Handling methods of laboratory mice and rats

Hanteringsmetoder av försöksmöss och råttor

Frida Lydén

Uppsala 2016

Ethology and Animal Welfare – Bachelor's programme

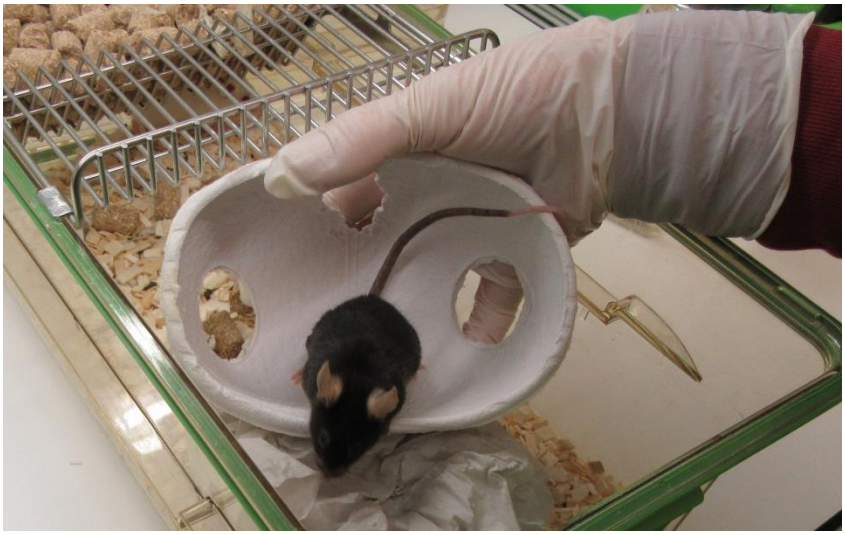

Photo: Helmersson, 2016

Studentarbete
Sveriges lantbruksuniversitet
Institutionen för husdjurens miljö och hälsa

Nr. 657

Student report
Swedish University of Agricultural Sciences
Department of Animal Environment and Health

No. 657

ISSN 1652-280X

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Student report 657, Uppsala 2016

Biology - Bachelor degree project, EX0520, 15 ECTS, G2E
Ethology and Animal Welfare – Bachelor's programme

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Key words: Handling methods, Mice, Rats, Welfare, Laboratory routines
Nyckelord: Hanteringsmetoder, Möss, Råttor, Välfärd, Laboratorierutiner

Series: Student report / Swedish University of Agricultural Sciences
Department of Animal Environment and Health
no. 657, ISSN 1652-280X

Swedish University of Agricultural Sciences
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Abstract

Stress and anxiety in laboratory mice and rats can affect not only their welfare, but also research results. It is also known that daily routines such as handling can be stressful. However, the question of whether different handling methods are more or less stressful is perhaps more interesting, and there is not much published research on the subject.
When writing a scientific paper, it is always essential to document all relevant parameters and details in the methods, in order for the results to be credible, and to make sure that the experiment can be replicated and accurately peer reviewed. If different handling methods can cause different amounts of stress and anxiety, thus possibly affecting research results, then handling methods ought to be included as relevant information in article methods.
The aim of this literature review is to investigate how different handling methods can affect both animal welfare and research quality.
Furthermore, to analyse to what extent handling is reported in article methods, the method sections of ten recently published articles from Nature Neuroscience and Nature Immunology respectively - two high impact factor journals using mice and/or rat models - were reviewed and compiled.
The literature review showed evidence of alternative handling methods sections, such as using tunnels or cupped hands when picking up mice and rats, having positive impacts on animal welfare and consequently possibly on data reliability, when compared to stressful traditional handling methods such as lifting by the tail.
The data compiled from article methods showed that reports of handling methods were lacking across all reviewed articles, regardless of journal or year.
It is concluded that using the least stressful handling methods, identified in the reviewed literature, goes in accordance with the refinement and reduction principles of the 3Rs. Methods such as using tunnels or cupped hands when lifting laboratory mice and rats, instead of lifting by the tail or body, should therefore be recommended. It is also suggested that handling should be added as a method criteria in checklists and guidelines such as NC3Rs ARRIVE guidelines or journals' own methods checklists that are used by authors.

1. Introduction

There are many different aspects of the welfare of laboratory animals, yet the research on their welfare is mostly focused on their health, while dimensions such as handling and interactions between the animals and their handlers are often overlooked (Spangenberg & Keeling, 2016). This can be paralleled to farm animals, where research has focused on welfare in relation to productivity, but also on the human-animal interactions (e.g. Pearce *et al*., 1989; Cransberg *et al*., 2000; Breuer *et al*., 2000; Hemsworth, 2003). Just as with laboratory animals, farm animal handlers deal with large quantities of animals that need to be handled in practical ways, but at the same time the animals' welfare needs to be in focus.

Two of the most common vertebrates used for research are mice (*Mus musculus*) and rats (*Rattus norvegicus*) (Deacon, 2006). In Sweden alone, 233,222 mice and 40,068 rats were used for research in 2012, out of a total of 720,572 animals, not counting catch and release of fish (Jordbruksverket, 2013). In the EU, 11.5 million animals were used for research in 2011, with mice accounting for 61% of the total use and rats following at 14% (European Commission, 2013). Together with rabbits they represent more than 80% of the total number of animals used for research purposes in the EU.

1.1 Mice and rats' biology and behaviours

To achieve good husbandry and welfare for laboratory animals, it is imperative to understand each species' natural biology and behaviours and how they relate to how the animals are kept in captivity (Brain, 1992; Olsson *et al*., 2003).

Mice and rats are both highly adaptable animals, which is probably what has led them to become so successful at procreating, surviving and spreading globally and also what led to their domestication (Latham & Mason, 2004). Their adaptability is also what has made them such common species in research (Latham & Mason, 2004). Being highly genetically adaptable with higher mutation rates than other mammals, along with their fast breeding rates, are all traits that help explain why they are so widely used as laboratory animals (Latham & Mason, 2004).

