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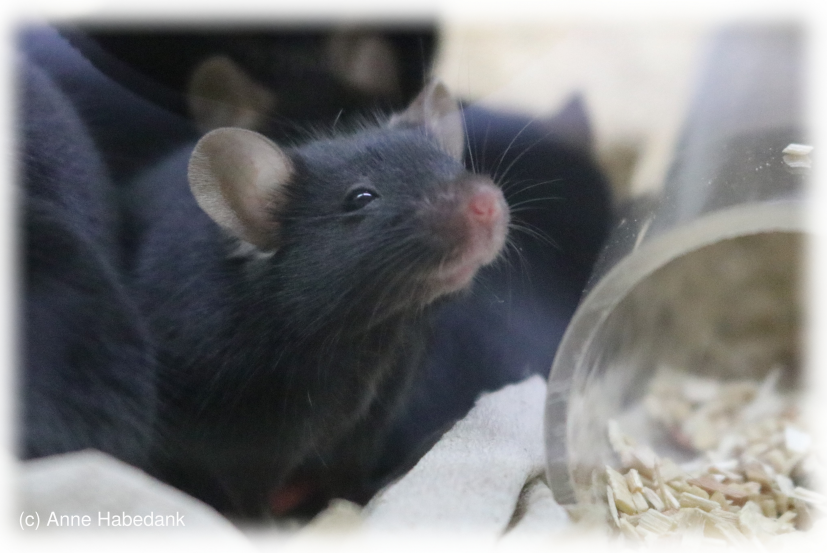
der Freien Universität Berlin

# Choice tests as a means for severity assessment from an animal's point of view

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## Abbreviations

| | |
|---|---|
| CPP | conditioned place preference |
| ITI | intertrial interval |
| microSD card | micro Secure Digital Memory Card |
| MoPSS | Mouse Position Surveillance System |
| RFID | radio frequency identification |

# 1 Introduction

In Germany alone, 2 million animals were used in 2019 for experiments, about 1.5 million of them were mice (Bundesministerium für Ernährung und Landwirtschaft 2020). Statistics from the European Union reveal that between 2015 and 2017, over 9.5 million animals annually were used for experiments, over 60 % of them were mice (over 5.7 million) (Report from the Commission to the European Parliament and the Council 2019). Numbers as high as these illustrate that we are not able to replace all animal experiments yet. It is therefore crucial to refine the experiments for the benefit of those animals still part of animal experimentation. This means that experimental and housing conditions should be set in such a way that they will cause the least possible harm, suffering, or pain for the animals (Russell and Burch 1992 (new edition); Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes).

However, the assessment of the actual severity of the harm, suffering, or pain induced by research methods is a very complex issue. Mostly, severity assessment is based on physiological, biochemical, or behavioural measures, e.g., weight (Smith et al. 2018; Talbot et al. 2019), temperature (Smith et al. 2018; Pereira et al. 2018), corticosterone levels (Leenaars et al. 2019; van der Mierden et al. 2020; Bach et al. 2019), facial expression by usage of grimace scales (Langford et al. 2010; Hohlbaum et al. 2018; Ernst et al. 2019; Andresen et al. 2020) or alterations in typical behaviour (nest-building in rats: Schwabe et al. 2019; locomotion in rats: Zieglowski et al. 2020; wheel running behaviour in mice: Häger et al. 2018; Weegh et al. 2019). Interpretation of these data can itself be very demanding, as for example, increase as well as decrease in weight might be a sign of reduced welfare (e.g., increase: obesity, Zou et al. 2020; unchanged: tumour-growth, Zhang et al. 2014; decrease: colitis, restraint stress, Talbot et al. 2019). In addition, severity assessment is often influenced by an anthropomorphic view, based on how we think the animal would feel in this situation. However, our perception of what is best for the animal might be misdirected. For example, aiming for a complete (di)stress-free environment for the animal to ensure good animal welfare might be a false conclusion because also a hypostimulation can impair animal welfare (Korte et al. 2007; van Praag et al. 2000). Thus, it is difficult to judge from the outside how stressful a specific experimental method is for the animal itself (see also Habedank et al. 2018 in Chapter 2).

But how can we gain information on how the animal perceives the severity of a situation? Can we "ask" the animals how they feel? Indeed, we can. But we have to use an indirect approach: One option is to use a cognitive bias test (Parker et al. 2014; Novak et al. 2015), another option are choice or preference tests (Habedank et al. 2018): The animals get to choose between two (or more) options. The chosen option is then assumed to be the preferred (or less avoided), and thus, the less severe one. In this manner, preference tests could yield a ranking of experimental methods from the lowest to the highest perceived severity.

In the following the three main types of choice tests will be introduced in detail: conditioned

place preference tests, T-maze tests and a home cage based preference tests (for more details see Habedank et al. 2018 in Chapter 2). Because mice are the animals most used for animal experiments, elaborations will focus on them, including the pitfalls when developing preference tests aimed to investigate the mice's perspective. (Note that "choice test" and "preference test" are always used synonymously in the following.)

## 1.1 Conditioned place preference test

The conditioned place preference test (or conditioned place aversion test, respectively) is commonly used to assess the effect of a stimulus with regard to its rewarding (e.g., food) or aversive (e.g., drug withdrawal, foot shock) effect. The test is based on classical (Pavlovian) conditioning: A previously neutral environmental cue (conditioned stimulus, CS) is paired with the motivationally significant stimulus (unconditioned stimulus, US). This association is learned by repeated presentations in daily conditioning sessions. As a result, presentation of the CS alone is able to evoke a similar response as the US. Thus, if the US is rewarding, animals will spend more time close to the CS. If the US is aversive, they will avoid also the CS. This effect can be measured in a final preference test, in which the animal can choose between a spatial location far or close to the CS. This is usually conducted by placing the animal in a setup with two adjacent compartments, of which one contains the CS and the other a neutral stimulus.
The conditioned place preference test (CPP) is very common for testing the effect of drugs (Cunningham et al. 2006a; Cunningham et al. 2006b; Tzschentke 1998; Tzschentke 2007; Wang et al. 2014). However, the CPP is also used to assess other reinforcers like home cage odours (mice: Fitchett et al. 2006), food (mice: Takeda et al. 2001; Imaizumi et al. 2000), or male aggression (mice: Martínez et al. 1995). In a next step, it should be feasible to use the the test for severity assessment and compare different experimental procedures with regard to their effect. In a similar manner, the access to running wheels was already investigated (rats: Masaki and Nakajima 2008; Lett et al. 2001; hamsters: Antoniadis et al. 2000).
We worked on the development of a CPP protocol using experimental procedures as US. However this proved to be challenging and research in this topic is still ongoing. For this reason, no results on CPPs for severity assessment will be presented here.

## 1.2 T-maze test

The T-maze (or Y-maze) preference test can be used to compare different food or fluid options. With regard to severity assessment, this can be useful to test food rewards under experiment situations, i.e., outside the home cage environment. Usually, mice are food restricted to increase the motivation to participate in an experiment (Sharma et al. 2010b; Deacon 2006). However, food restriction can alter behaviour (mice: Goltstein et al. 2018; Fu et al. 2017; rats: Heiderstadt et al. 2000; Maniscalco et al. 2015) and is itself considered mildly severe (Krüger et al. 2018). Finding a desirable reward might increase the mice's motivation without restricting food, and thus, it could contribute to reduce severity.
The T-maze consists of a T-shaped maze with a start arm and two arms branching off typically in a right angle from the start arm. It can also be filled with water, and in this version mice have to remember the arm which contains a platform to escape the water (Belzung et al. 2001; Guariglia and Chadman 2013). In case of the Y-maze, these goal arms have a wider angle (120°),

leading to the same angle between all arms. The T-maze is most commonly used for assessing the effect of drugs (Correa et al. 2015; Ito and Canseliet 2010), diseases (Belzung et al. 2001; Zhuo et al. 2007; Granholm et al. 2000; Lione et al. 1999) or genetic alterations (Belzung et al. 2001; Mayeux-Portas et al. 2000) on cognitive abilities of mice. There are typically three types of experiments conducted in the T-maze: Investigation of spontaneous alternating behaviour, position discrimination or stimulus discrimination.
The first, spontaneous alternating behaviour, investigates the natural tendency of mice to visit the arm not visited during the previous trial. This is a test referring to the working memory (remembering the own actions) and can also be conducted without placing a reward in the goal arm (Deacon and Rawlins 2006; Wenk 1998; Shoji et al. 2012). In the Y-maze, spontaneous alternation can be tested without distinct trials, and thus, without experimenter interference: The animal is placed inside the maze, where it is then free to explore for several minutes ("continuous alternation", Hölter et al. 2015).
Position discrimination tests on the other hand reward the visit of one particular arm (left or right). This test aims at the reference memory, i.e., the mice have to remember the information on the rewarded arm for a longer time (Deacon 2006; Sharma et al. 2010b; Wenk 1998).
In a similar manner, this is also done for stimulus discrimination, however, here the position of the rewarded arm can change and there is an additional non-spatial cue (e.g., colour, odour) which provides the information on the rewarded arm for each trial (Lione et al. 1999; Granholm et al. 2000; Mayeux-Portas et al. 2000).
For those three test types described above, there are multiple protocols describing an effective procedure. To our knowledge, there is no such protocol for preference testing with the T-maze. Although similar to a stimulus discrimination test in a T-maze, the preference test uses two different rewards (one in each goal arm), which might have an influence on the behaviour of the mice. Thus, before the mice's preference for different food rewards could be tested, a protocol for a T-maze preference test had to be developed. This, in itself, proved to be difficult and is explained in detail in publication 1 (Chapter 3).

## 1.3 Home cage based preference test

The home cage based preference test is suitable to compare different housing conditions, e.g., temperature (Gaskill et al. 2009; Gaskill et al. 2011; Gaskill et al. 2012), ventilation (Baumans et al. 2002; Krohn and Hansen 2010), type (Kirchner et al. 2012; Blom et al. 1996) and height (Freymann et al. 2015; Freymann et al. 2017) of bedding material, cleaning cycle (Godbey et al. 2011) or an enriched environment (de Weerd et al. 1997; Loo et al. 2005; Lewejohann and Sachser 2000). In short, each housing condition is presented in a different cage. By connecting these cages, the mice get free access to the different options, and they can choose in which cage they spend their time. The cage or condition the mice spend most of their time in can then be regarded as the preferred one.
Although there are already many studies which investigate the preferred housing conditions, many questions are still unanswered, e.g., preference of different levels of brightness or humidity. In addition, although it should be common knowledge by now that an enriched environment is preferred by mice, there are only a few studies comparing the modality of enrichment (shelter: Loo et al. 2005; nesting material: Ago et al. 2002; de Weerd et al. 1997). Especially regarding various structural elements (e.g., shelter, tube or an additional platform) or "active" enrichment

(e.g., running wheels or puzzles containing food) little is known. Thus, there is still a need for home cage based preference tests to investigate optimal housing conditions.
However, despite all the research that has already been done in this field, there was no time and cost efficient method available to conduct a home cage based preference test (for a more detailed comparison of existing methods see the Introduction of publication 2 in Chapter 4). Therefore, a new automatic, radio-frequency identification (RFID) based tracking system was needed to facilitate testing. The system should be open-source (if possible) and easy to built so that other research groups could also adopt this method.
Interestingly, the main challenge turned out to be the speed of the mice, as they moved faster through the RFID antennas than detection was possible. As a result, many prototypes were tested which aimed at slowing the mice down: barriers from below and above, flap doors and at some point also automatic doors. The latter was already far away from our original intention to keep it simple. Not only did it involve more complex technical designs but it also required some habituation steps before the mice could participate in an actual home cage based preference test.
In the end, the speed problem was solved by a combination of methods: The technical equipment (meaning the RFID readers) was improved to be as sensible as possible, and barriers were added from above and below in the connecting tunnel between the cages to slow the mice down. Moreover, an analysis program was developed in R which (on the basis of logical reconstruction) can find missing RFID detections in the data set and add them. This is very important for the time stamps, and therefore, also for the correct analysis of the stay time in each cage. We called the whole package – barriers, technical equipment and analysis program – the Mouse Position Surveillance System (short "MoPSS"). This system is explained in detail in publication 2 (Chapter 4).

## 1.4 Aim of this Dissertation

The aim of this dissertation was to investigate the feasibility of choice tests (conditioned place preference test, T-maze test and home cage based preference test) with regard to severity assessment and refinement of experimental and housing conditions. When starting with the research, however, it became clear, that first of all, working protocols (conditioned place preference test and T-maze preference test) or a feasible method for data collection (home cage based choice test) were needed. For all our investigations, it was fundamental that the methods themselves were kept as unstressful for the animals as possible.
In the case of the conditioned place preference test, this research is still going on. For the T-maze preference test, however, we tested two different protocols (publication 1). For the home cage based preference test, we developed an automatic, RFID-based system ("MoPSS"), and showed its practicability for choice tests (publication 2).

# 2 Literature Review: Severity Assessment from an Animal's Point of View

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# 3 || Publication 1: Alternate without alternative: Neither preference nor learning explains behaviour of C57BL/6J mice in the T-maze

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# Alternate without alternative: neither preference nor learning explains behaviour of C57BL/6J mice in the T-maze

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**Abstract**
In rodents, the T-maze is commonly used to investigate spontaneous alternating behaviour, but it can also be used to investigate preference between goods. However, for T-maze preference tests with mice there is no recommended protocol and researchers frequently report reproduction difficulties. Here, we tried to develop an efficient protocol with female C57BL/6J CrL mice for preference tests. We used two different designs, adapting habituation, cues and trial timing. However, in both experiments mice did not show any preference, although we used goods which we knew mice find rewarding. Instead, they alternated choices indicating that exploratory behaviour overruled preference. We argue that this behavioural strategy has evolved as an adaptive trait in saturated conditions where there is no need to take the reward immediately. Therefore, we deem the T-maze unsuitable for preference testing with the procedures we used here.

**Keywords**
T-maze, Y-maze, preference, mice, reward, choice, alternation.

## 1. Introduction

The T-maze is a behavioural test using a maze with a start arm (sometimes connected to a start cage) and two choice arms branching off at the same point from the start arm. In the classic design the arms lie exactly opposite

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each other, so that they form a T together with the starting arm. In the Y-maze variation, the arms branch off from the start arm at a steeper angle so that the overall shape of the apparatus is y-shaped. During a T-maze test, an animal is placed either in the start cage or directly inside the maze at the beginning of the start arm. At the end of the start arm, the animal has then to choose between entering the left or the right arm. Depending on the setup, in addition to the spatial position the arms can provide further cues, e.g., visual (mice: Lione et al., 1999; broilers: Buckley et al., 2011), tactile (compare Cunningham et al., 2006) or olfactory cues (Mayeux-Portas et al., 2000). Also, none, one or both arms can contain a reward, which can be food (Crusio et al., 1990; Deacon & Rawlins, 2006; Deacon, 2006), shelter (Pilz et al., 2020) or a platform (in case of the water T-maze, Granholm et al., 2000; Belzung et al., 2001; Guariglia & Chadman, 2013).

The T-maze is an important behavioural test to assess the effect of drugs (mice: Correa et al., 2015; rats: Lohninger et al., 2001), genetic alterations (mice: Granholm et al., 2000; Mayeux-Portas et al., 2000) or diseases (mice: Belzung et al., 2001; rats: Sánchez-Santed et al., 1997; Wu et al., 2018). It is often used to assess spontaneous alternating behaviour, spatial memory and/or discrimination of stimuli (Dember & Fowler, 1958; Wenk, 1998; Belzung et al., 2001; Dudchenko, 2004; Deacon & Rawlins, 2006; Deacon, 2006; Sharma et al., 2010b). Spontaneous alternating behaviour describes the tendency of rodents to choose the arm they did not visit in the preceding trial. This kind of behaviour occurs spontaneously and is not necessarily related to a resource being exploited in the preceding trial (mice: Gerlai, 1998; gerbils: Dember & Kleinman, 1973; rats: Sánchez-Santed et al., 1997). In position discrimination tests (also: spatial memory tests), only one spatial location, either the left or the right arm, is baited (mice: Lione et al., 1999; Granholm et al., 2000; Belzung et al., 2001; Sharma et al., 2010a; Guariglia & Chadman, 2013; Pioli et al., 2014). Thus, the spontaneous alternating is a way to evaluate the working memory (which location was last visited?), while the position discrimination test evaluates the reference memory (Deacon & Rawlins, 2006), similar to the conditioned place preference test (Wenk, 1998; Sharma et al., 2010b; Shoji et al., 2012; Hieu et al., 2020). In a further modification of the position discrimination, the T-maze can also be used as general discrimination test, using additional cues instead of merely the spatial one to provide information on the baited arm (mice: Lione et al., 1999; Granholm et al., 2000; Mayeux-Portas et al., 2000; broilers: Buckley et al., 2011).

Note that with different tasks different memory types are tested: For alternating behaviour, the working memory is important (remembering which arm was last visited). For position or stimulus discrimination behaviour, the working memory is also important (which cue was rewarded?) but between testing days, this information has to be retrieved from the reference memory (Sharma et al., 2010b).

