"
BfR	**B**undesinstitut **f**ür **R**isikobewertung
BMEL	**B**undes**m**inisterium für **E**rnährung und **L**andwirtschaft
CRF	**C**orticotropin-**R**eleasing **F**actor
Directive 2010/63/EU	**Directive 2010/63/EU** of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes
EE	**E**nvironmental **E**nrichment
ETS No. 123	**E**uropean **T**reaty **S**eries No.123 - European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes
EU	**E**uropean **U**nion
FELASA	**F**ederation of **E**uropean **L**aboratory **A**nimal **S**cience **A**ssociations

GAS	**G**eneral **A**daptation **S**yndrome
GenTG	**G**esetz zur Regelung der **Gent**echnik
GMO	**G**enetically **M**odified **O**rganism
GV-SOLAS	**G**emeinschaft für **V**ersuchstierkunde- **S**ociety **O**f **L**aboratory **A**nimal **S**cience
HPA	**H**ypothalamic- **P**ituitary-**A**drenal Axis
IVC	**I**ndividually **V**entilated **C**age
LAREF	**L**aboratory **A**nimal **R**efinement & **E**nrichment **F**orum
MBU	**M**ouse **B**ehavior **U**nit of the Translational Animal Research Center of the Universitätsmedizin of the Johannes-Gutenberg University Mainz
MWM	**M**orris **W**ater **M**aze
NC3R^{s}	**N**ational **C**entre for **R**eplacement **R**efinement & **R**eduction of Animals in Research UK
P1	**P**ostnatal day **o**ne
PRT	**P**ositive **R**einforcement **T**raining
SPF	**S**pecific **P**athogen **F**ree
Three Rs	**R**eplacement, **R**eduction and **R**efinement

1 INTRODUCTION

The laboratory mouse (*Mus Musculus)* is the most commonly used animal species in biomedical research. 7 million mice were part of experiments in the European Union in 2011 (European Commission 2013). For 2015, the Federal Ministry of Food and Agriculture (BMEL) of Germany presented figures showing that 72,83% of all vertebrates used in experiments in Germany were mice, totaling over 2 million (Bundesministerium für Ernährung und Landwirtschaft 2015). Due to new achievements in genetic engineering, it is possible to provide mice specifically genetically modified for many fields of research. Likely, this will result in a renewed increase of laboratory mice (Ormandy et al. 2009; Franco 2013). Furthermore, research into lawful and humane breeding and keeping conditions of animals used for experimentation becomes increasingly important. This dissertation seeks to contribute to this debate.

Thirty years ago, the EU attended to the matter of laboratory animal research trough the Directive 86/609/EEC. It was amended in 2010 and lead to the Directive 2010/63/EU, which strengthens the aspect of welfare of these animals still indispensable in biomedical research. The three-Rs of laboratory animal science, *Replacement*, *Reduction* and *Refinement* are embedded in the EU legislation (Directive 2010/63/EU Article 4).

The Three Rs were developed in 1959 by Russel and Burch and published in their book "The Principles of Humane Experimental Technique". On the first page they proclaim that “the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for a successful animal experiment” (Russell & Burch 1959).

Replacement demands the use of alternative methods instead of animal experiments whenever possible. Alternative methods replace the sentient being by in vitro methods, such as cell culture, perfused organs or silico methods i.e. computer simulation. *Reduction* aims to reduce the number of used animals to the lowest amount that is needed to obtain precise information. At the same time, it is essential that the number of animals is high enough in order to prevent a duplication of testing.

The third R, *Refinement,* is the one that has the potential to ameliorate the welfare of those mice, which are kept, bred and used in laboratory animal science. Refinement aims for a decrease in pain, suffering, distress or long lasting harm of laboratory animals. For example, Refinement was achieved by establishing a culture of care, improving enrichment factors or providing high surgical care, such as close monitoring and appropriate pain management (Jennings et al. 1998). The focus in the Three R debate is mostly on Replacement and Reduction. This emphasis is put on a political point of view, as the first two Rs may lead to a decrease in the number of laboratory animals. Nevertheless, a crucial point must not be forgotten: Refinement is the R that really affects living animals.

Refinement strategies concerning the breeding of laboratory mice are especially important regarding new developments in genetic engineering (Mertens & Rülicke 2007). It is not only that the number of animals increases (Ormandy et al. 2009; Franco 2013), but a lot of smaller institutes start breeding on their own and no longer receive their animals from commercial breeders. This trend poses a real threat for standardized and fruitful rearing of infant mice. As the Directive 2010/63/EU (31) points out, “animal-welfare considerations should be given the highest priority in the context of animal keeping, breeding and use.”

One major aim of this study is to identify factors that contribute to successful breeding and rearing of laboratory mice. A considerable amount of literature has been published on environmental enrichment (EE) and agrees on its fundamental role in animal welfare. The first focus is on investigating different approaches of EE that can be easily standardized within laboratory animal keeping. This study compares the influence of three different

enriched breeding cages on infant survival rate and pub development. The findings obtained will be published and are already submitted to a journal. (Leidinger et al. 2017b)

The second focus of this study is on keeping of laboratory mice. One major concern of animal keeping is to maintain a good health status, which is as well embedded in the Directive 2010/63/EU (Annex Ⅲ, Section A: 3.1.). One hazard factor is the weekly relocation of mice from a dirty cage to a clean one. This poses a risk of spreading pathogens across different cages (FELASA Working Group 2007). In response to this, part two seeks to find a hygiene-management-system for cage changing by thinking out of the box. This study therefore sets out to assess the possibility of training mice to voluntarily change cages on their own. For this approach, a positive reinforcement training of mice was established. Based on these findings a video article was published in March 2017. (Leidinger et al. 2017a)

The results are expected to contribute to the standardization of the breeding and keeping of laboratory mice on a high level of animal welfare.

1.1 THE ECOLOGY OF WILD AND LABORATORY *MUS MUSCULUS*

The most common used laboratory mice in biomedical research are descendants of the house mouse (*Mus musculus*). In some cases, the American white-footed mouse (Peromyscus leucopus) and the deer mouse (Peromyscus maniculatus) are used as mouse models, but as they do not belong to the family of Muridae, they are not further discussed.

The domestication of mice started at the beginning of the 17th century, when two English scientists, William Harvey and Robert Hook carried out experiments with mice in order to investigate their reproduction, blood circulation or the biological consequences of an increase in air pressure (Guénet et al. 2012b). At the same time, it became quite popular to breed mice as a hobby, all around Europe. In this way the so-called “fancy mice” were created, whose large variety in fur color is of special interest to their breeders, until this day (American Fancy Rat & Mouse Association). In addition, English merchants imported mice from Asia. These three sources provided the basis for the commercial breeding of laboratory mice. Due to this mixture one may well refer to stock or strain when talking about different kind of laboratory mice (Weiss et al. 2014b; International Committee on Standardized Genetic Nomenclature for Mice 2016). The first inbred mouse strain was bred in 1909 by Clarence Cook "C.C." Little, an American researcher, by intercrossing mice after coat color (Hutton 1969). The result is the still existing DBA/2 with a light brown coat color. Just ten years later Miss Abbie Lathtrop an American rodent fancier, established the most common laboratory mouse strain today, called C57BL/6, better known as Black 6 or in laboratory jargon just Blackis (Guénet et al. 2012a). Today over 450 inbred strains of mice are available, and some of them contributed substantially in gaining biomedical knowledge (Beck et al. 2000). That becomes evident when keeping in mind that almost 90% of the 106 Nobel Prizes awarded for Physiology or Medicine included research relying on different mouse models (Beck et al. 2000; AnimalResearch.Info 2015).

Nowadays, from an ecological point of view, representatives of the species *Mus musculus* are mainly living in three different environments. Wild mice have either a feral, non-commensal pathway independent from humans, or live as commensals associated with humans (Gray and Hurst 1997; Bronson 1979). The third variety of living conditions is in captivity and depending on their human caretakers, as pet or laboratory mouse. In order to understand the species-typical needs of the laboratory mouse, it is important to have a closer look at the ancestral wild house mouse (Brain 1992).

1.1.1 BEHAVIORAL CHARACTERISTICS OF *MUS MUSCULUS*

Wild representatives of the species *Mus musculus* display a large flexibility in their behavior. This predisposition is one of the main causes for being such a successful and wildly distributed species. It has even managed to grab a place on the list of the world's most invasive species (Lowe et al. 2000). Wild mice are nocturnal, meaning their activity phase is from dusk till dawn (Reichstein 1978). Their natural repertoire of behaviors includes running, climbing, jumping, burrowing and if absolutely necessary they turn out to be good swimmers (CFHS/FSCAA 2016; Stehr 1980; Ballenger 1999). Compared to their domesticated relatives they show a high manifestation of anxious behavior and are not at all calm towards humans (Blanchard et al. 2001; Fonio et al. 2012; Chalfin et al. 2014).

Wild mice live in a social family organization with hierarchical structures. A family consists of one dominant male mouse and several female mice together with their offspring (Reichstein 1978). Depending on the food deposits one family can be composed of up to 50 individuals (Jenrich et al. 2010; Macdonald 2004). Wild mice are territorial and do not move further than 15 meters away from their home (Ballenger 1999). Concerning the population density, there is a wide variation comparing the commensal populations with 10 mice/ $1m^2$ and feral populations with one mouse/ $100m^2$ (Ballenger 1999).

After almost 400 years of specific breeding the majority of behavioral repertoire of their ancestors is still present in laboratory mice (Jennings et al. 1998; Hutchinson et al. 2005). They still are nocturnal animals with a strong basic need for living in a group with established dominant hierarchies (Van Loo et al. 2004b; Hutchinson et al. 2005). In domesticated laboratory mice some characteristics may be emphasized such as the tameness towards humans. Other characteristics are deemphasized such as aggressive territorial defensive behavior as well as the likeliness of infanticide (Chalfin et al. 2014; Ebert & Hyde 1976; Jakubowski & Terkel 1982). One recent study provides evidence for a larger divergence in female laboratory mice from their ancestors than in their male conspecifics. The same study suggests that female laboratory mice almost completely lost some behavioral characteristics, such as freezing or spontaneous jumping (Chalfin et al. 2014).

1.1.2 ENVIRONMENTAL FACTORS FACED BY THE WILD *MUS MUSCULUS*

When choosing a habitat in a cultural landscape, one of the most decisive factors for the commensal house mouse is the close association with humans. In general mice prefer a dry and temperate habitat but due to this coexistence the house mouse inhabits regions from arctic to tropics (Weiss et al. 2014b). Commensal mice chose living areas in premises close food source like sheds or storage areas.

Their counterpart are feral mice living in the wilderness. If mice live independent from humans, they inhabit woodland and meadows where they live in cracks of rocks or make underground burrows. These burrows provide shelter and consist of different functional chambers for dwelling and nesting or the food storage. These chambers are all interconnected by a complex network of tunnels (Jennings et al. 1998; Berry 1970). In order to escape predators and not end up "like a mouse in a trap" the burrows have several exits (Ballenger 1999).

In the wild, seeds, fleshy roots, leaves, stems and insects are part of its diet (Ballenger 1999; Silver 1995). Regarding its eating habits as an omnivore, commensal mice are able to adapt well to people's nutrition. Mice are animals at the lower end of the food chain. Meaning, mice do not only experience the pressure of finding food, but have to hide from their predators as well. These include a lot of different small mammals like foxes as well as birds of prey and owls (Gerlach 2005; Brehm 1927).

1.1.3 ENVIRONMENTAL FACTORS IN KEEPING OF THE LABORATORY MOUSE

In laboratory animal science, the scientist is choosing the habitat for the mouse. General aspects are the reliability and reproducibility of the study outcome, as should be animal welfare considerations (Macarthur Clark & Zurlo 2012). However, there is not much freedom of choice, since the protection of animals for scientific purposes is highly regulated and restricted by the Directive 2010/63/EU. Before starting an animal experiment, the scientist has to ensure, the existence of an administrative license under article 11 of the German Animal Welfare Act. This article defines the requirements for the keeping and breeding of vertebrates i.a. for experimental purpose (German Animal Welfare Act). As a result, the animals are either bred in house or received from a commercial breeder. In general it is prohibited to take animals from the wild or to use ownerless or feral domestic animals for experimental purposes. The authorities responsible may grant exemptions from this if the objective of the experiment cannot be achieved using other animals. (Directive 2010/63/EU 2010 Art. 9; Animal Welfare/Laboratory Animal Ordinance 2013 Art. 20, Art.21).

The compulsory minimum standards for keeping of animals used for experimental and other scientific purposes is listed in Appendix A to Convention ETS No. 123, revised by the EU. On this basis, the Federation of European Laboratory Animal Science Associations (FELASA) published the “EUROGUIDE on the accommodation and care of animals used for experimental and other scientific purposes” (Federation of European Laboratory Animal Science Associations 2010). The Society for the Study of Research Animals (GV-SOLAS), also published recommendations, based on the German Animal Welfare Act and the German Animal Welfare/Laboratory Animal Ordinance 2013.

In the keeping of laboratory mice the most appropriate husbandry system is chosen with respect to the purpose of the study and the terms of hygiene requirements (Baumans 2005). The microbiological quality of experimental animals can critically influence animal welfare and the validity as well as reproducibility of research data. It is therefore important for breeding and experimenting facilities to establish a laboratory animal health monitoring (HM) programs as an integral part of any quality assurance system (Mähler Convenor et al. 2014). Husbandry systems vary from open systems with cages just covered by a grid to prevent the mice from breaking out, in which the whole room is the smallest hygiene unit or the whole barrier. On the next higher level of hygiene the lids are closed with a filter and around 30 cages are stored together in a constant climate, ventilated cabinet. A very high level of hygiene, which can be considered as the future standard housing, is provided by specific-pathogen free (SPF) units where the cages are individually ventilated (IVC) and are free from specific pathogens. IVCs have aspiration holes that connect to a pipe system which drains the extracted air and thereby prevents a contamination of surrounding cages. The highest possible hygiene regime can be carried out by keeping the mice in isolators, where any contact with the mice is just realizable through attached large plastic arms one can slip in.

For the common laboratory mouse, that means it will live in a polycarbonate cage with bedding material at around 22° C (±) 2°C, with a light-dark cycle of 12:12, a relative humidity from 45% to 65% in a room with a ventilation of 15-20 air changes. The minimum cage dimensions listed in ETS No. 123 are 330 cm^2 with a height of 12cm and a maximum stocking density of three mice with a body weight of less than 30g. From this stocking density, it can be deduced that laboratory mice have at the most 10% of the space they would use in wilderness. Fresh water is always provided in water bottles and food pellets are provided ad libitum in a feeding rack. Laboratory animal diet must ensure a balanced supply with the essential nutrients. Depending on the scientific issue, e.g. cancer research, food must be inspected for estrogens or other hormones. This will be essential for reliable study outcomes (Nygaard Jensen & Ritskes-Hoitinga 2007; Reardon 2016).

All those legal requirements are not evidence based but have their foundation in historical and practical perceptions (Würbel 2001). Some efforts are implemented in the ETS No. 123 e.g. providing of nest building material is requested as a minimum standard for laboratory mice. Another substantial benefit is that commercially available mouse cages have a wire bar lid that enables the mice to satisfy their need for climbing.

1.1.3.1 REFINEMENT IN LABORATORY ANIMAL SCIENCE

"Member States shall ensure refinement of breeding, accommodation and care, and of methods used in procedures, eliminating or reducing to the minimum any possible pain, suffering, distress or lasting harm to the animals" (Article 4.3 Directive 2010/63/EU).

A precise definition of Refinement is provided by the NC3Rs as "*methods which minimize suffering and improve animal welfare".* One Refinement strategy is to enrich the kept animals' environment. As described above nesting material and a grid are minimum requirements in the keeping of laboratory mice. They are basic forms of environmental enrichment (EE). Enrichment in general is a method to add species-specific stimuli to the mice's environment and therefore refine animal husbandry. The almost empty cage restricts normal dynamic behavior in mice (Latham & Mason 2004). It is highly important to consider and understand the species-typical behavior and the needs of the species that should be enriched (Hutchinson et al. 2005). Enrichment aims to enable the animals to display a large variety of their normal behavioral repertoire and should be evaluated critically. The list of possible enrichment factors is long. Just to name a few: environmental enrichment such as deformable substrate like nest building material (**Figure 1 C57BL/6JRj mouse interacting with nest building material**), social enrichment such as group housing, nutritional enrichment such as variation in diet plans and last but not least cognitive or behavioral enrichment such as animal training.

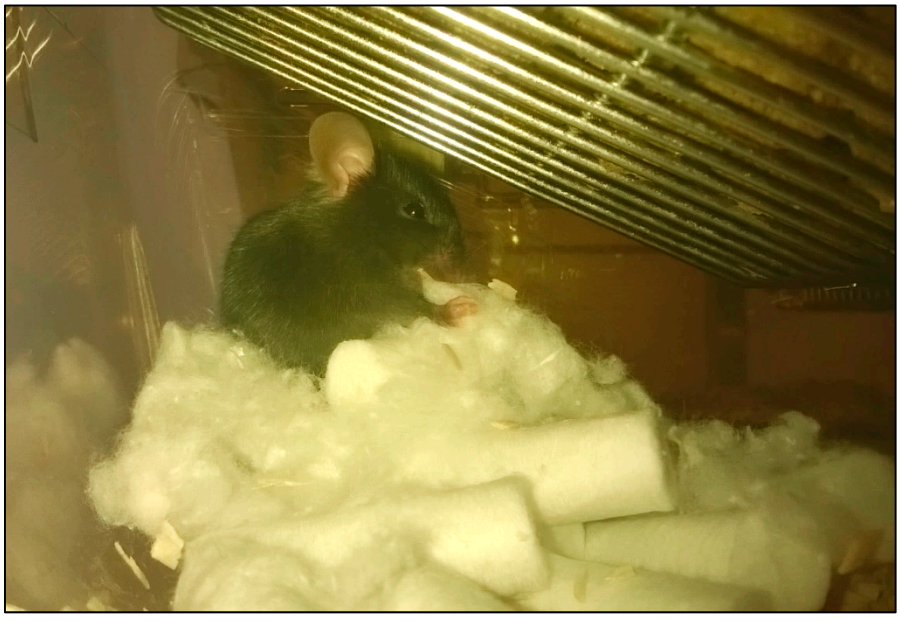

Figure 1 C57BL/6JRj mouse interacting with nest building material

One controversy with enrichment is that literature differs to which amount and especially what kind of enrichment is improving the welfare of laboratory mice. The easiest way would be to ask the animal what it wants, but obviously that is not so easily possible. But there are several different ways in indirectly asking an animal. To find out what enrichment is desired,

behavioral observations or choice tests can be performed (Sherwin 2007). But it is just expedient to observe an animal's behavior in an artificial environment if the behavior was previously analyzed in a natural or natural-like environment. All expressed behaviors under natural conditions taken together contribute to the species-specific ethogram (Hage et al. 2014). By comparing the displayed behavior pattern in artificial environment with the ethogram, it is possible to draw conclusion about the adequacy of the offered conditions. In choice tests, as the name implicates, the animal is faced with a choice. It is fair to assume that animals will choose the environment that contributes positively to their welfare. Two variants of choice tests exist: preference tests and consumer demand studies (Sherwin 2007). In preference tests the animals have to discriminate one environment against another (Sherwin 2007). One possible scenario could be two interlinked cages, one that is enriched with a mouse house and another that is enriched with nest building material. Evaluating the time the mice spent in each cage, allows conclusions to be drawn about the strength of the mice's desire for a mouse house or nest building material. Preference tests tell us which presented variant is better than another, but the degree of importance cannot be concluded by this method. Consumer demand studies provide exactly this solution. Previous research shows that consumer demand studies can be translated in animal studies (Kagel et al. 1975). One possible scenario could be to place an item of special interest for a mouse behind a door. At first it should be rather easy for the mouse to open the door and reach the item. Step-by-step the investigator makes it harder for the mouse to open that door. The effort the mouse is putting into opening the door reflects the strength of its desire to attempt the item.