Mice and rats are small prey animals with highly developed olfactory and hearing senses, often used to detect predators (Latham & Mason, 2004). They are also very sensitive to touch, tactile contact being especially important as mice and rats both have strong thigmotactic tendencies (Simon *et al*., 1994; Lamprea *et al*., 2008). This means they will often avoid open areas and try to uphold physical contact with solid objects such as walls, particularly when stressed and anxious (Simon *et al*., 1994; Latham & Mason, 2004; Lamprea *et al*., 2008). These are important factors to keep in mind despite the fact that the mice and rats used for research today are often described as very adaptable and "tame" (Latham & Mason, 2004).

1.2 Handling: how is it relevant and why is it important

Different handling and lifting interactions can influence not only the welfare of laboratory animals, but also the results of research conducted on said animals (Balcombe *et al*., 2004; Castelhano & Baumans, 2009). Despite these possible implications on any and all

scientific research where laboratory animals are used, not much research comparing handling methods - and particularly not on recommended alternative methods and their implications - seems to be published. For this reason it would be interesting and valuable to review the more recent research that does exist on the subject in an attempt to gain an overview of the topic.

The standard practice when lifting laboratory mice has long been, and still is, to pick them up by the base of the tail (Fig. 1) (Deacon, 2006; NC3Rs, 2016b). Rats, being heavier than mice, are usually also grasped by the body, but are still generally grabbed by the base of the tail like mice (Deacon, 2006; NC3Rs, 2016b). According to Deacon (2006), this practice is stressful for both mice and rats (Deacon, 2006).

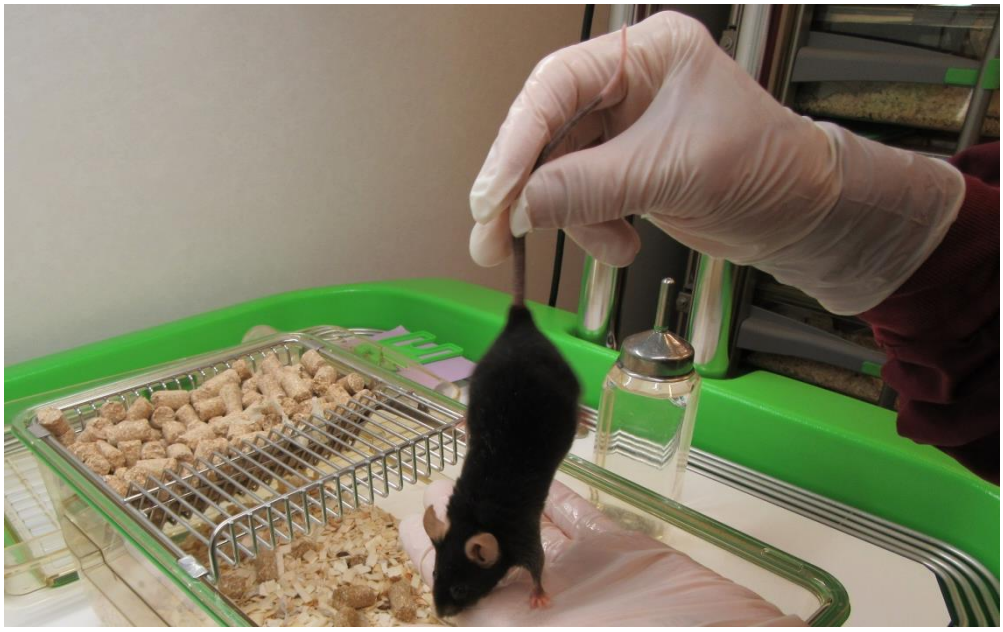

Fig.1. Traditional method of lifting mice. Photo: Helmersson, 2016.

Invasive procedures implemented on laboratory mice and rats in experimental studies are often well (e.g Statens jordbruksverks föreskrifter och allmänna råd (SJVFS 2012:26) om försöksdjur, senast omtryckt genom SJVFS 2015:38, saknr L150), or at least somewhat (e.g 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[1]) regulated and restricted by the authorities in charge, and often approval from some form of ethical board has to be acquired before the experiments commence (Balcombe *et al*., 2004). However, routine procedures that the animals are subjected to regularly, such as handling, may not be as regulated and are often overlooked (Balcombe *et al*., 2004).

The need to handle laboratory mice and rats regularly is unavoidable; it is necessary when moving them between home cages and testing areas, during experimental procedures and in routine care such as cage cleaning and supervision, making it an essential part of their lives (Bateson, 2014). In their review on stress implications of daily routine practices for laboratory mice and rats, Balcombe *et al*. (2004) analysed 80 published studies and came

[1] JEU L 276 22.9.2010, s X, Celex 32010L0001

to the conclusion that routine procedures that are considered to be non-aversive, such as being picked up and handled during cage cleaning, are indeed associated with stress for the animals. They also found that handling effects can considerably alter the animals' immune status which could have vital implications on research results (Balcombe *et al*., 2004). The animals' pain and fear, resulting in stress and distress, may make the data gathered from experiments much too varied to be sufficiently reliable (Balcombe *et al*., 2004).

Handling is in itself stressful and as such, amongst other things, induces increases in heart rate and body temperature that can last for up to 90 minutes (Harkin *et al*., 2002; Sharp *et al*., 2003; Balcombe *et al*., 2004). The importance of laboratory animals being habituated to handling early on, and the fact that even simple manipulations can cause stress if the animals are not used to handling, has been known and recommended for a long time (Kvetnansky *et al*., 1978; Maurer *et al*., 2008). While this is widely accepted for both the animals' welfare and the reliability of animal experiments, as stressed animals can affect research results in unwanted ways (Milligan *et al*., 1993), articles stressing the importance of getting the animals used to humans rarely mention any actual handling methods and do not discuss or question traditional handling methods. In studies by Meijer *et al*. (2007), Cloutier *et al*. (2012) and Fridgeirsdottir *et al*. (2014), where habituation to handling was examined in order to assess how handling impacts different areas, only one handling method - lifting the mice or rats by the tail - was used.

The housing and environment of laboratory animals is thoroughly planned and as standardised as possible to avoid deviations that could affect the results of studies conducted on the animals, or make it difficult to replicate a study (Clough, 1982; Wolfer *et al*., 2004). Despite this, studies have shown that environmental conditions and interactions can affect the data results even when the housing conditions provided are as similar as possible (Chesler *et al*., 2002). This could suggest that how we house and care for laboratory animals is not only about what our eyes can see, and that perhaps other factors besides standardised cages and animal rooms matter (Bilbo & Nelson, 2001; Wurbel, 2001; Chesler *et al*, 2002). However, it can be hard to determine what factors have affected the animals when these variations do occur (Latham & Mason, 2004). Indeed, studies have showed deviations in behaviour and test results, despite mice being sourced from the same line and breeders, where housing and husbandry routines were as standardised as possible. This was concluded to be due to different handling employed by personnel, amongst other things (Chesler *et al*., 2002; Wahlsten *et al*., 2003).