In a modification of the discrimination test, the T-maze is also used as a preference test: The arms are provided with different goods, and the animal is required to choose between them. This form of preference test seems to be easily performed with a variety of animal species (mice: Roder et al., 1996; Correa et al., 2015; Cutuli et al., 2015; wild mice: Nunes et al., 2009; rats: Patterson-Kane et al., 2001; Ras et al., 2002; Denk et al., 2004; van der Plasse et al., 2007; Cunningham et al., 2015; Hernandez-Lallement et al., 2015; Wadhera et al., 2017; Leenaars et al., 2019; pigs: Rooijen & Metz, 1987; hens: Dawkins, 1977; broilers: Buckley et al., 2011; zebrafish: Hieu et al., 2020; fruit flies: Fujita & Tanimura, 2011). Preference is usually assessed by offering the goods in the choice arms of the maze but in some cases, it might be useful to use stimuli which are associated with the to-be-tested goods instead, e.g., in tests for social preference, the real mouse might be replaced by urinary stimuli (Nunes et al., 2009; compare also Fitchett et al., 2006). It also has to be kept in mind that offering the goods itself can lead to saturation and/or influence the choice in the next trial (Kirkden & Pajor, 2006), in the same way as humans might prefer milk after eating something spicy (Nasrawi & Pangborn, 1990).

Preference tests in T-mazes can be performed with discrete or continuous choices: In a discrete measurement task, an animal has to perform multiple trials in which it can choose between the left or the right arm (mice: Tellegen et al., 1969; rats: Patterson-Kane et al., 2001; Ras et al., 2002; van der Plasse et al., 2007; Pioli et al., 2014). In a continuous measurement task, the animal stays in the T-maze for a defined period of time and the time the animal spends in the left or the right arm is used to ascertain preference (mice: Roder et al., 1996; Cutuli et al., 2015; wild mice: Nunes et al., 2009; Correa et al., 2015; compare also Pennycuik & Cowan, 1990; using a U-shaped maze and wild mice).

There are various protocols and recommendations on the conduction of T-maze tests for behavioural measures such as memory and discrimination.

However, there is to date no protocol for T-maze preference tests: The protocols focus either on spontaneous (unrewarded) alternation (Wenk, 1998; Deacon & Rawlins, 2006), rewarded alternation (Deacon & Rawlins, 2006; Shoji et al., 2012; Wenk, 1998) or position discrimination (Deacon, 2006; Shoji et al., 2012). A short comparison of different protocols is given in Table 1.

In general, for spontaneous alternation, no food restriction or habituation is needed. Animals should just be well-habituated to their environment and the handling, before they are placed into the maze. Protocols for rewarded alternation and position discrimination are more complex and differ in their recommendations. Often, food restriction to 85% of free-feeding weight is recommended, although Deacon & Rawlins (2006) at the same time state that well habituated animals should also perform the T-maze without food restriction (Deacon & Rawlins, 2006). For rewarded alternation, forced trials are recommended, in which animal are only allowed to visit one arm by blocking the other. In the following trial, animals get a free choice with both arms accessible. If the animals visit the previously blocked arm, they made an alternating choice. In position discrimination, on the other hand, no forced trials are conducted, and trials are always free choice. Also, rewarded alternation and position discrimination differ with regard to the recommendations made about cleaning: While for rewarded alternation tasks, cleaning seems to be more common, for position discrimination Deacon (2006) explicitly states that not cleaning maximizes the learning potential (Deacon, 2006). However, protocols for both types of tests differ greatly in their recommendations for habituation procedure (individuals or group, duration, free exploration or trials, reward or no reward) and intertrial interval (immediately or more than 10 min). All protocols recommend at least ten trials per day, but depending on the intertrial interval this leads to differing test durations from 50 min (Shoji et al., 2012) to several hours (Deacon, 2006). None of the protocols gives instructions with regard to testing time, and only one of the protocols (Shoji et al., 2012) provides an example for testing time, but only to emphasise that the tests should be repeated in the same time frame (their example is between 9:00 am and 6:00 pm, with lights 7:00 am–7:00 pm). Searching original studies instead of protocols, the time frame of experiments (if stated) varies, e.g., starting 2 h into the dark phase (Locurto et al., 2002), 3 h before the end of the light phase (Guariglia & Chadman, 2013), 3 h into the light phase (Derenne et al., 2014) or in general ‘during the light phase’ (Moy et

**Table 1.**
Comparison of T-maze protocols by Deacon & Rawlins, 2006; Deacon, 2006; Shoji et al., 2012 and Wenk, 1998.

| Protocol | Species | Test | Food deprivation | | Habituation | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Start | % of free-feeding weight | To reward | To test room | To maze: phase 1 | To maze: phase 2 |
| Deacon & Rawlins, 2006 | Mice | Rewarded alternation | Overnight | >85%, 90–95% is ideal | 1 h before dark phase: 2 ml milk/mouse in hc | 5–10 min | Whole group, 4 × 3 min with 10 min gaps, 4 days, food in maze | Individuals, ? runs for ? days |
| Shoji et al., 2012 | Mice | Rewarded alternation | 1 week before training | 80–85% | Daily: 8 sucrose pellets/mouse in hc | >30 min | Whole group, 30 min, 1 day, sucrose pellets in maze | Individuals, 5 × 5 min per maze compartment (30 min), ? days |
| Wenk, 1998 | Rats | Rewarded alternation | During test | 85%, allow about 5 g weight gain/week | 10 mg food reward/day for a few days before training | ? | Pair of animals (cage-mates), for ? min, 3–4 days, reward in maze | Individuals, for ? min, both arms rewarded, 7–10 days |
| Deacon, 2006 | Mice | Position discrimination | Overnight | >85%, 90–95% is ideal | 1 h before dark phase: 2 ml milk/mouse in hc | ? | Individuals, 6 trials, for ? days, food/drink in maze | Individuals, 1 trial, 1 day, both arms rewarded |
| Shoji et al., 2012 | Mice | Position discrimination | 1 week before training | 80–85% | Daily: 8 sucrose pellets/mouse in hc | >30 min | Whole group, 30 min, 1 day, sucrose pellets in maze | Individuals, 5 × 5 min per maze compartment (30 min), ? days |
| Deacon & Rawlins, 2006 | Mice | Spontaneous alternation | - | - | - | 5–10 min | - | - |
| Wenk, 1998 | Rats | Spontaneous alternation | - | - | - | ? | - | - |

**Table 1.**
(Continued.)

| | Test | | | | | |
|---|---|---|---|---|---|---|
| | Forced trials | Trials | Cleaning | ITI | Goal arm | Cues |
| Deacon & Rawlins, 2006 | (= test) first trial: forced trial, followed by free choice trial | 10/day | Optional between trials: soapy water, alcohol solution (10% is common) or other | Repeat after the 10th animal | The arm opposite the arm accessible in the forced trial (first trial); randomized for each trial, session, animal | ? |
| Shoji et al., 2012 | (= test) each forced trial followed by a free trial | 10/day (max. 50 min) | Between mice: with super hypochlorous water (pH 6–7) | Immediately | The arm not visited during the forced trial | Spatial |
| Wenk, 1998 | (= test) forced trial, followed by free choice trial | 10/day | ? | 0 s to minutes | Randomly varied on each day | Spatial |
| Deacon, 2006 | - | 20–40 | No cleaning to maximize the learning potential | >10 min (otherwise alternation) | The arm opposite the first arm | Paintwork, floor texture or objects |
| Shoji et al., 2012 | - | 10–20/day (max. 50 min) | Between mice: with super hypochlorous water (pH 6–7) | Immediately | Invariable across sessions | Spatial |
| Deacon & Rawlins, 2006 | - | ? | Optional between trials: soapy water, alcohol solution (10% is common) or other | ? | The arm opposite the arm visited last trial | - |
| Wenk, 1998 | - | 10/day | ? | 0 s to minutes | The arm opposite the arm visited last trial | - |

ITI = intertrial interval, hc = home cage, ? = not described.

al., 2008; Shipton et al., 2014). However, day time might influence motivation to gain food (Acosta et al., 2020; Koch et al., 2020) and should therefore be considered carefully.

Thus, there is not 'one perfect test design' with regard to rewarded alternation or position discrimination but various ways to perform it, depending on the research question. However, this makes it difficult to develop a protocol for preference tests. Personal correspondence with other researchers resulted mainly in reports of difficulties in reproduction of the T-maze test, especially when trying to alter the existing protocols for preference tests. In general, varying success rates might be caused by differences in strain performances (Gerlai, 1998; Moy et al., 2008). However, there are various additional factors which might influence results, e.g., differences in handling technique (base of the tail compared to cup or tube handling, Hurst & West, 2010; Gouveia & Hurst, 2017), stress (Mitchell et al., 1985), habituation (Deacon & Rawlins, 2006; Rudeck et al., 2020), level of food restriction (Richman et al., 1986).

One interesting solution for the factor handling is provided by Zhang et al. (2018), who developed an automated T-maze system (Zhang et al., 2018). Here, no handling is involved, and thus, influence of the researcher is reduced. Taking it one step further, Pioli et al. (2014) introduced an automated T-maze which is even home cage based. Here, mice can conduct the test when active and most motivated to work for the reward, which also makes food restriction superfluous (Pioli et al., 2014). However, this automated T-maze is designed for single housing (there is only a companion animal behind a partition), which might not be the desired husbandry condition. In addition, this automated T-maze is meant for spontaneous alternation tasks and it would probably need adjustments for preference tests with regard to, e.g., cue presentation and change of presentation side.

Thus, a working protocol for the conduction of a T-maze preference test is still needed. Here, we performed two experiments in search for such a protocol: In experiment 1, we investigated the preference between two fluids (apple juice vs. almond milk). In experiment 2, we changed the test design and offered one arm containing millet and bedding, and one arm containing only bedding. For both experiments, we used C57BL/6J mice because this is the mouse strain most commonly used; therefore, a working protocol would have the greatest impact for the research community. In addition, we tried to develop a protocol without food or water restriction because this condition itself might change the preference of the mice (see also in the discussion).

## 2. Material and methods

### *2.1. Animals*

A group of thirteen female C57BL/6J CrL mice was purchased in December 2017 at the age of 3 weeks from Charles River, Sulzfeld. This group was used in experiment 1 ('group 1'). Another group consisting of twelve female C57BL/6J CrL mice was purchased in June 2019 at the age of 4 weeks from Charles River, Sulzfeld. This group was used for experiment 2 ('group 2'). We used females because they show less aggression in groups and we needed these large group sizes for other home cage based experiments.

For both groups applies that all mice within a group had different mothers and different nurses to ensure maximal behavioural variability within the inbred strain. At the age of five weeks, transponders were implanted, a procedure performed under anaesthesia and analgesia (for details see the Appendix). Mice were always handled by tube handling. Both groups took part in multiple other experiments, including the development of an home cage based automated tracking system and conditioned place preference tests. By the time the T-maze test was performed, they were around 12 months (group 1, start in November 2018) or 11 months old (group 2, start in April 2020). In the sense of the 3R, we decided to use these groups despite their rather old age. Especially, because the repeatability of activity measures increases with the age of the mice (Brust et al., 2015), and performance levels of C57BL/6J mice in visual detection, pattern discrimination and visual acuity tasks are not decreased with 12 months (Wong & Brown, 2007). It has to be noted that by the start of the experiment 2, eleven of twelve mice in group 2 at least partly lacked their whiskers. This is important as it might influence their tactile-guided behaviour, for example, novel object recognition or open field activity (Haridas et al., 2018; Tur & Belozertseva, 2018). However, this should not have influenced the mice's ability to perceive visual, olfactory or spatial cues (left or right body turn) and to act on them. In addition, as barbering is a model for a disorder (trichotillomania), it is also important to note that mice which barber show no difference in learning ability itself, with the exception of a extra dimensional shift task (Garner et al., 2011). Here, however, only simple learning was required.

### *2.2. Housing*

One group of mice was kept in two type IV macrolon cages (L $\times$ W $\times$ H: 598 $\times$ 380 $\times$ 200 mm, Tecniplast, Buguggiate, Italy) with filter tops. The

two cages were connected via a Perspex tube (40 mm in diameter). This cage system was chosen because of other research purposes, and mice had lived in it since they were around 2 months (group 1) or 3 months old (group 2). Food (autoclaved pellet diet, LAS QCDiet, Rod 16, Lasvendi, Soest, Germany) and tap water (two bottles each cage) were available ad libitum in both cages. Cages were equipped each with bedding material (Lignocel FS14, spruce/fir, 2.5–4 mm, JRS, J. Rettenmaier & Söhne, Rosenberg, Germany) of 3–4 cm height, a red house (The MouseHouse, Tecniplast), papers, cotton rolls, strands of additional paper nesting material, and two wooden bars to chew on. Both cages also contained a Perspex tube (40 mm in diameter, 17 cm long), which was used for tube handling.

Room temperature was maintained at 22 ± 3°C, the humidity at 55 ± 15%. Animals were kept at 12 h/12 h dark/light cycle with the light phase starting at 7:00 am (winter time) or 8:00 am (summer time), respectively. Between 6:30 and 7:00 am (winter time) or 7:30 and 8:00 (summer time) a sunrise was simulated using a Wake-up light (HF3510, Philips, Hamburg, Germany). Once per week, the home cages were cleaned and all mice were scored and weighed. In this context, mice also received a colour code on the base of their tails, using Edding 750 paint markers, to facilitate individual recognition.

### *2.3. T-maze setup*

For the T-maze test, a start cage (type III, L × W × H: 425 × 266 × 155 mm, Tecniplast) filled with 1 cm bedding was connected via a tube to the T-maze. The tube contained an automated door. In experiment 1, the connection between the start cage and the T-maze resembled part of the setup used for habituation so mice were already habituated to it (compare Figure 1a and Figure 1b): a 15 cm tube with an radio frequency identification (RFID) antenna between cage and door, and a 6 cm tube with a light barrier between door and maze. If the mouse interrupted the light barrier in front of the door or was detected by the RFID antenna, the door opened for 5 s. For experiment 2 (without automated habituation), the tube connected to the start cage was 14 cm long and contained an RFID antenna, followed by the automated door and a 1 cm long tube (see Figure 1c). Here, the door also opened for 5 s whenever the transponder of a mouse was detected. There was no light barrier on the other side of the door because this time mice were not allowed to return to the start cage by themselves.

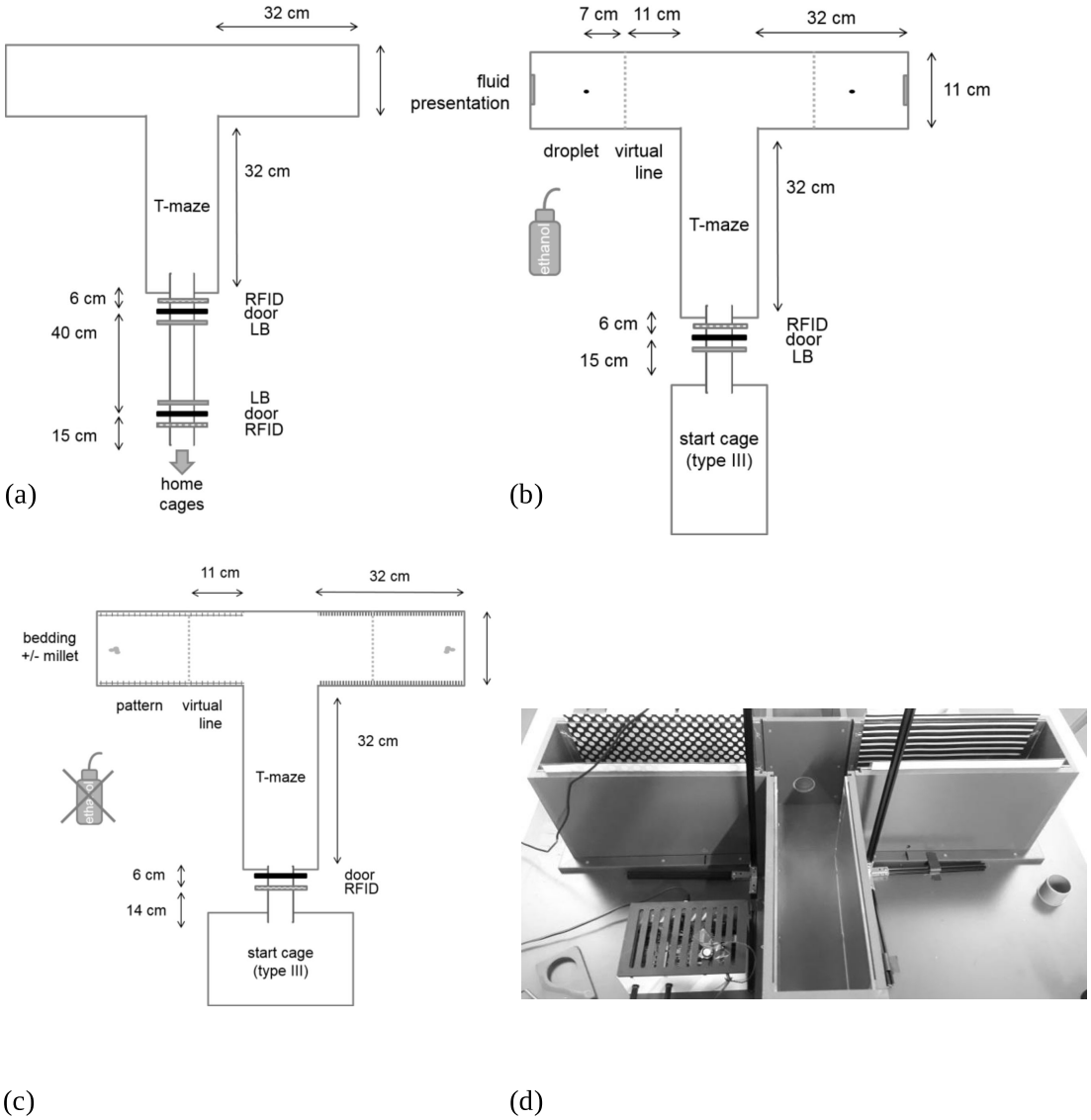



**Figure 1.** T-maze setup as a schematic drawing for experiment 1 habituation (a) and test (b), and test of experiment 2 (c). (d) Photo of the experiment 2 setup, the box on the bottom left contains the Arduino, which operates the automatic door, the device to its left (with the hole) is an example of the RFID antenna and light barrier constructions. LB, light barrier; door, automatic door; RFID, radio frequency identification antenna.