For example, laboratory mice have a strong desire for nest building material. The strong necessity of acquiring nest building material is partly because they need to maintain their thermoneutral zone. The preferred temperature of mice is higher than the 22°C programmed in animal facilities and by building a nest, they can achieve a place with a warmer microclimate (Gordon et al. 1998; Gaskill et al. 2009; Gaskill et al. 2012; Gaskill et al. 2013a). Also, giving the mice the opportunity of building a nest, which is an archaic need still remaining in domesticated mice is a necessity per se (Van De Weerd et al. 1998).

Assuming an appropriate Refinement method has been found, it must be verified whether it contributes to the other two Rs, as well (Replacement, Reduction). Furthermore another determinate that arouses before ameliorating the life of laboratory animals is to be evaluated after changing the mice's living conditions. Has enrichment got the power of improving research or does it deteriorate the quality of research? The first "R" Replacement could be neglected in this point as enrichment is about the living conditions of still kept and used animals. The second R, Reduction should be taken into account if the group size is influenced by adding enrichment due to higher variation. Experts are currently divided over whether enrichment will lead to a decrease or an increase in group size. One side is arguing that "*potentially greater variation among data, requiring more animals per group for statistical significanc*e" (Hutchinson et al. 2005) while the other side takes into consideration that an "*inadequate environment might increase individual differences, as indicated by the occurrence of abnormal behaviours such as stereotypies, resulting in variable and individual coping responses"* (Bayne & Würbel 2014). Other studies provide indicators that neither a decrease or an increase in group size is to be estimated (Wolfer et al. 2004; Würbel 2007). It must be kept in mind that several studies report an alteration in brain development and behaviors such as an increase in anxious behavior in mice, kept without enrichment (Würbel 2001; Würbel 2007; Wolfer et al. 2004). Finally the conclusion drawn already almost 20 years ago *"Happy animals make good science"* (Poole 1997) is reflected in various studies and the current opinion of the European Directorate-General for Environment (European Commission Environment DG 2016; Bayne & Würbel 2014; Garner 2004; Würbel & Garner 2007). Considering all of this evidence, it seems that one step towards Refinement methods, could be realized by adding standardized enrichment factors.

1.1.4 REPRODUCTION AND BREEDING OF MUS MUSCULUS

In the following data for *Mus musculus* in general is presented. Distinct differences between wild mice and laboratory mice are highlighted.

1.1.4.1 REPRODUCTION DATA

The success of the species *Mus musculus* is highly linked to their fast reproduction cycle. Reproduction data crucially depends on genetics as well as on environmental factors. Wild mice differ from domesticated mice, large mice differ from small mice, and inbred strains differ from other inbred or outbred stocks (Gaskill & Pritchett-Corning 2015). When comparing feral mice with commensal mice and domesticated mice, one difference is that feral mice breed seasonal whereas the others show the same high reproduction rate throughout the year (Berry 1970). The phenomenon is founded in the fact that feral mice live in much more instable conditions and have to adapt to the environmental circumstances (Bronson 1979).

The sexual maturity is reached by females around 6-8 weeks after birth (Jenrich et al. 2010; Weber & Olsson 2008) and by males a little earlier around 4-5 weeks after birth (Weiss et al. 2014c). In general terms, it can be added that a small litter size and favorable environmental conditions contribute to infant development and early maturity (Yoon 1955; Reichstein 1978; Hill et al. 2008). Female mice are polyoestric with an oestrus cycle of four to six days and they have a spontaneous ovulation (Hardy 2012; Berry 1970). Wild mice are polyandry which means that female mice mate with several male mice and they discriminate between dominant and subdominant males (Rolland et al. 2003; Thonhauser et al. 2014). Successful mating is indicated by a vaginal plug, that reaches from the cervix uteri to the external genitals (Rugh 1991). The plug is composed out of excrete produced by the accessory sex glands of the male mouse (Weiss et al. 2014c). Whether the mating led to a gestation and offspring can be determined after a period of 19-21 days (Baumans 2004). Just after delivery, the dam has an attached post-partum oestrus (Berry 1970).

1.1.4.2 PUP DEVELOPMENT

Litter size varies between four to nine pups, strongly depending on the stock, environmental conditions and individual features of the dam (Hardy 2012). Mice are an altricial species. The neonates weigh around 1g, are hairless, blind, deaf and they have almost no ability to move on their own (Ewer 1968). During the first days, almost all perception of their environment will be due to the neonates' tactile feeling by the whiskers and through the skin. Approximately until two weeks after birth, the neonates strongly depend on their mother, for shelter, nutrition and thermoregulatory control (König & Markl 1987; Turner 2013). Around the fifth day of life they start to acoustically perceive their environment and one week later they open their eyes (**Figure 2 C57BL/6JRj Dam with 5-day-old litter**) (Weiss et al. 2014a; Weber & Olsson 2008).

Figure 2 C57BL/6JRj Dam with 5-day-old litter

By day six a coat of thin hair starts to cover the pups that is transformed into a dense fur by day twelve (Weiss et al. 2014a; Weber & Olsson 2008). As the motor skills improve rapidly, the pups start to extensively explore their environment by day seventeen and around this time they start to change their nutrition from milk to solid food (König & Markl 1987). The following days are characterized by a progressing autonomy of the litter and are defined as the weaning period (Martin 1984). The pups are weaned around day twenty-three, as soon as the dam and the litter lay close together without suckling or any other nursing behavior from the dam (König & Markl 1987). At weaning the pups weigh around ten grams but this is highly influenced by stock differences and environmental as well as individual conditions (Weiss et al. 2014a; Weber & Olsson 2008). In breeding of laboratory mice, pups are weaned at the age of three weeks, as this is the time the dam will have the next parturition, if mated during the post-partum oestrus (Weber & Olsson 2008). As mentioned in the literature review wild mice start leaving their natal territory around week five to six (Balcombe 2010).

1.1.4.3 MATERNAL BEHAVIOR

Maternal behavior of mice is characterized by staying close and huddling over their pups as well as by nursing, licking and grooming their offspring (Weber & Olsson 2008). Just several days after conception, the first behavioral indicator of a gestation can be observed as the mouse starts building a brood nest (König 2012). In the first three weeks after parturition the dam spends about 92% of her time occupying her litter (Weber & Olsson 2008).

Female laboratory mice show an inherent nursing behavior (Jakubowski & Terkel 1982). There is stock diversity in the way and in the rate of displayed maternal behavior but aggression towards the own or foreign offspring is not common (Weber et al. 2016; Weber et al. 2013; Shoji & Kato 2006). Whereas, in animal facilities dead pups and dams feeding on dead offspring is a phenomenon that is disturbingly often observed (Weber et al. 2016; Elin M Weber et al. 2013). The finding that high litter loss occurs predominantly in primiparous mice (Brown et al. 1999) is contrary to more recent literature suggesting that littler loss and parity were found not to correlate (Weber et al. 2013). In contrast to this, female wild mice have a rather high tendency of infanticide and there are valuable differences in maternal qualities regarding the age and the experience of the dam (McCarthy & Saal 1984). Infanticide is defined as ‘‘the killing of conspecific preweaning young’’ and does not distinguish between own and foreign offspring (McCarthy & Saal 1984). Around one third of

wild mice have an impulse for communal nursing but are very selective when it comes to finding the right partner (Weidt et al. 2014). Studies reveal that multiple sired litters as well as communal nursing have a positive effect on the offspring's development (Thonhauser et al. 2013; Firman & Simmons 2008; Auclair et al. 2014). The underlying reason is not yet fully understood but there are indicators for reduced infanticide by male mice as they cannot distinguish between own and foreign offspring (Auclair et al. 2014). However, there are no evidence that an advantage in genetic diversity is an underlying aspect for this behavior as inbred depression in mice is to be unattended and the survival probability of inbred litters is not smaller than in outbred litters (Margulis 1998).

One of the maternal habits correlates predominately with survival of the offspring. Appropriate nest building behavior seems to be crucial for the successful rearing of infants (Brianna N. Gaskill et al. 2013a; Ioannidis 2014; Wallace 1981; Weber et al. 2016). Just several days after mating, female mice start with the building of maternal nests which are double the size of a sleeping nest with a full dome and one or two entrances (König 2012). Feral mice build nest sites in special chambers of their burrow and for commensal mice any hidden and protected spot seems appropriate to build nests out of material provided from their current environment. (Ballenger 1999). Feral mice use soft plants, grass and hair and when living with humans mice use cloth, paper or any other shredded and soft material to build a maternal nest (Schmeil 1970; Alcock 1979; Ballenger 1999). The need to perform nest building behavior is still the same for laboratory mice as for their wild ancestors (Estep et al. 1975). One main reason for the strong correlation of nest building and offspring survival may have something to do with the constant climate inside the nest that is several degrees higher than the surrounding area and essential for the mice's neonates with no abilities to maintain a constant body temperature (Lynch & Possidente 1978). Hence, looking at the breeding of laboratory mice, the correlation of nest building and offspring survival further supports the idea that the dams' maternal qualities are associated with the displayed nest building behavior. Presuming that maternal qualities are as well influenced by the dams' wellbeing and stress level it might be revealed that nest building behavior contributes to good maternal qualities. Furthermore, several studies suggest that nest building behavior is an indicator of wellbeing in mice (Würbel & Garner 2007; Gaskill et al. 2012; Brianna N Gaskill et al. 2013a; Gaskill et al. 2011). Together with other recent studies they propose that nest building material is a biologically relevant enrichment. Additionally, by manipulating the material the mice gain control over their environment (Olsson & Dahlborn 2002; Friend 1991). More evidence for this correlation provides the finding that less anxious behavior, which is a parameter for wellbeing as well, is measured when mice are provided with nest building material (Kulesskaya et al. 2011).
All the reviewed studies support the hypothesis that environmental enrichment and especially providing nest building material is the key to efficient breeding with a high standard of animal welfare.

1.1.4.4 BREEDING TECHNIQUES

Selective breeding is a method to interfere in natural reproduction with the intention to modify the genetic code of a species. In 1856, the first detailed investigation of inheritance was performed by the Austrian monk Gregor Mendel during the cultivation of peas in the monastery garden (Orel 1996; Medel 1865). Although he did not know about genes and chromosomes, he laid the foundation for future breeding techniques.

There is special vocabulary for the selective breeding in animal facilities. Various special terms for mice stocks and strains, such as inbred, outbred, transgene and several breeding techniques like permanent monogam, intermitting monogam, permanent polygam or intermitting polygamy are described below. Firstly, there is a need for distinguishing between the term stock, used for a specific, closely related breeding population, the term strain that

should be solely used for an inbred breeding population and the term line describing a certain pedigree stock descending from a single breeding pair (Lutz et al. 2012). The definition of an inbred strain is the continuously selective mating with solely sister- brother pairs for at least 20 generations (Festing & Staats 1973). Thus, mice of one inbred strain have a nearly genetically identical genome which is a valuable factor for standardization and reproducibility in biomedical research. Whereas for outbred stocks it is not about individual uniformity. The population should remain constant with common phenotypic characteristics but with genetic polymorphism and large variety in allelic forms of the individuals (Hardy 2012; Hartl 2000; Hartl & Clark 2007).

With the upcoming new technologies to develop genetic modified animals, selective breeding is fastened and the number of mice kept and used for scientific purpose rises. When genetic modification by selective breeding reaches its limits, it comes to, genetic engineering with the use of biotechnology. For the engineering of transgenic mice a DNA injection into its genome must be performed, that results either in a gain or a loss of genetic information (Rülicke et al. 2007). This bears genetically modified organisms (GMO) that are, by definition not emerged from natural or selective breeding, and along with their offspring they are regulated under the Genetic Engineering Law (GenTG). Thus this law stipulates a safety level 1 for working with GMOs, when there is no danger of impact for humans and the environment. However the German Animal Welfare Act demands no more than the usual license under article 11 for the breeding of mice, if no adverse phenotype is to be expected. (Weiss et al. 2014d) But as transgenic mice are often specific disease models, extreme caution is necessary when it comes to welfare assessments and the implementation of the three-Rs (Mertens & Rülicke 2007; Rülicke et al. 2007). The German Animal Welfare Act demands in paragraph 7.2.2 an official approval for the breeding of mice if it is associated with pain, suffering, distress or long-lasting harm for the offspring. In this case the breeding is classified as an animal experiment and falls under the corresponding regulations (German Animal Welfare Act). Therefore, it is necessary to create common regulations to further implement a severity assessment and phenotype characterization of genetically modified mice (BfR 2016). Thus, the breeding of mice with high litter losses might be a welfare problem itself, the breeding of transgenic mice must be strictly surveyed and evaluated (Weber et al. 2013).

Another difference between wild and laboratory mice gets obvious when looking at mating. In the wild mice chose their mating partner freely favor and dominant over subdominant males. Furthermore, mice tend to mate with multiple partners (Rolland et al. 2003; Thonhauser et al. 2014). In commercial breeding, mating with more or less of an individual choice is replaced by sophisticated mating systems with a prime importance on economic considerations. In addition, it must be considered what kind of stock, strain or line is to be created and can vary a lot (Hardy 2012). In their book Weiss et al. 2014e illustrate four major forms of mating systems in commercial breeding. In monogam mating, just one female and one male are kept together, whereas in polygam mating several females and one male are kept together in one breeding cage. If detailed knowledge of maternal descent and birthdate of the offspring is not relevant a polygam mating system is the most economic. In contrast to permanent mating, where the male stays in the cage and the postpartum oestrus leads to a new gestation, in intermitted mating the male is taken out of the cage after a positive mating and just returns after weaning. The Whitten Effect facilitates the breeding organization by keeping the females at least one night before mating in an olfactory range to males. This will lead to a synchronization of the oestrus cycle and increase the copulation rate (Dorsch 2012; Whitten 1956). In order to estimate if a mating occurred the plug check is performed and depending on the stock or strain a gestation can be confirmed with a reliability of 80-90% (Hardy 2012; Rugh 1991). The described breeding techniques where just a brief introduction and only scratching the surface of a wide field ranging from traditional breeding to genetic engineering with the use of biotechnology.

Thus it cannot be emphasized often enough how important the implementation of Refinement methods in the breeding of laboratory mice is.

1.2 ANIMAL TRAINING

1.2.1 THE HISTORY OF ANIMAL TRAINING

The relationship of humans and animals has been driven by the motivation of people to make the best use of the available resources. Around 60000 years ago the first human – animal relationship was promoted, when wolves began to accompany groups of primordial humans (Kotrschal 2012). From then till now people profit extensively from their fellow creatures in various ways, for example by using them as powerful working animals, as loyal companions or in animal experiments. Over the last millennia people established an intensive keeping and utilization of lots of species, e.g. dairy cows, fattening pigs or laying hens to cover their needs. The keeping of the aforementioned livestock animals does not require a good working relationship. In contrast to that, when looking at guard dogs, herding dogs, carriage horses or war horses the situation is completely different. In cases in which people require the voluntary collaboration of animals, one fundamental aspect is a sustainable interspecific cooperation. Such a relationship of trust requires an in-depth knowledge of the physiology and ethology of the cooperating animal. Ancient evidence for human-dog interactions is provided by cave paintings of hunters with dogs from the Neolithic period. The importance of this bond is stressed by the fact, that one of the first letters in ancient Mesopotamia was the one for “hunting dog” (Nachtwey 2016). This hunting connection to humans was not limited to dogs as the ancient Egyptians used tamed cheetahs for hunting (McGreevy & Boakes 2007). One major historical development of training animals besides hunting was the use of animals for military purposes. This has extended from the famous war elephants of Hannibal in 200 BC to operant conditioned war dolphins of the U.S. Navy Marine Mammal Program (NMMP; Cambridge Center for Behavioral Studies, Inc.). In addition, the tame and trained exotic animals of travelling menageries had a high entertainment value. In 1831 the first the performance of two trained lions could be seen in “Les lions de Mysore” at the Cirque Olympique in Paris (Heine 1837). In the thirties of the last century Burrhus Frederic Skinner, an American psychologist and behaviorist analyzed the lever-pressing behavior of rats (Skinner 1938). The emerging curiosity in understanding animals led to the award of the Nobel Prize for Physiology or Medicine in 1973 to Karl von Frisch, Konrad Lorenz and Nikolaas Tinbergen "for their discoveries concerning organization and elicitation of individual and social behavior patterns" (Nobelprize.org). Furthermore training of laboratory monkeys is already established in laboratory animal facilities and leads to more relaxed animals that cooperate during various procedures (LAREF 2007; Whittaker et al. 2001). Additionally, nowadays animal training is a hobby for a lot of people, especially with companion dogs or in equestrian sports. Animal training has a long history and has not just made its way into science but also in our daily life as eight million dogs are living in German households (Statista 2016). In this introduction of animal training and its history it becomes obvious that animal training is broadly adaptable to a wide array of animal species.

