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JUSSI HELPPI

Effects of environmental factors on reproductive performance of laboratory mice

EFFECTS OF ENVIRONMENTAL FACTORS ON REPRODUCTIVE PERFORMANCE OF LABORATORY MICE

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ABSTRACT

With an increasing need to produce more transgenic strains of mammalian laboratory models, it has become essential to focus on production and breeding efficiency so that more strains can be produced with fewer animals. This is both a practical and an ethical issue. The aim of this doctoral project was to study whether higher ambient temperature, different dietary phytoestrogen levels, and novel bedding material (cotton cloth) could improve reproduction response in embryo donors, as well as in recipient females used for producing transgenic mice (*Mus musculus*). The main hypotheses were that high temperature, low phytoestrogen and cotton cloth bedding would all improve reproductive outcomes. The focus was mainly on pregnancy rates as well as embryo and sperm yield and quality as indicators of successful reproduction. I demonstrate that ambient temperatures of up to 28°C can be tolerated by mice without an adverse effect on their early reproductive performance. However, the shift from 28°C to 30°C results in a significant drop in both male and female reproductive performance. High phytoestrogen content in mouse diet seems to benefit early embryonic development (high yield and good quality embryos), but conversely yields the lowest number of pups born. Significantly more pregnancies were observed in the wooden chip bedding compared to cotton cloth bedding. I conclude that higher ambient temperatures than currently recommended could be used when producing transgenic mice. Furthermore, high levels of phytoestrogens in mouse diet may not be as disadvantageous as often stated, and in certain cases they may even support good reproduction. Additionally, I was able to demonstrate that a cotton cloth cannot be recommended as a sole replacement for bedding and nesting material. The present data offers a better understanding of the limits and possibilities of some of the factors influencing the productivity of mice housed in individually ventilated cages and used to generate transgenic mouse lines. This could potentially lead to improved welfare and fewer animals being used for experiments.

Universal Decimal Classification: 591.133.1, 591.16, 591.53.063, 591.613, 591.615, 599.323.451

CAB Thesaurus: laboratory animals; transgenic animals; mice; reproduction; reproductive performance; sexual behaviour; pregnancy rate; embryos; embryonic development; spermatozoa; environmental factors; temperature; diet; plant oestrogens; litter; wood chips; cotton; animal welfare

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As a manager of laboratory animal facilities, I have always been interested in good animal welfare specifically, and efficient and successful work protocols in particular. It has been my great pleasure to combine my interest in pragmatic problem solving with this research project to be able to produce practical and meaningful solutions that could help animal facilities to manage their colonies better.

As this project has been ongoing for quite a while (read: decades) I need to thank so many people who have supported me during this process. Your support has been expressed in many different ways, including mentoring my earlier self, patiently waiting that I get my act together, proof-reading and critically commenting my manuscripts, and probably most importantly, just being there for me during the last few decades.

I was very lucky to be a part of the wonderful department of Applied Zoology at the University of Kuopio under the leadership of late Mikko Harri – that department was a unique environment for a young student to learn and grow, and it still saddens me immensely that it does not exist any longer. My first steps as a starting scientist I took under the caring wing of Liisa Nurminen, and I do believe that without her constant positive enforcement I would have never even started my PhD in the first place. During the early years of my PhD path Jaakko Mononen, my first original PhD supervisor, was a fundamental guiding light for me. He kept me on track even though I often could produce nothing for long periods. And he still is a great example and a major influence for me – thank you so much for believing in me Jaska.

I left Finland in 1996 to Germany and worked at the EMBL for three years – although during those years I could not proceed with my research projects, the time at EMBL probably shaped me by most during my professional career. I learned to work hard alongside some of the best scientists in the world. I learned to trust my abilities, and at the end of those three beautiful years, I knew who I was and what I wanted.

In 1999 I started to work for MPI-CBG, and since 2001 I've been happily living and working in Dresden. I'm extremely grateful to MPI-CBG to have allowed me to pursue my research interest for all these years, even though my work has taken the better of me so many times. My absolute sincere thanks will go to Ivan C. Baines for being a such a wonderful boss, a colleague and a friend – without his direct support, patience and advice I would have never made this far. Ivan, you are a star!

After I finally managed to put some time aside to start running my research projects, two people played a significant role in my success to be where I'm today.

Ronald Naumann has worked together with me now for nearly two decades, and without his insight and expertise in transgenic mice, none of my projects would have been what they become. I could have never had the access nor the ability to run the experiments with the colonies used to produce transgenic mice without Ronald ever so kindly agreeing to support practically all of my research ideas. Ronald, my dear friend and colleague, thank you!

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At the beginning, I struggled with my writing. Writing in Finnish was never a problem for me, and I used to enjoy it a lot. But writing in English was much more difficult, as I could not produce the text "perfectly" from scratch. But that was completely overturned by the great scientific writing courses held by Iain Patten. I learned from Iain that I can write! He thought me how to structure my writing process in a way that I did not get blinded any more by trying to polish my text too early. Because of him, I learned to love writing! He also reviewed many of my drafts for my publications, as well as this thesis. Thank you so much, Iain!

A big thanks must be given to Raine Kortet as well – my two-decades journey meant that my university was not any more what it used to be. My old department did not exist any longer, my old mentors and supervisors were either retired or elsewhere, and even the university had changed its name. So to successfully bring this project to an end he gracefully accepted to become my main supervisor – thousand thanks, Raine!

I once thought, many years ago, that I could try to defend my PhD in the same year my wife Arantxa earned hers. I could not have been more wrong – there was no way for me to match her level of commitment and excellence. But I'm very happy that I'm getting there at the and, and without her constant never-ending love and understanding I would not be writing this. ¡Te quiero muchissimo!

To my lovely kids Carlota & Oscar – you offered me my sanctuary at home after stressful and sometimes very hard working days. There is nothing that gets ones head out of the work mode better than small kids who seek your attention. I hope you succeed in anything you want to achieve in your life – and maybe the fact that I managed to defend my thesis as an "old" man shows you that, if sufficiently motivated, you can get anything done. If not earlier, then later!

Of course, I would not be here without the constant love and support from my parents throughout my life – my late mother thought me that with passion one can reach the stars, and my dad always served as an example that with hard work and commitment you can get far with your life. To my sister Katri, and my brother

Mikko – although we don't see each other that often, I can proudly call you my family. And from family one can draw strength.

Simply - thank you all!

So, what's next? Finally to learn to play the saxophone maybe?

Dresden, 27th July 2020
Jussi Helppi

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on data presented in the following articles, referred to by the Roman Numerals I-III.

I **Helppi, J**., Schreier, D., Naumann, R., Zierau, O. 2016. Mouse reproductive fitness is maintained up to an ambient temperature of 28℃ when housed in individually-ventilated cages. *Laboratory Animals*, 50(4): 254-263.