When an experimental study is carried out and a scientific paper is written for publishing purposes, it is always essential to document all the relevant parameters and details of the methods. This is done in order for the results to be deemed credible, and to ensure that the experiment can be replicated and accurately peer reviewed. Despite this, after reading numerous articles during my three years of studies, I have gotten the impression that reports of how the animals used in the experiments are handled - during both routine care and the actual experiments - are often lacking or completely absent in the methods report. To be able to confirm or discard this impression, a review of published articles needs to be made to examine exactly how they report the handling of their animals.

1.3 History

In order to gain insight and better understand and appreciate present-day research, it can be valuable to go back in history and look at how older literature describes handling of laboratory mice and rats. The following are three examples of published books specifically about laboratory animals that have commonly been used as teaching material for students and researchers (E. Spangenberg, Swedish University of Agricultural Sciences, personal communication, May 18, 2016).

Försöksdjurskunskap (Öbrink & Waller, 1996)
Published in Sweden in 1996, this book was intended for and used as teaching material for researchers, students, laboratory assistants and other personnel dealing with laboratory animals in Sweden. In the book, the authors address the relationships between animals and humans quite thoroughly, stating that most animals have a genetically founded protective behaviour, making them flee from and avoid humans. They continue stating that by handling laboratory animals gently, they can be taught to control their flight instinct. The authors stress that careless handling or too many changes in staff and/or routines can cause this flight instinct to reactivate, bringing biological repercussions. They also mention that getting the animals used to necessary routines will result in a lesser risk of acute stress moments, something that is deeply unwanted for the research. Finally, they present an interesting point, stating that pheromones is an important form of communication for mice and rats, and that it is not unlikely to think that the animals can determine who is entering the room very quickly based on their smell. This could mean that if the animals have a prior experience of being handled by certain people in a more stressful way than others, this could cause stress and anxiety from those people even just being in the same room, and not just when the animal is being handled. In the chapter on handling and restraining methods, the authors state that it is important to hold the animals in such a way that they feel security and trust towards their handler. They continue by showing images of how the capturing and handling of mice and rats should be done. According to the images, mice are to be picked up by the base of the tail, and if further fixation should be needed they can be grabbed by the skin of the neck. For rats, it is simply stated that they are not to be picked up by the tail, but no further information or alternatives are discussed.

Principles of laboratory animal science (Van Zutphen *et al*., 2001)
This book from 2001 is written "for the graduate student or young scientist who wishes to become a competent researcher." and according to the authors it covers all the main aspects relevant to laboratory animal science and animal experiments. This book also states that the best way to pick up mice is by the base of the tail. Rats should be picked up by placing a hand around the chest with the thumb placed under the chin and the index finger around the neck to safely secure the head. An alternative way is to lift the rat around the shoulders and support it with the same hand, using the other hand to support the hind part of the body. Picking rats up by the base of the tail is mentioned as less preferable, but still an option.

Guide to the care and use of experimental animals (Olfert *et al*., 1993)
To use an example of a book created by an independent organisation, the Guide to the care and use of experimental animals was created in 1993 by the Canadian Council On Animal Care. This is a council that claims to be "responsible for setting, maintaining, and overseeing the implementation of high standards for animal ethics and care in science

throughout Canada" (Canadian Council On Animal Care, 2016). In their book there is no mention of handling methods, but when writing about physical restraint they do mention that it is known that the quality of the restraint will influence the animals' response. They go on to say that the level of distress each animal experiences in the same situation is different, and that it may affect experimental results.

These three different books, from different countries and origins but meant for similar audiences, conclude quite well how these traditional handling methods have long been universal.

1.4 The 3Rs and ARRIVE guidelines

The NC3Rs is a scientific organisation that dedicates itself to the 3Rs; Replacing, Refining and Reducing the use of animals in research, principles that were developed over 50 years ago (NC3Rs, 2016a). The 3Rs are currently incorporated in the European Union, the respective Animal Welfare Acts in the United States, and in various other legislations worldwide (Törnqvist *et al*., 2014). With more and more guidelines, legislations and other practices incorporating it, the 3Rs is an important and widely used framework in improving animal welfare (Törnqvist *et al*., 2014).

In 2010, NC3Rs published their reporting guidelines, the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, on their website and in the online journal PLOS Biology (NC3Rs, 2016c). They were developed as a tool to improve animal based research, to maximise published information and minimise unnecessary studies (Kilkenny *et al*., 2014). The guidelines are intended to be used by any authors, editors or peer reviewers and consist of a detailed checklist of items describing relevant information that all published articles using animals should include, such as characteristics of the animals used, details of housing and husbandry, and the experiment methods (Kilkenny *et al*., 2014).

In 2005, Baturaite *et al*. discussed handling methods and their associated stress with habituation to handling. With refinement as one of the important purposes to keep in mind when designing and performing experiments, they concluded that it is important to identify the least distressing handling method, and to see if habituation to handling varies depending on method (Baturaite *et al*., 2005)

Just as with pain, increased stress and anxiety in laboratory mice and rats will lead to increased variability within the groups used in experiments (Miller & Leach, 2015). This increased deviation may in turn lead to the requirement of a higher number of animals to conduct the experiments (Miller & Leach, 2015). Therefore, minimizing stress and anxiety in every way possible is vital from both an animal welfare and scientific legitimacy perspective (Miller & Leach, 2015).

If handling methods can affect the animals' stress levels, thus influencing the research results, thereby possibly resulting in the need to use a larger quantity of animals, then not using the least stressful handling methods identified could counteract the reduction and refinement in the 3Rs. This could also mean contradicting legislations that have incorporated them.

2. Aim and questions

The aim of the literature review is to summarise published articles about handling methods for laboratory mice and rats and to illustrate how different handling methods affect both animals and research. The aim of the review of publications in scientific journals is to highlight to what extent handling methods are mentioned in scientific publications written about other subjects where mice or rats are used in their models.