1.2.2 LEGAL REGULATION OF LABORATORY ANIMAL TRAINING

The emerging knowledge about animals and their needs consequently leads to the adaption of several laws on animal handling and training programs. At European level the Directive 2010/63/EU on the protection of animals used for scientific purposes gives the countries the order to “… set up habituation and training programs suitable for the animals, the procedure and length of the project.” in animal facilities (Annex Ⅲ, Section A: 3.7.). In the Appendix A of the commission recommendation of 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (ETS No.123) it says that “… for some species, for example dogs and non-human primates, a training programme to encourage co-operation during procedures can be beneficial to the animals, the animal care

staff and the scientific programme." and that "Where appropriate, staff time should be set aside for talking to, handling, training and grooming animals." (ETS No. 123 Appendix A (2006) Section 4.10). At national level the Directive 2010/63/EU is transposed to the German Animal Welfare Act that demands in paragraph 11,(3),2 measures to habituate and train certain animals according to their keeping and further usage (German Animal Welfare Act). Those regulations require subsequent research about how they can be successfully implemented. An in-depth knowledge about the needs of different species of laboratory animals is essential.

1.2.3 METHODS OF ANIMAL TRAINING

"Teaching, it is often said, is an art, but we have increasing reason to hope that it may eventually become a science" (Skinner 1951).

Animal training cannot be understood without a basic understanding of learning and conditioning. The science that focuses on how individuals learn is called applied behavior analysis (Heidenreich 2007). The training of animals is a form of associative learning where the animal learns to connect two stimuli (classical conditioning) or it learns to connect its reaction with the following consequences (operant conditioning) (Myers 2014). The three scientist that mainly contributed to this field of applied behavior analysis were Ivan Pavlov, Edward Thorndike and Burrhus Frederic Skinner.

Ivan Pavlov, a Russian psychologist, derived the theory of classical conditioning from his investigations about the digestive system and digestive glands in animal studies (Pavlov & Thompson 1902). A simple example of classical conditioning is "Pavlov's dog". First there is the provision of food as an unconditioned stimulus and the dog is salivating as an unconditioned response. The next step is the actual conditioning, where a neutral stimulus is converted into a conditioned stimulus. Therefore, a bell is rang shortly before the food is presented. In the course of several repetitions of this procedure the ringing of the bell is now converted into a conditioned stimulus that evokes the conditioned response of salivation even without the presentation of food. (Pavlov 1927)

An extension of classical conditioning is the operant conditioning. Hereby individuals learn to set their own behavior and the following consequences into context (Gerrig & Zimbardo 2008). Thus, Edward Thorndike, an American psychologist introduced "the Law of Effect" stating that if a certain behavior of an animal was immediately followed by a satisfying consequence, the animal is likely to show this exact behavior progressively. On the other hand, if a certain behavior was immediately followed by a discomfort for the animal, the animal is likely to show the behavior less often. (Thorndike 1911)

On the basis of Thorndike's scientific findings Burrhus Skinner, an American psychologist and behaviorist was the first to intensively investigate the theory of operant conditioning. The literal translation of "operant" according to Skinner is to affect the environment. He performed a lot of experiments with the model of the operant conditioning chamber, the so called "Skinner box" (Kompaktlexikon der Biologie 2001). The Skinner box is a tool to investigate positive and negative reinforcement as well as positive and negative punishment. In a potential experimental setup for investigating a rat, the box contains a lever that the rat can interact with, two different sources of light, a feed dispenser and an electrified floor. Given that, the rat is supposed to learn to press the lever when the green light shines and not to do anything if the red light glows. An untrained rat will press the lever randomly and not depending on a previous condition. Using positive reinforcement, the rat gets access to the food dispenser every time it presses the lever after the glowing of the green light. If the rat sits on an electrified floor, the green light glows and the rat presses the lever the power is turned off, that would be negative reinforcement. The scenario when the red light glows, the rat presses the lever and the consequence would be to turn on the power would be

considered as positive punishment. The fourth possibility of operant conditioning is negative punishment, in this context the restriction of the access to food, if the rat is pressing at the glimpse of the red light. Summarizing, reinforcement is a mean to strengthen a certain behavior, whereas punishment leads to a weakening of the chance a certain behavior will be displayed in future. “Positive” means something is added to the rat’s environment, whereas “negative” means that something is taken away (Gerrig & Zimbardo 2008).

Comparing reinforcement training and punishment, one quite obvious disadvantage of punishment is that it evokes a discomfort, without providing a positive alternative for the animal. Thereby it can lead to massive side effects like distress, fear, aggression or apathy, which all hinder the wellbeing of an animal (Heidenreich 2007). Furthermore, the long-term impact of training on behavior is stronger with reinforcement training than with punishment (Schaefer et al. 2000). Looking more closely at the two variants of reinforcement there is evidence that the application of a positive reinforcement program is superior to a combination of positive and negative reinforcement. Hence, the same study demonstrated that a task was fulfilled more with higher accuracy and in shorter time. (Murrey 2007) Additionally, this method can be applied to shape an animal’s behavior. Shaping is the step by step reinforcement of all those behaviors of the animal that correspond to the desired behavior of the experimenter resulting in a new behavior pattern (Gerrig & Zimbardo 2008).

1.2.3.1 CLICKER TRAINING

One of the first obstacles with positive reinforcement training (PRT) Skinner noticed was the problem of timing. He postulated that even one second of delay between the displayed behavior and the reinforcement in form of a food reward, prevents a linkage of the two events for the trained animal (Skinner 1951). In the same discourse he proposed to establish a “conditioned” reinforcer that is much faster in rewarding a specific behavior. To established a conditioned reinforcer, Skinner advised to give a clear signal, like the click sound of a cricket, and provide the animal with food directly afterwards. Repeating this several times and the cricket becomes a conditioned stimulus. Two students of Skinner, the couple Marian Breland (today Marian Breland Bailey) and Keller Breland introduced the term of the “bridging stimulus”. A conditioned secondary reinforcer (click) bridges the time gap until the primary reinforce (food reward) can be obtained by the animal (Bailey & Gillaspy 2005). The following application model illustrates the concept of clicker training. First you have to think of a task, for example you want to teach a dog to sit down. To establish the conditioned secondary reinforcer, you continuously click and feed the dog immediately after the sound. You will know, if this step succeeded, by clicking and waiting a little longer before providing food. If the dog is now looking for its reward during the retardation, the conditioning is completed. In a next step you wait until the dog sits down occasionally. The exact moment its bottom touches the ground you click and reward the dog with a treat. Repeating this several times is going to trigger the dog to not sit down occasionally anymore but more often and with the intention of being rewarded. By this method the Brelands trained over 15.000 animals of 140 species (Bailey & Gillaspy 2005).

1.2.4 EFFECT OF TRAINING ON ANIMAL WELFARE

Looking at the three Rs, animal training mainly belongs to the category of Refinement. Refinement of daily husbandry routines as handling programs as well as the introduction of cognitive enrichment have the potential to be stress reducing for laboratory animals (Cloutier & Newberry 2008).

1.2.4.1 THE STRESS REACTION

Stress can be divided into negative stress- distress, neutral stress and positive stress- eustress. Distress occurs if an animals is confronted with a situation in which it experiences physical or psychological discomfort that leads to a disturbance of its physical or psychological homeostasis (Fraser et al. 1975). Over hundred years ago Walter Cannon, an American physiologist described the fight-or-flight response, as an individual's reaction to an acute stressor (Cannon 1915). Such short-term stressors activate the sympathetic-adrenal medullary system and lead to a release of adrenaline and noradrenaline out of the adrenal medulla (Von Borell 2000). The second activated system is the hypothalamic- pituitary-adrenal (HPA) axis, describing the interaction of three hormone producing glands: the hypothalamus, the pituitary and the adrenal glands. The starting point is a stressor activating the hypothalamus to release corticotropin-releasing factor (CRF). CRF stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). The effect of ACTH is to trigger the adrenal gland to release metabolites especially the stress related corticosteroids.
In 1981, János H. B. "Hans" Selye, an Austrian-Canadian endocrinologist defined the physical reaction to long term stressors as the general adaptation syndrome (GAS) (Selye 1981). He described a 3-stage set of physical responses to stressors, beginning with the nonspecific mobilization phase or alarm phase, followed by the resistance phase and the exhaustion phase. The nonspecific mobilization phase equates to the fight-or-flight response and results in an increase of blood pressure, heart rate and respiratory rate. In addition, the activation of the HPA axis leads to the secretion of several messenger substances. The following resistance phase, is characterized by persistence against the stressor, the physical defense and the intensity of the stressor are at level. In the course of the third phase, the stressor overruns the defense mechanisms of the body and the three glands lose their capacity to secret hormones. The body is weak and highly vulnerable to illnesses. (Cohen et al. 2013) A recent study summarizes the effect of chronic stress as followed "Cardiovascular, metabolic, reproductive, digestive, immune, and anabolic processes can be pathologically affected, subsequently leading to myopathy, fatigue, and hypertension, decreased growth rates, gastrointestinal distress, suppressed immune function, and, ultimately, impaired disease resistance. (…) leads to structural and functional changes in the brain, and, when extreme conditions persist, permanent damage can result." (Clayton & Tynes 2015).
Distress in laboratory animal facilities is not limited to experiments but may occur in daily routine, like cage cleaning or handling (Cohen et al. 2013; Balcombe et al. 2004; Brown et al. 2006).

1.2.4.2 REDUCTION OF DISTRESS DUE TO ANIMAL TRAINING

Due to enhanced interaction with caretakers and animals, animal training can be seen as extended handling program. A study on rats comparing the results of behavioral tests with intensively handled rats compared to the control group, provides significant results of a benefit for welfare due to handling (Cloutier et al. 2013). They go even further and call the handling an interspecific "social enrichment" for rats. Mice, the most used species in animal research, may be more complicated to handle, perhaps this is due to the fact that they are more foreign to humans than rats or because of their inherent timidity, but with some effort this gap will easily be overcome (Deacon 2006). But there is evidence that "non-aversive tunnel handling can substantially improve mouse performance in behavioural tests compared to traditional tail handling." (Gouveia & Hurst 2017)
This is further validated by one study comparing the performance in behavioral experiments with mice, that were handled intensively as young mice. These mice showed an in improvement in navigation skills which was most likely because of an established human-mice relationship and less fearful and distressed mice (Fridgeirsdottir et al. 2014). Another important finding of this study was that intensively handled mice displayed less variability in

their performances in the experiments. If the variation is smaller, less animals have to be examined in experiments. Thus, handling would contribute not just to the Refinement aspect but would lead to a reduction of animal numbers. But it should be kept in mind, that handling must be applied thoughtfully and can result in a stress reaction if the circumstances are not right (Jennings et al. 1998).

Furthermore PRT has the power to contribute to animal welfare as cognitive enrichment (Milgram 2003). The term cognitive enrichment was further explained as “A task (or tasks) providing opportunities to use cognitive skills to solve problems and control some aspect of the environment; and whose use is associated with an increase in one or more validated indicators of positive well-being and/or a decrease in one or more validated indicators of negative well-being” (Clark 2013). Due to the implementation of PRT the animal gains the possibility to control and predict its environment and it has the possibility to adapt to the present conditions. This is highly important, as a loss of control is a significant cause of distress in animals (Bassett & Buchanan-Smith 2007). Studies investigating PRT with zoo animals found that the training reduced the animals fear toward humans, and has therefore the potential to contribute to animal welfare (Ward & Melfi 2013; Westlund 2014). Another study stresses the advantage of this training method as the animal has the choice to participate in PRT, and cannot be forced to do it (Heidenreich 2012). Different studies achieved results showing that cognitive tasks contribute to the welfare of farm animals such as pigs, goats and cows (Meyer et al. 2010; Hagen & Broom 2004; Langbein et al. 2007; Puppe et al. 2007). Furthermore, the positive effect on stress-reduction and welfare could be demonstrated in non-human primates (Graham et al. 2012).

1.2.5 CLICKER TRAINING – A SPECIAL FORM OF POSITIVE REINFORCEMENT TRAINING

As mentioned before, the Brelands succeeded in training a huge number of different animal species using clicker training. For chicken and working elephants there exist scientific investigations on PRT showing how smart they are in clicker training (Fagen et al. 2014; Hazel et al. 2015). Rodents as well can be easily trained with PRT using a clicker as a bridge stimulus. One prominent example are the giant-pouched rats of the APOPO organization that help saving lives in Africa. They are trained to detect tuberculosis in patient samples or to indicate the location of land mines. (Apopo 2016; Glanzman 2014; Cengel 2014).

In laboratory animal facilities clicker training is implemented in the husbandry of laboratory monkeys and several studies try to establish common training programs for squirrel monkeys and long-tailed macaques (Gillis et al. 2012; Roth 2012). It is now ten years since a study was carried out to assess the effect of PRT with a secondary reinforcer (click, whistle) on variables indicating acute stress (Lambeth et al. 2006). The focus was on Chimpanzees that were trained to voluntarily present a leg for an intramuscular injection. Those individuals that participated voluntarily showed significant lower levels in white blood cell counts, absolute segmented neutrophils and glucose levels. The assessment of the hemograms imply that the training of chimpanzees results in much less stressed individuals during invasive procedure.
Taken together, these findings suggest for clicker training to play an important role in promoting a lasting welfare improvement to animal facilities.

1.3 OVERALL AIM OF THE STUDY

The overall aim of this study was to investigate different Refinement strategies concerning the breeding and the keeping of laboratory mice. This study consists of two parts, one investigating Refinement strategies in breeding. The other part seeks out to contribute to the

wellbeing of animals in the daily routine of keeping. Both studies pursue the common goal to achieve methods to reduce the lifelong experience of pain, suffering, distress or long lasting harm for laboratory mice. Hereby, we hope to contribute to the three R-principle of Russel and Burch that is a requirement of the new common legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU).

1.3.1 PARTICULAR AIM OF THE BREEDING STUDY

This part of the study addresses the breeding conditions of laboratory mice, in order to fulfill the legal requirement for the implementation of Russell and Burch's three Rs for animals used for scientific purposes. Research in the field of Replacement and Reduction is huge, but there is only a small amount of research regarding Refinement, and even less on Refinement concerning the breeding of laboratory mice. The study presented in the following committed to the problem of animal welfare in the breeding of laboratory mice. The relevance of environmental enrichment in the breeding cage was the central topic. The aim was to approach to a "gold standard" that would provide ideal condition for a successful breeding and rearing of the offspring. We hypothesized that the dams would profit of a super-enriched condition and suffer from an impoverished condition. Furthermore, we speculated that the survival rate and the development of the offspring would reflect the degree of wellbeing of the dam. As high litter loss causes a need to breed more animals it is counteracting the goal of a reduction of the number of animals used for biomedical research. Therefore this study contributed to two Rs of Russell and Burch's.

1.3.2 PARTICULAR AIM OF THE CLICKER TRAINING STUDY

The commitment to Refinement strategies in laboratory animal husbandry is a major goal in fulfilling the Three R principle of the Directive 2010/63/EU. There is evidence that gentle handling protocols play a crucial role in improving the wellbeing of laboratory mice. The aim of this part of the study was to introduce clicker training as an escalated handling program as well as a form of cognitive enrichment for laboratory mice. Clicker training has distributed among companion animals and found its way into the keeping of laboratory monkeys and non-human primates. The guidebook "Making lives easier for animals in research labs" (LAREF 2007) points out the following in the chapter 7.2. Injection and Blood Collection—How to Minimize Stress Reactions: *"Rodents (…) are the toughest animals for me to give injections without stressing them unduly. There seems to be no way of rewarding them except for their release—so it seems impossible to develop a positive reinforcement training technique for them"*. We took up this challenge and tried to develop a clicker training protocol that is suitable for the training of laboratory mice. Clicker training is a special form of PRT using a secondary reinforcer (click) to build up a time bridge between the strengthened behavior and an upcoming reward. Our primary aim was that this protocol can be transferred into the daily routine of animal facilities as well as in experiments conducted with mice. Furthermore, we sought to compare indicators of wellbeing between a trained and a control group. We hypothesized that clicker training has the potential to contribute to animal welfare in the keeping of laboratory mice. However, the background of this study is to improve techniques contributing to the health status of the animal facility. By thinking out of the box, the study's preliminary approach is to get an idea of the mice's cognitive abilities to learn. The interpretation will help to estimate if mice can be taught to voluntary cage change to a clean cage on their own, without a direct interaction with the animal keeper. A positive outcome will pave the way for a new hygiene management system.

2 MATERIAL AND METHODS

2.1 ETHICS STATEMENT

All animal procedures were approved by the Ethics Committee of the local governmental authorities (Landesuntersuchungsamt Rhineland-Pfalz, approval ID G 15-1-061) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

2.2 MATERIAL

Table 1 Register of material and equipment

Material /Equipment	Company	Comments/ Descriptions
C57BL/6JRj mice	Janvier Labs, Le Genest-Saint-Isle, France	registered international breeder
BALB/cJRj mice	Janvier Labs, Le Genest-Saint-Isle, France	registered international breeder
IVC Rack	Tecniplast, Buguggiate, Italy	Single Sided IVC Rack Dims: 1528 x 500 x 1995 (2090) #Part 0011.2GM70CPSU
Ventilated cabinet	ZOONLAB GmbH, Castrop-Rauxel, Germany	UniProtect Luftstromschrank
Mouse cages	Tecniplast, Buguggiate, Italy	Type II long, filter-top cages; SealSafe Plus, polyphenylsulfone, 365 mm L x 207 mm W x 140 mm Greenline
Bedding material	Lab & Vet Service GmbH, Vienna, Austria	Aspen bedding material
Mouse house	Tecniplast, Buguggiate, Italy	Red polycarbonate houses
Tissue	SCA Hygiene Products GmbH, Wiesbaden, Germany	"Tork"
Food	Ssniff, Soest, Germany	Ssniff M-Z Extrudat,
Target Stick with Clicker	TRIXIE Heimtierbedarf GmbH & Co. KG, Flensburg, Germany	
Mouse tunnel	Thyssenkrupp, Essen, Germany;	PVC 100 mm x 40 mm

iChoc White Vanilla	EcoFinia GmbH 32020 Herford, Germany	Ingredients: raw cane sugar*, cocoa butter*, rice-drink-powder* 18%, tiger nut-semolina*, Bourbon vanilla*, *products from organic farming (ÖKO-Kontrollstelle: DE-ÖKO-013)
Forceps	Aesculap AG, Tuttlingen, Germany	Anatomical forceps " BD025R" or any other tool to fixate chocolate
"Pool"	Ø	Stainless steel water container 120cm in diameter, 60 cm deep
"Pool liner"	Ø	Black inlay (with dimensions to fit the container) - from standard 0.8 mm thick pool liner
"Camera System"	Camera: Panasonic Marketing Europe GmbH, Wiesbaden, Germany Software: Noldus Information Technology, Oberreifenberg, Germany	Camera: analog video camera Panasonic, 720 vertical line resolution, with lens Computar 4.5-12.5 mm, 1:1.2 Software: EthoVision® XT

2.2.1 ANIMALS AND HOUSING

All mice were kept in a central animal facility that is certified according to article 11 of the German Animal Welfare Act, defining the requirements for the keeping and breeding of vertebrates i.e. for experimental purpose (Kreisverwaltung Mainz- Bingen, Aktenzeichen: 41a/177-5865-§11 ZVTE). The animal facility works according to a SPF hygiene concept. The microbiological and parasitological testing takes place twice a year on the basis of the "FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units" (Mähler Convenor et al. 2014). The health status of the mice is maintained due to the implementation of strict hygienic measures and a quarantine system. The mice were housed in type II long, filter-top cages (Tecniplast, Buguggiate, Italy; SealSafe Plus, polyphenylsulfone, 365 mm L x 207 mm W x 140 mm Greenline). The mice were maintained under a 12:12-h light/dark cycle (lights on 06:00–18:00) in a temperature- and humidity-controlled animal room (22±2°C, 55±5%). Food (ssniff M-Z Extrudat, ssniff, Soest, Germany) and water supply were provided ad libitum. At the end of both studies a veterinarian examined the mice. All mice displayed a good health status and were provided for other scientific research.