II **Helppi J**, Naumann R, Zierau O. 2020. Phytoestrogen-containing diets offer benefits for mouse embryology but leads to fewer offspring to be produced. *Laboratory Animals*, doi: *10.1177/0023677219898486. [Epub ahead of print]*.

III **Helppi J**, Naumann R, Asikainen M, Mononen J, Zierau O. 2017. Novel Bedding Material Results in Poor Pregnancy Rate with Female Mice Used as Fosters for Producing Transgenic Mice. *Scandinavian Journal of Laboratory Animal Science*, 43(3): 1-5.

The above publications have been included at the end of this dissertation with their copyright holders' permission.

AUTHOR'S CONTRIBUTION

I) Jussi Helppi was the main author. He participated in study conception and design, acquiring and modelling data, analysis of data, writing the article, and revising the article.

II) Jussi Helppi was the main author. He participated in study conception and design, acquiring and modelling data, analysis of data, writing the article, and revising the article.

III) Jussi Helppi was the main author. He participated in study conception and design, acquiring and modelling data, analysis of data, writing the article, and revising the article.

CONTENTS

1 INTRODUCTION

1.1 GENERAL

The laboratory mouse (*Mus musculus*) has become one of the main animal models in modern biomedical research. It is a small mammal and can thus be housed in relatively large quantities in laboratory animal facilities. The mouse finally overtook the rat (*Rattus norvegicus*) as the key animal model of choice when its genome was sequenced early in 2002 (Mouse Genome Sequencing et al., 2002), but even more importantly, the technologies were already developed in the early 1980s to precisely engineer its genome (Glaser et al., 2005; Palmiter et al., 1982). This led to a dramatic increase in the production of genetically modified laboratory mice worldwide. All existing techniques used to produce genetically modified mice require a reliable and high yield of fertilized embryos that can then be genetically modified and transferred to a recipient mouse (Vintersten et al., 2008). This is a delicate process typically including hormone treatment of young female mice to produce a high yield of embryos and surgery in recipients under general anaesthesia. This process can only be successful if the mice used are breeding well.

It is to be assumed that good animal welfare leads to better animals, and better reproductive performance (Balcombe, 2006; Gaskill et al., 2013b; Gonder and Laber, 2007; Moberg, 1985). Animal welfare is tightly linked to the environment in which the animals live (Broom and Johnson, 1993). Wild mice can adapt and modify their own environment. They can burrow underground to protect themselves from excess heat or cold, and they can build a nest in an attempt to protect their newborn litter from temperature fluctuations. They can move through temperature and humidity gradients and thus find an optimal environment for different needs, such as resting or foraging. This behavioural manipulation of the environment is greatly limited in laboratory animal facilities (Gordon et al., 1998). This was already a challenge with conventional cage systems (open top cages) but has become even more challenging with the dramatic increase in the use of individually ventilated cages (IVC) in recent years (Baumans et al., 2002). Ultimately it is the responsibly of people who are in charge of the care of animals in such facilities to provide the suitable environment by adjusting the physical conditions (e.g. temperature), the diet composition (e.g. phytoestrogen content), as well as environmental enrichment (e.g. bedding material).

1.2 HOUSING CONDITIONS AND INDIVIDUALLY VENTILATED CAGES

During the last two decades, there has been a significant change from conventional mouse housing with open cages towards barrier housing using IVCs (Baumans et al., 2002). The micro-environment in IVC systems relies on a cage that is tightly sealed against the elements from the animal room, and thus requiring active forced ventilation, resulting in up to 100 air changes per hour, to maintain a suitable environment for mice (Baumans et al., 2002). The sealed cage together with active ventilation creates an effective barrier between the micro-environment of each cage and the macro-environment of the animal room (Rosenbaum et al., 2010). This barrier protects mice from disease-causing agents, such as bacteria and viruses, that could be present in the room environment or carried by people entering animal rooms. This barrier also protects animal care staff by significantly reducing mouse allergens in the animal rooms (Feary et al., 2019). IVC systems are thus also called micro-isolators, as they create an isolator-type barrier between the inside and outside of the cage. Furthermore, this barrier can be used not only to protect mice (to keep them healthy) but also to house mice that are infected with pathogens that could potentially harm humans (zoonoses), therefore protecting operators from those pathogens.

Overall, the increasing use of IVC systems to house mice, together with an increased understanding of the technical requirements of animal facilities, has improved the level of control over several environmental factors, such as temperature, humidity and ventilation. This has undoubtfully created benefits for mouse husbandry. Although most newly built mouse facilities tend to opt for ICV systems due to the aforementioned benefits, their impact on mouse welfare, production of genetically modified mice and reproduction has still not been thoroughly studied.

1.3 GENETICALLY MODIFIED MICE AND REPRODUCTION

The production of genetically modified mice was already established decades ago, and the very first technique utilized DNA microinjection into pronuclei of a mouse embryo (Mouse Genome Sequencing et al., 2002; Gordon and Ruddle, 1981). This technique, and its many variations, is still widely used today (Nagy, 2003; Behringer, 2014; Vintersten et al., 2008; Kumar et al., 2009) for transgenesis (stable integration of foreign DNA into a mouse genome). The procedure to introduce the transgene into a mouse has remained largely unchanged since the 1980s, and typically includes the following steps (according to Fielder and Montoliu (2011)):

- Cloning, testing and purification of the transgene DNA
- Superovulation and mating of mice to produce fertilized embryos
- Microinjection of DNA solution into pronuclei

- Transfer of injected embryos into oviducts of pseudopregnant females
- Genotyping of offspring to identify founders

As the DNA randomly integrates into the mouse genome with one or many, even hundreds, of copies, individual offspring that carry the transgene are unique and can function as founders of a new transgenic strain. The microinjection itself is performed with fertilized one-cell-stage embryos. Typically, the embryos are collected from young 3- to 4-week old superovulated females after overnight mating with a fertile male. The embryos are then further cultivated in a dish, and subsequently the DNA solution (purified gene construct, plasmid or bacterial artificial chromosome) is microinjected into the pronucleus of the developing embryo. After several steps of washing and incubation, the embryos that survive the process are then transferred to pseudopregant recipients, who then give birth to new genetically modified mice (so called founders). The process is highly standardised and can be found in several publications and laboratory manuals (Nagy, 2003; Kumar et al., 2009)

Typically, when producing new genetically modified strains, two distinct types of mouse strains are needed (Nagy, 2003). Firstly, an inbred strain of the required genetic background is normally used to produce embryos that can then be modified genetically, e.g. by microinjecting DNA into the pronucleus of the developing embryo. Inbred strains are defined as strains that have been bred for more than 20 generations as brother x sister mating, making them both genotypically and phenotypically uniform (Hedrich, 2012). This uniformity leads to lower variability between individuals, thus reducing the overall number of animals needed for the experimental work. However, due to the inbreeding itself, inbred mice tend to be inferior breeders with lower fertility and smaller litters (typically less than 8 offspring per litter). The most commonly used inbred strains are the different substrains of the C57BL/6J or C57BL/6N, as these have become the most frequently used background for genetically modified strains (Fontaine and Davis, 2016). Once embryos have been successfully modified, they are then transferred to a pseudopregnant recipient. Pseudo pregnancy can be caused by mating a fertile female with a sterile vasectomised male. After copulation, the female mouse behaves hormonally as if it were pregnant and can therefore be used to receive embryos. Recipient strains that are proven good mothers (large litter size, low pre-weaning mortality, robust) are typically outbred stocks such as IRC (CD1). Outbred mice are bred to minimize inbreeding and are thus genetically heterozygous. This often leads to better fertility and larger litters (typically more than 10 offspring in a litter), and they are therefore well suited as recipients.