Questions for the literature review:
Have any new ideas and suggestions on handling methods on laboratory mice and rats emerged during the last years (2000 and onwards), and what implications do they have?
Are there any recent recognised best practice recommendations regarding handling methods on laboratory mice and rats?

Questions for the review of handling method reports:
How many of the scientific papers report handling methods of the mice/rats used?
Has the method reporting changed compared to 10 years ago?

3. Method

I chose to do a literature review where I present all information found based on searches of articles relevant to the aim and questions. To find the identified articles, the databases Web of Science and Google Scholar were used. Different combinations of the following keywords were used when searching the databases for articles: "laboratory mice, laboratory rats, handling methods, handling techniques, welfare, anxiety, stress, restraint, sampling, refinement, 3R". These searches gave me 58 results out of which I ended up using 42 articles. Some of the articles found using the keywords also led me to other relevant articles via their reference sections. Additionally, websites with relevant statistics and organisations were used.

I chose Nature Neuroscience and Nature Immunology when reviewing reports of handling methods; two journals with high impact factors which publish articles using mice and rats as animal models. From each journal I reviewed the latest 10 articles that used mice or rats. I also reviewed 10 articles from each journal published 10 years ago for comparison in order to establish if method reporting has changed. I used the previously mentioned ARRIVE guidelines (Kilkenny *et al*., 2014; NC3Rs, 2016c) when reviewing the articles to evaluate if they reported handling methods or not, specifically following the three sections under the guidelines' category "Housing and Husbandry" in "Methods", and excluding the ones not relevant for mice and rats, making the following categories:
a. Housing; type of facility, type of cage, bedding material, number of cage companions
b. Husbandry conditions; light/dark cycle, temperature, type of food, access to food and water, environmental enrichment
c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.

Based on these three housing and husbandry guidelines and seeing as the guidelines do not specifically mention handling, I decided that any mention of handling methods in the articles reviewed would be marked with an "X" under category "c", while reports of

housing and husbandry would be marked with an "X" under their respective categories. This made the maximum amount of X possible for each category as follows:
a. Housing: maximum number of "X" = 4
b. Husbandry: maximum number of "X" = 5
c. Handling: maximum number of "X" = 1
Total maximum number of "X" per article = 10

3.1 Delimitations

When choosing to write about laboratory animals, I decided to focus only on mice and rats, the most common vertebrates used in research, so as to not risk ending up with a review much too extensive. I decided to use articles published in the 21st century to make the review new and relevant, but parallels will be drawn with older research as well. I have kept a global perspective when choosing articles and not just focused on specific countries, since the topic affects laboratory animals worldwide.

4. Results

4.1 "Taming anxiety in mice" as a ground breaker

When the article by Hurst & West was published in 2010, it opened up a discussion about implications of different handling methods in laboratory environments that had not previously been acknowledged to the authors' knowledge. They theorised that since picking up laboratory mice by the tail is such an extensively used method, the responses of the animals regarding stress and anxiety might be viewed as 'normal', if they are even regarded at all (Hurst & West, 2010). Based on the amount of citations the article has had since its publishing (cited by 113 in June 2016) and the fact that all other articles published after 2010 found relevant for this literature review have cited Hurst & West (2010), one could definitely argue that it has had a large impact factor and can be seen as ground breaking in that it opened up for more research on the implications of different handling methods on laboratory mice and rats.

In the study by Hurst & West (2010), lifting by the base of the tail was compared to lifting in cupped hands (Fig. 2) or using a tunnel. The mice were handled with the three different handling methods for 60 seconds once a day for nine days (Hurst & West, 2010). The study had clear results: mice handled by the tail urinated and defecated the most during handling, had more risk assessment behaviours and showed more anxiety when subjected to tests. They also had the least amount of voluntary interactions with their handler and would generally only approach them briefly and cautiously (Hurst & West, 2010). On the other hand, the mice handled with tunnels or cupped hands quickly developed a willingness to voluntarily enter the tunnels or climb the hands and arms of their handlers, with the tunnels being the quickest response to develop (Hurst & West, 2010). The authors claim that past handling experience, individual differences and sex differences were all minor influences that did not affect the end results, making them very clear (Hurst & West, 2010). Interestingly, mice that had only been accustomed to tunnel handling were just as positive in their responses to their first time of being cupped by hand as the mice that had exclusively been accustomed to cupping (Hurst & West, 2010).

In their study, Hurst & West (2010) suggest that when an inspection is necessary where just tunnel or cupped handling is not possible, such as an abdominal inspection, first picking up the mice by using tunnel or cupping, and then lifting them by the tail once in the hand, is a less aversive method than picking them up by the tail straight away. This could mean that what causes the mice the most amount of stress and anxiety is the actual chasing and capturing in the cage, something that makes a lot of sense from an evolutionary perspective for a prey animal, no matter how domesticated (Hurst & West, 2010).

In 2013, Gouveia & Hurst followed up the previously mentioned study by Hurst & West (2010) by adding another element to the comparison of tunnel and tail handling. They investigated if the positive handling responses in the previous study were only prevalent if the tunnels used were familiar to the mice, or if unfamiliar tunnels could induce the same results. Their results showed that just as Hurst & West (2010) had found, the mice handled with a tunnel were more interactive with their handler and showed less anxiety compared to mice being picked up by the tail. Furthermore, Gouveia & Hurst (2013) could confirm these results even when the tunnels were unfamiliar, concluding that the reduction in stress and anxiety compared to being lifted by the tail is significant regardless of previous familiarity with the tunnels.

Following in the footsteps of the findings of Hurst & West (2010) and Gouveia & Hurst (2013), several studies emerged and were motivated to examine different handling methods and their effects on each study's specific area.

In an experiment by Miller & Leach (2015), tail handling and tunnel handling of mice was compared for a new pain assessment score technique. In their study, Miller & Leach (2015) concluded that since the two handling methods did not impact the pain assessment method in question, tunnel handling should be used as a common practice over tail handling as recommended by Hurst & West (2010). They stated that since doing so will have no influence on the implementation of the pain assessment score, the established least stressful and anxiogenic method ought to be used (Miller & Leach, 2015).