Breeding study

For the breeding study 70 primiparous timed-pregnant C57BL/6JRj mice were purchased on gestation day 14 from a registered international breeder (Janvier Labs, Le Genest-Saint-Isle, France). The day the plug was detected was considered gestation day 0. The commercial breeder enriches the breeding cages with nest-building material. During the study, the mice were individually housed and provided with different amounts of cage enrichment; aspen bedding material (Lab & Vet Service GmbH, Vienna, Austria), red polycarbonate houses (Mouse House, Tecniplast, Buguggiate, Italy) and tunnels (Thyssenkrupp, Essen, Germany; PVC 100 mm x 40 mm) as shelter and tissue papers (Tork, SCA Hygiene Products GmbH, Wiesbaden, Germany) to perform nest building behavior. The cages were stored in an IVC-

mouse rack (Single Sided IVC Rack, Tecniplast, Buguggiate, Italy) but due to the filter top cage, they were not connected to the ventilation. The weekly cage change was always performed by the same female animal keeper. The mice were transferred by the method the animal keeper was most familiar with, in this case lifting the mice by the tail stock.

Clicker training study

For the clicker training study 3-week-old BALB/cJRj mice were purchased from a registered international breeder (Janvier Labs, Le Genest-Saint-Isle, France). The total amount of 48 mice consisted of 24 female and 24 male mice. They were housed in separate-sex groups of four mice. All cages were enriched with aspen bedding material (Lab & Vet Service GmbH, Vienna, Austria), one red polycarbonate house (Mouse House, Tecniplast, Buguggiate, Italy) as shelter and one tissue paper (Tork, SCA Hygiene Products GmbH, Wiesbaden, Germany) to perform nest building behavior. During the training period of four weeks, the 12 filter top cages were stored in a constant climate, ventilated cabinet (UniProtect Luftstromschrank, ZOONLAB GmbH, Castrop-Rauxel, Germany). Afterwards the mice were transferred to another building. The short transport of three km happened with a licensed transport vehicle and the mice stayed in their home cages. In the second building, the mice were kept on rack system. The weekly cage change was solely performed by the same experimenter. All mice were transferred by putting a hand under the mouse's belly and covering it with the other hand.

2.3 EXPERIMENTAL DESIGN

2.3.1 TREATMENT PROTOCOL FOR THE BREEDING STUDY

The data of the breeding study was obtained from a study that was originally designed for another scientific purpose. The statistical planning was conducted to fit for the other study. Hence this is a retrospective description of disquieting findings. Due to the fact that, the findings are too important to ignore them, they will be reported nevertheless.

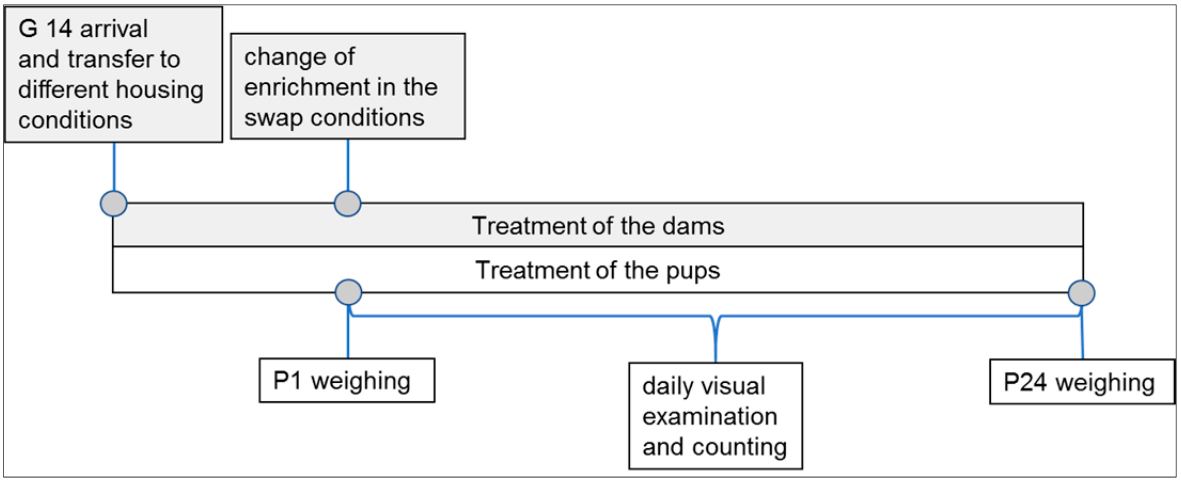

Figure 3 Timeline of the breeding study

(Leidinger et al. 2017b)

Treatment of the dams

The timed-pregnant mice arrived at the animal facility on gestation day 14. The same day they were randomly divided into three separate groups and transferred to the breeding cages. Three different house conditions were provided. One condition the “standard condition” was according to the SOP of the animal facility and consisted of 250 g of bedding material, one mouse house and one tissue paper. The “super-enriched condition” consisted of 350 g of bedding material, two mouse houses, three tunnels and four tissue papers. In the “impoverished condition” mice were kept in a cage with 75 g of bedding material without any further enrichment.
Two additional groups of five dams each underwent a variation in housing conditions. In the “impoverished swap condition” enrichment was added to impoverished cages. In contrast, enrichment was taken away from the cages of the “super-enriched condition”. In both groups the change occurred on postnatal day one (P1), leading to a standard housing condition.
In all groups, the dams were undisturbed until delivery.
(Leidinger et al. 2017b)

Treatment of the pups

The neonates were counted and weighed at P1 and P24. On P24, only the randomly chosen pups designated for the originally planned study were weighed. To ensure that no dead pups were overlooked, pups were inspected for skin colour, movements and wounds, as those are indicators of vitality (Olsson & Dahlborn 2002). Daily visual inspections of the neonates were performed and pictures were taken occasionally. In order to record survival rates, the pups were counted daily during the first week and upon weaning. Both the visual inspection of the pups as well as the counting could be performed in the impoverished condition quite often, without opening the cage. The standard condition limited the sight of the pups, leading to a disturbance of the dam while taking proper measurements. This was even more pronounced in the super-enriched condition. Sometimes a lot of cage enrichment had to be removed to get a glimpse of the pups.
(Leidinger et al. 2017b)

2.3.2 TREATMENT PROTOCOL FOR THE CLICKER TRAINING STUDY

Determination of a suitable primary reinforcer

The study on clicker training performed with the BALB/cJRj mice skips the determination of a suitable primary reinforcer as it has been established in a pilot study. As this thesis intents to give the overall picture it is thus described in the following.

The protocol is designed for three consecutive days. First a range with different food rewards should be available. Due to the strict hygiene management in animal facilities it is often required that the food reward should be autoclavable or at least stand a fumigation with H_2O_2. In addition, the reward should meet the food safety standards for animal feed or food that is suitable for human consumption. On the first day, a petri dish with several small pieces of various kinds of rewards should be prepared (**Figure 4 Example of a petri dish with different food rewards**; list of rewards, beginning at the top of the circle and then in a clockwise direction: dark chocolate, white chocolate, green gummy bear, cashew nut, raisin, milk chocolate; center: walnut). (Leidinger et al. 2017a)

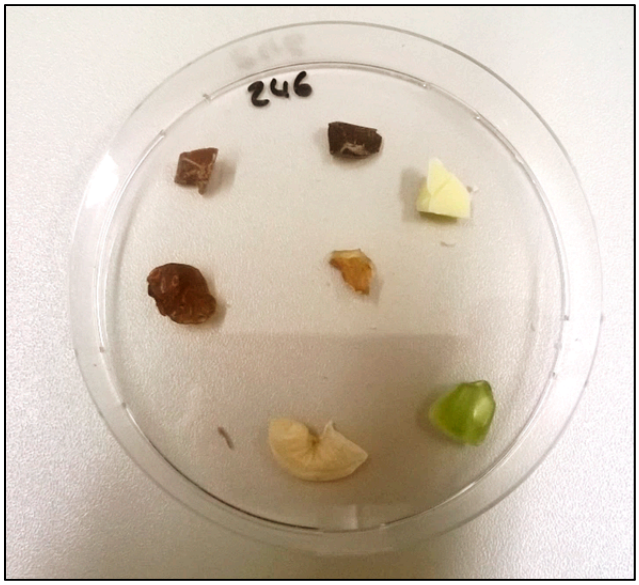

Figure 4 Example of a petri dish with different food rewards*; list of rewards, beginning at the top of the circle and then in a clockwise direction: dark chocolate, white chocolate, green gummy bear, cashew nut, raisin, milk chocolate; center: walnut (Leidinger et al. 2017a)*

The petri dish is inserted in the home cage of the mice. If the mice are not yet used to the experimenter, they should be left alone. After a period of ten minutes the experimenter should check for leftovers. If the mice ate up everything, the timespan must be shortened until the most preferred primary reinforcer can be determined. On the following days the mice should be offered small amounts of the determined reward. To keep it simple, the second reinforcer can be added to the home cage. Thereby all mice of the cage will get an affection to reward. (Leidinger et al. 2017a)

Clicker training

In this study, the period of the clicker training is followed by a period of behavioral tests. The week prior to training is reserved for the acclimatization phase of the mice (**Figure 5 Timeline of the clicker training study**).

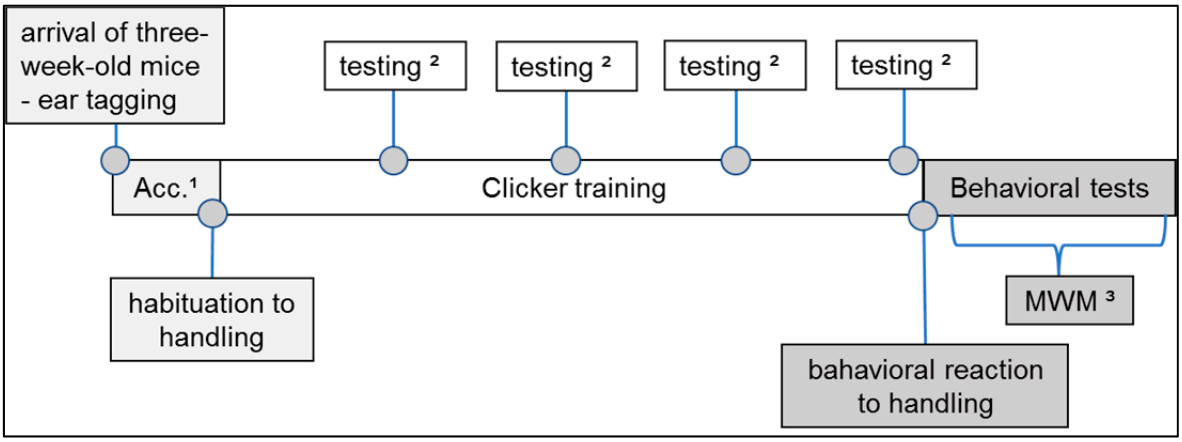

Figure 5 Timeline of the clicker training study *[1]Acclimatisation; [2]Testing of the learned behavioral pattern; [3]Morris Water Maze*

General rules for all training sessions

As the animals were kept in groups of for mice, three cages of each sex were randomly chosen to be part of the trained group. The other 12 female and 12 male received no positive reinforcement training. There are some general rules that were respected in every training session. All mice were handled in a calm and gentle manner. Each mouse was taken out of a cage by putting a hand under the mouse's belly and covering it with the other hand. The protocol of *"cupping on the open hand"*(Hurst & West 2010) was very useful. Two additional cages were prepared with some bedding material, one for each sex. The reward was prepared by warming the chocolate until it was malleable and then putting it around one end of a sterile forceps. A timer and the clicker-target-stick combination were arranged.

The home cage of the mice being trained was transferred to a quiet training place. This place was the laboratory desk of the same animal room the mice were kept in. The order of the trained mice was generated randomly on the day of training and was changed the following day. A training session with one mouse lasted about five minutes each day; Monday till Friday. At first, all unnecessary items had to be removed from the home cage so that it was suitable as training area. As all mice were trained individually this included most of the bedding material, the mouse house, the nest building material as well as the cage mates. The cage mates, of the mouse being trained, were transferred to the additional prepared cage. During every training session, 30 seconds of positive reinforcement were followed by 15 seconds of break. To receive the second reinforcer, the reward was offered to mice for about one second. A discontinuous pattern of rewarding was executed. From the first to the 10^{th} correct performance of a behavior, the mice were rewarded every time. From the 11^{th} to the 21^{st} performance the reward was presented every second time and afterwards merely every third time. After four minutes of training the mice received a jackpot reward, meaning they were allowed to gnaw three seconds on the reward, for the next correct performance of the reinforced behavioral pattern. (Leidinger et al. 2017a)

Linking the secondary reinforcer with the food reward

In the first training session, a connection of the secondary reinforcer with the food reward was created. This occurred with the help of a mouse tunnel. Mouse tunnels were not a part of the environmental enrichment for the mice in this study. Hence, two days prior to the first training session a mouse tunnel was added to each cage of the trained groups. During the first training session the tunnel was placed next to a wall. In doing so not only giving the mouse an opportunity to hide, but in addition taking advantage of the innate thigmotaxis of mice. As soon as the mouse entered the tunnel for the first time, it was clicked with the clicker-target stick combination and the reward was presented at the end of the tunnel. The mouse fed on the reward while sitting in the tunnel. It was clicked continuously for 15 seconds while the mouse was sitting in the tunnel. After the mouse had left the tunnel the procedure started again. (**Figure 6 "Position of Mouse Tunnel**. To enhance the probability of the mouse entering the tunnel, the tunnel is placed next to a cage wall." (Leidinger et al. 2017a)) (Leidinger et al. 2017a)

***Figure 6** "**Position of Mouse Tunnel**. To enhance the probability of the mouse entering the tunnel, the tunnel is placed next to a cage wall." (Leidinger et al. 2017a)*

Running through a tunnel

On training days two to five the reinforced behavioral pattern was "running through a tunnel". The tunnel was added to the training cage. As soon as the mouse entered the tunnel, it was clicked and the reward was presented at the end of the tunnel. As long as the mouse was in the tunnel, it was clicked and the reward was presented in the same manner. This was repeated for consecutive 30 seconds. During a pause of 15 seconds the tunnel was taken out of the cage. Then the tunnel was then added again and it was clicked immediately when the mouse re-entered the tunnel and the reward was presented at the end of the tunnel. The mouse was allowed to gnaw for up to one second, then the reward was taken away. As soon as the mouse started re-entering the tunnel on its own, the reward was presented in front of the end of the tunnel (**Figure 7 Presenting the Reward**). (Leidinger et al. 2017a)

***Figure 7 "Presenting the Reward**. The appropriate position for presenting the reward outside the end of the tunnel is shown." (Leidinger et al. 2017a)*

Following a target stick

In the second week, the ball of the target stick played a key role in the training sessions. The reinforced behavioral pattern was “following a target stick”. As soon as the mouse showed interest in the ball, it was clicked and the reward was presented next to the ball (**Figure 8 Training Success**; ***Figure 9* Rewarding**). The mouse was only rewarded after it touched the ball with its nose. After the mouse had linked the performed action to the reward, the position of the ball was changed during the training session (**Figure 10 Alternating Target Stick Positions in the Second Week of Training**)**.** The next challenge was only started if the mouse succeeded this last task. For this next challenge the ball was placed in the cage like before, but shortly before the mouse was able to touch the ball, the position of the ball was slowly changed. It was clicked and rewarded when the mouse had crossed the distance and was touching the ball. This procedure was repeated until the mouse followed the ball reliably. Then, the distance the mouse had to cross was slowly extended. (Leidinger et al. 2017a)

Figure 8 *"**Training Success**. A trained mouse follows the target stick while being trained." (Leidinger et al. 2017a)*