The T-maze itself consisted of grey plastic and had three arms, each 32 cm long and 11 cm wide, with 20 cm high walls (see Figure 1d). On either side of the arms a mark was made outside the T-maze so that a virtual line could be drawn 11 cm from the central arm during video analysis. If a mouse crossed this line with its whole body (but not yet with its tail), this was defined as a choice being made.

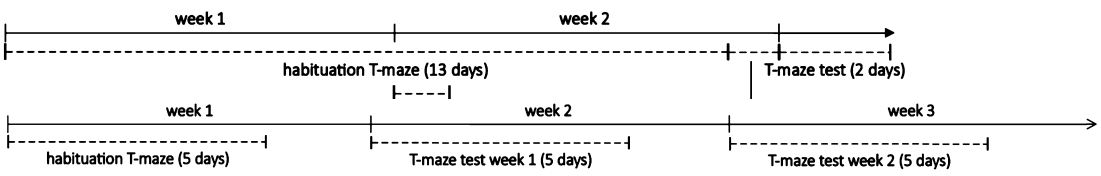
For video recording, in both experiments a webcam (C390e, Logitech, Lausanne, Switzerland) was mounted above the maze on a metal beam construction. The connected computer was placed near the T-maze in such a way that the experimenter could observe the mouse in the T-maze via the computer screen.

### *2.4. T-maze test*

In the first experiment, the T-maze test was used to compare the preference for two fluids. Mice performed discrete choices between the two arms, which contained a droplet of either almond milk or apple juice. Because insufficient habituation might slow the performance in the maze (Deacon & Rawlins, 2006) and might be one of the main problems, we conducted a thorough habituation phase: For about two weeks, mice had free access to the T-maze via a connection to the home cage. After one week, fluids were presented for 24 h inside the home cage. (As the mice drank extensively from the almond milk bottle during that time, a longer presentation seemed unnecessary.) After thirteen days, mice were moved to the testing room, to habituate to it before the start of the actual T-maze test.

The preference test was then performed on two days, with five test trials per mouse per day (based on the protocol of Deacon (2006) which recommends a larger break approximately after five trials), and a side change after the seventh trial to control for side preference (see Figure 2). The test was conducted between 9:00 am and 7:00 pm (lights 7:00 am–7:00 pm), similar to the example provided by the protocol of Shoji et al., 2012. The mice had the choice between almond milk and apple juice, with 20 $\mu$l of fluid as a

(a)




(b)

**Figure 2.** Timeline of experiment 1 (a) and experiment 2 (b). In experiment 2, no habituation to the experimental room was necessary because it took place in the husbandry room. In addition, no habituation to the options (millet with or without bedding material) was necessary because mice were familiar with it from previous experiments.

reward in the respective arm. As an intramaze olfactory cue, we applied some of the fluid onto a cellulose sheet at the end of the arms. In addition, for the first seven trials the spatial intramaze (left/right) and extramaze cues (position in experimental room) remained the same (before presentation side was switched). Between mice, the maze was cleaned with ethanol. During trials, an additional light was added (for more details on the procedure of experiment 1 see Appendix). In this experiment, we expected the mice to prefer the arm with almond milk based on observations made during the initial presentation of the fluids (see Appendix) and results from consumer demand preference tests made in our laboratory (Kahnau et al., data not shown).

In a second experiment we changed the design in several points (see Table 2): active (manual) habituation instead of passive habituation for 3 min on five consecutive days, daily repeated trials instead of block-wise trials, no ethanol disinfection of the maze between mice, no additional light for the T-maze, and intramaze visual cues supplementary to olfactory cues. Also, the choice was now not between two fluids but between millet (0.05 g mixed with bedding material) or no millet (a visually similar amount of bedding material). We changed the reward because we conducted pre-tests in which mice fed more readily on millet than on almond milk outside their home cage. Thus, to increase the likelihood that mice would actually consume their reward, we now used millet. Note that this preference test design now also resembled a learning test because only one arm was baited.

Habituation to the T-maze and the preference test were conducted between 8:00 and 11:00 am (lights 8:00 am–8:00 pm), to keep the test close to the dark phase, and thus, to the active phase, for all animals. To reduce the testing time per day, the preference test was performed on five consecutive days with two trials per mouse per day (leading to the same amount of trials as in experiment 1) and a side change after the sixth trial. Then, after this proved not to show the hoped-for results, a second week was added (see Figure 2b): Again the test was conducted on five consecutive days but this time three trials were conducted per mouse per day (i.e. one trial more than there were options, to have one additional 'test' trial in case the first two function as exploration), and this week, there was no side change. Thus, the visual cues and spatial intramaze (left/right) and extramaze cues (position in experimental room) provided the same information. A comparison of the timeline of both experiments can be found in Figure 2 (for more details on the procedure of experiment 2 see Appendix). In this experiment, we expected

**Table 2.**
Experimental design of the T-maze tests conducted in experiment 1 and 2.

| | | Experiment 1 | Experiment 2 | |
|---|---|---|---|---|
| | | | Week 1 | Week 2 |
| Habituation procedure | Method | Passive | Active | |
| | Duration | 13 days | 5 days | |
| | Habituation trial | 1 (on day 1) | No | |
| General test setup | Cleaning | With 70% ethanol | No | |
| | Illumination | 171–350 lux | 18–50 lux | |
| | Options | Almond milk vs. apple juice | Millet + bedding vs. bedding | |
| Test procedure | Duration | 2 days | 5 days | 5 days |
| | Trials/day/mouse | 5 | 2 | 3 |
| | Side change | After trial 7 (day 2) | After trial 6 (day 4) | No |
| | Cue | Odour | Pattern (+ odour) | Pattern + side (+ odour) |

Further explanations on the procedures, e.g., on the illumination levels in the T-maze arms, can be found in the Appendix.

the mice to prefer the arm with millet based on observations in pre-tests (see Appendix) and enrichment experiments made in our laboratory, in which mice were willing to work (e.g., lift a flap, turn a flap or move a ball) to get access to millet (Hobbiesiefken et al., data not shown).

### *2.5. Statistical analysis*

In short, for the T-maze preference test video recordings were analysed with the help of BORIS (Behavioral Observation Research Interactive Software, Version 7.9.8; Friard & Gamba, 2016), noting the time points (a) when the mouse was placed into the start cage (only experiment 2), (b) when the mouse entered the maze, (c) when the mouse crossed the virtual line in one of the choice arms, 11 cm into the arm, and (d) when it entered the handling tube to be returned to the start cage or the home cage. Each behaviour was only counted when the mouse had all four paws on the bedding of the start cage (only experiment 2) or the whole mouse (except the tail) had entered the maze, the tube, or crossed the virtual line (both experiments).

All time points and choices were filled into a table and further managed with the help of R studio (experiment 1: Version 1.1.383, experiment 2: Version 1.2.1335, using R 3.4.0 or higher). For each mouse, choices were pooled (experiment 1: for both days, experiment 2: per week), and the percentage of choices for one option was calculated. Examined were side preference (left vs. right), the option preference (almond milk vs. apple juice in experiment 1, millet vs. no millet in experiment 2), alternating choices (same arm as before vs. different) and pattern (only experiment 2, dots vs. stripes). The analysis of alternating was done by labelling the choices according to whether the arm chosen in this trial was also the arm chosen in the trial before. The first day of both weeks, respectively, were excluded from this labelling.

The results from all mice were then used for significance testing: To test for normal distribution, the Shapiro–Wilk test was performed in R. The data was normal distributed ($p > 0.05$); therefore, a t-test was used to compare the percentages of the mice with a random chance level of 0.5. In all statistical tests, significance level was set to 0.05, and result values are given as mean and standard deviation. (For more details on the analysis, especially with regard to the passive habituation of experiment 1, see Appendix.)

### 2.6. Ethical approval

All experiments were approved by the Berlin state authority, Landesamt für Gesundheit und Soziales, under license No. G 0182/17 and were in accordance with the German Animal Protection Law (TierSchG, TierSchVersV).

The second experiment was preregistered at the Animal Study Registry (DOI: 10.17590/asr.0000213).

## 3. Results

### 3.1. Experiment 1

#### 3.1.1. Passive habituation

After one week, all mice except for one visited the T-maze frequently. After nine days, all thirteen mice did so. As in retrospect was noted that the RFID registration system might have had a malfunction (although this was not the case when tested before), some passages might have not been detected. However, as the system could not add additional passages, this only means that there might have been more passages to the T-maze then registered, and habituation might have been even better than the RFID data showed.

#### 3.1.2. Trial duration and intertrial interval in the T-maze

In most cases, mice self-initiated the trials: Only in two out of 143 trials (habituation trials and miss-recorded trials included), a mouse did not start the trial by itself within the set start time and had to be guided by tube handling into the maze.

Habituation trials included a visit in both arms. From the time point when the mice entered the T-maze to the time point when the mice had crossed the virtual line in both arms, on average $17.2 \pm 11.6$ s passed (minimum: 7.5 s, maximum: 45.5 s). For the preference test trials, average duration was $4.46 \pm 2.93$ s (minimum 1.25 s, maximum: 25.9 s). Note that in this experimental setup, the way back to the start cage was not blocked so mice could return to the start cage and later on re-visit the maze. The numbers given here are only from those times when a mouse entered the maze and actually crossed one of the virtual lines. Mean intertrial interval (ITI), including cleaning time of the maze and the time until the mouse decided to enter the maze once again, was $204.9 \pm 81.8$ s ($= 3.4$ min), ranging from a minimum of 137 s to a maximum of 506 s.

### *3.1.3. Preference testing*

It was not possible to compare the intake of the offered fluid droplet between apple juice and almond milk on the basis of the video recordings as it was only detectable for the opaque almond milk whether it disappeared. Still, we assessed when the animals spent some time investigating the droplet (licking or intensely sniffing it). This was observed in 74 of 139 trials (including only one time during a habituation trial), representing barely more than half of the trials. 75.67% of these observed behaviours were performed towards an almond milk droplet.

Comparing the choices of the mice for the arm with apple juice or the arm with almond milk, mice chose in $52.8 \pm 9.9\%$ of the trials the arm with almond milk. This indicates no preference ($t = 1.028$, df $= 12$, $p = 0.3242$, see Figure 3). Mice showed also no side preference: The left arm was chosen on average in $49.5 \pm 14.1\%$ of trials ($t = -0.13145$, df $= 12$, $p = 0.8976$). As the T-maze test is often used to test for spontaneous alternation (Deacon, 2006), we then analysed the data with regard to alternating choices. Indeed, mice chose in $64.4 \pm 13.5\%$ of trials the arm which they did not choose during the last trial ($t = 3.8442$, df $= 12$, $p < 0.003$).

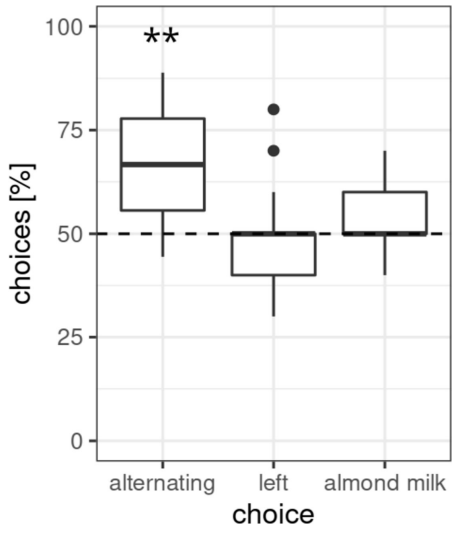



**Figure 3.** Percentage of choices for the arm not visited in the preceding trial (alternating), the left arm and the arm containing almond milk. Thirteen female mice chose 10 times (5 per day) between an arm containing the odour and a 20 $\mu$l droplet of almond milk or apple juice. Presentation side was randomized across the group, and switched after trial seven. $^{**}p < 0.01$.

### 3.2. Experiment 2

### 3.3. Active habituation

Mice were familiar with automated doors from previous experiments. However, in this new setup they seemed to experience the door as something new, so that on day one of habituation, only one mouse went into the maze on its own. Nevertheless, on the fifth day of habituation all mice went into the maze by themselves within the time frame of three minutes.

### 3.4. Trial duration and intertrial interval in the T-maze

Time spent by the mice in the start cage before entering the maze ranged between 1.7 and 159.5 s (on average 21.27 $\pm$ 22.71 s). Inside the maze, the mice took only 3.6 $\pm$ 1.7 s to make a choice and enter one of the goal arms far enough to cross the virtual line (min 1.4 s, max 14.5 s). There, mice spent about 47.4 $\pm$ 33.09 s in the arm before entering the provided tube. After preparing the arms again for the next trial, the mouse was returned to the start cage. This intertrial interval lasted on average 19.4 $\pm$ 8.8 s (min 4 s, max 107 s, caused by an error during the preparation), measuring the time between the mice being taken out of the arm and starting the new trial. Including the time between making the choice and leaving the arm would add the approximately 47 s spent in the goal arm.

### 3.5. Preference testing

In week 1 (two trials per day, side change after trial six), mice chose in 43.3 $\pm$ 8.9% the arm containing millet, which meant that they significantly preferred the arm without it ($t = -2.6018$, df $= 11$, $p < 0.05$, see Figure 4). There was no side preference (left arm chosen in 50.0 $\pm$ 12.8%, $t = 0$, df $= 11$, $p = 1.00$) and no pattern preference (dots chosen in 55.0 $\pm$ 10%, $t = 1.7321$, df $= 11$, $p = 0.11$). However, mice also significantly alternated between arms (63.9 $\pm$ 15.8%, $t = 3.0446$, df $= 11$, $p < 0.05$).

In week 2 (three trials per day, no side change), mice chose in 53.4 $\pm$ $-11.4$% the arm containing millet ($t = 1.0155$, df $= 11$, $p = 0.33$, see Figure 4b). There was no side preference (left: $t = -0.6603$, df $= 11$, 47.8 $\pm$ 11.7%, $p = 0.52$) or pattern preference (dots: 45.6 $\pm$ 10.9%, $t = -1.4062$, df $= 11$, $p = 0.19$) but mice significantly alternated between trials (67.9 $\pm$ 13.4%, $t = 4.5993$, df $= 11$, $p < 0.001$). When looking at the individual trials (see Figure 5), percentage of alternation was especially apparent in the

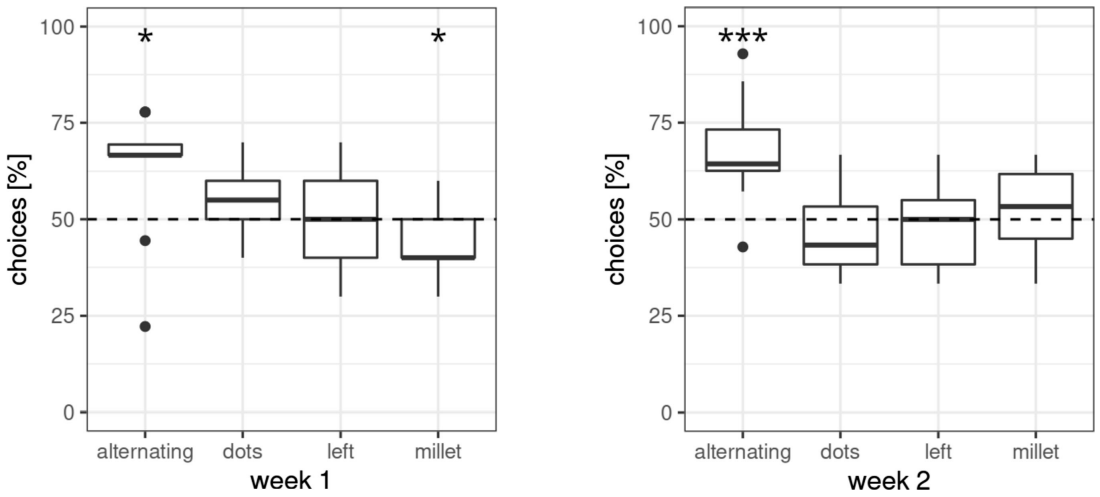



**Figure 4.** Percentage of choices for the arm not visited in the preceding trial (alternating), the arm marked with dots, the left arm and the arm containing almond milk. One group of 12 female mice chose between an arm containing bedding mixed with millet and an arm only containing bedding. Presentation side and pattern (dots or stripes) was randomized across the group. (a) In week 1, two trials were performed per day (10 in total), and after trial six, presentation side was switched. (b) In week 2, three trials were performed per day (15 in total), and presentation side was kept as last used in week 1. $^{*}p < 0.05$, $^{***}p < 0.001$.