Similar to the comparison by Miller & Leach (2015), Ghosal *et al*. (2015) compared cup handling and tail handling on mice and their effects on measurements of glucose tolerance. Here, the authors saw that the cup handled mice showed both reduced anxiety behaviours and improved glucose tolerance in comparison with the tail handled mice (Ghosal *et al*., 2015). They concluded that metabolic studies such as glucose tolerance are highly influenced by stress. As such, selection of handling methods and their differences in stress impact on the animals are highly relevant for such studies and for reduction in the number of animals needed (Ghosal *et al*., 2015).

In a study on affective states on mice and how different levels of anxiety can affect this, using exploration based cognitive bias with positive/negative outcome predictions, Novak *et al*. (2015) used the findings of Hurst & West (2010) and tail handling and cupped handling, hypothesising that the tail handled mice might display a more negative cognitive bias. The authors found no significant difference in how the tail handled and cupped mice acted in their exploration mazes (Novak *et al*., 2015). However, the authors discussed the possibility of the cognitive test in their particular experiment not being sensitive enough or appropriate for detecting changes in the animals' affective state caused by different handling methods, or that it simply did not detect differences in affective states specifically

induced by handling (Novak *et al*., 2015). Arguably, the results of this study are in no way to be interpreted as a definitive conclusion that the different handling methods have no influence on affective state in mice, but rather that it may be a subject so complex that it requires further studies.

In 2015, a report on ways to improve the welfare of rodents in epilepsy and seizure models was published (Lidster *et al*., 2015). One of the 27 compiled recommendation points dealt with handling, stating that research personnel should be sufficiently qualified and competent for appropriate handling of the animals (Lidster *et al*., 2015). It also concluded that picking up mice by the tail should be avoided and recommended using the less aversive tunnel or cupped handling methods instead, citing the findings of Hurst & West (2010) and Gouveia & Hurst (2013).

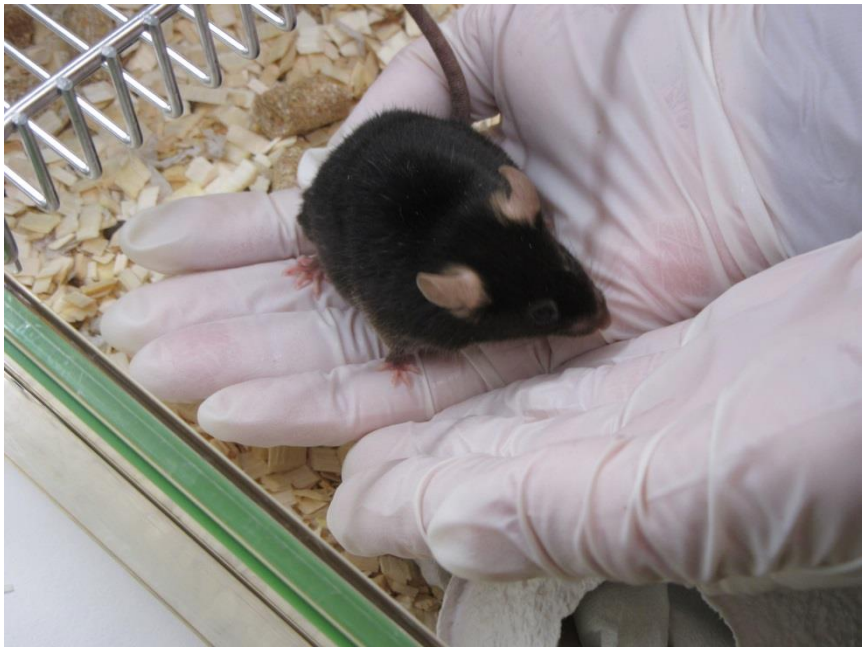

Fig.2. Example of lifting a mouse in cupped hands, one of the alternative methods compared by Hurst & West (2010) Photo: Helmersson, 2016.

In 2002, Chesler *et al*. studied laboratory environment conditions' impact on nociception responses of mice. They could conclude that the experimenters performing the tests were a more important factor than mouse genotype; environmental sources accounted for 42% of the variance of their trait, whereas genetic sources only accounted for 27% (Chesler *et al*., 2002). The authors theorised that how the animals are handled, and by whom, induces different levels of stress which in turn is what is responsible for the so called "experimenter effect".

In another study, Baturaite *et al*. (2005) compared the cardiovascular effects of four different handling methods on rats; being lifted by the scruff, encircling (supporting the rat's head and forelegs with one hand and its tail and lower body with the other), using a plastic cone restrainer, or lifting and holding by the tail. The results showed that the rats being held by the scruff had the most significant decrease in response duration, showing signs of the rats becoming the most habituated to this method, whereas they showed no signs of being habituated to the other three methods (Baturaite *et al*., 2005). The restraint

cone, used to hold rodents for injections, had the most striking effect and was the most distressing method for the rats (Baturaite *et al*., 2005). The authors discussed the possibility of this being due to the fact that the cone squeezes the rats' whiskers along the head, thus making it impossible for the rats to assess their surroundings for which the back and forth sweeping movements of the whiskers are essential. With the cone also being very restrictive and tight it is perhaps not that surprising that this method was the most stressful for the rats (Baturaite *et al*., 2005). This study concluded that out of the four methods compared, holding by the scruff was the best method of handling, possibly due to pleasant memories being triggered from when pups are carried by their mother (Baturaite *et al*., 2005).

In 2008, Maurer *et al*. found long-term effects of gentle handling on rats. They compared a group of 21-day old rats who were gently stroked, hand fed and talked to twice daily during their fourth and fifth week of life, with a control group who received no human contact apart from routine care. Their results showed that the gentled group showed significantly higher values in the different "tameness towards humans" assessments and that these significant differences were present for up to 6 months despite the gentle handling only being limited to two weeks during the rats' youth (Maurer *et al*., 2008). The rats in this study also recognised the specific people who had conducted the gentling 5 months earlier and favoured these people over unfamiliar people. This could be paralleled to laboratory mice and rats recognising laboratory technicians or researchers who handle them in more or less stressful ways. If the rats in the experiment conducted by Maurer *et al*. (2008) could recognise specific handlers after 5 months, it is possible to imagine that animals subjected to routine stressful handling weekly or even daily also learn to recognise their handlers and possibly start feeling stressed as soon as they enter the room.