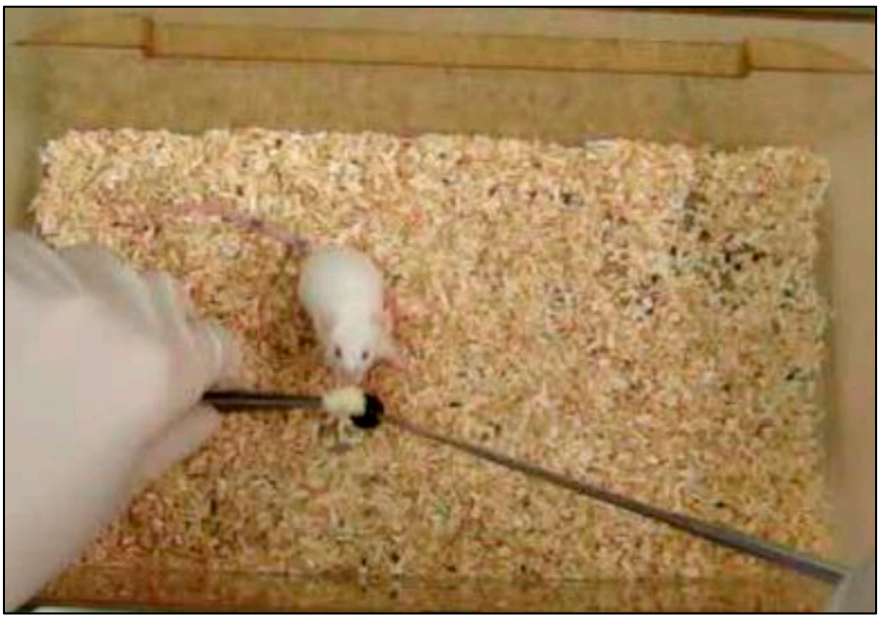

Figure 9 *"**Rewarding**. A mouse is rewarded next to the target stick during the training session" (Leidinger et al. 2017a)*

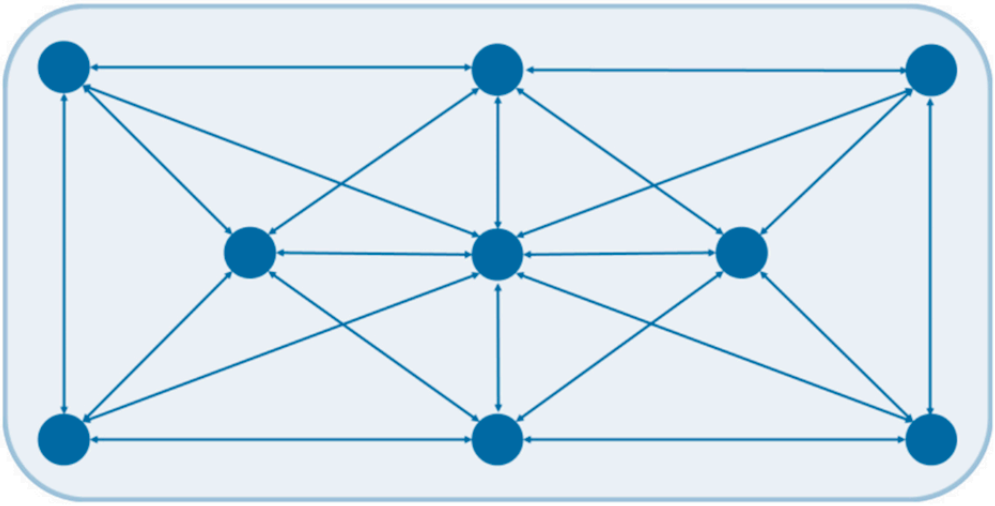

***Figure 10 "Alternating Target Stick Positions in the Second Week of Training**. The positions of the training stick are indicated in this schematic drawing." (Leidinger et al. 2017a)*

Following the target stick to the experimenter's hand

In the third week, the experimenter's hand played a significant role in the training sessions. The reinforced behavioral pattern was "following the target stick to the experimenter's hand". The experimenter placed one hand in the training cage while holding the clicker/target stick combination and the reward in the other hand. The ball at the end of the target stick was placed next to the hand. As soon as the mouse showed interest in the ball, it was clicked and the reward was presented next to the ball. When the mouse had successfully met this challenge, the ball was moved closer to the hand. Finally, the ball was placed on the palm of the hand. It was clicked and rewarded while the mouse was sitting on the palm of the hand (**Figure 11 Training Success**).
(Leidinger et al. 2017a)

***Figure 11 "Training Success**. A mouse has followed the target stick onto the hand of the trainer." (Leidinger et al. 2017a)*

Behavior triggered by scruff holding

The mouse was lifted out of the cage by cupped hands and gently placed on a grid. Then it was restrained with one hand. Therefore, the scruff of the neck was grasped with two fingers and the base of the tail was restrained with the little finger and the thenar eminence. The mouse was restrained in this position for 15 seconds. It was recorded, if the mouse displayed spontaneous urination, defecation, and vocalization.

Morris Water Maze

Note: Due to the concept of refinement, we evaluate the floating behavior not in the forced swim test, but in the Morris Water Maze (MWM). Following the severity assessments for laboratory animals, the MWM is less severe than the forced swim test. Furthermore, it is possible to gain additional information from MWM.

The Morris Water Maze was performed following the protocol of the Mouse Behavior Unit of the Translational Animal Research Center of the Universitätsmedizin of the Johannes-Gutenberg University Mainz (MBU) (**Figure 12 Setup of the Morris Water Maze in the MBU**). This protocol is a modification of a standardized protocol and is described in the following (Weitzner et al. 2015).

Figure 12 Setup of the Morris Water Maze in the MBU *(Day 3 to Day 8) - A C57BL/6JRj mouse has reached the platform*

The test was performed from Wednesday to Friday, Monday to Friday and Monday to Wednesday in three consecutive weeks. Two days with a visible platform were followed by eight days with a hidden platform including the probe trial on the last day without a platform. There were some general requirements. A tank with a diameter of 120cm was filled with water of drinking water quality. The water temperature of 25°Celsius was guaranteed by a heating rod, that was put in the water at the end of the test day. As the test was performed with white BALB/cJRj mice, the tank was covered with a black inlay. The light was adjusted to a luminosity of 500 lux at water surface. The wall of the room was covered with huge printings of symbols. There are four trials per mouse per day and every time the mouse was placed into the water facing another direction. The mice were carried to the tank in cupped hands. Then they were gripped by the tail and put in the water facing the wall of the tank. As soon as the mice were in the water the experimenter left the room. The provided platform was round shaped with a diameter of nine cm. The platform was located one finger's width underneath the surface of the water. In order to make it visible a thin pole of roughly 15 cm was adjusted in the center and a painted table tennis ball was attached to its end. A video tracking software was used to record the trials (EthoVision® XT).

Supplementary to the applied protocol, each time a mouse displayed floating behavior was recorded. "*Floating is defined as lack of swimming, and can include minimal movement of one leg, sufficient to keep the head above water*."(Crawley 2007). If the floating behavior was displayed for over three seconds it was recorded. Moreover, it was recorded when a mouse vocalized as it was lifted.

Each trial had a maximum duration of 90 seconds or shorter if the mouse was reaching the platform earlier. If a mouse failed to reach the platform in 90 seconds it was guided to the platform. The mice remained on the platform for 15 seconds in addition. Afterward the mouse was transferred to a cage that was placed on a heating plate. This cage was enriched with tissue paper which was removed when it was too wet. Each mouse had a pause while four other mice performed the test. Finally, they were returned to their home cage and left alone for the rest of the day. The male mice were tested prior to the female mice on each day.

Visible platform

On day one the platform was made visible with the table tennis ball and was located in the north-east of the tank. In order to prevent the mice from looking behind the tank's edge, it was just filled to one-fifth of its maximum capacity.

On day two the procedure was the same but the platform was located in the south-east of the tank.

Hidden platform

Days three to eight had the same schedule. The pole and the table tennis ball were removed, so that the platform was hidden. The tank was almost completely filled with water, so that the mice were able to look behind the tank's edge.

Probe trial

The probe trial was performed on the last day after all animals were tested with the hidden platform. For this trial, the platform was removed. The trial had a duration of 90 seconds for all mice.

2.4 STATISTICAL ANALYSIS

Statistical analyses were performed by using GraphPad Prism Version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). The results of the data were expressed as the mean ± SEM in column bar graphs. In the survival curve data were expressed as a percentage. Results are expressed as mean ± S.E.M. All data was checked for normal distribution (D'Agostino & Pearson omnibus normality test). For normally distributed data, ANOVA tests were performed. For not normally distributed data the Mann-Whitney U-test was performed. One time an unpaired t-test with Welch's correction was performed. For comparison of survival curves, the log rank test was used. In this study, differences were labelled as not statistically significant at a P value of ≥ 0.05 (ns) and statistically significant at a P value of < 0.05 (*). A P value of < 0.01 (**) was considered highly significant, and a P value of < 0.001 (***) very highly significant.

3 RESULTS

3.1 ANALYSIS OF THE BREEDING STUDY

The entirely passage “3.1 ANALYSIS OF THE BREEDING STUDY” including all figures is a citation of “**Leidinger et al., 2017b. Pup mortality in laboratory mice - a neglected welfare problem.** Submitted to: Laboratory Animals” and is not further declared as citation. (Figure numbers may vary from the original publication.)

Litter size on P1 and P24 in three different housing conditions

The pups born in three differently enriched cages (standard, super-enriched and impoverished) were first counted on the day after birth (P1). We could not detect a statistically significant difference in the litter size of the mice born in the different environments (**Figure 13 Litter size and pup loss on P1**). The mean litter size in the standard group was 6.3±1.3 pups, in the impoverished group was 5.8±2.6 pups and in the super-enriched group was 5.2±2.5 pups.

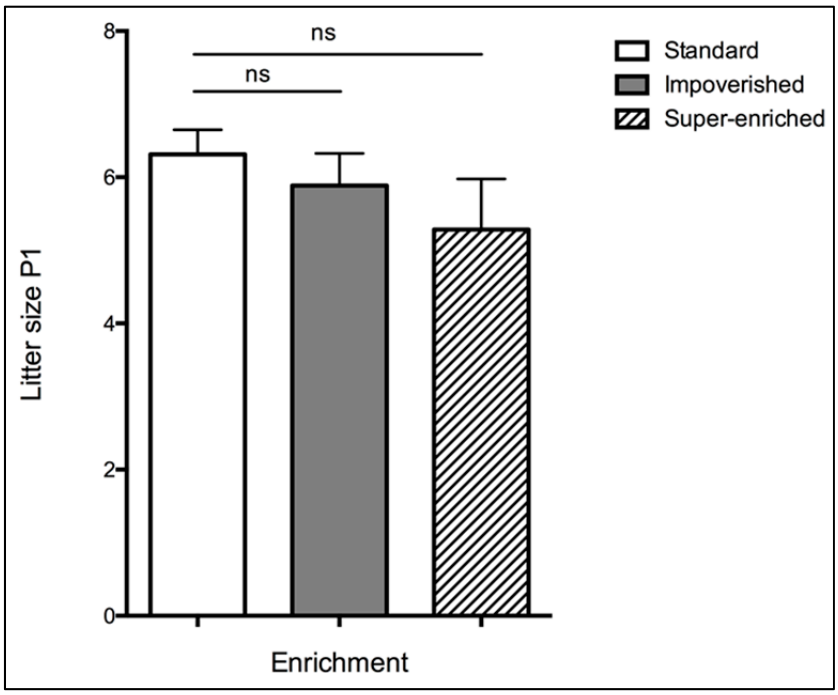

Figure 13 Litter size and pup loss on P1. *C57BL/6JRj dams were housed and gave birth in three different cage environments (standard, impoverished, super-enriched). The pups were counted on P1 (standard n=16, impoverished n=35, super-enriched n=14). The litter sizes at P1 are shown as mean ± S.E.M. ns= not statistically significant, p ≥ 0.05. The results were compared using one-way ANOVA.*

On P24 (at weaning), the picture had changed. The mean litter size of the impoverished group on P24 was 3.1±3.3, which was significantly reduced compared to the mean litter size of the group in the standard housing condition (p= 0.0053, 6.1±1.7). In the super-enriched environment the mean litter size was slightly but not significantly reduced to 4.9±3.0 (**Figure 14 Litter size and pup loss on P24**).

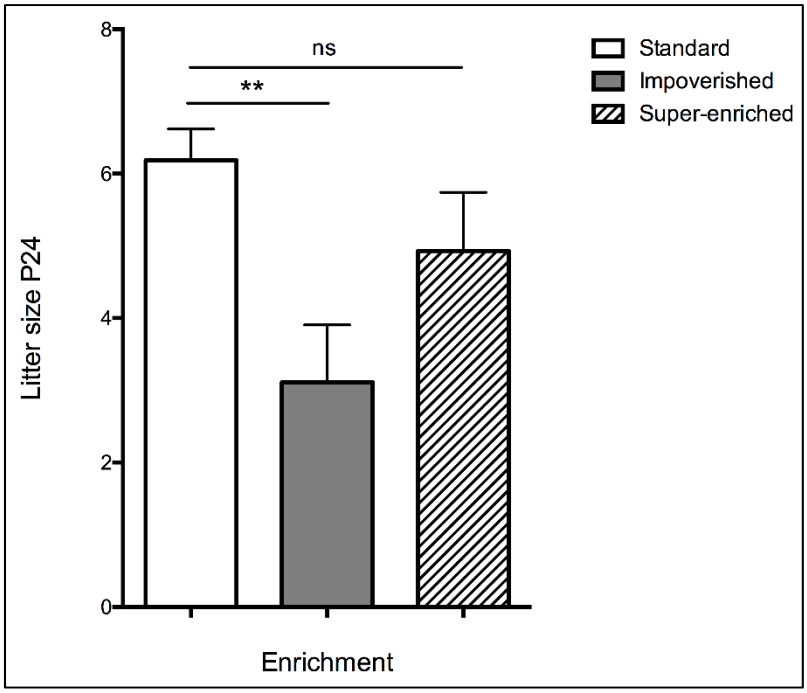

Figure 14 Litter size and pup loss on P24. *C57BL/6JRj dams were housed and gave birth in three different cage environments (Standard, Impoverished, Super-enriched). The pups were counted on P24 (Standard n=16, Impoverished n=18, Super-enriched n=14). The litter sizes at P24 are shown as mean ± S.E.M. ns= not statistically significant= p ≥ 0.05; p < 0.01 (**). The results were compared using one-way ANOVA.*

A closer look revealed that in half of the cases the entire litter died; in the other half, only individual pups died (**Figure 15 proportion of litters that were lost completely in relation to litters where only single mice died**).

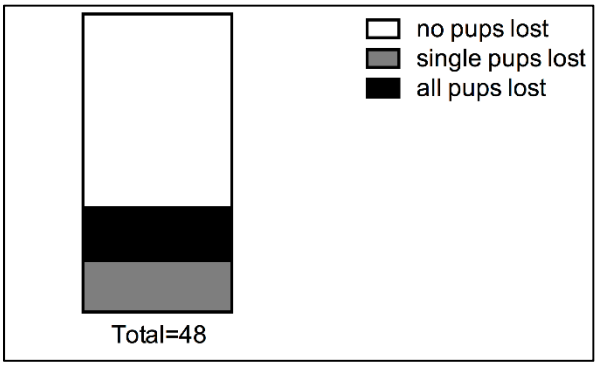

Figure 15 Shows the proportion of litters that were lost completely in relation to litters where only single mice died. *(no pups lost n= 31 litters; single pups lost n= 8 litters; all pups lost n=9 litters)*

Pup weight

In contrast to the litter size, the mean pup weight of the impoverished group was already significantly reduced on P1 (p=0.0017, **Figure 16 Pup weight on P1**).

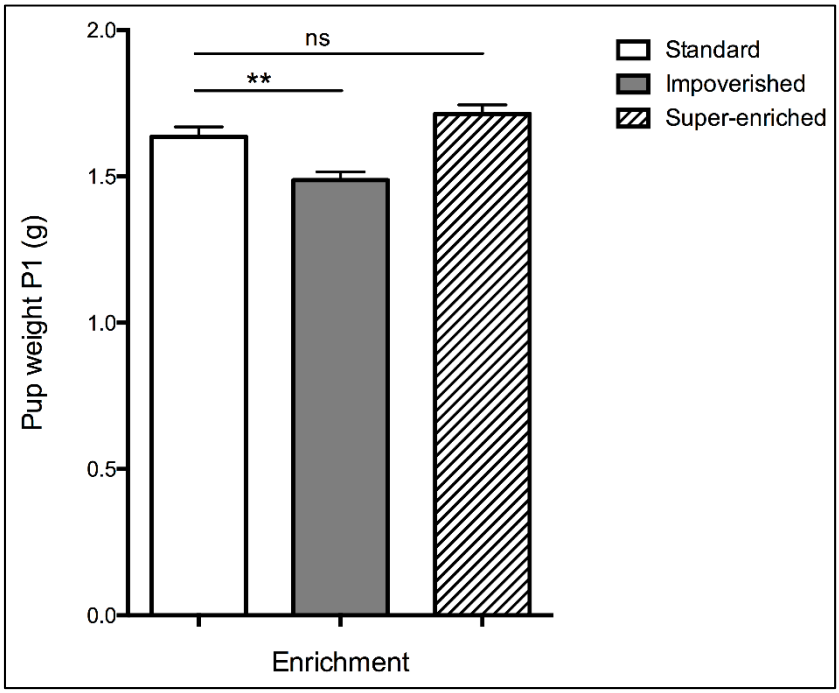

Figure 16 Pup weight on P1*. C57BL/6JRj dams were housed and gave birth in three different cage environments (Standard, Impoverished, Super-enriched). The pups were weighed on P1 (Standard n=75, Impoverished n=89, Super-enriched n=68). The weights are shown as mean ± S.E.M. ns= not statistically significant= p ≥ 0.05; p < 0.01 (**). The results were compared using one-way ANOVA.*

On P24 at weaning, the mice were weighed again. At this later time point, the reduced weight of the impoverished group is even more striking (p<0.0001, **Figure 17 Pup weight on P24**). The mean weight of the weanlings of the impoverished group was only 8.1g±1.4 g, compared to 10.6g±1.6 g in the standard group. The mean weights of the standard and the super-enriched groups (10.2g±1.0 g) were not significantly different.