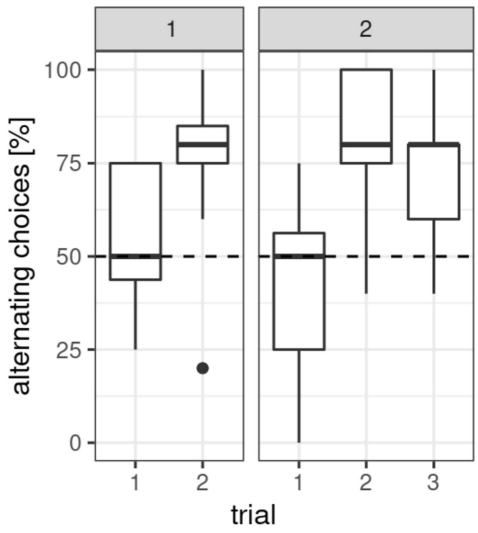



**Figure 5.** Percentage of choices for the arm not visited in the preceding trial (alternating) across trials for week 1 (left, two trials per day) and week 2 (right, three trials per day). One group of 12 female mice chose between an arm containing bedding mixed with millet and an arm only containing bedding. Presentation side and pattern (dots or stripes) was randomized across the group. In week 1, two trials were performed per day (10 in total), and after trial six, presentation side was switched. In week 2, three trials were performed per day (15 in total), and presentation side was kept as last used in week 1.

second and third trial but not in the first, which was compared to the last trial on the day before (week 1: trial 1 52.1 ± 19.8%, trial 2: 73.3 ± 27.4%; week 2: trial 1 43.8 ± 24.1%, trial 2 81.7 ± 19.9%, trial 3 73.3 ± 17.8%).

## 4. Discussion

### *4.1. Habituation*

Mice took on average about 10 s (experiment 1) or 4 s (experiment 2) to make a choice after starting the trial. This implies that mice were well habituated: As Deacon & Rawlins (2006) describe, a trial duration longer than two minutes can indicate insufficient habituation, and here, mice were much faster. However, the two minutes Deacon & Rawlins (2006) use as a bench mark usually include the time from placing the animal in the start area of the maze to the actual choice (Deacon & Rawlins, 2006). We here provided the animal the opportunity to self-initiate the test, which probably conduced to a shorter trial time because trials started apparently when the animal itself was motivated.

However, it is possible that animals were not habituated enough for the preference test itself: Judging on the basis of their behaviour in experiment 1, mice tested the fluid drop only in half of the trials. This might be an indication for insufficient habituation, as during pre-tests before the second experiment, mice fed on millet in an unfamiliar surrounding only after several sessions of habituating to it. In addition, we observed during the pre-tests that millet was consumed more willingly in general than almond milk. Therefore, in experiment 2, one week of active instead of passive habituation to the T-maze was conducted, and we used millet as a reward. Here, all mice fed on the millet when choosing the respective arm. Thus, feeding behaviour in the maze seems to be influenced by both the habituation method and the type of reward.

### *4.2. Lack of preference or reward-aimed behaviour*

In preparation of experiment 1, when offering the two fluids in the home cage for habituation, the twelve mice as a group drank nearly 500 ml of the almond milk in 24 h, whereas they drank only about 200 ml of the provided apple juice. This implies a strong preference. However, no fluid preference was found in the T-maze preference test.

In the same manner, mice should have preferred the rewarded arm (bedding and millet) over the unrewarded arm (bedding only). It is not likely that mice did not revisit the arm because they were sated on millet: In maximum, they could have consumed three times 0.05 g, and in another experiment from our research group, mice received about 0.8 g millet per day and were still willing to work for it (e.g., lift a flap, turn a flap or move a ball, Hobbiesiefken et al., data not shown).

There are various possible reasons for this lack of preference, the main ones being the influence of the cues, and the usage of different foraging strategies, which will both be discussed in the following.

### *4.3. Missing cues*

One explanation for the lack of preference might be a missing perceivable cue on where to find the preferred good. In the first experiment, in addition to spatial information (at least during the first seven trials) an odour cue was provided. However, between the trials, the maze was cleaned with ethanol to erase odour cues. This was done because intramaze odour cues of previous decisions might influence the next choice (rats: Means et al., 1992). Nevertheless, the ethanol itself might have left an odour, masking the olfactory cue of almond milk and apple juice.

We investigated this theory by not cleaning the maze between mice in experiment 2. Although we did not provide an additional olfactory cue on a cellulose sheet as in experiment 1, it can be assumed that the options (millet or no millet) naturally include an olfactory cue. In addition, a visual cue (wall pattern) and a spatial cue (no side change in week 2) were provided. Thus, mice should have had the possibility to learn which of the two arms was the rewarded one. However, this also did not lead to a preference for the rewarded arm.

### *4.4. Foraging strategies*

As the setup of experiment 2 is in general similar to simple learning tests (operant conditioning, learning the relationship between behaviour and its outcome), mice should be able to learn the position of the millet. For optimal foraging, animals should adopt in this scenario the win–stay/lose–shift strategy, meaning that they should stay (or return to) where they found food before and change position when they did not find food (Shettleworth, 2010).

However, it seems we observed a similar result here as described in the study by Locurto et al. (2002), in which offspring of a C57BL/6 and DBA/2J

cross easily learned the win–shift strategy but did not exceed chance levels when requested to perform win–stay (Locurto et al., 2002; also Locurto, 2005). This is in contrast to other studies which successfully report using the T-maze for discrimination tests (spatial or visual) which includes learning of the win–stay strategy (Lione et al., 1999; Granholm et al., 2000; Belzung et al., 2001).

*4.4.1. Memory dependency*

One premise for showing the win–stay strategy would be remembering what was done last time to find food. As trials were performed on multiple days, remembering the last choice made on the day before (which would refer to the reference memory) seemed not possible for the mice, so that the first choice was always based on chance (see analysis of trials, experiment 2, Figure 5). With only two trials per day, a preference based on working memory might also have been disguised in week 1 of experiment 2. However, in week 2, there were always three trials per day. This means even if the mice had not remembered the position of the millet from the day before, after two trials of sampling, the third trial should have been based on a preference. As a result, it could have been expected that a) all third trials were made towards the millet arm, and b) the preference for millet in total was at least in 2/3 of the trials. However, this was not the case as alternation levels in the third trial were similar to the second, and portion of chosen millet arms was about 1/2.

*4.4.2. Partial feeding and refilling*

Another factor that might prevent the win–stay strategy could be that mice found the reward already lying in the arm, instead of receiving a reward when entering the arm (experiencing the arm as empty but then getting food). As a result, when leaving the arm after eating all the millet, they might have memorised this arm as empty.

This might correspond to the findings of Herrmann et al. (1982), who performed a three-table task with rats (without being previously food restricted): After some exploration time in the apparatus, rats received their reward on one of the three tables. If they were allowed to completely feed on the food, rats were able to learn win–shift but not win–stay. If they were only allowed to feed partially, win–stay behaviour was faster shown than win–shift (Herrmann et al., 1982). This indicates that the animals remember whether the feeding place was emptied or not, and it could explain why mice seldom returned to the arm in which they had experienced food beforehand. Thus,

one way of improving the procedure could be to allow only partial feeding in the goal arms.

Another possibility would be to 'show" the mice that the feeding place is refilled. This is inspired by conditioned place preference tests and the study of Goltseker & Barak (2018): Here, conditioned place aversion was only induced when mice were placed in an empty compartment first, and then experienced the onset of the aversive stimulus (in this case: cold water flooding). Conditioned place aversion was not induced when the mice were placed in an already flooded compartment (Goltseker & Barak, 2018). This implies that timing plays an important role for association formation.

However, experiments like the Lashley III maze (Smith et al., 2017) or the cheeseboard task (Lopez et al., 2010) work without partial feeding or the experience of refilling.

#### *4.4.3. Other motivations*

Another factor that might prevent manifestation of the win–stay strategy might be that mice had other motivations than to search for a preferred fluid or a food reward in the maze. To our knowledge, there are no studies investigating this in mice, although this is well-known for birds: As described by Dixon et al. (2013), additional motivations can influence behaviour and the results of preference tests. Here, results of the conditioned place preference test were undermined by the motivation of the birds to search for food or to stay in the more familiar compartment (the one experienced last) (Dixon et al., 2013). This could also be the case here for mice, as further discussed in Section 4.5.4.

However, it cannot be said that mice showed no preference in their behaviour at all. Instead, they showed a clear preference for the arm which they had not visited during the last trial, a behaviour known as 'spontaneous alternation'.

### *4.5. Influences on spontaneous alternation*

Spontaneous alternation behaviour is a common phenomenon in the T-maze (Dember & Fowler, 1958; Deacon & Rawlins, 2006; Sharma et al., 2010b). Although we do not know, what the main cause of the alternation behaviour shown in our experiments is, there are many theories on the factors that influence spontaneous alternation (also reviewed in Richman et al., 1986). In the following we will shortly discuss some of them.

*4.5.1. Arrangement of maze arms*

In the T-maze goal arms are opposite from each other, forcing animals to make a 90° body turn, while in the Y-maze, the turns are 120°. Some studies use both mazes, assessing alternation in the T-maze, while conducting discrimination tasks with the Y-maze (Shipton et al., 2014). On the other hand, when using the Y-maze for spontaneous alternation, animals are usually placed in a start arm to freely explore the maze without interference of the experimenter or distinct trials (called 'continuous alternation', Hölter et al., 2015).

Alternation decreases when both arms lead towards the same goal (Dember & Fowler, 1958). Also, if the arms are positioned not opposite to each other but in parallel, spontaneous alternation is reduced (Novak et al., 2016a, b). Thus, the setup of the T-maze might not be ideally for preference tests.

*4.5.2. Choice of cues*

In mice, influence of spatial and non-spatial cues seems to differ between strains and tasks. C57BL/6J, for example, did not exceed chance level in a spatial discrimination task using extramaze cues but were slightly better in a non-spatial proprioceptive task (left vs. right turn). BALB/cByJ, on the other hand, performed well in both tasks (Crusio et al., 1990). In a different experiment, performing a spontaneous alternation task, C57BL/6J mice seemed to rely mainly on extramaze cues, and had in general higher alternation levels than, e.g., DBA/2 (Gerlai, 1998). In addition, in a more recent study with C57BL/6J × Sv129 mice, it was found that distal visual (extramaze) cues might overshadow proximal (intramaze) cues (Hébert et al., 2017).

In our experiments, we used C57BL/6J mice, and we provided several cues: In both experiments for most of the trials (except for those after the side change) the spatial intramaze cues as well as the non-spatial (proprioceptive) cues were the same. In addition, we provided an olfactory (experiment 1) and a visual (experiment 2) intramaze cue. Moreover, in experiment 2 odour trails from previous trials could have functioned as a cue, in which the maze was not disinfected between the trials. However, as the other studies suggest, all intramaze cues might have been overshadowed by extramaze cues. Although we did not artificially add extramaze cues, we did not change the environment, and therefore, extramaze cues (e.g. position and colour of the walls) could have worked also as sufficient cues. However, it is evident that

the mice did not use any of the provided cues to choose the supposedly more rewarding arm.

Instead, the cues might have influenced alternation as it is discussed that animals might be driven to explore the stimulus which is less familiar, i.e., to which they were not exposed last (Richman et al., 1986). Thus, additional motivations during the test might have masked the motivation to gain food reward.

#### *4.5.3. Intertrial interval*

In general, spontaneous alternation behaviour seems also to be intertrial interval (ITI) time (and thus, memory) dependent. However, regarding which ITIs support spontaneous alternation and which do not, the literature is mixed. Here, in experiment 2, ITI was about 19 s but never longer than 2 min, and in experiment 1, ITI lasted about 3.5 min. This fits to the description made by Deacon (2006) for mice. We can also confirm that for long ITIs alternation drops to chance level (Durantou et al., 1989; Deacon, 2006): Comparing alternation proportions of individual trials for experiment 2 revealed less alternation behaviour during the first trial of each day. Thus, the last choice of the day before (with an ITI $>$ 21 h) seems not to be relevant for the first choice, reflecting that the behaviour is based on the working memory, not the reference memory (Sharma et al., 2010b).

However, one of the problems of comparing the influence of ITIs might be that studies use different definitions what they exactly consider to be the intertrial interval. For example, Locurto (2005) regards the ITI as the time between two trials but with one trial consisting of two forced choice trials and one free choice trial, meaning the time between the forced choice and the free choice trials is not considered (Locurto, 2005).

#### *4.5.4. Food reward and food deprivation*

It is also discussed whether food reward itself influences alternation behaviour, and if so, under which circumstances. Apparently, at least in rats alternation levels are reduced with increasing food deprivation (Richman et al., 1986). This is also implemented in more recent studies with mice, which conduct discrimination tasks with food restriction but alternation tasks without (Shipton et al., 2014). Returning to the topic of the foraging strategies, this implies that the animals switch to win–stay strategy (and away from alternation) only when the motivation to gain food is high enough. In other words: Below a specific food deprivation level, the motivation to explore

what was not experienced in the preceding trial might be higher than the motivation to gain food (Richman et al., 1986). This exploration behaviour could be driven by additional needs, for example, search for shelter (Pilz et al., 2020) or an escape out of the maze (which is commonly used for the Lashley III maze).

In this context, it has also to be kept in mind that it was shown already in the 1960s that conditioned stimuli are not equally effective for all kinds of unconditioned stimuli, for example, gustatory and olfactory stimuli are more easily associated with internal discomfort than audio-visual stimuli (Garcia & Koelling, 1966). This learning phenomenon is probably caused by an evolutionary advantage of facilitated association of specific stimuli. In a similar manner, evolution might have favoured learning mechanisms which cause mice to prefer the win–shift strategy under *ad libitum* food conditions and the win–stay strategy under food restricted conditions. Thus, asking the mice to choose a food rewarded arm over an empty arm might be a completely different question under different feeding conditions.

*4.5.5. Arousal*

It has to be mentioned that an additional important factor for alternation seems to be fear or stress. Under the key word 'optimal arousal theory' multiple studies can be found, which investigate the effect of a mild stressor (open field test), food shock or water presence (water-escape T-maze instead of dry T-maze) on the alternating behaviour (rats: Means, 1988; Comer & Means, 1989; mice: Mitchell et al., 1984; Mitchell et al., 1985; Bats et al., 2001). In general, this theory suggests that individuals seek the optimal arousal, which is shaped in an upside-down U-curve. Thus, when an animal is not aroused it would seek something arousing, for example, a less familiar environment. When the animal is already 'too much' aroused (behind the peak of the curve), however, it would seek the less arousing stimuli, meaning a more familiar environment. This theory tries to explain why after experiencing a mild stressor, mice perseverated their choices instead of alternating (Bats et al., 2001). Mitchell et al. called it the 'punishment paradox' (Mitchell et al., 1984).

Transferring these observations to our experiments, we could conclude that the procedure before and during the T-maze was probably not stressful as our mice did not perseverate but alternate. What we observed was rather the 'alternating paradox', meaning alternating although perseverating was reinforced.

## 5. Conclusion

It is obvious that the T-maze as used in this setup was not suitable to investigate preference or reward-aimed learning in C57BL/6J mice. Instead, mice alternated their choices in 60–70% of the trials. Although the main reason behind this alternation behaviour remains unclear, we can at least validate the statement by Deacon & Rawlins that well habituated animals run the T-maze alternation test well without food restriction (Deacon & Rawlins, 2006). It might be possible to increase performance by imposing deprivation on the animals. However, as we were interested in preference under un-restrained conditions, we deem the T-maze as used here not suitable for our research question. Researchers interested in the T-maze as a means for preference assessment should therefore take caution when designing their tests.

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## Appendix

### *A.1. Transponder implantation*

At the age of five weeks, transponders (FDX-B transponder according to ISO 11784/85; group 1: Planet-ID, Germany; group 2: Euro I.D., Germany) were implanted under the skin in the neck of the mice. To do so, in group 1 all mice obtained an analgesic (Meloxicam) two hours before the procedure. The transponder implantation itself was performed under isoflurane anaesthesia. RFID (radio frequency identification) transponders were injected directly behind the ears subcutaneously in the neck, so that they were rostrocaudal oriented. After transponder implantation, mice were placed in a separate cage with bedding and sheets of paper, and monitored until they were fully awake again. Then they were returned to their home cage. In group 1, two mice lost their transponders after the first implantation, and for those two mice the transponder implantation was repeated at the age of 8 weeks.

For group 2, the administration time of the analgesic was altered to the evening before the procedure because we hoped to reduce transponder loss this way: By administering the Meloxicam earlier, the analgesic effect was expected to cease before the dark phase after the implantation (active phase), and mice would be more hesitant to focus on the injection side. Implantation of the transponders was performed in the same way as in group 1. In group 2, no transponder was lost.