There are several reports on the impact of handling when it comes to identification and biopsy procedures on laboratory mice and rats. In three different studies, procedures varying in invasiveness, such as toe clipping, ear punch, mouth and rectum swabs etc., were compared (Cinelli *et al*., 2007; Schaefer *et al*., 2010; Paluch *et al*., 2014). Although constructed differently, all three studies claimed that the most distressing aspect, regardless of what procedure was performed, was always the actual handling in itself (Cinelli *et al*., 2007; Schaefer *et al*., 2010; Paluch *et al*., 2014). The studies did not go into detail about the specific handling methods used.

On a similar note, a study published earlier this year about stress associated with gavage on mice suggested that the mice experienced a more profound stress from the handling and restraint rather than the actual insertion of the gavage needle (Kärrberg *et al*., 2016). The study made many conclusions on the importance of habituation, but not about handling methods. It is mentioned that the mice restrained were done so by "scruffing", i.e. grabbed by the skin of the neck (Kärrberg *et al*., 2016). It would have been interesting to also know the methods employed to pick up the mice.

4.2 Analysis of scientific papers' methods descriptions

The results of the method reports review showed that handling was never mentioned in any of the journals or years looked at (Tab. 1-4). The results also showed that overall, reports of housing and husbandry aspects have increased in both journals compared to 10 years ago (Fig. 3).

Tab. 1. Reports of housing, husbandry and handling methods in articles published 2016 in the scientific journal Nature Neuroscience. Mice were used in all articles except for Cosker *et al*. and Hollis Il *et al*., where both mice and rats were used.

Article	a. Housing	b. Husbandry	c. Handling
Zhang *et al*. (2016)			
He *et al*. (2016)	XX	X	
Cosker *et al*. (2016)	X	X	
Hollis II *et al*. (2016)	X	X	
Peixoto *et al*. (2016)			
Qi *et al*. (2016)	XX	XX	
Padilla *et al*. (2016)		XX	
Rossi *et al*. (2016)	X	XX	
Pereira *et al*. (2016)	X	XX	
Falkner *et al*. (2016)	X	XX	

Tab. 2. Reports of housing, husbandry and handling methods in articles published 2006 in the scientific journal Nature Neuroscience. Mice were used in all articles except for Diano *et al*., where both mice and rats were used.

Article	a. Housing	b. Husbandry	c. Handling
Bellone *et al*. (2006)			
Majdan *et al*. (2006)		X	
Tropea *et al*. (2006)			
Tsankova *et al*. (2006)	X	XX	
Stranahan *et al*. (2006)	X	XXX	
Andermann *et al*. (2006)	XX	X	
Ramirez-Castillejo *et al*. (2006)			
Diano *et al*. (2006)		XX	
Walter *et al*. (2006)			
Gooley *et al*. (2006)	XX	XXXX	

Tab. 3. Reports of housing, husbandry and handling methods in articles published 2016 in the scientific journal Nature Immunology. Mice were used in all articles.

Article	a. Housing	b. Husbandry	c. Handling
Mathewson *et al*. (2016)			
Wang *et al*. (2016)	X		
Vannella *et al*. (2016)	X		
Esterházy *et al*. (2016)	X		
Apostolidis *et al*. (2016)	X		
Xing *et al*. (2016)	X		
Martin *et al*. (2016)	XX		
Beyer *et al*. (2016)	XX		
Xia *et al*. (2016)			
Gallegos *et al*. (2016)	XX	XX	

Tab. 4. Reports of housing, husbandry and handling methods in articles published 2006 in the scientific journal Nature Immunology. Mice were used in all articles.

Article	a. Housing	b. Husbandry	c. Handling
Martins *et al*. (2006)	X		
Kallies *et al*. (2006)			
Hamilton *et al*. (2006)	X		
Staton *et al*. (2006)	X		
Cooper *et al*. (2006)	X		
Shinohara *et al*. (2006)			
O'Leary *et al*. (2006)			
Gazit *et al*. (2006)	X		
Sun *et al*. (2006)			
Yoshida *et al*. (2006)	X		

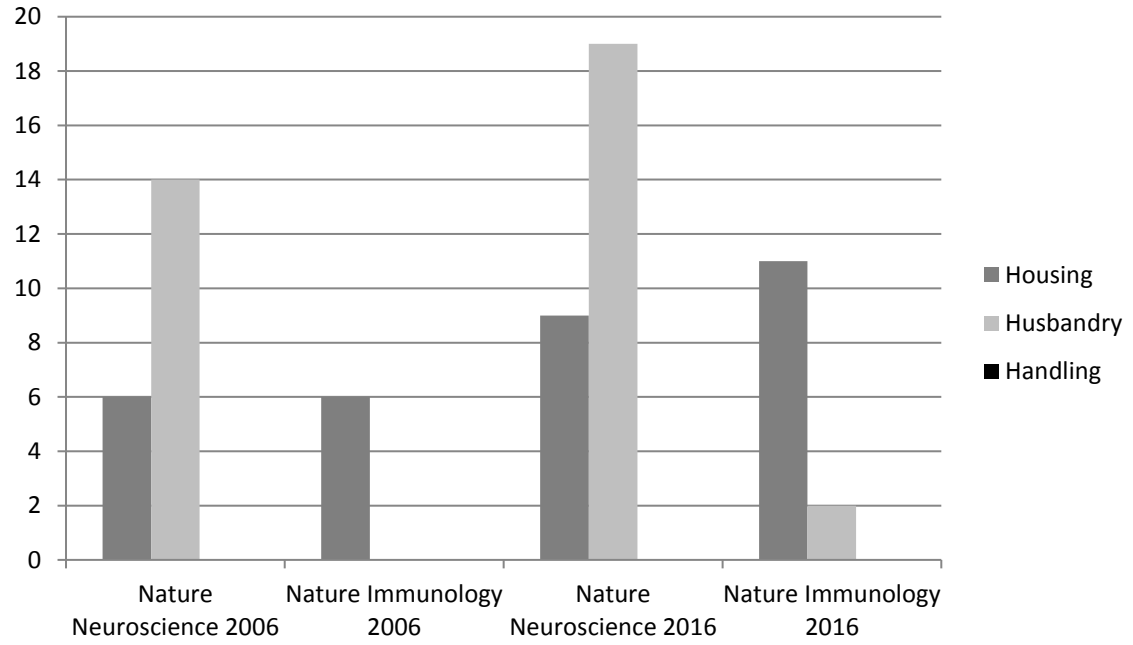

Fig. 3. Total combined number of "X" for reports of housing, husbandry and handling methods of the 10 articles reviewed in each journal each year. Maximum number of "X" possible for each article being 4 (housing), 5 (husbandry) and 1 (handling), making the total maximum number of "X" possible per article 10.