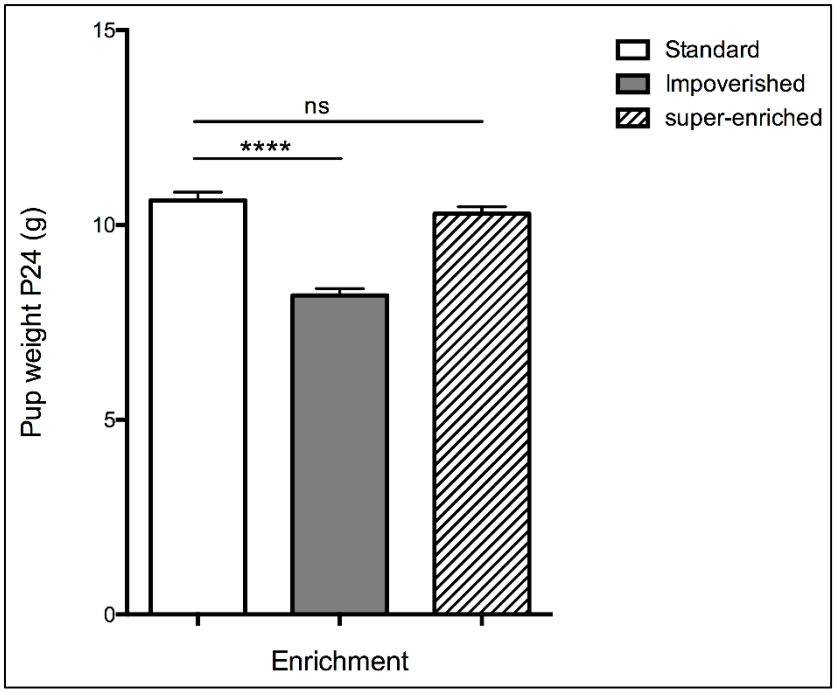

Figure 17 Pup weight on P24. *C57BL/6JRj dams were housed and gave birth in three different cage environments (Standard, Impoverished, Super-enriched). The pups were weighed P24 (Standard n=57, Impoverished n=72, Super-enriched n=37). The weights are shown as mean ± S.E.M. The results were compared using one-way ANOVA. ns= not statistically significant= $p \geq 0.05$; $p < 0.0001$ (****)*

Pup development

Visual examinations of the offspring confirmed very small neonates in the impoverished group on P1 (**Figure 18 Visual examinations on P1**). Furthermore, the development of the pups in the impoverished environment was delayed overall, as they were still hairless on P7 (**Figure 19 Visual examninations on P7**).

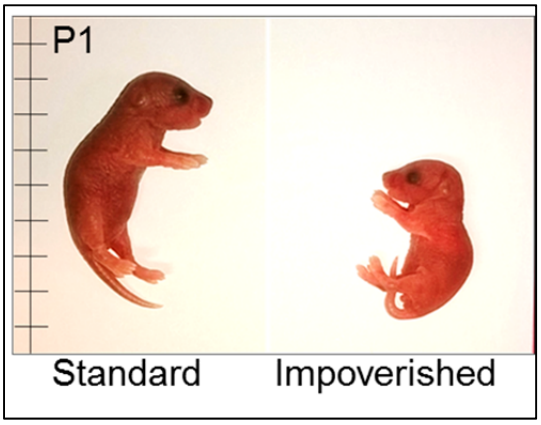

Figure 18 Visual examinations on P1 *showed the reduced size of the impoverished pups.*

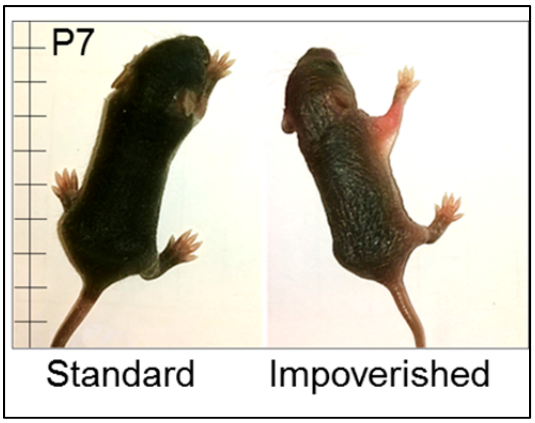

Figure 19 Visual examinations on P7*. During regular monitoring, it also became obvious that the impoverished pups still remained hairless on P7.*

Survival

In the next step, we took a closer look at the chronological sequence of pups dying. For the first 7 days, we inspected the animals daily. We also counted the pups at weaning. Overall, we saw a high survival rate for the standard group (**Figure 20 Effect of changed enrichment on the survival** - solid black line, 98% survival), and a slightly reduced survival of the super-enriched group (**Figure 20 Effect of changed enrichment on the survival** - grey solid line, 88%). For the impoverished group, the survival rate was significantly reduced (**Figure 20 Effect of changed enrichment on the survival** - solid red line); only 43% of the born pups survived till P24. In all groups, the highest rate of infant mortality was observed on P2 and P3. From the impoverished group, one individual pup died between P8 and P24. No pups in the other groups died later than P4.

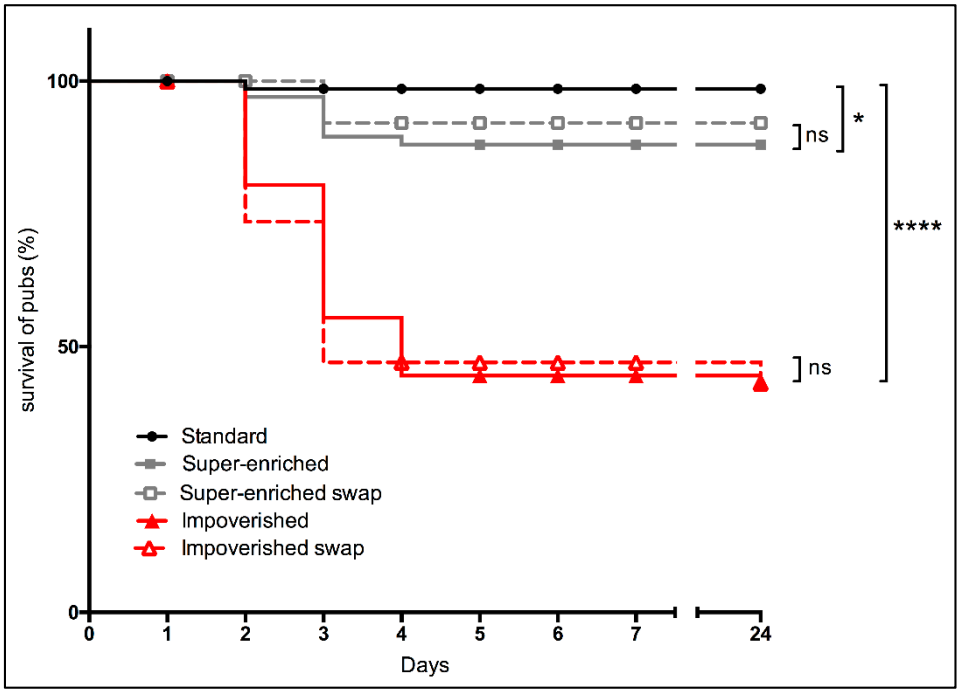

Figure 20 Effect of changed enrichment on the survival. *The survival of the C57BL/6JRj pups in the different housing conditions was monitored daily during the critical phase between P1 and P7, and on P24 (standard: black solid line, super-enriched: grey solid line, impoverished: red solid line). Two additional groups (pups of 5 dams each) underwent changes in their enrichment at P1 (from super-enriched and impoverished to standard, dashed grey and red lines). The survival curves were compared using a log rank test.*

The effect of changed environment

To investigate whether a change of environmental enrichment after birth has an effect on the survival of the animal, animals from the super-enriched and impoverished groups had to undergo a change to the standard housing condition at P1. The swapping of housing conditions on P1 did not significantly ameliorate the survival rate compared to groups of the same condition that did not undergo a variation in environment (**Figure 20 Effect of changed enrichment on the survival** - dashed lines, super-enriched swap group, 92%; impoverished swap group, 43%). Further, the time of mortality looked very similar. Here again, the majority of pups died before P4. After swapping the environment from impoverished to standard, a single pup died between P8 and P24.

In contrast, it became obvious that changing the enrichment from impoverished to standard led to a higher weight gain. These pups were able to close the gap in weight by the age of 3 weeks (**Figure 21 Pup weight in relation to a variation in housing conditions –** standard: : 10.4±1.6g; impoverished swap: 10.57±0.4g). The weight of the impoverished again was significantly reduced (**Figure 21 Pup weight in relation to a variation in housing conditions** - impoverished 9.0g±1.4g). Changing from a super-enriched to standard environment did not seem to have an effect on the weight (**Figure 21 Pup weight in relation to a variation in housing conditions** - super-enriched: 10.46g±0.9g; super-enriched swap 9.9g±1.5g).

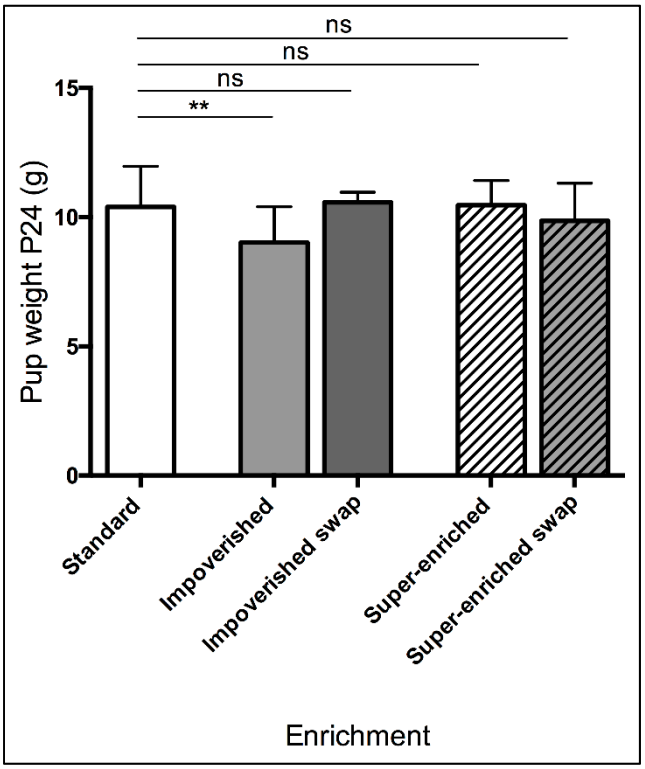

Figure 21 Pup weight in relation to a variation in housing conditions. *C57BL/6JRj dams were housed and gave birth in three different cage environments (Standard, Impoverished, Super-enriched). Two additional groups (pups of 5 dams each) underwent changes in their enrichment at P1 either from Super-enriched or Impoverished to Standard. The pups were weighed on P1 (not shown) and P24. (Standard n=32, Impoverished n=16, Super-enriched n=21, Impoverished swap n=9, Super-enriched swap n=28). The weights are shown as mean ± S.E.M. The results were compared using one-way ANOVA.*

The entire passage “3.2 ANALYSIS OF THE CLICKER STUDY” including all figures is a citation **of Leidinger et al., 2017a. Introducing Clicker Training as a Cognitive Enrichment for *Laboratory* Mice**. **Journal of Visualized Experiments, (121), pp.1–12.”** and is not further declared as citation. (Figure numbers may vary from the original publication.)

Success of the training protocol

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 (…). 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" (…). The vast majority of the trained mice-all female mice and 83% of the male mice-followed the target stick (**Figure 22 Success of Training Protocol; Figure 28 BALB/cJRj following the target stick over a bridge between two cages**).

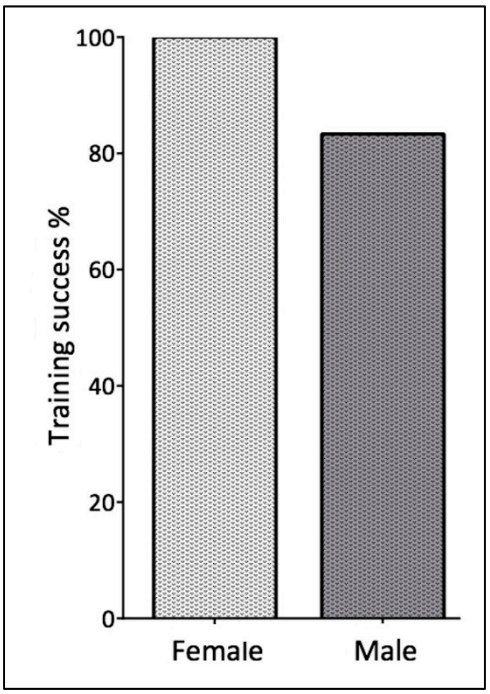

Figure 22 Success of Training Protocol*. A cohort of 12 BALB/c inbred strain mice of each sex was trained. By the end of training session week 2: "Following a target stick," all female mice and 83% of the male mice successfully overcame the challenge.*

Sex differences of the performance of the respective behavior

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 23 Repetitions of Respective Behavior after One Week of Training**).

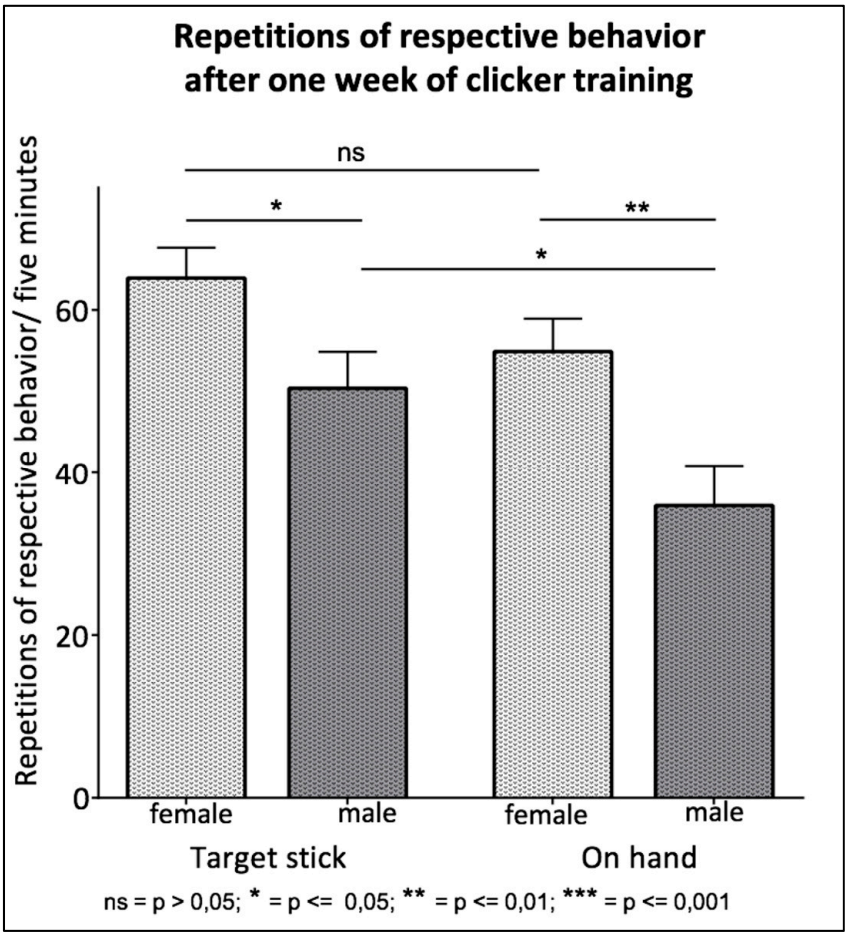

Figure 23 Repetitions of Respective Behavior after One Week of Training*. A cohort of 12 BALB/cJRj in bred mice of each sex was trained. Repetitions of Respective Behavior after One Week of Training. A cohort of 12 BALB/c inbred mice of each sex was trained. On the last day of training session week 2: "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.)*

To check whether the training had a positive effect on the wellbeing 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 m**ice of each sex served as a control group. Trained mice displayed a significantly lower frequency** of anxiety related behaviors than untrained mice (**Figure 24 Displayed Behavior Triggered by Scruff Holding**).

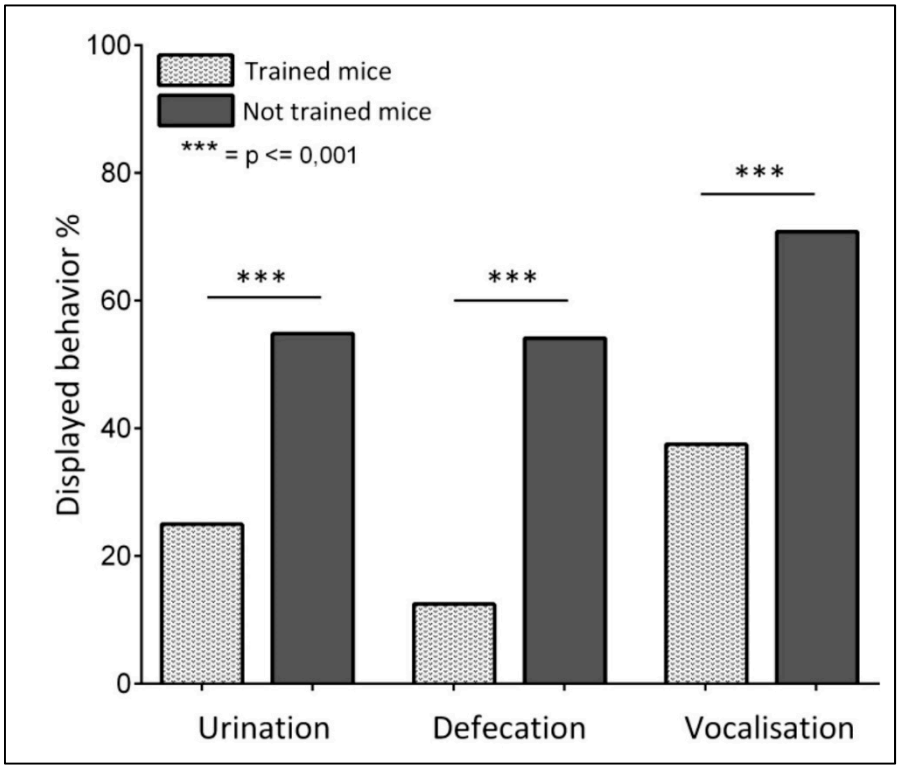

Figure 24 Displayed Behavior Triggered by Scruff Holding*. A cohort of 12 BALB/c inbred mice of each sex was trained following the clicker training protocol. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as a control group. The mice were singlehandedly restrained (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.*

Similar results were obtained when recording the vocalization linked to handling while performing the Morris Water Maze Test (…). The trained mice squeaked significantly less than the untrained mice (…) and the total number of squeaks per mouse was significantly reduced (**Figure 25 Displayed Behaviors during the Morris Water Maze Test**).