### *A.2. Experiment 1*

#### *A.2.1. Tested goods*

Two fluids ware compared, namely almond milk (3 g sugar per 100 ml; Mandel drink, Alpro, Düsseldorf, Germany) and apple juice (100%, 10 g sugar per 100 ml, made out of concentratel Solevita, Lidl, Kremmen, Germany).

We chose fluids because their odours can work as additional cue without previous conditioning.

During the eighth day of habituation, the two fluids were presented inside the home cage system: One of the usually two water bottles in each cage was replaced by a bottle containing one of the test fluids (500 ml). Originally, it was planned to present the bottles for a few days with randomised positions. However, after the first 24 h the bottle with the almond milk was nearly empty. As there was no leakage of the bottle, we have to assume that the mice drank all of the missing fluid. Health of the mice seemed to be unaffected but we noted excessive urination inside the home cages and the T-maze. Therefore, presentation of the two fluids was immediately stopped.

*A.2.2. Habituation to the T-maze*

Mice were habituated passively to the T-maze for 13 days, during which they could enter the T-maze whenever they were motivated. To do so, the tube between the two home cages was interrupted by a junction, which had a connection to the T-maze via a tube (40 mm diameter). RFID antennas were installed to receive information on the mice visiting the T-maze. Because mice were too fast for the RFID antennas, they were slowed down by two doors. After a first 15-cm-long tube followed one door, then a 40-cm-long tube, a second door, and a 6 cm long tube leading into the T-maze. Each door was directed by an Arduino micro-controller and surrounded by a light barrier (outer side, leading to the home cage or the maze) and an RFID antenna (inner side, leading to next door). Doors opened for 5 s when the transponder of a mouse was detected by the RFID antenna or a mouse interrupted the light barrier. In addition, with the help of two RFID readers also the direction of movement was reconstructable. In this manner, mice could move freely in and out of the T-maze, while their individual stay time was monitored via the RFID readers and stored onto an SD card by the Arduino.

Every day (except for the weekends), the maze was detached from the cage system, washed with water and then cleaned with 70% ethanol to "reset" odour conditions. After the 13th day of habituation to the T-maze, mice cages were transported from their husbandry room to the experimental room. Here, mice had one day to habituate to the new environment before the start of the experiment. Note that this was also already an extended habituation time as the common T-maze protocols recommend from 10 min up to over 30 min for habituation to the test room.

*A.2.3. Preference test*

In experiment 1, the T-maze test (not habituation) took place in an experimental room, and an additional light was placed above the T-maze. Light conditions for the left arm were 171 lux, 201 lux for the right arm, 350 lux for the start/central arm, and 264 lux for the spot between the choice arms.

T-maze testing was conducted on two consecutive days. Mice were habituated to the test room before (see above) and performed five test trials per day, with an additional habituation trial beforehand on trial day 1. Test duration was approximately 40 min per mouse, lasting about 9 h per day for the whole group.

The order of tested mice was randomised for both trial days. In addition, presentation side of fluids was randomised for the mice so that for half of the mice almond milk was presented in the left arm and apple juice in the right, and for half of the mice the other way round. For trial day one, presentation side of the fluids did not change between trials. On trial day two, two trials were performed with fluids presented in the same arm as the day before, while in trials three to five presentation sides were reversed to control for a potential side preference.

Before each mouse, 1 ml of the test fluids was administered on a cellulose sheet and stuck to the walls at the end of a choice arm as an odour stimulus. In addition, a fluid droplet of 20 $\mu$l was placed on the floor of the respective arm as a reward.

All trials were recorded with a video camera (C390e, Logitech, Lausanne, Switzerland) and iSpy 64 (version 7.0.3.0). Before each mouse, maze and start cage were cleaned with 70% ethanol and bedding in the start cage was replaced by new bedding. Then, the additional light was switched on, and the automated system controlling the door was started.

The first trial of the first day was the habituation trial: A mouse was taken out of the home cage by tube handling and placed into the start cage. The mouse had now 5 min to initiate a trial by going through the tube into the T-maze. If the mouse did not enter the T-maze during this time, it was lifted by the handling tube, allowing the mouse only to leave the tube into the tube leading to the maze by blocking the other tube entry. It was then waited until the mouse had entered both arms of the maze (crossed the virtual line with the whole body but not yet with its tail). After additional 30 s, the mouse was returned to the start cage with the help of the handling tube. Before the start of the next trial, the light and the automated system controlling the door

were switched off; this prevented the mice from entering the maze, while the floor was cleaned with 70% ethanol.

After drying the maze and replacing the droplet on the floor, light and automated system were turned on again and the mouse could re-enter the maze. The mouse had 3 min to do so before it was guided by tube handling into the maze. During the test trials, it was waited until a mouse had entered one of the arms (crossed the virtual line with the whole body but not yet with its tail), before it was returned to the start cage with the handling tube. After the last trial, the mouse was returned to its home cage. Between mice, the whole maze including the walls were cleaned with 70% ethanol.

On day two, there was no habituation trial. In addition, a side switch of the fluids took place after trial two; therefore, between trials not only the floor but the complete maze was cleaned with 70% ethanol. Also not only the droplet on the floor but also the cellulose sheet at the end of each arm was renewed.

For video recording, a webcam (C390e, Logitech) was mounted above the maze on a metal beam construction. The connected computer was placed near the T-maze in such a way that the experimenter could observe the mouse in the T-maze via the computer screen.

*A.2.4. Additional notes on the analysis*

During passive T-maze habituation, the two Arduinos automatically saved all RFID detections and additional events (door opened/closed or light barrier interrupted) onto an SD card. Each record included a time stamp (hours, minutes and seconds since start of the Arduino, provided by a real-time clock), milliseconds passed since start of the Arduino, type of event, and the unique RFID transponder number. With the help of R studio (Version 1.1.383), the data sets recorded by the two Arduinos were then tagged with a number for each Arduino and merged. To analyse mouse visits to the T-maze, position changes were extracted (whenever a mouse was detected first by one reader and then by the other), excluding all additional events and RFID detection duplicates (if the same mouse was detected multiple times). Using the time stamps it could then be analysed how long each mouse stayed inside the maze.

In total, 143 trials were analysed, containing 13 habituation trials. Of the 130 preference test trials, five could not be assessed due to camera problems (camera recording stopped unnoticed for one mouse), leaving 125 trials.

Originally, it was planned also to take into account whether the mouse had consumed the reward droplet on the floor or not. However, for apple juice this was not possible: Because of its transparency (in comparison to almond milk), the wet floor left behind looked too similar to the apple juice droplet itself. Therefore, we instead assessed whether the mouse spent some time (>1 s) in which it its behaviour suggested licking or intensely sniffing the droplet.

### *A.3. Experiment 2*

#### *A.3.1. Tested goods*

In pre-tests we observed that millet seems to be a better working reward than almond milk: While mice showed no interest in almond milk when offered in a separate cage filled with home cage bedding, mice immediately fed on millet grains. In addition, after a few sessions of habituation, mice also fed on millet in an empty type-III macrolon cage within one minute after entering the cage. We therefore expected mice to do so in the T-maze after the habituation trials as well.

As the aim of this test was mainly to establish a working protocol for the preference test, we decided against comparison of millet and another reward. Instead, we tested millet against "nothing". In this manner, the test design also resembled a simple learning test. To control for the visual (or exploratory) effect, we provided millet mixed with a specific bedding material in one arm and bedding material (without millet) in the other arm. As bedding material we used the same bedding material as in the home cage (Lignocel FS14, spruce/fir, 2.5–4 mm, JRS, J. Rettenmaier & Söhne, Rosenberg, Germany) as this was a definitely neutral (familiar) cue. Mice were already habituated to the millet in the course of other experiments (including the pre-tests).

#### *A.3.2. Habituation to the T-maze*

While in the last experiment, mice were habituated passively to the T-maze, in this experiment mice were manually habituated to the maze: Mice were placed individually into the maze setup for a short time period on five consecutive days.

This time, the T-maze was installed in the same room in which the mice were usually kept, so no transportation was necessary. In this husbandry room, no other groups of mice were kept during this experiment. Habituation trials were performed between 08:00 and 11:00 in the morning. After

preparation of the setup, the filter top of the home cage system was removed and mice had 10 min to habituate to the illumination change.

For habituation to the maze, mice were taken individually and in a randomized order out of the cage and placed into a start cage, which contained only bedding material and was connected to the T-maze via a tube with an automated door (similar to the setup in the last experiment). Mice had already experiences with automated doors, thus, no habituation to the door was needed. Starting at the moment the mice entered the T-maze, they had 3 min to explore the whole maze. A return to the start cage was blocked by the automated door. If a mouse did not enter the maze within 3 min, it was retrieved by tube handling and held in front of the connection tube with the end to the start cage closed. If it then again did not enter the T-maze within the next 7 min, it was placed directly inside the maze. After 3 min of T-maze exploration, mice were returned to their home cage.

During habituation, maze arms were empty and without visual cues. The maze was not disinfected between mice but it was cleaned (using paper and water) whenever defecation or urination were observed. The exploration behaviour inside the maze was recorded by a video camera (C390e, Logitech) mounted above the maze on a metal beam construction.

It has to be noted that one mouse of the twelve received only four days of habituation: On day one it showed unusual behaviour which might have been correlated with health issues, and therefore, was excluded. As its behaviour returned to normal within two hours (and the maze test does not cause any severity), and the veterinarian had no objection, we decided to start habituation with this mouse on day two. In the course of the following three weeks (one habituation week and two test weeks) there was no unusual behaviour observed.

*A.3.3. Preference test*

In experiment 2, the test took place in the husbandry room and no additional light was added. This led to illumination levels of 18 lux minimum at the end of both arms and 50 lux maximum at the start arm. In experiment 2, choice arms of the maze were covered with patterns.

While in the last experiment preference tests were conducted block-wise (two days with five trials each), in experiment 2 preference tests were conducted on five consecutive days, with only two trials (week 1) or three trials (week 2) per day. This test design should enable an improving habituation to the test with every experimental day. It also allowed flexible addition of test

days if necessary (e.g., if mice had not fed on the millet due to still insufficient habituation).

Between habituation trials and test trials, there was a two day break. Just like the habituation, the preference test took place in the same room in which the mice were usually kept. Tests were performed between 08:00 and 11:00 in the morning, taking approximately 6 (week 1) to 8 (week 2) min per mouse. After preparation of the setup (installing laptop and cameras), the filter top of the home cage system was removed and mice had 10 min to habituate to the illumination change.

For the preference test trials, in one of the maze arms 0.05 g millet mixed with bedding material and in the other maze arm a similar amount of bedding material was placed. Walls of both arms were decorated with patterns: either white dots on black ground or white and black stripes. (Patterns are designed according to the description of Cunningham et al. (2006), except that the colour was inverted.) Combination of pattern, treatment and side were randomized across mice. In week 1, presentation side of the millet was kept the same for six trials, and then the side was switched (similar to experiment 1). In week 2, no side change was conducted.

Each experimental day, following a randomized order a mouse was taken out of the cage and placed individually into a start cage. The start cage contained only bedding material and was connected to the T-maze via a tube with an automated door. The mouse now had 3 min to initiate a trial by entering the T-maze. If a mouse had not entered the maze within 3 min, it would have been retrieved by tube handling and held in front of the connection tube with the end to the start cage blocked. Entering the maze, the mouse had the choice between the rewarded (millet and bedding material) and the unrewarded arm (bedding material). As soon as the mouse crossed a virtual line which was 11 cm into the arm, this was considered a choice. The mouse was given time to feed on the millet, while leaving the arm was prevented by the experimenter's hand holding the handling tube. As soon as the mouse entered the tube, it was returned to the start cage and the procedure was repeated. After the second trial (or third trial, week 2) the mouse was returned to the home cage.

Between the two trials of the same mouse, the maze was not cleaned. Between different mice, the maze was not disinfected but it was cleaned (using paper and water) whenever defecation or urination were observed. Both trials were recorded by a video camera (Logitech C390e, Switzerland)

mounted above the maze on a metal beam construction. As in experiment 1, the connected computer was placed near the T-maze in such a way that the experimenter could observe the mouse in the T-maze via the computer screen.

*A.3.4. Additional notes on the analysis*

In total, 300 trials were analysed (10 per mouse in week 1, 15 in week 2). Of the 300 preference test trials, one missed the time point of the mouse entering the start cage because the video recording started too late.

Originally, it was planned to also take into account how long the mouse spent eating on the millet. However, as in all but two cases the millet was eaten completely (at least as far as visible) and mice had very different feeding speed, we decided against it.

*A.4. Data set*

The data sets of both experiments containing the mice's choices for all trials can be found here: https://doi.org/10.5281/zenodo.4621082.

# 4 Publication 2: O mouse, where art thou? The Mouse Position Surveillance System (MoPSS) - an RFID based tracking system

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# O mouse, where art thou? The Mouse Position Surveillance System (MoPSS)—an RFID-based tracking system

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**Abstract**

Existing methods for analysis of home cage-based preference tests are either time-consuming, not suitable for group management, expensive, and/or based on proprietary equipment that is not freely available. To correct this, we developed an automated system for group-housed mice based on radio frequency identification: the Mouse Position Surveillance System (MoPSS). The system uses an Arduino microcontroller with compatible components; it is affordable and easy to rebuild for every laboratory because it uses free and open-source software and open-source hardware with the RFID readers as the only proprietary component. The MoPSS was validated using female C57BL/6J mice and manual video comparison. It proved to be accurate even for fast-moving mice (up to 100% accuracy after logical reconstruction), and is already implemented in several studies in our laboratory. Here, we provide the complete construction description as well as the validation data and the results of an example experiment. This tracking system will allow group-based preference testing with individually identified mice to be carried out in a convenient manner. This facilitation of preference tests creates the foundation for better housing conditions from the animals' perspective.

**Keywords** Behavior · Preference test · mice · Laboratory animals · Home cage · Group housing · Automated recording · Tracking · RFID · Refinement

## Introduction

Preference tests are increasingly used to improve the housing and living conditions of laboratory animals. Such test procedures allow the animals' point of view to be directly involved in the refinement process. In order to get a meaningful impression of the choices made, the tests should largely reflect normal laboratory conditions and allow to record the choice behavior without interference by an experimenter. This is at best realized using home cage-based preference tests (Habedank, Kahnau, Diederich, & Lewejohann, 2018). For mice, the apparatus for such a choice test usually consists of two (Kawakami et al., 2012; Kirchner, Hackbarth, Stelzer, & Tsai, 2012; Loo, Blom, Meijer, & Baumans, 2005) or more (Ago, Gonda, Takechi, Takeuchi, & Kawakami, 2002; de Weerd, Loo, Zutphen, Koolhaas, & Baumans, 1997; Godbey, Gray, & Jeffery, 2011) connected cages, directly connected via tubes or with a center cage. Animals are given continuous access to the options presented in each cage. In order to measure preference, either the nest position (Loo et al., 2005; Baumans, Schlingmann, Vonck, & van Lith, 2002) or the compartment in which the animals spent more time (Blom et al., 1992; Freymann, Tsai, Stelzer, & Hackbarth, 2015, 2017; Godbey et al., 2011; Kawakami et al., 2012; Kirchner et al., 2012) is then monitored and regarded as the favored one (Habedank et al., 2018).

Thus, home cage-based preference tests are based on binary or multiple choices, and they are designed to rank

Supplementary Material such as 3D printing templates, Arduino code, the R evaluation script, and raw data of the validation experiment can be found under: https://zenodo.org/record/4650404

If there is interest in the video recordings of the validation experiment, please contact us via e-mail.

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preferences, not to assess the strength of preference or the "demand" for this resource (Kirkden & Pajor, 2006). In this manner, the preference of mice was already investigated regarding bedding material (Blom, Tintelen, Vorstenbosch, Baumans, & Beynen, 1996; Kirchner et al., 2012), the provided amount of it (Freymann et al., 2015, 2017), nesting material (Ago et al., 2002; de Weerd et al., 1997), shelters (Loo et al., 2005), cage change interval (Godbey et al., 2011), ventilation (Baumans et al., 2002; Krohn & Hansen, 2010), temperature (Gaskill, Rohr, Pajor, Lucas, & Garner, 2009, 2011; Gaskill et al., 2012) and environment (Kawakami et al., 2012). Further husbandry conditions, which to our knowledge are not yet fully investigated in this manner are, e.g., brightness, humidity, and different items of enrichment such as structural elements or equipment for active engagement.

When conducting a home cage-based preference test, it can be distinguished between the active (dark) and the inactive (light) phase to analyze the data (Freymann et al., 2015; Lewejohann & Sachser, 2000). This is especially important if the tested cage conditions are predominantly associated with active (e.g., running wheel) or inactive behavior (e.g., nesting material). Social species of laboratory animals such as mice are usually kept in groups. Social conditions are likely to influence the choice of individual mice; for example the sleeping temperature might be influenced by the presence of other animals (Gordon, Becker, & Becker, 1998). Thus, generally speaking, animals that are living in groups under normal laboratory conditions should also be tested in groups. However, measuring the preference of a group of mice is a far greater challenge than measuring singly housed mice, and thus, many of the preference studies investigated individual mice instead of groups (Blom et al., 1992, 1996; de Weerd et al., 1997; Kawakami et al., 2007, 2012). When testing groups (Freymann et al., 2015, 2017; Godbey et al., 2011; Gaskill et al., 2009, 2011, 2012; Kirchner et al., 2012), individuals in one group can influence each other (Loo, de Groot, Zutphen, & Baumans, 2001; Shemesh et al., 2013; Valsecchi & Galef, 1989), so that the results from one group might have to be counted as a single unit. More recent advances in statistical methods allow including "group" as a random factor in the model, but still the total number of animals might have to be increased to account for such group effects.