5. Discussion

Although I believe a literature review was an appropriate choice of method for this particular subject, one weakness was the fact that there were a few articles I did not manage to gain access to, due to them being in a foreign language and/or not available through the library access of the Swedish University of Agricultural Sciences. However, all in all I did manage to access the majority of the articles I initially considered relevant, so this limitation was not deemed major.

Despite being few, the reviewed articles comparing handling methods show consistent results: mice picked up and handled using cupped hands or a tunnel instead of by the tail have a much greater will to interact with the handler and show less signs of anxiety. (Hurst & West, 2010; Gouveia & Hurst, 2013). The evidence of positive impact on the animals' welfare suggests that the methods used when handling laboratory mice and rats should be emphasized, and not just their amount of habituation to handling when young, which has historically been the discussed factor. Seeing as data gathered from experiments can become unreliable if the animals used are stressed, handling methods are factors affecting not just the animals' welfare, but also all research where they are used (Balcombe *et al*., 2004).

In the introduction of this review, studies about habituation to handling where only one handling method (lifting by the tail) was ever used were mentioned as examples (Meijer *et al*., 2007; Cloutier *et al*., 2012; Fridgeirsdottir *et al*., 2014). These studies were examining how handling affects the animals' acute stress response, their fear of humans and their results in a water maze respectively (Meijer *et al*., 2007; Cloutier *et al*., 2012; Fridgeirsdottir *et al*., 2014). Based on the results of this literature review, I find it reasonable to believe that had the authors of these articles used a different handling

method, such as tunnel or cupped handling, they might have ended up with different results. Regardless, they are good examples of the fact that lifting mice and rats by the tail is still common practice, seeing as the three articles are all published relatively recently.

This review suggests that the article on handling effects on mouse anxiety by Hurst & West (2010) has had an impact on how handling methods are discussed and viewed as relevant in other studies. Even though tunnel handling and/or cupping may not be best practice at most facilities as of yet, they are certainly starting to be recommended by individual authors (Ghosal *et al*., 2015; Lidster *et al*., 2015; Miller & Leach, 2015).

Gariepy *et al.* (2002) found that handling effects are influenced by both individual experiences beyond infancy and genetic background of different mouse strains. This could be important to keep in mind when handling different strains of laboratory animals, so as to always consider that strains as well as individual variations could make it unsuitable to handle each animal the same way, even though handling with the lowest possible stress implications should always be recommended. The fact that Hurst & West (2010) used three different common strains (two inbred and one outbred) of mice in their study made the results more reliable. Even though it would be interesting and valuable to compare more strains, it still means their results can be relevant for more than just one specific strain and makes them more likely to be significant and applicable for the welfare of all laboratory mice – or laboratory animals as a whole, for that matter.

As mentioned in the introduction of this review, mice and rats have strong thigmotactic tendencies and strive to avoid open areas without anything to press up against as much as possible, especially in stressful situations (Simon *et al*., 1994; Latham & Mason, 2004; Lamprea *et al*., 2008). This fact alone justifies the reasoning behind the evaluation of using objects such as tunnels to lift mice by Hurst & West (2010), in my opinion. By using any sort of physical object when lifting an animal, it will automatically have something to hold on to from inside the cage; a method that keeps natural behaviours such as thigmotaxis in mind.

Although the conclusions of many of the studies reviewed were based on mice, using tunnels or other objects for handling in order to reduce stress and anxiety should be viewed as equally applicable to rats, especially considering the thigmotaxis of both species, discussed above.

In one of the studies reviewed, Baturaite *et al*. (2005) concluded that the best handling method out of the four compared was holding the rats by the scruff. While this may be a valid conclusion, it would have been interesting to do the same experiment, but adding the two less stressful methods used on mice by Hurst & West (2010), i.e. handling with tunnels and cupped hands, making the study even more comprehensive.

A study on the impact of the 3Rs on research in the pharmaceutical industry collected data from 36 reduction projects' work between 2006 and 2010 (Törnqvist *et al*., 2014). The results showed that major animal reduction had been achieved by using different strategies including improved method development. This shows great promise for the possibility of even further reduction and refinement by continuing to improve and update the work with the 3Rs, for example by adding alternative handling methods to the discussion.

Interestingly, even though NC3Rs' ARRIVE guidelines checklist does not mention handling methods, they do mention it on their website, where they clearly recommend using the tunnel or cupping methods when handling mice and rats, citing the findings of Hurst & West (2010) and the follow-up article by Gouveia & Hurst (2013) (NC3R, 2016b).

This is perhaps not surprising after examining the funding for these studies and finding that Hurst was awarded a studentship from NC3Rs in 2009. As positive as it was to find the handling method recommendations on their website, seeing them being implemented into the ARRIVE guidelines would be even better as this could hopefully result in authors and journals starting to add it to their recommended methods.

The abovementioned is also true for the report by Lidster *et al*. (2015), mentioned in the results as being one of the articles that recommend using tunnel or cupped handling instead of lifting by the tail. After further examination it was made clear that the lead author of this article is employed by NC3Rs (NC3Rs, 2016d). Again, seeing as Hurst was awarded a studentship from NC3Rs in 2009, this makes the recommendation by Lidster *et al*. (2015) quite expected, but still a valuable contribution to spreading the discussion of alternative handling methods.

Because of extent limitations, I had to make the review of article methods quite simplistic, and it can argued that the ten articles reviewed in each journal each year is not nearly enough to draw a significant conclusion. However, this can be viewed as a pilot study for future more extensive reviews. There are still many unanswered questions and aspects that I would like to delve deeper into. If possible it would be interesting to conduct a wider review focused purely on articles' method reporting and analyse a larger quantity of articles from different scientific journals.