A good quality source of both inbred and outbred mice, either from in-house breeding or from a vendor, is a prerequisite for successful and effective production of genetically modified mice. Consequently, by carefully following the production of genetically modified mice and the factors that may affect its success, we have a

unique opportunity to investigate mouse reproduction in general, as it captures many of the important steps of mouse breeding. We could look into the young mouse response to hormone treatment as well as the mating success with a male, including the sperm quality. The development of the embryos, as well as the robustness of the embryos after microinjection, can tell us a lot about what could affect mouse embryonic development. After transfer to pseudopregnant adult recipients, we can observe the further development of embryos all the way to live offspring.

1.4 ENVIRONMENTAL FACTORS AND MOUSE BREEDING

1.4.1 General background

The basic physiological needs (Maslow, 1943) of mice housed in animal facilities have been extensively covered by animal welfare laws, regulations and recommendations (EU, 2010; GV-SOLAS, 2014; National Research Council., 2011). From this, it follows that basic needs such as access to water and food, shelter and a suitable environment are aready a given. We know that under standard housing conditions using common cages and housing systems provided with appropriate bedding and nesting material, mice are doing reasonably well (Makowska and Weary, 2020). Animal welfare can be measured in many different ways, including behavioural measures such as responces to defined stimuli, or physiological measures such as heart rate, body temperature and hormones (Broom and Johnson, 1993; Whittaker et al., 2012; Burman et al., 2014). These are all very suitable short-term indicators of animal welfare in response to the environment. Long-term indicators, on the other hand, can offer much greater insight into how animal facilities can optimise their housing systems. One of the most practical variables to test the long-term effects of an environment on animal welfare is reproductive success. Although mice can reproduce well under standard housing conditions, we can still safely assume that the environment offered could be further enriched, and thus the breeding performance could be improved (Bayne, 2018). Finding an optimal combination of different environmental factors, such as ambient temperature, diet, and bedding material, could lead to better breeding performance and thus reduce the number of mice needed for breeding and experimentation.

1.4.2 Temperature

To set the appropriate ambient temperature for experimental animals is one of the fundamental ground truths we apply to any animal housing conditions. Thermoregulation of mammals has been intensively studied in the past (Gordon, 1993;

Bligh and Johnson, 1973) and has played a key role in our concepts of modern physiology (Bligh and Johnson, 1973), but research in the field has waned in recent past (Gordon, 1993). Only in recent years have we seen a resurgence of interest in understanding the effect of thermoregulation, specifically ambient housing temperature (Reitman, 2018; Gordon, 2017).

Mice are typically housed at about 22°C room temperature, as reflected in the recommendations from different countries and regions around the world. In Germany, for example, it is expected that mice are housed between 20°C and 24°C (GV-SOLAS, 2014), whereas in the USA, the guide sets the standard of 20°C to 26°C (National Research Council., 2011). Interestingly, the most common housing temperature for mice falls exactly within the temperature range humans find most comfortable (Karp, 2012). Therefore, the decision to keep mice in common room temperatures could have been driven mostly by human interest in feeling comfortable rather than by optimising the temperature for the mice. Cooler ambient temperatures that are suitable for man may become even more challenging for mouse thermoregulation when animals are subject to drafts caused by IVCs. Remarkably, the effect of different ambient temperatures on mouse breeding and welfare when housed in IVCs has hardly been studied at all. A PubMed search (performed July 2, 2020) with the key words *individually ventilated cages, mouse, temperature* yielded only three publications that discussed the effect of the temperature on mouse husbandry and breeding (Rosenbaum et al., 2010; David et al., 2013b), one of them being Article I in this dissertation (Helppi et al., 2016).

A number of recent studies have suggested that indeed both mice and studies using mice could benefit from higher housing temperatures than currently recommended (David et al., 2013b; Gaskill et al., 2009; Gordon, 2017). The thermoneutral zone of a mouse, i.e. the ambient temperature range in which the metabolic rate of an animal is minimal, is between 28°C and 34°C (Gordon, 1993). There is ample evidence that, in certain housing conditions or experimental setups, higher temperatures of up to (and beyond) 30°C could be beneficial (Yamauchi et al., 1983; Karp, 2012; Hylander and Repasky, 2016). Indeed, it has been suggested that housing mice according to the current standard of about 22°C could even lead to chronic cold stress (David et al., 2013a; David et al., 2013b). This could undoubtedly question the validity of some experiments in the past where mice have been housed under suboptimal conditions, thus potentially affecting their physiology and the results of the experiments. Although it has been increasingly suggested that mice would do better in warmer ambient temperatures, the practice in laboratory animal facilities is yet to change. Perhaps even more importantly, the recommendations and laws governing mouse housing may need to be reviewed.

1.4.3 Diet and phytoestrogens

As mice housed in captivity have no option to seek nutrition themselves, the diet offered plays an important role in their development and well-being. There is a wide variety of manufactures, all offering different types of diets for different needs. We understand the basic needs of mice in laboratory animal facilities, and selecting a diet from a known manufacturer following their advice is a safe way to make sure that the nutritional needs of mice are met (National Research Council (U.S.). Subcommittee on Laboratory Animal Nutrition., 1995). Still, environmental conditions could affect the way mice need to be fed. Also, whether the IVC system with active ventilation would lead to slightly different nutritional needs is not yet fully understood. More importantly, the effects of different diets on reproductive performance and embryology when producing genetically modified mice has not been widely studied. It can be assumed that the composition of the diet can play a significant role in mouse welfare, and thus a given diet can have a direct impact on mouse reproduction.