Of the available methods to analyze a home cage-based preference test, most do not carry the capability to sufficiently cope with implicit challenges of choice tests. For example, monitoring only the nest position (Baumans et al., 2002; Loo et al., 2005) causes little costs with regard to equipment and time, but provides mainly information on where the mice spent their inactive time and thus does not reflect temporal distribution of individual preferences. The most common analysis of home cage-based preference tests is therefore done by video recordings (Ago et al., 2002; Gaskill et al., 2009, 2011; Godbey et al., 2011; Kawakami et al., 2007). However, video analysis is very time-consuming, especially when it is necessary to distinguish between individuals. For this reason, some research groups only analyze part of the recordings instead of a continuous tracking (every 5 min: Kawakami et al., 2007; every 10 min: Gaskill et al., 2009, 2011, 2012; every 60 min: Godbey et al., 2011), whereby the time saving is at the expense of the accuracy of the measurement. Analysis of the videos in a more automated manner by using video tracking software (Nath et al., 2019; Noldus, Spink, & Tegelenbosch, 2001; Rao et al., 2019) is by now not advanced enough to ensure decent tracking of individual mice in the husbandry cage.

However, there are other techniques which allow automated tracking: For example, in the connecting tunnels, light barriers can be implemented to record whenever an animal changes cages (Blom et al., 1992, 1996). This method allows easy continuous tracking without much analysis effort. However, this approach is not suitable for group housing because aside from lacking individual detection, the determination of direction of passages is erroneous if sensors can be triggered by more than one animal. Similar problems would also arise if using digital scales below the cages combined with an automated tracking program (Krohn & Hansen, 2010).

To combine automated and individual detection, telemetry can be used by either implanting a rather large, battery-powered transponder (Kawakami et al., 2012) or injecting a smaller, passive transponder for radio-frequency identification (RFID) (Freymann et al., 2015, 2017; Kirchner et al., 2012). The latter method is also very commonly used not just for choice tests but to record general patterns of mice (Bains et al., 2016; de Chaumont et al., 2019; Freund et al., 2013; Weissbrod et al., 2013), rats (Redfern et al., 2017) and birds (Bridge et al., 2019).

All in all, there have been several systems described which automatically track the position of mice. However, these systems are often based on proprietary equipment, only commercially available and expensive (Actual Home Cage Analyzer by Actual Analytics and AstraZeneca: Bains et al., 2016; Redfern et al., 2017; a sorting system by PhenoSys: Winter & Schaefers, 2011; PhenoWorld and other TSE products: Castelhano-Carlos, Costa, Russig, & Sousa, 2014; Linnenbrink & von Merten, 2017). In addition, most of these systems are not designed for preference tests, and thus would need reconfiguration to meet the demands of home cage-based preference tests. This is also the case for tracking software like the closed-source software EthoVision (Noldus et al., 2001) or the non-proprietary software MAPS (Endo et al., 2018), AnimApp (Rao et al., 2019), DeepLabCut (Nath et al., 2019), and

MiceProfiler (de Chaumont et al., 2012), which are not set to track mice in a common husbandry cage with a grid top and optional enrichment. There is further development of the MiceProfiler combined with RFID; however, for this method, two transponders have to be implanted, which is a disadvantage (Weissbrod et al., 2013). Another system, the Mouse Tracker (de Chaumont et al., 2019), uses only one RFID transponder but also does not work in a common husbandry cage. One promising approach is a system that was actually developed for home cage-based preference tests, called the DoubleCage (Tsai, Nagelschmidt, Kirchner, Stelzer, & Hackbarth, 2012). However, this system is also based on proprietary equipment, not freely available and has limited accuracy. Another approach is a study conducted with birds, but they use non-implantable transponders and is geared to detect animal species moving slower than mice (Bridge et al., 2019). Thus, for a home cage-based preference test with group-housed mice, a reliable, low-cost, adaptable, and time efficient analysis method is still missing. (An overview of the described methods so far and their advantages and disadvantages is summarized in Table 1.)

For this reason, we developed an automated system based on RFID that is affordable for everyone (all in all <150 euros), not based on proprietary software or equipment (except for the RFID readers), easy to (re)build, and suitable for individual tracking in group-housed mice: the Mouse Position Surveillance System (MoPSS). It consists of an Arduino MKR WIFI 1010 microcontroller and two RFID controllers with two antennas (with the RFID controllers as the only proprietary hardware we used). In order to read an RFID signal, the transponder has to stay within the electromagnetic field of the antenna for around 30 ms. Mice are capable of very fast movements, and can reach up to 18.0 m/min without training on a treadmill (Billat, Mouisel, Roblot, & Melki, 2005), 23–31.8 m/min after training (Hollinski et al., 2018), 67 m/min on a running wheel (Bono, Adlam, Paterson, & Channon, 2006) and possibly even higher velocities during short sprints and jumping. Therefore, additional barriers were added in the connecting tube between the cages in order to slow down the movements in the vicinity of the antennas. Here, we provide the experimental validation of the system with a group of 7-week-old female C57BL/6J mice as well as the complete implementation description: To facilitate the rebuilding of the MoPSS in other laboratories, we supply the construction plan, the Arduino code, and the 3D print design of the barriers. We also describe an additional analysis method for the data which uses logical reconstruction to further improve the obtained data. With the help of this paper, the MoPSS can be rebuilt by any laboratory and/or altered with regard to example, other species).

## The Mouse Position Surveillance System (MoPSS)

### General principle

The basic experimental setup consists of two cages that are connected by a Perspex tube (40 mm in diameter) passing two RFID antennas (see Fig. 1). As the system relies on RFID, all animals need to have an RFID transponder implanted. We recommend placing it under the skin in the neck region. For best reading performance, the transponder must be implanted lengthwise (rostrocaudal). When a mouse moves through the tube and enters the magnetic field emitted by the RFID antenna, the transponder is read and the transponder number, antenna number, and current timestamp are saved onto a microSD card (32 GB). For the analysis, a mouse detected at the left RFID antenna is counted as being in the left cage, and a mouse detected by the right RFID antenna is counted as being in the right cage. It is possible to subtract the transition duration so as to not add it to one of the cages. However, as mice usually pass very quickly through the tube, we argue that the passage time is neglectable. The main challenge while developing the apparatus was that the mice were too fast for the RFID detectors, i.e., they spent less time than necessary within the read range during the read cycle. In addition, if multiple mice were in the range of the same antenna, interference led to poorer detection as well. Therefore, we added two barriers inside the connecting tube, each obstructing approximately 40% of the tubes' diameter and thereby forcing the mice to slow down in the vicinity of the antennas while passing the barriers.

### Electronics

The MoPSS system consists of an Arduino MKR WiFi 1010 microcontroller with an attached Arduino MKR SD PROTO SHIELD holding a microSD card (Samsung, South Korea) for data collection and control of the RFID reader modules. A small lithium-polymer battery is attached to the Arduino with a 3D-printed mount (Supplement File: MoPSS_Battery_Holder.stl) including a dedicated switch integrated in the housing, to allow disconnecting the battery.

Two RFID reader modules (RFIDRW-E-TTL, Priority 1 Design, Australia) and two external antennas (RFIDCOIL-49A, Priority 1 Design, Australia) are used for reading the RFID signals. In order to protect the antenna coils, a support that fitted exactly around the Plexiglas tubes was used, first premade and later self-built using a 3D printer (files available in the Supplement).

The mainboard for the MoPSS system is built on a perfboard and provides the connections between the

**Table 1** Described methods available for home cage-based preference tests

| | **Home cage-based preference tests with mice** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Baumans et al. (2002); Loo et al. (2005) | Blom et al. (1992, 1996) | Krohn and Hansen (2010) | Kawakami et al. (2007); Ago et al. (2002) | Godbey et al. (2011); Gaskill et al. (2009, 2011, 2012) | Kawakami et al. (2012) | Kirchner et al. (2012); Freymann et al. (2015); Linnenbrink and von Merten (2017) | |
| Method | Nest position | Red light sensors | Digital scale | Video recordings | Video recordings | Telemetry | RFID antenna | |
| Group housing | ✓ | – | – | – | ✓ | – | ✓ | |
| Individual tracking | – | – | – | ✓ | ✓ | ✓ | ✓ | |
| Continuous tracking | – | ✓ | – | – | – | ✓ | ✓ | |
| Home cage compatibility | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | |
| Open source | ✓ | ✓ | – | ✓ | ✓ | – | – | |
| Comments | | | | | | | | |
| | **Activity monitoring** | | | | | | | **One-way sorting system** |
| | Bains et al. (2016); Redfern et al. (2017) | de Chaumont et al. (2019) | Weissbrod et al. (2013) | Noldus et al. (2001) | Rao et al. (2019); Nath et al. (2019) | Endo et al. (2018); de Chaumont et al. (2012) | Bridge et al. (2019) | Winter and Schaefers (2011); Linnenbrink and von Merten (2017) |
| Method | RFID antenna + video recordings | RFID antenna + video recordings | RFID antenna + video recordings | Video tracking | Video tracking | Video tracking | RFID antenna | RFID antenna + doors |
| Group housing | ✓ | ✓ | ✓ | ✓ | – | ✓ | ✓ | ✓ |
| Individual tracking | ✓ | ✓ | ✓ | ✓ | – | ✓ | ✓ | ✓ |
| Continuous tracking | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Home cage compatibility | ✓ | – | – | – | – | – | ? | ✓ |
| Open source | – | ✓ | ✓ | – | ✓ | ✓ | ✓ | – |
| Comments | | | Two RFID transponders used | Cage has to be nearly empty | Cage has to be nearly empty | Cage has to be nearly empty | Optimized for birds | |

Methods are sorted by their purpose: used in home cage-based preference tests, used for activity monitoring but in general applicable for preference tests and used as a one-way sorting mechanism, which would either have to be re-programmed, or of which two would have to be used, for each direction one. The capabilities of the systems were derived from the papers and what the authors described there

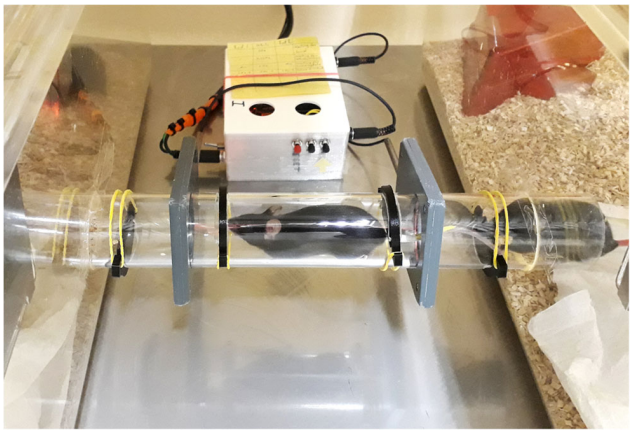

**Fig. 1** Setup of a home cage-based preference test using the MoPSS. Two cages are connected via a tube with four barriers and two RFID antennas

Arduino and the RFID modules. Three LEDs for visual feedback, and three push buttons for user input and reset are added. The mainboard also provides pin header connections for the push buttons, antenna barrel connectors, and the power connector (Fig. 2).

The box for the MoPSS system is printed using polylactic acid (PLA) and consists of a bottom unit with a cutout for easy access to the microSD card and mounting holes for the buttons, etc. A lid with venting holes for the box is also included (Supplement File: MoPSS_Case.stl and MoPSS_Lid.stl).

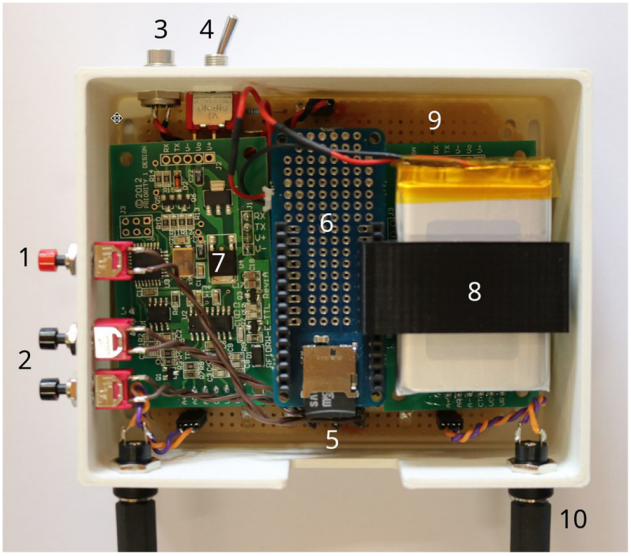


**Fig. 2** Inner workings of the MoPSS: 1 reset button, 2 button B1 and B2 for user input, 3 power connector, 4 battery on/off switch, 5 microSD card, 6 MKR SD SHIELD, Arduino below, 7 RFID reader module, 8 lithium-polymer battery with holder, 9 mainboard, 10 antenna connector

## Barrier construction

Barriers were implemented to slow the mice down while moving through the 31-cm-long tube (diameter: 4 cm). To achieve this, we applied four barriers: For both RFID antennas, a barrier from below (5 cm from the end of the tube) and a barrier from above (10 cm from the end of the tube) are inserted (see Fig. 3). To install the barriers, 5-mm-wide slits have to be cut into the tube. Barriers block about 40% of the tubes' diameter and are 4 mm wide. The barriers are made with a 3D printer (Ultimaker 3 Extended, Ultimaker B.V., The Netherlands) using Ultimaker black PLA as material. They are designed with two hooks on either side, so they can be easily inserted into the tube and fixed with a rubber band. The barrier template for the 3D printer is offered (Supplement File: Barrier.stl). In addition, to facilitate the cutting of the tube, a 3D template is provided (Supplement File: Gauge_Tunnel_Barriers.stl), which assists in drawing exact cutting lines onto the tube.

## Transponders

We use transponders according to ISO 11784/85 (FDX-B transponders, Euro I.D., Germany). The transponder needs to be implanted rostrocaudal for optimal detection sensitivity. The best read performance is achieved when the RFID transponder is oriented lengthwise (0°/180°) to the antenna where read ranges of approximately 4 cm can be achieved. If a transponder were oriented transversely (90°/270°) to the antenna, the read range would approach 0 cm. For more details on the transponder implantation procedure, see section Experiment 1, Animals.

## Software

The Arduino and RFID reader modules each run different software. The RFID modules use proprietary software while the software for the Arduino is available in the Supplement.

**RFID modules** The RFID modules are connected to an antenna each in order to read the unique number of the RFID tag that is within read range and transmit this tag number to the Arduino.

As soon as an RFID tag enters the read range of the antenna, the tag number is read by the RFID module and transmitted to the Arduino. However, the tag number is only transmitted when the tag newly enters the read range.

In order to eliminate interference between the two RFID antennas in close proximity, we decided to enable only one RFID reader at a time for 100 ms, alternately switching between both. As a consequence, every time an RFID reader is re-enabled, any tag it reads will be automatically transmitted because the tag appears as "new" to the RFID

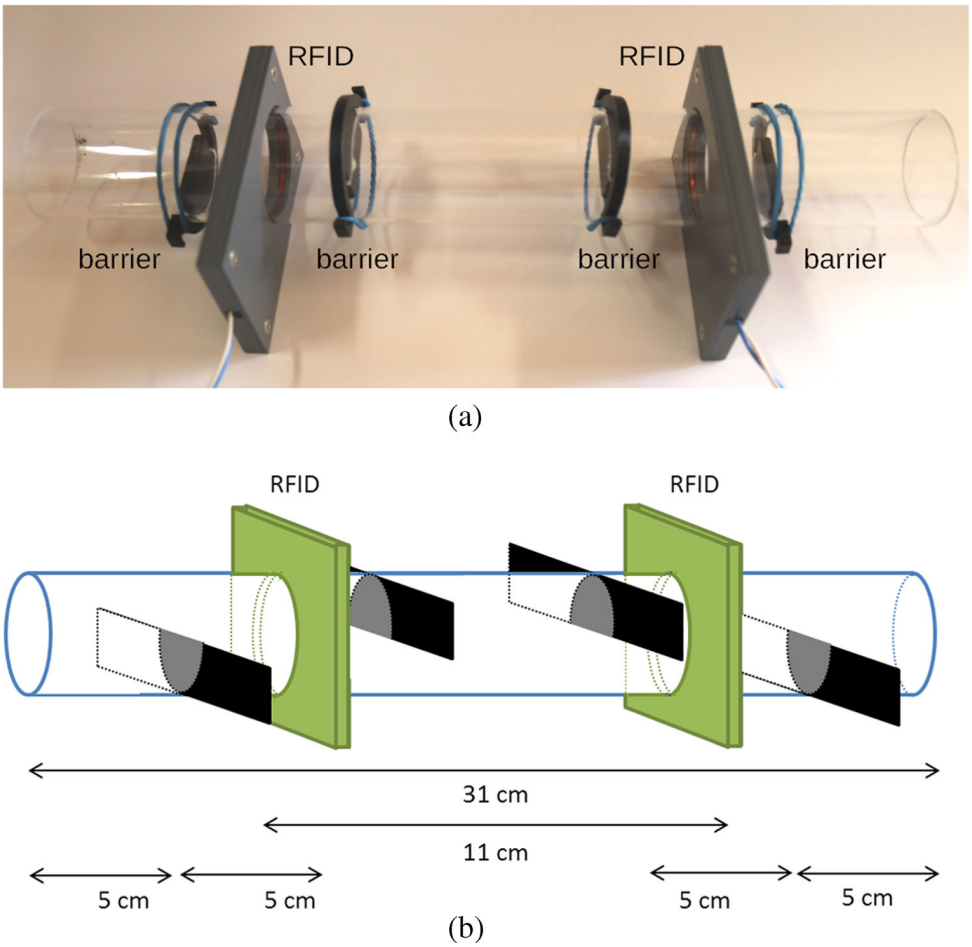


**Fig. 3** Picture of barrier construction (**a**) and schematic drawing (**b**) of barrier construction. *RFID* RFID antennas, *black* barriers

reader. This enables us to easily detect when an RFID tag is no longer within the read range of the reader.