Unfortunately, none of the article methods reviewed mentioned handling methods (Tab. 1-4). But the fact that reports of both housing and husbandry conditions have improved in both of the journals reviewed compared to 10 years ago (Fig. 3) could be an indicator that things are moving in the right direction and that the addition of handling methods is not an impossibility.

The results of the method reports review showed interesting differences between scientific journals in that Nature Neuroscience had much higher reports of husbandry conditions than Nature Immunology (Fig. 3). Although these results were not the focus of this review, they are indeed a basis for future discussions about the implications of different scientific journals and fields having such diverse methods reports.

Another interesting angle for future research is what education lab technicians and others who handle laboratory animals on a daily basis have, and how that can affect the animals' welfare as well as research results. The requirement of competent personnel with adequate education and training is already regulated in the EU (Directive (EU), 63/2010). However, examining if laboratory facilities across the EU actually comply with this, and comparing animal welfare with personnel experience and/or attitudes would be interesting.

Although this review was focused on the specific handling methods and not on sampling and identification techniques, I do want to mention the latter briefly. It often goes without saying that when choosing methods of identification, the least invasive method should always be chosen to prevent pain, suffering and distress. Whilst I fully agree with this, after reading several articles claiming that the most distressing aspects of the procedures for the animals is actually the handling, and not the procedure in itself (Cinelli *et al*., 2007; Schaefer *et al*., 2010; Paluch *et al*., 2014), it makes the issue much more complex in my eyes. For instance, when using pen markers on tails or fur as identification, the markings have to be renewed regularly (Dahlborn *et al*., 2013), requiring repeated handling that the animal might not be able to habituate to (Hurst & West, 2010). However, when using toe clipping as an example, this only has to be done once. I have not gone into depth reviewing

the credibility of the articles that do exist on the long-term effects of these procedures on the animals' suffering and distress, but based on the handling implications it definitely makes it an interesting and multifaceted issue worthy of further discussion and examination. Ultimately, it shows how important the discussion of handling is in other regards.

Refining or suggesting new practices which are more ethologically appropriate could potentially help improve both animal welfare and the reliability of research conducted on said animals. To encourage authors to start mentioning handling methods in any meaningful way in their methods, some form of best practice recommendations could be established, possibly based on the most recent research reviewed in this paper. This way authors would simply be able to mention that they have followed the best practice recommendations in their methods. The ARRIVE guidelines, which are already being used by many should be updated to include handling methods and possibly even specifically recommend habituating all mice and rats to tunnel or cupped handling based on the positive implications it has been shown to have. Regarding animal welfare and article guidelines, it is possible that independent scientific journals might argue that handling methods are not important enough to add if there is no clear scientific credibility benefit too. However, the ARRIVE guidelines created by NC3Rs definitely have an animal welfare perspective to take into account. With this in mind, I believe this review has shown that there is enough evidence to claim that handling methods is a relevant parameter to add to their guidelines.

It is my hope that as a potential consequence of this literature review, organisations such as NC3Rs and scientific journals with their own methods checklists, will consider adapting their guidelines to include the importance of handling methods in some way. I believe both animal welfare and research credibility could benefit from doing so.

Ultimately, it is my hope that this review can end up increasing awareness and improving the welfare of laboratory mice and rats for as long as we still need them for research.

6. Conclusions

The aim of this review was to summarise published articles about handling methods on laboratory mice and rats and to illustrate how different handling methods affect both animals and research. The aim of the review of publications in scientific journals was to highlight to what extent handling methods are mentioned in scientific publications written about other subjects where mice or rats are used in their models.

From the literature review of different handling methods, it could be concluded that handling methods such as using cupped hands or tunnels when lifting mice and rats, instead of lifting by the tail, have been proposed and recommended in several articles since the publication of Hurst & West (2010).

Handling methods such as using cupped hands or a tunnel instead of by the tail have shown positive impacts for the animals' welfare and thus possibly also on the reliability of research conducted on them.

No recent recognised best practice recommendations regarding handling methods on laboratory mice and rats seem to exist. However, individual authors are starting to

recommend using tunnels and/or cupped hands instead of lifting by the tail as proposed by Hurst & West in 2010.

From the review of articles in Nature Neuroscience and Nature Immunology, it could be concluded that reports of handling methods were lacking in both journals from both 2006 and 2016. It could also be concluded that reports of housing and husbandry conditions had increased in both journals from 2006 to 2016.

7. Simple summary

Daily laboratory routines such as handling can be stressful for laboratory mice and rats. Stress can also affect research results, which means that eliminating unnecessary stress is important for the animals' welfare as well as for the research we conduct on them. The articles written about handling stress in the past rarely mention in what way the animals were handled, and when they do, the traditional method of lifting the animal by the tail is used. It is known that lifting by the tail is stressful for mice and rats. The question of whether using different handling methods can be more or less stressful has not been studied much.
Handling methods are also important when writing and publishing scientific articles, where mentioning all relevant details in the methods is vital in order for the results to be credible and to ensure that the experiment can be replicated and reviewed by other researchers. If different handling methods can cause different amounts of stress and anxiety, and if this can affect the research results, then handling methods should be mentioned in all scientific articles that are published.
The aim of this literature review was to investigate how different handling methods can affect both the animals and research results, and to summarise what has been published on the subject over the last few years.
Furthermore, to see how many scientific articles mention handling methods, the method sections of ten recently published articles each from two high impact factor scientific journals that used mice and/or rats in their experiments, were reviewed.
The literature review showed that alternative handling methods (such as using tunnels or cupped hands when picking up mice and rats) are less stressful, compared to traditional methods such as lifting by the tail.
This review of article methods shows that no articles published in the identified scientific journals mentioned handling methods.
It is concluded that handling methods such as using tunnels or cupped hands when lifting laboratory mice and rats, instead of lifting by the tail or body, should be recommended, for the animals' sake as well as for the reliability of the research. It is also suggested that mentioning handling in the methods section should be a requirement for all articles that are published in scientific journals.

8. Acknowledgements

I want to thank my wonderful family and friends for all the help and support. Thank you to my supervisors Jenny Yngvesson and Elin Spangenberg, and to my critical partners. Thank you also to Katarina Cvek and Gunilla Helmersson for supplying me with literature and images. Most of all, thank you to my rats, who have taught me so much, and who have inspired and motivated me to fight for the welfare of their comrades who are used in research.

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