One of the most commonly used ingredients as a protein source in rodent diets is soy (Food and Agriculture Organization of the United Nations., 2004). It is abundantly available as a raw material, and it is relatively cheap. Soy contains a high concentration of phytoestrogens, a group of substances that share some similarities with vertebrate oestrogens, and can therefore produce oestrogen-like effects (Cederroth et al., 2012). Phytoestrogens have been associated with a number of potential health benefits in humans (Patisaul and Jefferson, 2010; Bennetau-Pelissero, 2016), such as reducing the risk of certain cancers and osteoporosis. On the other hand, phytoestrogens can also have adverse effects as endocrine disruptors, and they have been found to disrupt and affect rodent reproduction in many different ways, such as causing reduced fertility and infertility (Jefferson et al., 2009), reduced litter size (Delclos et al., 2009), defeminising female sexual behaviour (Kudwa et al., 2007), demasculinisation of male mice (Wisniewski et al., 2005), and low sexual receptiveness (Khan et al., 2008). This has led to a general recommendation in the field of laboratory animal science to reduce phytoestrogen in rodent diets or even completely remove it. These recommendations are generally based on information obtained from studies where mice and rats have been used as models of human health, which therefore had the aim of understanding the effects of phytoestrogens on human conditions and diseases. A PubMed search performed July 17 2020 with the key words *dietary, phytoestrogen, mouse, breeding* resulted in only three hits from the last two decades that studied the direct dietary effect of phytoestrogen on the breeding success of mice (Ramin et al., 2015; Jefferson et al., 2007), one of them being Article II in this dissertation (Helppi et al., 2020). Further about 70 publications can be found using the key word *reproduction* instead of *breeding*, but the vast majority of those handled specific genetic defects, medical conditions, and

single organ functions, but not the actual breeding success in husbandry situations. Hence, there have been practically no studies that have looked into the direct effect of (long-term) dietary phytoestrogens on mouse husbandry and breeding.

1.4.4 Cotton cloth as an environmental enrichment

The micro-environment of mice (environment within the cage) is not only defined by physical parameters, such as ventilation and temperature, but equally importantly by the materials and objects provided to the mice. It has long been known that the composition of bedding material plays an important role in animal welfare (Gaskill et al., 2013a; Hess et al., 2008; Robinson-Junker et al., 2017). The current recommendations also go beyond just offering bedding material to mice, as nowadays it is expected that additional environmental enrichment also be provided (Bayne, 2018). These can vary tremendously in material compositions and shape, with commonly provided enrichment materials including nesting material and nest boxes to allow extensive nest building, tubes and containers to provide better separation of individual mice and offer more options for locomotion (climbing, borrowing etc.), and provision of material suitable for gnawing (Bayne, 2018; Olsson and Dahlborn, 2002). New materials that enrich and improve the cage environment, and consequently mouse welfare, are constantly sought.

A potentially interesting concept was developed by Japanese researchers proposing the use of a large washable and reusable cotton cloth instead of traditional bedding material, such as wooden chips or shavings (Kawakami et al., 2007; Kawakami et al., 2012). By replacing the bedding material with a cloth, one could offer in one easy way both bedding material and environmental enrichment. The cloth could enable the mice to build nests within the folds of the material, it could protect them from draught from ventilation, it would offer physical barriers and distancing between individuals, and it would lower the level of dust within the cage. Furthermore, the cloth would offer substantial benefits for the laboratory animal facility management, including significantly reduced waste and potentially longer cage changing intervals with less frequent requirements for cage changes and washing.

1.5 AIMS OF THE PRESENT RESEARCH

To optimise the environmental conditions of mice housed in IVCs and used to produce transgenic mice, I sought to explore whether we could improve the welfare of mice by manipulating certain parameters, such as temperature, bedding material and diet. I hypothesized that some of the current recommendations and guidelines for temperature, diet composition, and bedding material are not optimal for mod-

ern IVC systems, as many of the past studies have been performed with conventional housing systems (open cages with wire bar lid).

The dependent variables of the present experiments were all related to breeding performance, such as pregnancy rate, embryo yield and quality, sperm yield and quality, and number of live offspring. These were expected to help us understand how to optimize the use of modern IVCs for mouse housing to effectively produce transgenic mice, and also to provide indicators of mouse well-being.

The overall goal was to contribute to the revision of the current recommendations and guidelines used for housing mice in laboratory animal facilities to better match modern housing systems. The results of this study could lead to improved animal welfare and fewer animals being used for experiments, thus directly applying the widely accepted principles of the three R's (Reduction, Refinement, Replacement) (Russell and Burch, 1992) in laboratory animal work.

The specific study objectives were as follows:

1. To explore whether higher ambient temperatures than commonly recommended would influence the reproductive performance of mice used as embryo donors (I)

2. To determine whether long-term exposure to different levels of phytoestrogens in mouse diets could affect the reproductive success of mice used as embryo donors and recipients (II)

3. To examine whether a novel bedding material (cotton cloth) could be used as a replacement for standard bedding material for recipient mice after embryo transfer (III)

2 MATERIALS AND METHODS

Only a general overview of the materials and methods is presented here – for more detailed information please refer to the original papers (I-III) attached.

2.1 GENERAL BACKGROUND

Each of the three studies (I-III) presented here was performed at the Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany. It is to be noted that none of the three studies used animals only for the purpose of this research. Moreover, each study was designed to utilize already existing approved workflows established and used at the MPI-CBG. In practical terms, this meant that mice already destined to be used for the production of genetically modified mouse strains (transgenic mice) were subjected to different environmental conditions (temperature, enrichment, diet). Hence, each experiment was part of a standard production of transgenic mice, and each successful experiment resulted in mice that were the foundation for several established transgenic strains. The study presented here utilised the combination of female donor mice for embryo collection and males used for the mating, as well as female recipient (foster) mice that received the embryos after they were manipulated. The technique used for generating transgenic mice throughout these studies was DNA microinjection into the pronucleus of a developing embryo.

All mice used in these studies were housed in an IVC system (type 1145T; Tecniplast, Buguggiate, Italy). Each cage was provided with aspen bedding (unless experimentally changed, see paper III) and nesting material (Tapvei, Paekna, Estonia). They were routinely changed once per week under laminar flow (InterActive Cage Changing Station; Tecniplast). Pelleted autoclaved mouse diet (Harlan Teklad 2018S; Harlan, Indianapolis, IN, USA) and filtered and acidified (pH 3.0) water were provided *ad libitum*. Some groups in study II received experimental diet. The standard room climate, unless experimentally changed (see paper I), was maintained at 22±1°C, and at a relative humidity of 55±10%. Light–dark cycle was 12:12 h with lights on at 05:00 h.

Dependent parameters varied slightly between the three studies and are shown in Table 1.

Table 1. Variables used to define the reproductive performance under different study conditions.

	Study:		
Endpoints:	**I) Temperature**	**II) Phytoestrogen**	**III) Cotton cloth**
Mating success (plug positive)	x	x	
Total yield of embryos	x	x	
Yield of good quality embryos	x	x	
Yield of injectable embryos	x	x	
Yield of transferable embryos	x	x	
Yield of sperm	x	x	
Quality of sperm	x	x	
Number of pregnancies		x	x
Number of pups born		x	x
Number of pups weaned		x	x

2.2 TEMPERATURE STUDY (I)

Female donor mice (C57BL/6JOlaHsd) were ordered weekly from the vendor (Harlan, Horst, The Netherlands) at an age of three weeks, placed directly in either the control or the experimental room, and subsequently used for collecting embryos. Mice were housed under four different ambient temperatures (22°C, 25°C, 28°C or 30°C) with 22°C set as the control treatment and other temperatures as experimental treatments. Male mice (C57BL/6JOlaHsd) were ordered before the beginning of the experiments at an age of eight weeks, placed singly in cages and test-bred once with fertile females. A pool of 30 males was set up for each treatment, and males were used for breeding on average three times per week. Female mice were superovulated and mated with males. Embryos were collected at the day of the plug check and counted from all mated mice.