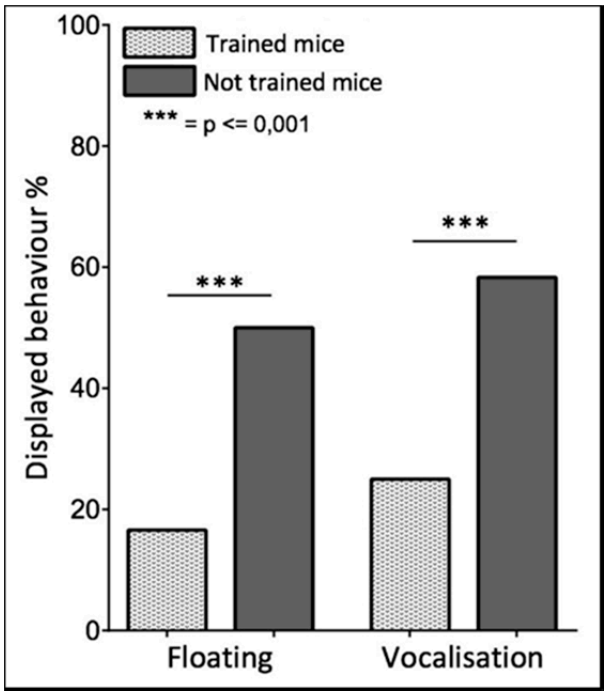

Figure 25 Displayed Behaviors During the Morris Water Maze Test. *A cohort of 12 BALB/c inbred mice of each sex was trained following the clicker training protocol. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as a control group. A Morris Water Maze Test was performed 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.*

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. Floating is a depression related behavior in the Porsolt (forced) swim test. This depression-related floating behavior turned out to be significantly reduced in the trained group in the Morris Water Maze (**Figure 26 Prevalence of squeaks per mouse**).

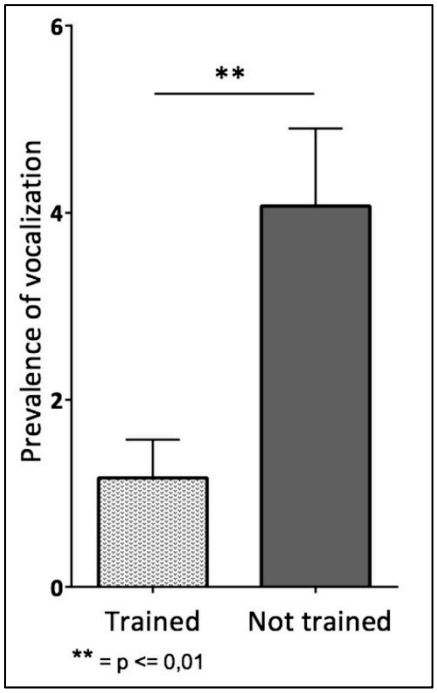

Figure 26 Prevalence of squeaks per mouse. *A cohort of 12 BALB/c inbred mice of each sex was trained following the clicker training protocol. An untrained but gently handled cohort of 12 BALB/c inbred mice of each sex served as control group. A Morris Water Maze Test was performed with all mice after 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.*

4 DISCUSSION

4.1 DISCUSSION OF THE BREEDING STUDY

This report begins by discussing **Refinement aspects of the empirical findings about the breeding success of laboratory mice** when challenged with individual different environmental conditions. “Successful breeding plays a crucial role in providing the millions of mice required for research every year. Hence, breeding success is one factor that could indicate a poor wellbeing, it should receive the attention that it deserves.” (Leidinger et al. 2017b) Reported litter losses of up to 50 percent are particularly worrying and perturbing implications for a neglected animal welfare issue in the breeding of laboratory mice (Weber et al. 2013). “As long as there is no environmental stress, our data do not seem to support a high pup mortality rate for primiparous dams in enriched environments in comparison to our standard and super-enriched environments.” (Leidinger et al. 2017b) We focused on environmental enrichment as it is very important in the breeding of mice. It enables the mice to perform archaic behaviors, still remaining after domestication (Dawkins 1990; Dawkins 1998; Weerd et al. 2006). Prior studies have noted the importance of environmental enrichment in the breeding cage and its beneficial effect on maternal behavior (Bult & Lynch 1997; Tsai et al. 2003). In addition, a strong relationship between nest building behavior of laboratory mice and a successful breeding and rearing has been reported in further literature (Gaskill et al. 2013b; Gaskill et al. 2013c; Lynch & Possidente 1978).

Our study’s set-up includes five different groups of randomly divided dams of the inbred strain C57BL/6JRj. One limitation of our study is that it solely focused on the investigation of primiparous dams. This might be a confounding factor as the influence of parturition on maternal quality and breeding success is controversially discussed in literature (Brown et al. 1999; Weber et al. 2013). The dams were kept under different conditions of environmental enrichment. The poorest enriched dams, the “impoverished” group, were kept in cages with only a small amount of bedding material. Our evaluated findings show that keeping dams in impoverished cages resulted in the highest value of litter losses with up to 50 percent (**Figure 20 Effect of changed enrichment on the survival**). These findings match those observed in earlier studies (Whitaker et al. 2009). In half of the cases, the dams lost individual pups, which could lead to the assumption that the remaining pups should have a higher access to maternal resources, which in general seems to lead to an enhanced weight. Moreover, one would assume that the pups dying are the weakest and therefore also probably the lightest. Both these observations would lead to higher average weights. Instead, the difference in weight on P24 became even more evident (**Figure 17 Pup weight on P24**). The reduced weight of the weanlings in the impoverished environment seems also to be consistent with previous research (Whitaker et al. 2009). The control group, further referred to as “standard group” was enriched in accordance to the minimum requirements of the FELASA- guidelines and showed litter losses of just two percent. Contrary to our expectations, this study found a significant higher litter loss in the “super-enriched” group with a survival rate of 88 percent. In the super-enriched condition the breeding cages were super-enriched with almost double the items of the standard group. An explanation for this is that the daily counting of pups under these conditions produced the highest rate of disturbance. Due to the super-enrichment and in contrast to the other two conditions, it was often necessary to remove a lot of cage items and to produce more confusion to get a reliable look at the pups. “Further, in some cases the nest built in an entirely closed mouse house had to be disturbed. Such stressors can possibly have a negative effect on the breeding performance (Peters et al. 2002).” (Leidinger et al. 2017b) In addition, inappropriate super-enrichment in the breeding cage could have led to this phenomenon.

The evaluation of the offspring development demonstrated the same pronounced difference between the impoverished condition and the other two groups. At birth there was no difference in litter size but already on postnatal day two (P2) there was a significant difference between the groups that became measurable as well as visible. The mean weight of the offspring born in the impoverished cages was significantly reduced compared to that of the other two groups. In the course of repeated visual examinations of the offspring a delayed infantile development of the pups in the impoverished group was striking. They were much smaller and it took longer until their bodies were covered with fur. Especially in the postnatal period newborn mice are strongly depending on their dams maternal abilities. Mice pups are altricial describing that they are not very well developed and rely entirely on the dam for warmth, food or transport (Bayne & V. Turner 2013).

At the age of three weeks, the pups were weaned and separated from their mother. At that time, there was still a significant gap between the weight of the weanlings of the impoverished group and those of the other two groups. This discrepancy may be due to a higher stress level in the dams kept in impoverished housing conditions. This thesis would be in agreement with studies that propose a correlation of stress and a recession in breeding performance of laboratory mice (Martin et al. 2009; Reeb-Whitaker et al. 2001). Under impoverished conditions the dams had no possibility to perform archaic and highly motivated behaviors such as nest building, hiding or simple interaction with anything at all. “Accordingly, it has been shown that enhanced nest-building activity is associated with higher weaning rates and that the quality of the nest at birth is critical for survival; this might be the major source of our observations” (Bult & Lynch 1997; Perrigo 1987). Without nesting material, additional energy from the dam is needed to keep the pups warm in a standard temperature laboratory environment, which is usually cooler than that preferred by neonatal pups (Elwood 1991).” (Leidinger et al. 2017b) Thus, it is likely that the dams’ basic needs in the resource-consuming phase of lactation were so disrupted that their remaining resources were not sufficient enough to perform adequate maternal behaviors, such as licking, grooming and arched-back nursing. Although, we could observe the aforementioned behavioral patterns as well in the impoverished condition, we had no long-time recording and are not able to estimate the extent of those behaviors. Taken together with the significantly higher litter loss in the impoverished condition the results give the impetus to think about cooling of the neonates due to a missing of nesting material or even infanticide by the dam. Yet some circumstances indicate otherwise. If the litter loss was due to a rapid cooling of the pups a balanced litter reduction in all impoverished cages would have been to be expected. In contrast, there was a high heterogeneity throughout the pup density in the impoverished condition. Infanticide is defined as ‘‘the killing of conspecific preweaning young’’ (McCarthy & Saal 1984). Previous research on this topic reveals that a restriction of the dams resources can provoke cannibalistic behavior of the dam resulting in a litter reduction (Perrigo 1987; Elwood 1991; König 1989). Laboratory mice have much less tendency to commit infanticide than their wild relatives (McCarthy & Saal 1984). We observed dams feeding on dead offspring but could not observe one case of infanticide which match those findings observed in earlier studies (Weber et al. 2013).

In order to understand to which extend the prenatal environment regulates the offspring’s development two additional groups were tested. These groups underwent a variation in housing condition. In one case, the variation consisted of adding enrichment factors to impoverished cages “impoverished swap group” and in the other case, enrichment factors were taken out super-enriched cages “super-enriched swap group”. For both groups, the change was performed on P1 and the new condition was identical to the “standard” group. The obtained data highlights that the postnatal period is highly influenced by the dam’s environment before delivery as litter loss has not changed compared to not swapped groups. Furthermore, this finding underlines that the main reason for pup mortality was most certainly not due to a cold distress. In contrast, the weight gap between the impoverished and the other groups could be closed after swapping. At weaning no difference in the weight of the

different groups could be measured. “These findings suggest that the breeding success was positively influenced by the changed conditions.” (Leidinger et al. 2017b) It emphasizes the general importance of environmental enrichment. “A delayed amelioration in maternal abilities could be a possible explanation for the still-high pup mortality rate yet an improved infantile development after swapping. Further, it might also be possible that the pups were already too weak to survive. This point is difficult to address because the majority of the deaths occurred very early.” (Leidinger et al. 2017b)

The fact that the standard condition obtained good values, and even better ones than a commercial breeder, is a good sign (Janvier Labs 2013). But a note of caution is due here since the breeding performance is just one indicator of wellbeing. A good breeding performance does not directly mirror a high animal wellbeing. This is in accordance with a review on the connection of performance characteristics and animal welfare for other domesticated animals (Müller et al. 1985).

A fateful constellation in the breeding of laboratory mice, is that high litter losses occur in the postnatal period, but the offspring is usually counted for the first time at weaning (Brown et al. 1999; Weber et al. 2013). Those findings are in agreement with ours showing that the highest prevalence of litter loss is from P1 until P4.

We can state that poor breeding performance creates serious welfare problems. On the one hand, the suffering of the dams must be extensive if it leads to enormous litter losses. On the other hand, there is the suffering of the offspring. There is reference in literature indicating the existence of conscious feelings in “moderately immature young newborn (…) mouse pups” (Mellor et al. 2008).

Furthermore, there are certain **Reduction aspects to find in the study on breeding success of laboratory mice**. Firstly, if more pups are born than weaned this inevitably leads to more litters to obtain the same number of animals. Consequently, more dams must be kept to obtain more litters. Secondly, the number of subjects to get statistically significant results is larger if the variety among subjects is higher. Stress is a major significant factor to the variability of laboratory mice. Hence, for inbred strains their genome is nearly equivalent, they are kept the same way and gain the same experience in daily routine of animal facilities. If they are stressed, animals will react with different coping strategies, thus leading to a heterogeneity among the subjects. Hence, animal numbers have to increase to compensate the growing variability (Festing et al. 1998; Howard 2002). To close the circle to the breeding performance of laboratory mice, the science of epigenetic is necessary. Several studies reveal, that the stress level of the dams alters the stress-related behavior of the offspring (Bayne & V. Turner 2013; Meek et al. 2000; König 1989; Weaver et al. 2004). To maintain the lowest number of animals for experimental research it is mandatory to check breeding facilities on the described weaknesses.

In addition, there is abundant room for further progress in determining a “golden standard” for the keeping and maintenance of breeding colonies.

“The data presented here might help to implement refinement strategies demanded in EU 2010/63 on a national basis, resulting in uniform general advice for refinement of breeding cages, thus improving both animal welfare and validity and reproducibility of data.” (Leidinger et al. 2017b)

4.2 DISCUSSION OF THE CLICKER TRAINING STUDY

The second part of this report is the discussion on the feasibility of the **practical implementation and on Refinement aspects in relation to clicker training of laboratory mice**. Own unpublished pilot studies with other mice strains implied that it is possible to train mice (C57BL/6J; B6 IFN g -/-; BALB/c; TCR-CL4). In accordance with that, it was no problem to perform clicker training with the subject mice of the particular study. All 12 females and 12 male mice of the inbred strain BALB/cJRj participated in the PRT and thus proved to have sufficient cognitive skills. Some other studies that have successfully trained mice in different ways lead to similar conclusions (Bathellier et al. 2013; Ehret & Dreyer 1984; Heffner & Heffner 1988; Novak et al. 2015). This finding is a little alarming as the “lowest developed mammals within laboratory animals” seems to have more in common with our companion animals than previously anticipated. Alone due to this new perspective a culture of care with new estimations of the mice’s needs is getting more and more inevitable.

In clicker training the mouse needs to participate in the training on its own accord and therefore the motivation of the animal is a core issue. The greatest challenge in using PRT with mice is to find the right reinforcer that is a suitable motivation for the subject mice. The fact that a cohort of mice consists out of different little personalities with different tastes, different expressions of appetite and different exploration behavior became obvious in our own empirical data and is inconsistent with data obtained in other studies (Crawley 2007; Loos et al. 2015). We overcame this difficulty by performing a choice tests, to be more precise a preference test with different kinds of foods before we started any kind of training. All BALB/cJRj mice liked white chocolate the most (**Figure 27 Habituated BALB/cJRj mouse eating white chocolate**). In the selection of the food reward we paid special attention to an organic, animal friendly and fair-trade predicate.

Figure 27 Habituated BALB/cJRj mouse eating white chocolate

The second major problem is grabbing the attention of the mice. The mouse must not be in contact with anything motivating it to do something else. Any source of items even bedding material, they can use to dig in, should be limited to a minimum amount during the training session. In the early stage of training confusing noise, odor, other people, and other mice should not be present in the training surrounding. If the distraction is limited to a minimum the mice will participate on their own accord. In later stages of the PRT the mice will connect the circumstances with the event of training and may even become impatient. A too long delay till the training starts should be avoided, as it can be frustrating for the mouse that a predicted event does not take place and can cause distress (Bloomsmith & Lambeth 1995).

Another "critical step within the clicker training 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." (Leidinger et al. 2017a)

The training can contribute to the process of Refinement in two different ways. Primarily, it serves as an escalated handling program, where the mice can build up a relationship of trust with their keeper or the scientist itself. Secondly, it is a cognitive enrichment for the mice. It is now well established from a variety of studies that gentle handling protocols benefit the mice's wellbeing (Fridgeirsdottir et al. 2014; Heredia et al. 2012; Maurer et al. 2008). The set-up of our study included behavioral experiments after the training period to predict the effect of training on the mice's tolerance of treatments. For the statistical analysis of the analyzed anxiety-related behaviors the mice were one hand restrained- grasping the scruff of the neck and the base of the tail with one hand (Seibenhener & Wooten 2015). The evaluation showed that spontaneous urination, defecation and vocalization were significantly less recorded in trained animals. In addition, we performed a Morris Water Maze Test that was evaluated regarding the demonstration of depression-related behavior. Floating at the surface of the pool, without any active movements is considered to be such a depression-related behavior (Can et al. 2011). Floating behavior was displayed by the untrained but gently handled mice with a significantly higher prevalence.

The condition that lead to "better" results in the trained group were either due to a closer mice-human relationship or due to more relaxed animals because of the provision of cognitive enrichment. Most likely both factors influenced the results. However, for the practical implementation of clicker training in laboratory facilities it does not matter, which part contributed the most. For the successive realization of this kind of training in animal facilities, it seems however evident that it contributes to more Refinement.

More broadly, research is also needed to determine the component of social enrichment for the mice if a mice-human relationship is created. This could be a great contribution to the problem of social isolation of single housed mice. Clicker training inherits a great potential to foster a kind cooperation between humans and mice. The training of animals to cooperate in animal experiments has been long established for monkeys and non-human primates (LAREF 2007). The clicker training of mice can be modified for different demands. Furthermore, we made considerable progress towards our goal of paving the way for a new hygiene management system where the mice are taught to change voluntarily from a dirty to a clean cage. At least some of the female mice in our study followed the target stick over a bridge into a new cage (**Figure 28 BALB/cJRj following the target stick over a bridge between two cages**). The other mice were too distracted by the bridge and didn't participate in the training any longer.

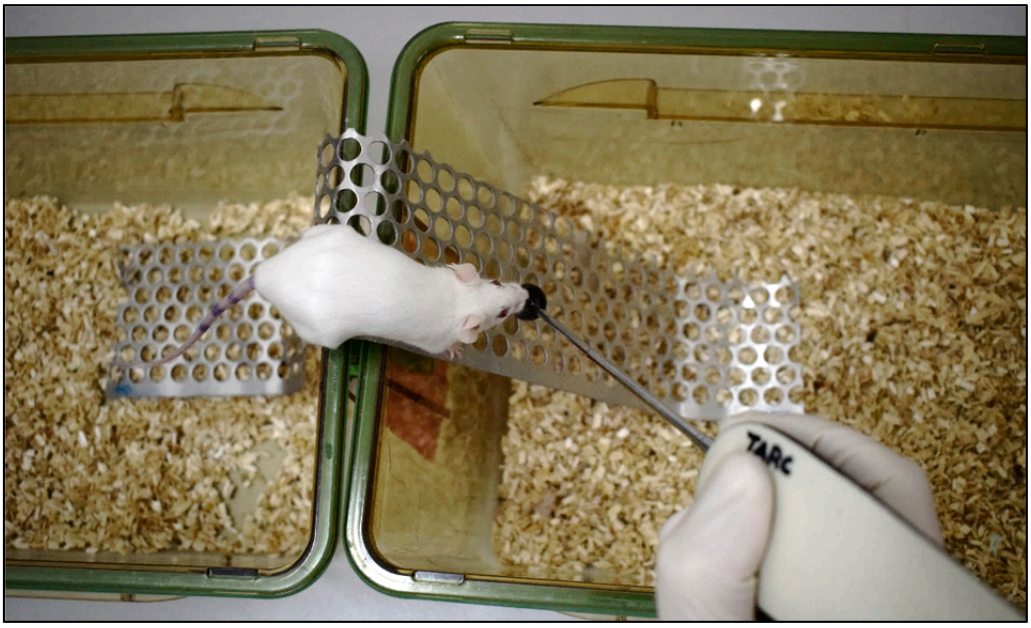

Figure 28 BALB/cJRj following the target stick over a bridge between two cages

A further study could assess the long-term effects of clicker training on this idea. We already thought about other approaches to this idea in building a tunnel-bridge between two cages. We propose that if the mice learn easily to pass through a shorter mouse tunnel, they can learn to do the same with a longer and curved one. Another scope could be to teach the mouse to hold still, for a photograph or even deeper imaging. This could eliminate the stress during restraining or even the risk of anesthesia.