**Arduino** The Arduino is handling the processing of the RFID tag numbers that are communicated by the RFID modules and adds additional functionality such as visual feedback and logging. Additionally, the Arduino controls charging of the battery that allows coping with short-term power loss.

During startup, the Arduino connects via Wi-Fi to the Internet in order to update the internal real time cock, which is then used during logging to provide accurate timestamps for all RFID tag detections. For the timestamps, the Unix time is used, which is easily processed in further analysis and indifferent to time zones. After successful synchronization, the Wi-Fi on the Arduino is no longer required and turned off, thereby greatly reducing power consumption. The battery allows independent operation of the Arduino, guarding the system in case of external power loss for roughly 26 h. Even though RFID capability is lost while running on battery power, the reader modules will restart without adverse consequences once power is restored. Battery power can also be used for the startup of the MoPSS system at a different location, for example, if there is no Wi-Fi available inside the animal facility.

The Arduino also controls the LEDs on the mainboard communicating the different states between power on and ready for operation. At the time of writing these are: “searching for Wi-Fi network”, “fetching time from network time protocol server;”, “ready for operation”, and “error during setup” indicating a faulty/missing microSD card, inability to connect to the network/synchronize the time. During operation, two red LEDs corresponding to the two RFID reader modules are also used to indicate the detection of a tag.

In the event of a successful RFID tag detection, the Arduino saves the data to the microSD card: the antenna number by which the tag was read (A1/A2), the current time (e.g., 1567081062), the tag number (e.g., 900_200000123456) and a flag (E) indicating that this detection corresponds to a mouse entering the read range. When the transponder is no longer detectable, an additional

**Table 2** Example of the recorded data provided by the MoPSS

| Antenna no. | Unix time | Tag number | **E**ntry/E**X**it flag |
|---|---|---|---|
| A1 | 1567081062 | 900_200000123456 | E |
| A1 | 1567081063 | 900_200000123456 | X |
| A2 | 1567081071 | 900_200000123456 | E |
| A2 | 1567081072 | 900_200000123456 | X |

entry is made containing the antenna number, current time, the tag number and the flag X to indicate an exit from the read range. See Table 2 for an example.

### Data evaluation

Although accuracy of the RFID detections was very high (see section Experiment 1 Validation, Results), there were still a few missed detections. We therefore conducted an in-depth analysis of the possible combinations of missed detections with the known detections to identify cage changes despite missing data. The resulting R script can systematically analyze raw data and reliably reconstruct cage changes in the few cases of missing detections. The complete description of this procedure can be found in the Supplementary Material.

## Experiment 1: Validation

In order to compare the accuracy of the MoPSS to manual video analysis, we performed a validation experiment using both methods in parallel.

### General procedure

A group of 12 young mice was habituated for 6 days to the MoPSS, including the barrier system in the connection tube before a 24-h video recording was performed. The video recording was then analyzed with regard to cage changes, and these were compared to the cage changes detected by the MoPSS.

### Animals

We chose C57BL/6J CrL mice because this is the most commonly used mouse strain. Twelve female C57BL/6J CrL mice, kept as one group, were used for this experiment. They were purchased in June 2019 at the age of 4 weeks from a commercial breeder (Charles River, Sulzfeld, Germany) and had different mothers and had different nurses to prevent any breeding-related effects. At 5 weeks of age, transponders (FDX-B transponder according to ISO 11784/85, Euro I.D., Germany) were implanted

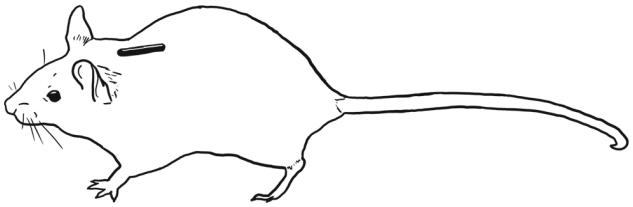

**Fig. 4** Schematic drawing transponder position. ©Anne Habedank

subcutaneously in the neck region (see Fig. 4). In order to prevent potential harm inflicted by the implantation procedure, the mice obtained an analgesic (Meloxicam) the evening before implantation. The transponder injection itself was performed under anesthesia (Isoflurane) and the RFID transponder was injected directly behind the ears subcutaneously in the neck, so that it was oriented rostrocaudal. After transponder injection, the mice were placed in a separate cage with bedding and paper for monitoring until they were fully awake again. They were then returned to their home cage.

### Housing

In the first weeks, the mice were kept in a type IV Makrolon cage (L × W × H: 598 × 380 × 200 mm, Tecniplast, Italy) with a filter top. Food (autoclaved pellet diet, LAS QCDiet, Rod 16, Lasvendi, Germany) and tap water (two bottles) were available ad libitum. The cage was equipped with bedding material (Poplar Granulate 2-3 mm, Altromin, Germany) of 3–4 cm height, two red houses (The MouseHouse, Tecniplast), four papers, four cotton rolls, 12 strands of additional paper nesting material, and four wooden bars to chew on. The cage also contained a Perspex tube (40 mm in diameter, 17 cm long), which was used for tube handling (Hurst & West, 2010; Gouveia & Hurst, 2013).

For the validation of the MoPSS, when the mice were 6 weeks of age, they were moved into two type III Makrolon cages (L × W × H: 425 × 276 × 153 mm, Tecniplast, Italy) with filter tops connected via a Perspex tube (40 mm in diameter, 30 cm long) containing barriers from above and below (blocking 40% of the tube diameter with a thickness of 4 mm; see description of barriers in the "Barrier construction"). The equipment described above for the type IV cage was equally split unto the two type III cages, except that only one cage contained the handling tube.

Room temperature was maintained at 22 ± 3 °C and the humidity at 55 ± 15%. Animals were kept at a 12 h/12 h dark/light cycle with the light phase starting at 8:00 a.m. (summer time). Between 7:30 and 8:00 a.m., a sunrise was simulated using a Wake-up light (HF3510, Philips, Germany). Once per week, the home cage system was cleaned and all mice were scored and weighed. In this

context, the mice also received a color code on their tails (using an edding 750 paint markers) to facilitate individual recognition during video recording.

## Procedure

With 6 weeks of age, the 12 female C57BL/6J mice were transferred into the test system, consisting of two cages connected with a tube containing four barriers and two RFID antennas (for details see "Housing" and "Barrier construction"). After 6 days of habituation to this setup, video recordings of the tube were made for 24 h. To ensure continuous recording of mouse movement, we installed a red light source, which was automatically switched on during the dark phase. The video recordings were conducted with a webcam (Logitech C390e, Switzerland) using the recording software iSpy 64 (version 7.0.3.0), which automatically cut the videos into blocks of 1-h duration. The webcam was positioned in a way that ensured a clear view of the connecting tube and the MoPSS, which signaled every RFID detection via two separate red LEDs.

Afterwards, we collected the recorded data from the MoPSS and compared the detected cage changes with the 24-h video recordings: We fast-forwarded the video recordings until a mouse was visible and, slowing down the video, then monitored whether the MoPSS signaled via a blinking LED that the RFID tag number of the mouse was detected. In some cases, more than one mouse passed through the tube and an additional evaluation whether or not all mice were detected was conducted: The recorded data from the MoPPS were examined to verify that all RFID tag numbers were recorded at the corresponding timestamp. All missing detections were noted.

As described in "Data evaluation", in addition to just using the data as it was saved by the MoPSS, we also developed a method to improve the received data by means of logical reconstruction (searching the recorded data for inconsistencies in the order of cage changes; for details see "Data evaluation" and the Supplements). In the process of evaluating the R script for this logical reconstruction, parts of the video recordings were watched again to compare the results of the script against the true events.

## Results

During the 24 h, 7382 detections were recorded, including 2804 cage changes. On average, there are more than twice as many detections as cage changes because mice do not always change cages but sometimes also just stick their nose inside the RFID antenna (poke) and then return to the cage they came from. After a manual comparison of the recorded detections with the 24-h video recordings, we found nine missed detections, meaning an event in which one of two antennas did not detect the mouse (situation B and C from section *Data Evaluation*, Supplements). This led to an error rate of 0.122% of all the cage changes. There was no cage change detected on video for which both antennas did not detect the mouse (situation D from section *Data Evaluation*, Supplements), which would have not been possible to reconstruct due to the missing timestamps.

After analyzing the data by means of logical reconstruction (as described in section *Data Evaluation*, Supplements), we were able to infer the nine missing detections automatically and correct the corresponding cage changes. In this manner, the error rate was reduced to 0%.

Analyzing the detections, we found that dwelling time between the readers was on average 1736 ms $\pm$ 8255 ms, with 87.33% of cage changes taking $\leq$3 s and 94.27% taking $\leq$5 s.

## Discussion

Validating the MoPSS' detection with manual video analysis, we confirmed that the MoPSS reaches a very high accuracy. After logical reconstruction, the MoPSS detection matches 100% with the results of the manual video analysis. The only divergence arises in the timestamps—when one of the two antennas missed the passage, the timestamp of the second antenna had to be taken (as explained in section *Data evaluation*, Supplements). However, we can assume that the mouse was missed by the antenna only because it moved too fast out of the antenna's read range (about 5 cm before and behind the antenna). Thus, we argue that the missing timestamp and the timestamp from the second antenna should be differing only by a few seconds from the correct time, and it is reasonable to use it to replace the missing timestamp.

Note that the error rates reported above are only results of one group of mice, and thus they might not be representative for other groups, especially when differing in age, strain, or sex. Still, we regard the chosen test group as the optimal one for its purpose: The main difficulty, as explained above, was the velocity of the mice, and that is why we used very young and thus fast animals. The mice had 6 days of habituation to adjust to the new barrier setup. However, it is possible that the mice were not at their highest possible speed. In the study by Bono et al. (2006), it is described that maximum continuous speed increased until day 17 of training for female C57BL/6J mice (10 to 11 weeks old). Hollinski et al. (2018) described an increase in maximum continuous speed up until week 8 of training. Nevertheless, these studies were conducted on running wheels, whereas for our experiment the maximum speed over a distance of approximately 8 cm in a straight line is the most relevant, as this is the range of the RFID antenna.

We believe our manual video analysis can be considered nearly flawless because, when in doubt, videos were played backwards or in slow motion. This also emphasizes the improvement the MoPSS is going to make, as an accurate analysis by video was very time-consuming.

Comparing the MoPSS' accuracy to the other available methods for home cage-based preference tests (which were described in the Introduction) proves difficult. First, accuracy can only be compared to manual analysis, which would make video recordings automatically the most accurate method. However, as we experienced during the development of MoPSS prototypes, especially when using group-housed mice, even manual analysis can be complicated. When mice climbed over each other, they were sometimes not distinguishable without the information provided by the RFID antennas.

Second, comparing the MoPSS' accuracy to other automated tracking systems is in some cases not possible because the studies do not provide any information on accuracy (Krohn & Hansen, 2010; Linnenbrink & von Merten, 2017) or any details on the tracking system except that they used one (Kawakami et al., 2012). We, on the other hand, reported very detailed how the accuracy was measured.

Third, of the remaining two automated tracking systems, the one described by Blom et al. (1992) only uses individually housed mice, which makes data acquisition far easier, but with the disadvantage that the transferability of gained results for group-housed mice remains questionable. In addition, Blom et al. (1992) and Tsai et al. (2012) use a correlation between relative dwelling times per cage based either on visual observations or automatically registered cage changes. This, however, does not provide general information on the error rate of the system; it merely states that there is no significant difference between the results. This, however, would change if a cage change was missed after the mice had stayed in this cage for several hours. The paper by Tsai et al. (2012) offers an error rate with 0.26% of misreported cage changes. In comparison, the MoPSS has an initial error rate (before logical reconstruction which corresponds to RFID reader accuracy) of 0.122% for missed detections. As explained in the section "Data evaluation", missed detections do not have to lead to a missed cage change if the first RFID antenna the animal was passing through was the one with the missed detection because only the second RFID antenna reports an actual change in position.

Fourth, it has to be noted that currently no automated tracking system can reach 100% accuracy at all times (without additional analysis of the data afterwards) because at this time, there are situations which cannot be identified by automated systems. For example, when a mouse passes through an antenna and another mouse passes the antenna at the same time, two RFID transponders are within the detection range and one RFID tag may obscure the other. However, this is a very rare scenario. Overall, we demonstrated that the MoPPS is equally accurate as video observation and much superior with regard to time taken for analysis.

## Experiment 2: Example data

### General procedure

Experiment 2 is an example of a home cage-based preference test conducted with the MoPSS. Please note that this preference test was performed to show that the MoPSS has the capability of tracking even a group of 12 mice easily. It is not our recommendation to conduct preference tests in such large groups, and because of that, the result of this experiment should not be generalized (see also *Discussion*). Two types of bedding material were compared, using one group of 12 mice. The preference test was performed in two consecutive rounds of 3 days each. Between rounds, the presentation side of the bedding materials was changed, starting the new round with freshly cleaned cages. The MoPSS was active during the whole duration of the experiment; however, only the second day of both rounds was used for analysis, providing the first day for habituation.

### Hypothesis

We conducted a home cage-based preference test comparing two bedding materials: Pure (cellulose, JRS) and Comfort White (cellulose, JRS). Both bedding materials were known to the mice because they were used before in a conditioned place preference test as the conditioned stimuli. In this test, mice had shown a significant preference for Comfort White bedding during the 10-min habituation as well as during the final test after conditioning. Now, we wanted to investigate whether this preference would persist if mice had not only 10 min but several days of continuous access to the bedding materials.

### Animals

Another group of 12 female C57BL/6J CrL mice was used for this experiment. This group was purchased in December 2017 at the age of 3 weeks from Charles River, Sulzfeld. The mice were born to different mothers and had different nurses in order to cope for any possible effects on behavior related to the prenatal and early postnatal phase within the inbred strain. With about 5 weeks, transponders (FDX-B transponder according to ISO 11784/85, Planet-ID, Germany) were implanted under the skin in the neck. The procedure was the same as for the group in Experiment 1,

except that Meloxicam was given 2 h before the procedure instead of the previous evening. In addition, for two mice, the transponder implantation had to be repeated at the age of 8 weeks because they lost their transponder immediately after the first implantation.

This group of mice took part in multiple testing of prototypes to develop an automated tracking system. By the time the home cage-based preference test was performed to gain example data with the MoPSS, they were around 19 months old. In between, mice had also participated in other experiments, e.g., T-maze preference tests and conditioned place preference tests (the latter were pre-registered at the Animal Study Registry: Lewejohann Lewejohann, 2019a, b the former took place before the launch of the Animal Study Registry).

## Housing

Outside experiments, mice were kept in two type IV Makrolon cages (L × W × H: 425 × 276 × 153 mm, Tecniplast, Italy) with filter tops connected with a Perspex tube (40 mm in diameter), which was equipped in the same way as the two type III cages described for the group in Experiment 1.

## Procedure

Because this group of mice was usually kept in a home cage system with two connected cages, those cages were identically equipped as always, except that we changed the normal bedding material for different ones: One cage was filled with Pure bedding (cellulose, Arbocel pure, JRS, J. Rettenmaier & Söhne GmbH + Co KG, Germany) and one with Comfort White bedding (cellulose, Arbocel comfort white, JRS, J. Rettenmaier & Söhne GmbH + Co KG, Germany) up to the same height of 3 cm. Both beddings consisted of cellulose, while the usual bedding consisted of conifer wood (spruce/fir). For a picture of the different bedding materials, see Fig. 5. The connecting tube was similarly designed as described in Experiment 1, however, we only added barriers from below to facilitate their passing through the tube. This group was older, and one mouse was unusually hesitant towards new objects, which had already been observed during several other experiments, and we did not want to exclude it.