The study included 440 donor mice divided into four different temperature groups as follows: 22°C group, 220 mice; 25°C group, 125 mice; 28°C group, 90 mice; and 30°C group, 60 mice. Pools of on average six donor mice were used for embryo collection daily. For practical reasons, two rooms were used, with one held constantly at 22°C and the other set to the desired temperature as follows: 25°C for two weeks, 28°C for two weeks, 30°C for two weeks, and finally back to 25°C for the last two weeks. Three days were used between the temperature changes to allow mice to adapt to the new temperature. The temperature in the room was controlled using an electric heater installed inside the supply air duct, and when insufficient, two additional stand-alone electric heaters with thermostats were used

within the room. The relative air humidity was maintained constant using an additional air humidifier. The ambient temperature and humidity were recorded using the building's in-built monitoring system with gauges installed in the supply and exhaust air ducts, and verified by placing temperature data loggers in different parts of the room, and inside the cages.

As indicators of reproductive performance, the success of the mating was observed (plug check), including embryo yield and quality and sperm yield and quality. Embryos were scored at different stages: I) immediately after collection from the donor mouse, II) during and after wash procedure, III) during DNA microinjection, and IV) after final incubation. Sperm was collected from selected (used) males and scored according to Hamilton Thorne Automated Sperm Analyser default settings for mouse sperm (count, motility and progressivity).

2.3 DIETARY PHYTOESTROGEN STUDY (II)

Donor mice (C57BL/6JOlaHsd) were maintained with three different diets obtained from Harlan Teklad: high phytoestrogen (High-PE, ~400mg/kg Genistein), low phytoestrogen (Low-PE, ~10mg/kg Genistein) and standard diet (Normal-PE, ~120mg/kg Genistein). Each feeding regime was followed for a minimum of two generations before mice were used for the experiments. Embryos were collected from hormone-primed donors, and subsequently transferred to pseudopregnant recipients (Hsd:ICR (CD-1®)). The yield and quality of embryos and sperm was analysed, as well as the production of offspring. Experiments with High-PE and Low-PE groups were performed on the same days with the same DNA (exact pairwise comparison). Experiments with the Normal-PE group were performed during the same time frame but not necessarily on the same days as High-PE and Low-PE groups. Typically, five females were used per experimental batch (within one day) for the High-PE and Low-PE groups. However, eight to ten were typically used per experimental batch for the Normal-PE group. This was to safeguard enough material for transgenic work. Each experiment was part of a standard production of transgenic mice.

Altogether, 108 donor mice were used from each of the High-PE and Low-PE groups, and 162 donor mice were used from the Normal-PE group. A total of 46 recipients received embryos from High-PE donors, 48 recipients from Low-PE donors, and 68 recipients from Normal- PE donors. Recipient mice were all fed with the standard diet (Normal-PE).

As indicators of reproductive performance, the success of the mating was observed (plug check) along with embryo yield and quality and sperm yield and quality. Embryos were scored at different stages: a) immediately after collection from the donor mouse, b) during and after wash procedure, c) during DNA microinjection, and d) after final incubation. Sperm was collected from selected (used) males

and scored according to Hamilton Thorne Automated Sperm Analyser default settings for mouse sperm (count, motility and progressivity). Additionally, the number of pregnancies of recipient mice and the number of pups born and weaned were observed.

2.4 COTTON CLOTH AS BEDDING STUDY (III)

Hsd:ICR (CD-1®) in-house bred female mice, 9-12 weeks of age, weighing 25-35g, received embryos from pronuclear injection experiments. Each cage was provided with either 160g of autoclaved aspen bedding and a full handful of nesting material (Tapvei, Paekna, Estonia) or an autoclaved cotton cloth (AGREBE Cotton Cloth, 55cm x 55cm, DuoMedix OHG, Hamburg, Germany). Cages from both groups were changed once per week under a laminar flow changing station. Cotton cloths were machine washed (90°C without detergent) and autoclaved, and subsequently reused. Aspen bedding was changed during the cage change weekly.

Altogether, 116 recipient mice were used (63 with aspen bedding, 53 with cotton cloth). Pregnancies were observed 15 days after the transfer and mice were scored 'pregnant' or 'nonpregnant'. On postnatal day three, the number of offspring was recorded. The offspring were weaned and the numbers of male and female pups were counted on postnatal day 21.

2.5 ETHICS OF THE RESEARCH

All animal housing, handling and experimental techniques for this study were in accordance with the principles set out in the Declaration of Helsinki, as well as in accordance with the ethical standards of the European and German Animal Welfare legislation. Experiments were planned and conducted to adhere closely to the "3R" (replacement, reduction, refinement) principles of animal welfare (Russell and Burch, 1992). The required licenses were obtained from the local governmental regulatory body (Landesdirektion Sachsen, permission numbers 24-9168.11-9/2005-1, 24-9168.11-9/2010-3, 24-9168.11-9/2012-1) and carried out according to the Institutional Animal Care and Use Committee guidelines as regulated by German Federal law governing animal welfare.

3 RESULTS AND DISCUSSION

3.1 WARMER AMBIENT TEMPERATURES CAN BE TOLERATED BY MICE (I)

This study demonstrated that embryonic reproduction performance was maintained at a high level all the way up to 28°C ambient temperature. Embryo yield and quality remained high up to 28°C (Table 2). Male reproductive performance (sperm yield and quality) was also evaluated under different ambient temperatures. The yield and quality of sperm gradually diminished with rising temperatures, effectively halving the sperm yield when the ambient temperature was raised from 22°C to 30°C (Table 3). Interestingly, sperm quality remained high up to 28°C.

Table 2. Summary of the results regarding embryo yield and quality at different ambient temperatures (Article I). Results are shown as mean ± SD. # = P<0.05. Statistical test: analysis of variance (ANOVA) followed by Tukey's honest significance difference. Data are shown in more detail in Article I.

Ambient temperature (°C)	22	25	28	30
Plug frequency (%)	58±25%	66±24%	66±18%	57±24%
Average embryo yield per female	35.0±12.8	36.2±13.7	36.8±12.4	24.3±12.6
Good quality embryos per female	32.1±12.7	32.8±12.9	33.3±12.6	21.7±12.0
Injectable quality embryos per female	17.3±9.0	16.3±5.9	15.8±7.7	6.0±4.1#
Injectable quality per embryo yield (%)	49±14%	47±16%	43±15%	24±16%#

Table 3. Summary of the results regarding sperm yield and quality at different ambient temperatures (Article I). Results are shown as mean ± SD. # = P<0.05. Statistical test: sperm count, significant linear regression; motility and progressivity, analysis of variance (ANOVA) followed by Tukey's honest significance difference. Data are shown in more detail in Article I.