“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 (Crawley 2007).” (Leidinger et al. 2017a) If the study will be repeated with different strains of mice it would be interesting to histologically compare the brain alteration of the trained and untrained individuals. This would allow us to get an impression of whether the training leads to more neuronal networking. As previously mentioned single housed mice should be included as well in order to evaluate if they profit from the mouse-human relationship. In addition, one could think about further behavioral experimentation to get a larger image of the impact of clicker training on mouse behavior.

Finally, there are certain aspects of the **Reduction of the animal number to find in clicker training.** Cognitive enrichment and a trustworthy human- mice relationship created by the clicker training have the same benefits for the mice as a thoughtful breeding management - they lead to a reduction of distress. And as previously explained it is further legitimated to claim that the prevention of distress leads to more homogeneity among subjects and finally ends in a reduction of subjects due to a smaller variability.

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. (Leidinger et al. 2017a)

5 CONCLUSION

An initial objective of the project was to identify lawful and humane breeding and keeping conditions of animals used for experimentation. The present results are significant in at least two major respects of the three R principle of Russel and Burch – Refinement and Reduction.

The first part of this study was designed to determine the ***effect of environmental enrichment on the breeding performance and offspring development of laboratory mice***. The findings of this study have a number of important implications for future practice. As assumed the keeping of mice without any enrichment is not just listed as requiring special approval but has a deeply negative effect on offspring survival and development. Furthermore, this can mirror a poor wellbeing of the dams. Super-enriched and standard enriched breeding cages did not differ as much as we estimated. Though one source of weakness in this study, which could have affected the breeding performance in the super-enriched cages, was the high disturbance when counting the offspring in this condition. More research is required to determine the efficacy of different kind of nest building material on pup survival. The highest percentage proportion of littler loss during the early postnatal phase emphasizes the importance of recording the litter size long before weaning. Only in this way a reliable evaluation of breeding performance is achievable. Hence, maternal suffering and the resulting, poor offspring development and high litter losses can be considered as neglected welfare problem.

In the second part of this study we ***overcame the challenge to develop a clicker training protocol that is suitable for the training of laboratory mice***. The protocol meets the requirements to be easily transferred into the daily routine of animal facilities as well as in ongoing experiments. The cognitive abilities of mice proved to be adequate in order to learn the imposed tasks. The assumption that clicker training, has the potential to contribute to animal welfare in the keeping of laboratory mice has been validated in this study. Several behavioral experiments were conducted after training and the trained group displayed less stress related behavioral patterns than the control group. Furthermore, the trained mice showed less depression like behaviors compared to the control group. In addition, animals that experience low levels of distress during their lifetime are less prone to develop detrimental coping strategies when confronted with stressors. This reveals a further argument for the implementation of cognitive enrichment. It can contribute to a homogenization of the animals' phenotypes. Less variation among subjects consequently leads to a lesser number of animals to gain statistical significant results. This will effectively contribute to the principle of Reduction. (Bayne & Würbel 2014) If the obstacle of the initial shyness regarding working with our small and foreign fellow creatures is once overcome, there is great potential in clicker training of mice. Thus, the broad implementation of positive reinforcement training in the keeping of mice strongly contributes to the 3R principle as it refines the husbandry conditions and experiments in biomedical research of mice.

Taken together, these findings support strong recommendations to the implementation of Refinement strategies in the breeding and the keeping of laboratory mice.

6 SUMMARY – REFINEMENT STRATEGIES IN BREEDING AND KEEPING OF LABORATORY MICE

Refinement strategies in breeding and keeping of laboratory mice play a pivotal role in assuring the best possible solution as long as animal based research is indispensable. Furthermore, Refinement is one of the three Rs proclaimed by Russel and Burch in 1959 that found their way into the current European legislative on the protection of animals used for scientific purposes.

In Germany a majority of 72,83 percent of all vertebrates used in experiments are mice, totaling over two million individuals. Almost all used laboratory mice are descendants of the house mouse (*Mus musculus*). The domestication of mice started at the beginning of the 17th century, when mice were kept and bred for different intentions and led to its proceeding domestication. In Great Britain mice were already in the focus as objects of study, but they were as well popular within amateur breeders that created the so-called “fancy mice”. Subsequent and more and more professional breeding led to the emerging of the first inbred mouse strains in 1909. This launched a cascade that enables researchers today to draw on a reservoir of over 450 inbred strains of mice. The necessity of mouse models becomes visible when keeping in mind that almost 90% of the 106 Nobel Prizes awarded for Physiology or Medicine included research relying on them. To understand the basic needs of laboratory mice it is essential to have a closer look at their ancestors. They are still living among us as wild mice with either a feral, non-commensal way of life or they live as commensals associated with humans. The domestication did not change much in the behavior of mice as they still are nocturnal animals with a strong basic need for living in a group. But some of the basic changes coming along with domestication are applicable for laboratory mice as well, as they are usually less aggressive and more easily to tame than their wild relatives.

The experimental aspect of the thesis consists of two parts that both contribute to gain knowledge about minimizing the lifelong experience of pain, suffering, distress or long-lasting harm for laboratory mice. The first part aims to refine the breeding methods and the second part is a new approach to create a mouse-human relationship as well as to add a new form of enrichment - cognitive enrichment.

The evaluation of different breeding conditions is consistent with data found in literature that in not profitable conditions postnatal litter loss reaches numbers up to 50 percent. In detail, the present study evaluated the impact of an impoverished, a super-enriched and a standard-enriched condition from the prenatal period until weaning of the offspring. A solid and constant breeding success was discovered in the standard enriched cage. The super-enriched condition did provide more unpalatable results concerning litter loss and pup growth. The most prominent finding was the detrimental high litter losses and the delayed pup development in the impoverished condition. The result that the highest prevalence of pup mortality occurred during the first four days after birth, leads to the conclusion, that if the usually counting of pups at weaning is going on, a huge amount of infant death is not recorded. To get a hint of how much influence the prenatal condition has on the maternal quality and thus on the offspring’s development, we tested a group with a change of the condition from impoverished and super-enriched to standard enrichment on P1. The swapping did not result in a prevention of perinatal litter loss. But one can estimate, that there was a certain effect of the variation in enrichment as the neonatal weight gap between the groups was closed until weaning. But not just a tribute to Refinement is approached but due to a better knowledge of the prevention of litter loss, a reduction of the animal number can be realized.

We successfully overcame the challenge to create a positively connoted mouse-human relationship and to introduce a PRT into the keeping of laboratory mice. Previous research provided the information that gentle handling protocols contribute to more relaxed mice. Enrichment is one major factor enabling mice to interact with their surroundings, and by

manipulating things, they gain control over their environment. Control and the possibility to predict what is likely to happen next have a deep impact on the stress level. This study concentrated on clicker-training with one intention of an escalated handling protocol and with another intention of providing a new form of enrichment- cognitive enrichment. In clicker-training positive reinforcement consists of a chain of two reinforcers. The primary reinforcer is the food reward and a second reinforcer- a click is added to build up a time bridge between the strengthened behavior and an upcoming reward. Our little fellow creatures differ in their personality, even though they have an equal genetic background and born and raised under the same conditions. The food reward must address the motivation of each mouse, because motivation is the underlying reason why mice are participating in training. Further the cognitive abilities of mice turned out to be more than enough to perform the assigned tasks. The clicker-training of mice turned out to be quite simple. The evaluation of different behavioral experiments stress that trained mice are more confident in the interaction with humans and show less stress related and less depression like behaviors. In addition, stress reduction leads to a lower variability among experimental mice which results in a reduction of the amount of needed subjects and leads to data with a higher quality. This study proposes that clicker-training improves Refinement as well as reduction and thereby contributes in the implementation of the 3R-principle of Russel and Burch.

7 ZUSAMMENFASSUNG – REFINEMENTSTRATEGIEN IN DER ZUCHT UND HALTUNG VON LABORMÄUSEN

Solange Tierversuche noch unersetzbar bleiben, ist die Weiterentwicklung von Refinementstrategien essentiell für das Wohlbefinden der Versuchstiere. Bereits 1959 publizierten Russel und Burch die Bedeutung des 3R-Prinzips „Replacement“, „Reduction“ und „Refinement“, das sich seit wenigen Jahren seinen Weg in die europäische Legislative in die „Richtlinie 2010/63/EU zum Schutz der für wissenschaftliche Zwecke verwendeten Tiere“ gebahnt hat.

Die intensive Auseinandersetzung mit der Haltung von Labormäusen ist ein entscheidender Faktor, da fast drei Viertel aller Versuchstiere Mäuse sind. In Zahlen bedeutet das, dass allein in Deutschland pro Jahr mehr als zwei Millionen Mäuse zu Versuchszwecken gehalten werden. Um die Bedürfnisse dieser kleinen Nager zu verstehen, muss man sich ihre Abstammung und die Lebensweise ihrer Vorfahren ansehen. Bis auf einige Ausnahmen stammen alle Labormäuse von der Hausmaus (*Mus musculus*) ab. Hausmäuse leben immer noch wie zur Zeit der Domestikation der Labormaus im 17. Jahrhundert entweder angeschlossen an den Menschen als Kommensalen oder sie bestreiten eine vom Menschen völlig unabhängige Lebensweise. Und auch Labormäuse haben sich nur wenig verändert. Sie sind nach wie vor sozial lebende nachtaktive Nagetiere. Allerdings sind auch die gängigen Folgen einer Domestikation an der Maus nicht spurlos vorbeigegangen, so sind sie weniger aggressiv und leichter zu zähmen als ihre wilden Artgenossen. Ein Grund für die Domestikation der Maus war damals bereits ihre hervorragende Eignung als Studienobjekt. Eine weitere Welle der Domestikation steht im Zusammenhang mit Hobbyzuchten, die insbesondere auf Fellfarbe selektierten und somit die „Fancy Mice“ schufen. Seit dem Beginn des 20. Jahrhunderts münden diese beiden Haupteinflüsse in professionelle Zuchten ein und führen schließlich dazu, dass Wissenschaftler heutzutage auf mehr als 450 verschiede Mausestämme zurückgreifen können. Für fast jedes Krankheitsmodell gibt es so die passende Maus. Die große Bedeutung dieser Mausmodelle für die Ergründung bestimmter Fragestellungen wird deutlich, wenn man sich bewusstmacht, dass mit dem Nobelpreis für Physiologie und Medizin ausgezeichnete Werke zu 90% auf der Arbeit mit Mäusen beruhen.

Der experimentelle Anteil dieser Arbeit beschäftigt sich zum einen mit der Zucht von Labormäusen während der zweite Teil die Lebensbedingungen in der Haltung von Mäusen untersucht. Beide Teile sind darauf ausgerichtet, Refinementstrategien zu ergründen, die dazu beitragen den Versuchsmäusen ein Leben ohne Schmerzen, Leiden oder Schäden zu ermöglichen.

Eine Neonatensterblichkeit von bis zu 50% in der Zucht von Labormäusen erregt Grund zur Besorgnis und macht weitere Untersuchungen auf diesem Gebiet notwendig. Der Ansatz der ersten Studie war es verschieden angereicherte Zuchtkäfige hinsichtlich Jungtierentwicklung und Jungtiersterblichkeit zu untersuchen. Hierfür wurden drei Grundausstattungen gewählt, eine verarmte, eine superangereicherte und eine Standardeinrichtung, die der Ausstattung nach den FELASA-Richtlinien entspricht. Der Zuchterfolg in den Standard eingerichteten Zuchtkäfigen war konstant gut und übertraf die angegebene Aufzuchtrate des kommerziellen Züchters. Zu unserer Überraschung brachte die Zucht im superangereicherten Käfig deutlich unbeständigere Ergebnisse mit einer reduzierten Wurfgröße. Dies könnte allerdings darauf zurückzuführen sein, dass für das tägliche Zählen der Jungtiere deutlich mehr Käfigeinrichtung entfernt werden musste, was einen negativen Effekt auf den Stresslevel der Mutter gehabt haben könnte. Ein prägnanter Unterschied zeichnete sich in den verarmten Zuchtkäfigen ab, da hier Wurfverluste von 50% und eine verzögerte juvenile Entwicklung zu beobachten war. Ein weiteres alarmierendes Ergebnis ist, dass sich der höchste Jungtierverlust direkt postnatal bis zum vierten Tag nach der Geburt ereignet hat. Da es jedoch gängige Praxis ist, die Jungtiere erst im Alter von drei Wochen bei der Trennung von der Mutter zu zählen wird ein möglicher Jungtierverlust nicht wahrgenommen. Um Auswirkungen der prä- und postpartalen Umwelt auf das Brutpflegeverhalten der Mäuse

evaluieren zu können, wurden Tests mit zwei weiteren Gruppen angestellt. Die Mütter saßen präpartal entweder verarmt oder superangereichert und die Käfige beider Gruppen wurden am Tag der Geburt so modifiziert, dass sie der Standardeinrichtung entsprachen. Dies hatte im Vergleich zu den Haltungssystemen ohne Veränderung keinen Einfluss auf die Rate der perinatalen Jungtierverluste. Aber die postpartale Veränderung der Umwelt, hin zur Standardeinrichtung, bewirkte eine Erholung des Wurfes im Hinblick auf die Gewichtszunahme. Nach der Veränderung konnte die Gewichtsdifferenz der Neonaten in den unterschiedlichen Enrichments (Umweltanreicherungen), bis zum Zeitpunkt des Absetzens, in der dritten Lebenswoche, ausgeglichen werden. Weitere Untersuchungen müssen durchgeführt werden um zu untersuchen, ob statt weiteres Enrichment, anderes Enrichment das maternale Verhalten positiv beeinflussen könnte und so zu weniger Jungtierverlusten führen könnte. Eine genaue aber möglichst tierschonende Dokumentation ist essentiell für den Einzug und die Evaluation von Refinement in Zuchten. Aber nicht nur eine Verbesserung der Lebens- bzw. Überlebensbedingungen kann so geschaffen werden, durch höhere Absetzraten kann auch die gesamte Tierzahl reduziert werden, da weniger Zuchtmütter benötigt werden.

Der zweite Teil der experimentellen Arbeit befasst sich mit der generellen Realisierbarkeit und den Auswirkungen eines positiven Verstärkungstrainings (PRT) in Labormaushaltungen. Studien belegen bereits, dass intensivierter Kontakt zu den Mäusen dazu beiträgt, dass die Tiere allgemein entspannter sind. Des Weiteren ist Enrichment ein Schlüssel zum Wohlbefinden von Tieren, da es durch Interaktion und eine gewisse Vorhersehbarkeit den Tieren Kontrolle über ihre Umwelt ermöglicht. Zum einen sollte die Etablierung einer Maus-Mensch Beziehung untersucht werden, und zum anderen wollten wir herausfinden, ob Clickertraining ein kognitives Enrichment für Mäuse sein kann. Clickertraining ist ein PRT mit zwei Verstärkern, einem primären, der Futter-Belohnung und einem sekundären, in diesem Fall der Click eines Clickers. Der sekundäre Verstärker dient dazu, die Zeit zwischen korrektem Verhalten und zeitverzögerter Belohnung zu überbrücken. Da sich unsere kleinen Mitgeschöpfe in ihrer Persönlichkeit sehr unterscheiden, obwohl sie fast genetisch identisch und unter den gleichen Bedingungen aufgezogen wurden, war es wichtig herauszufinden, für was sie sich motivieren lassen. Motivation ist der Schlüssel zur Partizipation von Tieren im PRT. Die kognitiven Fähigkeiten der getesteten Mäuse waren mehr als ausreichend, um die entsprechenden Aufgaben zu lernen. Die spätere Auswertung verschiedener Verhaltenstests ergab, dass trainierte Mäuse ruhiger im Umgang mit Menschen sind und auch weniger stressbezogenes und depressionsartiges Verhalten zeigten. Auch hier zeigt sich nicht nur ein Refinementeffekt, sondern wie auch im ersten Teil der Studie spielt das zweite R (Reduction) eine Rolle. Weniger gestresste Tiere führen zu konstanten und valideren Ergebnissen, die eine geringere Streuung aufweisen als die der Kontrollgruppen. Somit führt die Arbeit mit oder an entspannten Tieren zu einer Reduktion der Fallzahl. Schlussfolgernd kann Clickertraining in Mäusehaltungen deutlich zur Umsetzung des 3R-Prinzips beitragen.

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9 LIST OF OWN PUBLICATIONS

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In: 55th Annual Meeting of the Society for Laboratory Animal Science GV-SOLAS and 17th Advanced Training Course of the IGTP 11.-13.09.2017 in Köln
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Winner of the Young Scientist Travel Award

Poster presentations

Hohlbaum, K.*; **Leidinger, C**.* et al., (2017) * Equal authorship;
Resocialization of single housed male C57BL/6JRj mice after castration
RSPCA / UFAW Rodent and Rabbit Welfare Meeting – 2017 Surrey, England 14.11.2017;

Hohlbaum, K.*; **Leidinger, C**.* et al., (2017) * Equal authorship;
Resozialisierung von einzeln gehaltenen männlichen Mäusen nach Kastration (#58)
In: 55th Annual Meeting of the Society for Laboratory Animal Science GV-SOLAS and 17th Advanced Training Course of the IGTP 11.-13.09.2017 in Köln
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11 DECLARATION OF ORGINALITY

Hereby, I declare that the present thesis has been prepared by myself. I assure that I exclusively used the mentioned sources and facilities.

Berlin, 15.11.2017

Charlotte Sophie Leidinger

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