Ambient temperature (°C)	22	25	28	30
Average sperm count per male	2084±549	1575±454	1306±171	1009±247#
Motility (%)	72±8%	74±4%	69±8%	46±10%#
Progressivity (%)	22±4%	26±1%	20±2%	12±2%#

These results show that higher ambient temperatures are not only tolerated well by mice, but that the ambient temperatures between 25°C and 28°C may even be beneficial for certain aspects of mouse reproduction, such as plug frequency or embryos yield and quality. Mice apparently thrive well in higher temperatures than currently recommended (GV-SOLAS, 2014; National Research Council., 2011). This concurs with several studies published in recent years (Hylander and Repasky, 2016; Keijer et al., 2019) and strongly supports the suggestion that many experiments performed with mice in the past have been done in suboptimal thermal environments (Ganeshan and Chawla, 2017). In fact, there is a lot of evidence that mice housed at about 22°C could already be under chronic cold stress (Karp, 2012; David et al., 2013b). Based on the good reproduction observed up to and including the ambient temperature of 28°C, it can be concluded that significantly higher temperatures than commonly used could be employed for mouse housing. Furthermore, current recommendations are too inflexible as they do not acknowledge the fact that higher temperatures can indeed be beneficial for mouse welfare and experimental output.

Strikingly, at the ambient temperature of 30°C, all reproductive indicators used in this study dramatically deteriorated. Both the yield and quality of embryos dropped by about 35%, and the injectable quality of embryos went down by more than 60% (Table 2). This is physiologically interesting, as the lower threshold of the thermoneutral zone of a mouse lies at about 28°C (Gordon, 1993), and it suggests that mice housed in IVCs do not breed that well if they are being housed at their thermoneutral zone. Overall, this may not be so unusual, as it has been observed that mice housed singly actually prefer temperatures between 26°C and 29°C, and group housed mice favour temperatures only about one degree lower (Gordon, 1993; Gaskill et al., 2012). Housing temperatures around the thermoneutral zone may suit mice well physiologically when they are at rest, but may not offer enough opportunities to seek the optimal ambient temperature during more active behaviour patterns.

Although mice seem to prefer warmer temperatures than typically provided in animal facilities, their reproduction seems to be affected if they are housed close to their thermoneutral zone. In this study, the length of the adaptation was short (one to two weeks), but there is some evidence that mice may adapt to higher temperatures given enough time (Bronson and Pryor, 1983). The possibility for mice to acclimatize to different temperatures can be routinely organised by offering them greater freedom to influence their immediate environment. Under normal static (non-ventilated) cage conditions, mice can easily adapt to cooler ambient temperatures as long as they have been given sufficient bedding and nesting material. By using these during the resting period, for example, they can effectively create a microclimate that is warmer than the ambient temperature. Nesting material in particular can offer a great opportunity for mice to seek higher temperatures inside the nest compared to the actual ambient temperature (Gaskill et al., 2013a). The

protective function of nesting material may not be that effective in modern IVC systems with forced ventilation up to more than 100 air changes per hour, however, and may even be inadequate when the ambient temperature is closer to 20°C. In practice, this may mean that IVCs need to be run at somewhat higher temperatures than static open top cages to avoid excess cold stress. On the other hand, one has to be careful not to run them at too high ambient temperatures (very close to, or at the thermoneutral zone), as the behavioural management of the microclimate using nesting material becomes too limited to allow mice to effectively select their preferred temperature for different activities.

Furthermore, sperm motility also was reduced by about 35%, and sperm progressivity by 40%, at the highest temperature of 30°C. Low sperm quality at higher temperatures was not surprising, and has also been previously shown (Yaeram et al., 2006). Even short exposures to higher ambient temperature may lead to lower sperm quality (Zhu and Setchell, 2004). Interestingly, the absolute sperm count observed in this study seems not to be a significant factor on males' ability to impregnate female mice. Moreover, as long as the sperm quality stays high, even lower sperm count does not have a negative effect on mating ability. Similar results have been observed with human sperm, where the quantity of the sperm is not the most significant parameter predicting the success of a natural conception (Larsen et al., 2000).

Finally, there is increasing evidence that many experiments performed with mice may have been done in suboptimal thermal environments, leading to a suggestion that mice could perform better in warmer temperatures (Gaskill et al., 2009; Ganeshan and Chawla, 2017). Most commonly, mouse room temperature is set to 22°C, conveniently precisely the room temperature preferred by humans. This is also evident in current recommendations. Typical standard mouse room temperature recommendation in Europe for housing mice is between 20°C and 24°C (EU, 2010). The NIH Guide for the Use of Laboratory Animals endorses the temperature range from 20°C to 26°C, but recommends using the middle range (~23°C) as a standard (National Research Council., 2011). Deviations from these recommendations are typically not tolerated by the authorities unless specifically requested for specific projects or experiments. Changing the common housing temperature beyond the recommended range is practically impossible for many laboratory animal facilities. Yet, there are already many studies suggesting that housing mice in higher temperature may indeed be a better choice, both for mouse welfare and experimental results (Gaskill et al., 2009; Hylander and Repasky, 2016; Keijer et al., 2019). It is evident from this study that housing temperatures around typical room temperatures of 21-22°C may indeed be too low, but temperatures of 30°C or higher may also be too high. This has recently also been suggested by Keijer et al. (2019). In conclusion, the present study confirms that warmer ambient temperatures than commonly used and recommended can and should be considered while producing transgenic mice.

3.2 DIETARY PHYTOESTROGEN AFFECTS EMBRYOLOGY AND REPRODUCTION (II)

This study demonstrated that embryonic reproductive performance was best when mice were fed a diet rich in phytoestrogen (Table 4). Mating success (plug frequency) and embryo yield were highest for the group fed with a high phytoestrogen diet (400 mg/kg genistein), somewhat lower for the group fed with a low phytoestrogen (10 mg/kg genistein) diet, and significantly lower for the group fed with a standard diet (~120 mg/kg genistein). A similar tendency was observed with the quality of embryos where the highest yield of good and injectable quality embryos were obtained from mice fed with a high phytoestrogen diet. This disagrees with a number of previous studies. Jefferson et al. (2009) found that early embryo development is significantly disturbed when mice are neonatally treated with phytoestrogen. The same authors had previously demonstrated the general ability of phytoestrogen to disrupt female reproductive function (Jefferson et al., 2007; Jefferson et al., 2005). Ramin et al. (2015) found that embryo yield was disrupted when mice were fed with a phytoestrogen-containing diet. It has also been shown that blastocysts treated with phytoestrogen *in vitro* display disrupted development (Chan et al., 2007). Contrary to all these studies, high phytoestrogen content seemed to have a positive effect on embryo development in the present study. This could be because this study was a multi-generational study where mice were fed with the respective diets for several generations. Many other studies have investigated a short-term (up to only a few weeks) response to phytoestrogen (Jefferson et al., 2009; Ramin et al., 2015; Jefferson et al., 2007). Interestingly, Patel et al. (2017) studied the effects of phytoestrogen in trials lasting from one to eight months. They found that many of the effects of phytoestrogen on pregnancy rates and fertility occurred with the lower phytoestrogen content. Surprisingly, the highest phytoestrogen content dosed for eight months did not have any adverse effects on their reproductive success. It could therefore be speculated that long-term exposure to a different (or high) phytoestrogen levels in diet could eventually induce epigenetic changes (Dolinoy et al., 2006; Moller et al., 2010; Guerrero-Bosagna and Skinner, 2014) that cannot be observed in short-term studies. Furthermore, a short-term response (fewer embryos) has been observed with high phytoestrogen content similar to the standard diet of this study (~120 mg/kg genistein) (Ramin et al., 2015). The present study suggests that significantly higher phytoestrogen content (400 mg/kg genistein) could indeed be a much better choice if the intention is to produce a high yield of good quality embryos. It can also be questioned whether the change in diet in short-term experiments could in itself induce a stronger response than the diet composition (e.g. phytoestrogen content) itself. Furthermore, on average, about half of the embryos developed to proper injectable quality in laboratory settings across all groups, sug-

gesting that the major difference between different feeding groups occurred during mating and early development in mouse.

Table 4. Summary of the results regarding the embryo yield and quality for different dietary phytoestrogen content (Article II). Results are shown as mean ± SD. # = P<0.05 compared to Normal PE or Low PE. Statistical test: analysis of variance (ANOVA) followed by Tukey's honest significance difference. Data are shown in more detail in Article II.

Feeding group	HighPE	LowPE	NormalPE
Plug frequency (%)	85±15%#	79±18%	72±13%
Average embryo yield per female	32.0±9.7#	26.3±8.2	23.5±6.7
Good quality embryos per female	29.6±10.1#	24.8±8.4	21.5±6.5
Injectable quality embryos per female	16.3±5.4#	12.3±6.0	11.5±3.7
Injectable quality per embryo yield (%)	49±15%	46±17%	50±9%

Unexpectedly, the weakest performing group regarding embryo production was the one that was fed with the standard diet, whereas both high and low phytoestrogen groups generally performed better (Table 4). Although it can be reasonably suggested that high phytoestrogen in mouse diet does not negatively affect embryo yield and quality, the phytoestrogen content itself cannot fully explain the differences between the feeding groups. In the present study, the low phytoestrogen group performed better than the standard group with higher phytoestrogen content. Based on this study, it is not possible to conclude what may have caused the difference, but one could speculate that the general composition of the diet must have had some effect. The experimental diets for high and low phytoestrogen groups were based as closely as possible on the standard diet formula, the only difference being the phytoestrogen (genistein) content. However, experimental diets are typically produced in small batches, whereas standard diets are produced in large batches. This in itself may cause special diets to be more palatable or nutritionally superior. It can be argued that the source of the ingredients, and the way the diet is manufactured, can indeed have measurable effects on mouse reproduction. Therefore, this should be taken into consideration when diets are selected for mouse colonies. Finally, the role of potential epigenetic changes cannot be concluded based on this study, and would require further investigation.

Table 5. Summary of the results regarding the production of live offspring under different phytoestrogen content in diet (Article II). Results are shown as total or as mean ± SD where relevant. # = P<0.05. Statistical test: pregnancies and birth events, Pearson's chi square test; litter size, Kruskall-Wallis followed by pairwise comparison (Dunn-Bonferroni test). Data are shown in more detail in Article II.

	HighPE	LowPE	NormalPE
Donors used	108	108	162
Recipients (fosters) used	49	46	68
Pregnancies observed	57%[#]	77%	91%
Pups born	59[#]	118	351
Average litter size	3.7±1.8[#]	5.9±3.4	7.4±3.3
Pups born per foster	1.2	2.6	5.2
Embryos needed for one pup	19.2	9.7	4.9
Donors needed for one pup	1.8	0.9	0.5

Strikingly, the positive effect of high phytoestrogen content in diet for embryonic development was not translated into an ability to successfully produce live offspring, in fact quite the contrary (Table 5). Pregnancy rate was significantly higher in the standard diet group (91%) and lowest in the high-phytoestrogen group (57%). Recipient mice that received embryos from the high-phytoestrogen group had by far the lowest number of pups born (1.2 pups born per foster), followed by the low phytoestrogen group (2.6), and then, with a significant difference, the standard diet group (5.2). Recipients that received embryos from the high phytoestrogen group required 2-3 times more embryos on average to produce one live offspring compared to the other two feeding groups. This study may indicate that early development and implantation of embryos were especially compromised when embryos were obtained from the high-phytoestrogen group. Although there is some evidence suggesting that phytoestrogens may have some minor effects on embryo implantation in mice (Li et al., 2014), it is far from conclusive. There is some evidence that embryonic development is not disrupted in the recipient regardless of the phytoestrogen treatment of the donors (Jefferson et al., 2009), but at the same time, subcutaneously injected genistein has been found to disrupt embryonic development. How much influence the method of delivery (injected versus dietary) can have on reproduction is not yet know, but the effect has been shown by Rayyan et al. (2015). The striking effect of dietary phytoestrogen in the present study may also have been caused by the cumulative effect of multi-generation feeding. This would be in line with the suggestion that long-term exposure to phytoestrogen may

cause epigenetic effects (Moller et al., 2010; Guerrero-Bosagna and Skinner, 2014). Perhaps most significantly, this study used recipients that were fed with standard diet only. Therefore, it could be debated whether embryos from other feeding groups struggled to develop in a recipient that had been fed with a different diet. This hypothesis would warrant further studies.

This study shows that phytoestrogen-containing diets may be both beneficial and disadvantageous for the reproductive performance of mice. High dietary phytoestrogen content may be beneficial if one is interested in producing large numbers of good quality embryos. Strikingly, embryos derived from the high-phytoestrogen group failed to develop properly in recipients, resulting in significantly fewer live offspring. Clearly the diet composition may have significant effects on mouse reproduction and care should be taken when selecting or changing diets.

3.3 SIGNIFICANTLY BETTER REPRODUCTION WITH WOODEN ASPEN BEDDING (III)

Many efforts have been made in recent years to study the effect of environmental enrichment on mice (Bayne, 2018; Dean, 1999). It is widely accepted that provision of only cage and bedding is no longer adequate in standard housing and husbandry, and additional enrichment to support natural behaviour should be sought. This study demonstrated that the use of cotton cloth as a replacement for standard bedding cannot be recommended (Table 6). Significantly more pregnancies (43%) were observed with aspen bedding compared to cotton cloth (19%). This translated directly into fewer offspring being born for the cotton-cloth group, although once recipients got pregnant, the litter size was the same. This suggests that the cotton-cloth group had a higher incidence of disruption in early pregnancy. About 9% of offspring did not survive to weaning age in the cotton-cloth group, whereas 100% survival rate was observed in the aspen-bedding group. Curiously, the sex ratio favoured males in the cotton-cloth group, while the sex ratio was as expected in the aspen-bedding group. A skewed sex ratio may indicate elevated stress levels in recipients, as a similar relationship has been observed in a study with squirrels where elevated stress leads to more males being born (Ryan et al., 2012).

In a previous study, mice have been found to prefer cotton cloth as a bedding (Kawakami et al., 2007), but surprisingly in this study the cotton cloth resulted in poor breeding performance. This could have been partially due to the cotton cloth being offered as a novelty item to recipients after successful embryo transfer (under anaesthesia). Whether the cotton cloth could have been better accepted by the recipients if they had been raised with it cannot be concluded from this study. More importantly, it was observed by the caretakers that cages with cotton cloth ap-

peared dirtier, and instead of making a nest with the cotton cloth, as expected, mice were often found lying under the cloth directly on the plastic cage bottom. This may have suited adult mice but not been as optimal for newborn offspring. Furthermore, mice hidden under the cloth created difficulties for animal care, as mice could not be easily observed in routine daily check-ups, and cage changes were more laborious, as mice were difficult to catch.

Table 6. Summary of the results regarding the production of live offspring between aspen bedding and cotton cloth (Article III). Results are shown as total or as mean ± SD where relevant. # = P<0.05. Statistical test: pregnancies, Pearson's chi square test; pups born, Mann Whitney U test. Data are shown more in detail in Article III.

	Aspen bedding	Cotton cloth
Recipients (fosters) used	63	53
Pregnancies observed	43%	19%#
Pups born	87	32#
Average litter size	3.2±1.4	3.2±1.8
Pups weaned	87	28
Male:Female ratio	1.02	1.73
Pups born per foster	1.4	0.6

The objective of the study was to find out whether aspen bedding could be replaced by cotton cloth. This could have potentially resulted in a combined bedding and nesting material that would positively support the reproduction of mice. It was shown that this was not the case for recipients. Additionally, the cotton cloth was intended to offer significant benefits for the laboratory animal facility in terms of less waste to be produced, and possibly longer cage changing intervals. This also proved not to be the case. The cotton cloths wore out much faster than anticipated, and showed signs of major wear and tear after only a few weeks of usage. In conclusion, cotton cloth cannot be recommended as a sole replacement for aspen bedding.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

This dissertation dealt with a small selection of environmental factors that could influence the breeding efficiency of laboratory mice. It offers an insight into how some of the outcomes of breeding and reproduction used for the production of transgenic mice could be improved.

Higher housing temperatures than currently recommended can and should be considered for laboratory mice. The present study shows that higher ambient temperatures are not only tolerated well by mice but that temperatures between 25°C and 28°C may even be beneficial for certain aspects of mouse reproduction, such as plug frequency or embryo yield and quality. Current recommendations regarding housing temperatures for mice ought to be revised to allow animal facilities more flexibility to select the most optimal housing temperature. Furthermore, the effects of dietary phytoestrogen on mouse reproduction seem to be more complex than previously presumed. The present study shows that high phytoestrogen content in mouse diet does not negatively impact early reproductive performance and could potentially even improve embryo yield and quality. On the other hand, based on this study, high phytoestrogen content in donor mouse diet may lead to birth of fewer offspring to recipient mice. This could, at least partially, be due to dietary change, so care should be taken when selecting an appropriate diet, and when different diets are being used between donor and recipient colonies. Finally, using cotton cloth with recipient mice as a sole replacement for bedding material cannot be recommended.

We now know that warmer temperatures may be beneficial for mouse husbandry and breeding, and we know at which temperature the breeding performance starts to decline. As the reproductive success stayed fairly similar across temperatures from 22°C to 28°C, it would be interesting to find out at what lower temperature the reproductive success would start to decline. Additionally, it would be important to understand if added enrichment material or different kinds of bedding would increase the yield or quality of embryos without increasing the housing temperature from the standard 22 °C. Furthermore, as this study was performed at a constant relative humidity of 55±10%, it would be fascinating to study how high or low humidity would influence reproductive performance under different ambient temperatures. Recent unpublished observations from our facility suggest that especially high humidity may lead to lower embryo yield. The embryo production in this study was induced by hormonal treatment of young mice prior to their first natural ovulation cycle. Consequently, it would be interesting to study whether the effects observed would also be valid for naturally mated adult mice.

As the phytoestrogen in this study clearly did not have the predicated effect of disrupting embryonic development, further studies would be needed to understand the exact mechanisms responsible. It would be very interesting to study the long-term effect of phytoestrogen in diet, and whether it effects natural reproduction in the same way it affected hormonally treated mice in this study. As the role of the potential epigenetic changes cannot be estimated from the present study, it would be an interesting subject to study further. This study only used one inbred strain as embryo donors. Consequently, whether the effects observed in this study would be similar with other inbred strains, or even outbred stock, is not known, and would warrant further studies. Furthermore, as this study could not offer final conclusions regarding whether high dietary phytoestrogen content or simply the change in diet between donor and recipient colonies was the reason for poor breeding results, further research should be performed to study this.

Although it was clear from this study that cotton cloth was not readily accepted by pregnant recipient mice, it does not mean that cotton cloth (or similar enrichment) would not be potentially beneficial. It could be that the combination of standard bedding and a smaller cloth would be more preferable for mice. It would be very interesting to combine studies from different enrichments together with different ambient temperatures. Do mice need the same nesting or enrichment material, for instance, if the housing temperature were higher than currently recommended? One could assume that mice housed in colder ambient temperatures would benefit more from added nesting and enrichment materials, but if warmer ambient temperatures become more of a standard it could require some changes in the use of such materials. Thus, continuous efforts are still needed to find out what kind of enrichment materials are suitable under different housing conditions.

From a methodological point of view, when studying environmental effects on mouse reproduction, it would be beneficial to look into the whole process of reproduction and not only certain aspects of it. This has been effectively shown in Article II, where initial results on embryo development suggested an effect that was then reversed at later reproductive stages (pups born). Therefore, care should be taken when interpreting results from single outcomes of reproduction if general conclusions for reproductive success are to be drawn.

As a final conclusion, with the present data we now have a better understanding of the limits and possibilities of some of the factors influencing the productivity of mice housed in IVCs and used to generate transgenic mouse lines. This could potentially lead to improved welfare and fewer animals being used for experiments.

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JUSSI HELPPI

Good animal welfare leads to better animals, and better reproduction. Finding an optimal combination of different environmental factors, such as ambient temperature, diet, and bedding material, could lead to better breeding performance and thus reduce the number of mice needed for breeding and experimentation. This thesis provides novel insight into how some of the outcomes of breeding and reproduction used for the production of transgenic mice could be improved.

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