As it is possible that the spatial position in the room (and its light, noise, room air conditions) influences the preference of the mice (Blom et al., 1992), we performed two rounds, between which the presentation sides of the bedding materials were changed. This ensures a discrimination between side and bedding preference. The experiment lasted 7 days, with 3 days presenting bedding material Pure left and Comfort White right (round 1), then switching sides and presenting Pure right and Comfort White left to control for a spatial bias (round 2). On the first day of each round, the mice were placed into freshly cleaned and newly equipped cages, placing individual mice alternately into the left and right cage, dependent on the order they entered the handling tube. The first day was considered as a habituation day to get the mice accustomed to the new bedding material. The second day was then used for actual data recording. The third day was added for organizational reasons: After approximately 23 h of the third day, the mice were then taken out of the test setup and placed into a separate cage (which contained the spruce/fir bedding they usually had), while preparing the new setup. Mice were then placed into a freshly cleaned and newly equipped cage, this time with changed presentation sides of the bedding. Only the food was maintained; pellets of both cages were mixed and split for the two new cages. The tube connecting the cages as well as the barriers were not cleaned in between. In the second round (just as in the first


**Fig. 5** Bedding materials used during the experiment. Comfort White (**a**) and Pure (**b**) bedding material were compared in the home cage-based preference test and consist of cellulose. **c** Poplar Granulate bedding material consists of poplar chips. This bedding material was not used in the home cage-based preference test but was used during normal husbandry conditions

round), only the second day was analyzed, leaving the first for habituation.

## Statistical analysis

During the preference tests, RFID detections by the two RFID antennas were automatically saved by the Arduino onto a microSD card. Each record included a timestamp (synchronized before the start of the experiment via an Internet connection), antenna number (A1 or A2) and the detected RFID tag number. With the help of R studio (Version 1.1.383, requiring on R 3.0.1+), the data recorded by the Arduino were analyzed for missing detections (see section *Data evaluation*, Supplements). Following this procedure, cage changes were extracted. In the case of missing detections, in which one RFID antenna did not detect the cage change, the timestamp of the detection of the second antenna was used, arguing that the missing detection resulted from a mouse passing too fast through the tube, which should lead to a roughly similar detection timestamp for both antennas. We decided against subtracting the time spent in the tube from the stay duration. Thus, we calculated stay times for each mouse in each cage as times between cage changes when a mouse entered a new cage (only detections by the antenna passed second).

For each mouse, stay times in each cage were then summed up per day. As already mentioned, we analyzed only the second day of each round because the first day was considered habituation time. Thus, for the investigated 48 h, the percentage of time spent in each cage was calculated for each of the 12 mice. These percentages were then used for further analysis to compare side preference (left vs. right cage) and bedding preference (Pure vs. Comfort White, whereby presentation sides were switched after the first round). To test for normal distribution, the Shapiro–Wilk test was performed in R. The data were considered normally distributed ($p > 0.05$); therefore, a *t*test was used to compare the stay time percentages with a chance level of 0.5 (the expected relative stay time if mice had no preference for one of the two cages). In all statistical tests, significance level was set to 0.05, and result values are given as mean and standard deviation.

## Results

During the two analyzed days, the mice changed cages between 52 and 178 times per 24 h (100.75 ± 31.84 cage changes). Comparing the times the 12 mice spent in the two cages, we found that during the whole experiment, the mice stayed significantly longer in the right compartment, namely 57.49 +/− 3.83% of the time ($t(11) = -6.77$, $p < 0.001$, see Fig. 6a). For the different bedding materials, on the other hand, there was an even clearer preference: the mice stayed 72.76 ± 3.00% of the time in the compartment with Comfort White bedding ($t(11) = -20.19$, $p < 0.001$, see also Fig. 6c).

## Discussion

In this experiment, stay times of the 12 mice on Comfort White and Pure bedding material were compared, whereby stay time was only analyzed after 1 day of habituation, and the presentation side of the bedding was changed in-between to control for side preference. When looking at

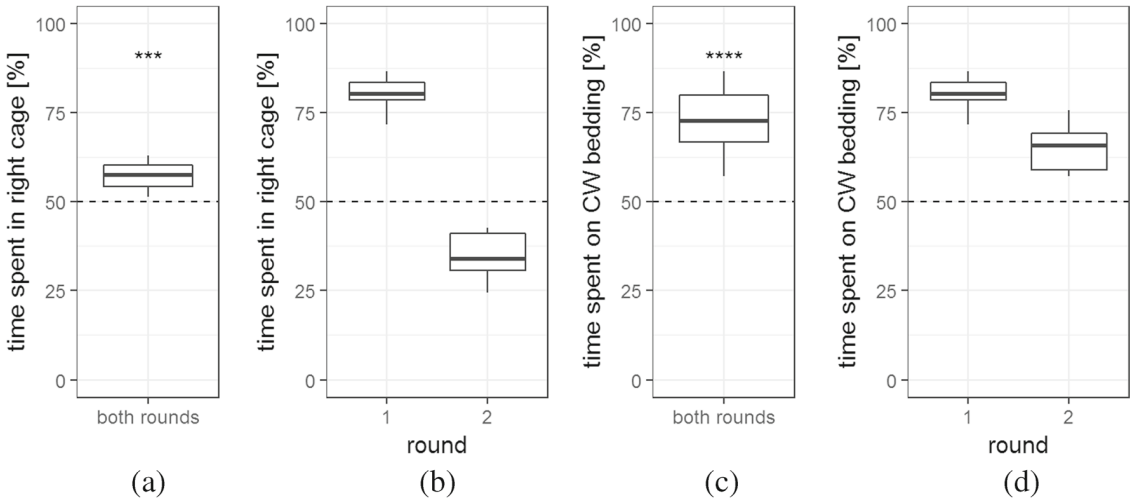


**Fig. 6** Time spent (%) in the two cages, analyzed by cage side and bedding material. Time spent in the right cage **a** in total (48 h), or **b** with regard to round (24 h). Time spent in the cage with the Comfort White bedding material **c** in total (48 h), or **d** with regard to round (24 h). Comfort White bedding material was presented in the right cage during the first round and in the left cage during the second round. CW = Comfort White *** $p < 1 \times 10^{-4}$, **** $p < 1 \times 10^{-9}$ *t* test comparison to chance level, $n = 12$

Fig. 6b, which compares the side preference on the second day of both rounds, side preference seems to be more distinct during the first round than the second. This was also reflected in a significant side preference, which could be due to spatial reasons (position in the room etc., Blom et al., 1992). Another explanation could be that the condition preference (for the bedding material) changed over time, becoming less strong and thus leading to a side preference when compared with the round before.

Nevertheless, the mice had a distinct preference for the cage with Comfort White bedding compared to the cage with Pure bedding. Thus, during this home cage-based preference test, we could confirm the results already obtained during the two 10-min observations of the conditioned place preference test: Comfort White bedding is preferred over Pure bedding by this group of 12 C57BL/6J mice.

The main purpose of this experiment was to test the new setup in a week-long experiment as well as to validate the bedding preference previously observed during a conditioned place preference (CPP) test. We have to emphasize that the result of this preference test cannot be generalized for C57BL/6J mice: Although we tested the preference of 12 mice, they were all together as one group in the test system and, thus, might be considered as only one independent sample. Indeed, it is possible that the mice influenced each other in their stay (a) by the behavior of dominant mice, (b) by avoiding or following individual mice, (c) or by preferring to not sleep alone over individual bedding preferences. As stated above, the bedding material was also familiar to the mice and as it was presented first in an experimental environment, it is possible that this might have had an influence. Thus, this test would have to be repeated with more groups with less and younger individuals for a more generalized conclusion. In any case, the preference test was successful in showing the feasibility of the MoPSS even with large group numbers under the experimental conditions of a home cage-based choice test. A study of home cage-based preference tests in which the MoPSS was used for several months to compare different enrichment is currently in preparation.

## Conclusions

In this paper, we offer the construction description to build an automated tracking system that can be used to facilitate the analysis of home cage-based preference test. We showed that the MoPSS is accurate even for fast mice and its error rate can be further reduced close to 0% with the help of additional logical reconstruction of the data. We also presented an example experiment with the corresponding results in which we compared two different bedding materials.

With this automated tracking system, analysis of home cage-based preference tests will become much easier: They will be less expensive, require less time for the data analysis, and will have much finer data resolution. The MoPSS is able to track individual mice and, therefore, it is suitable for group experiments. In our laboratory, the MoPSS is already being used to compare multiple enrichment conditions with regard to the mice's preference over several months.

We want to emphasize the great advantages of the MoPSS to existing systems: It is even able to detect fast animals and can be easily rebuilt. Currently, we are working on a further improved version with an RFID reader module without proprietary software and increased detection rates. In addition, in the near future, we will be adapting the MoPSS system to be suitable for larger animals such as rats and guinea pigs that require a tube diameter of more than 4 cm. On the basis of the construction description, it is also possible to adjust the MoPSS to other research questions. For example, we are working with a modified MoPSS onto which automated doors and levers or nose poke sensors can be added to test not only for preference but also for the strength of preference by letting the animals work for the access to the other cage (Lewejohann & Sachser, 2000; Sherwin & Nicol, 1995, 1996). Using only one RFID antenna, the MoPSS can also be used to record activity data in the home cage. In addition, the MoPSS might also be used to study group dynamics and the influence of individual group members on the position of the whole group.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.3758/s13428-021-01593-7.

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## Declarations

**Ethical approval** All experiments were approved by the Berlin state authority, Landesamt für Gesundheit und Soziales, under license No. G 0182/17 and were in accordance with the German Animal Protection Law (TierSchG, TierSchVersV).

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Experiments were not preregistered.

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# Supplements for "The Mouse Position Surveillance System (MoPSS)"

## 4.1 Glossary

Before going into details, we will have a short definition of the wording used here:

**detection:**
When a mouse moves through the tube, the RFID tag number of its transponder is detected by the RFID antenna. The RFID reader connected to the RFID antenna transmits the tag number to the Arduino, which can then save it onto the microSD card.

**RFID antenna:**
To simplify the explanation, we will use “RFID antenna” synonymous to “RFID reader”. Note that “first antenna” is always referring to the first antenna the mouse passes through when moving to the other cage, independent from direction. In the following sections, we will also refer to it as “A1”, irrespective of its position (left or right). In the same way, “second antenna” (A2) is referring to the second antenna the mouse passes through and consequently the antenna that is closer to the new cage.

**mouse:**
Technically, only the RFID tag number of the mouse’s transponder is detected by the RFID antenna. However, we will speak of “mouse”.

**cage change:**
Cage changes are determined by consecutive detections on both antennas, as caused by a mouse changing cages and consequently passing first A1, then A2. A “cage change” is synonymous to “side change”, “passage” or “transition” used in other studies.

## 4.2 Data Evaluation

During recording, RFID detections were automatically saved onto a microSD card by the Arduino. Each detection includes a timestamp (synchronized before the start of the experiment via an internet time server), antenna number (A1 or A2), and the unique RFID tag number of the mouse. The recorded data is then analysed for each mouse individually by identifying cage changes.
However, if a mouse while changing between cages is not detected by one or by both RFID antennas, the simple approach of looking at consecutive detections does not work anymore. In the following, we will explain how to handle these situations by deducing the mouse position

from the available data.

### 4.2.1 Four Situations for Data Acquisition

Four situations can be distinguished. (A schematic drawing for the following explanations is depicted in Fig. 4.1.)

A) **Both antennas detect the mouse**, this is the common/regular case.

B) **The first antenna (A1) does not detect the mouse, but the second antenna (A2) detects the mouse (~~A1~~ $\rightarrow$ A2).** In this case, the cage change is easily deductible since the mouse must have passed the first antenna in order to get to the second. The missing information is the point in time when the mouse passed/entered the first antenna.

C) **The first antenna (A1) detects the mouse but the second (A2) does not (A1 $\rightarrow$ ~~A2~~).** Here, the cage change is not immediately identifiable because a detection of the mouse on the first antenna (A1) does not necessarily indicate a cage change. Indeed, dwelling in the range of the antenna without completely passing through the tube is occurring commonly (see Tab. 4.1). The fact that the mouse has passed the tube is becoming obvious the next time the mouse returns and passes again through the antennas in reverse direction (A2 $\rightarrow$ A1). We know from observations, that mice usually do not spend prolonged time within the tubes (98.82 % of cage changes in the validation experiment took $\leq$ 10 s). Therefore, it can be inferred that a cage change must have taken place earlier when the mouse is detected at A2. Now that we know that we have missed a cage change, we can look at the previously recorded data and infer when this cage change most probably has happened. For this we use the timestamp of the last mouse detection at A1. This is the best approximation as to when the cage change happened.

D) **Both antennas do not detect the mouse (~~A1~~ $\rightarrow$ ~~A2~~).** In this case, there is no inference possible because there is no information on the time when the missed cage change happened, apart from the general time frame between two successful cage changes.

### 4.2.2 Handling Situations A-D

Our main focus was first, to find the cage changes in which one RFID antenna did not detect the mouse (B and C), and to correct possible false timestamps (C, as the time belongs to a new cage change when from the antenna's perspective the mouse appears for the first time on this side), and second, to identify cage changes which were completely missed by the antennas (D, wherever possible, as explained above). To achieve this, we developed an R script to help with the logical reconstruction of the data (available here: https://zenodo.org/record/4650404). At the end, in Table 4.1 it is shown how the output of the evaluation script of Experiment 1 (validation) looks like.
Our dataset contains a timestamp and the antenna number where the detection occurred. Apart from cage changes, around 62 % (see Table 4.1) of our data points consist of detections we considered "pokes". These are detections in which a mouse is recorded (multiple times) at the antenna without passing through it. This is due to dwelling near the beginning of the tube.
In short, the procedure of the R script is as follows (also depicted as a schematic drawing in Fig. 4.2):

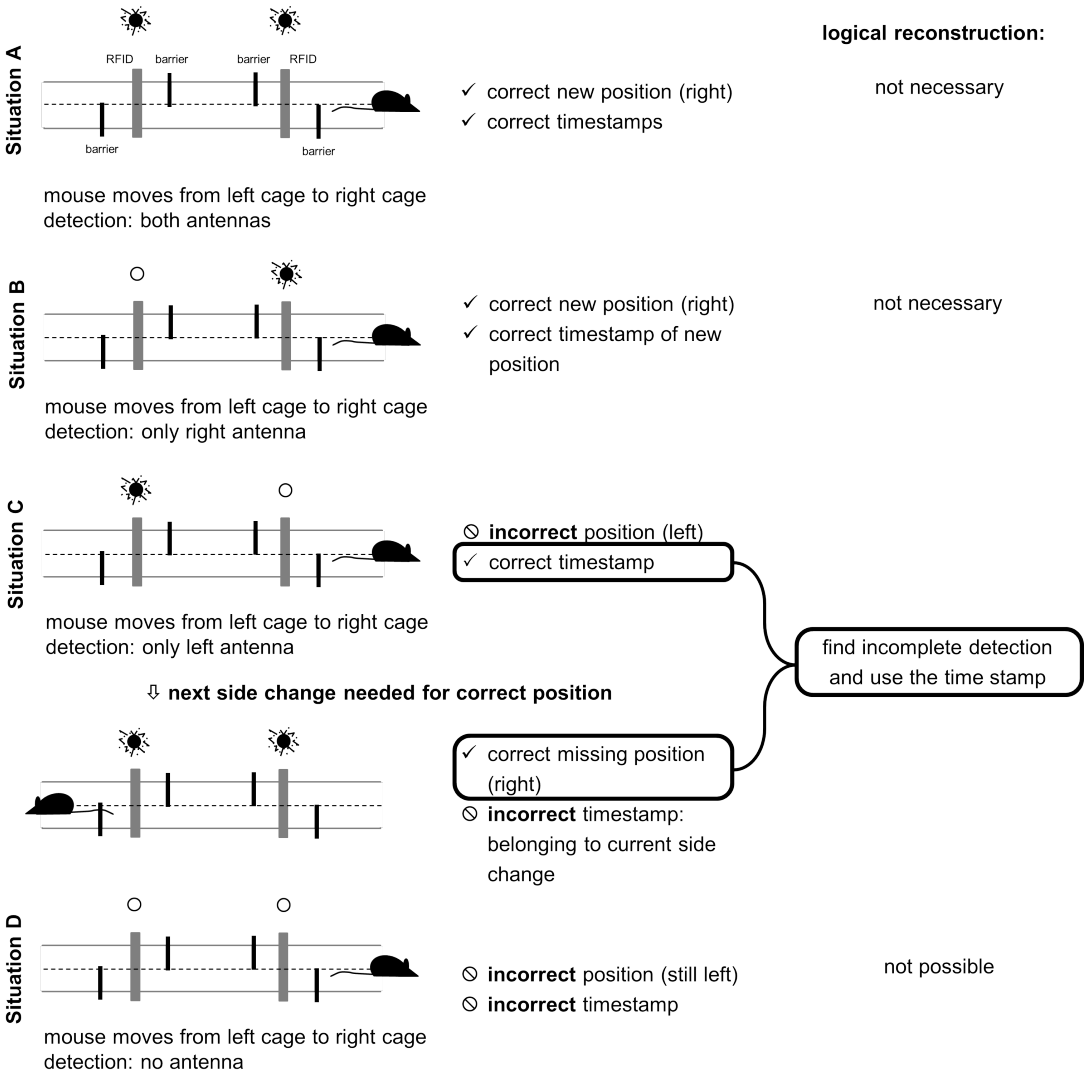



Figure 4.1: Four possible situations that might arise during cage changes and how to reconstruct the actual cage changes from them. For a more detailed description of how these situations are handle by the R script, see Fig. 4.2.

1) Whenever a mouse was detected by an antenna by which it was not detected before, this was identified as a cage change and labelled according to its duration: time passed between detection by the first antenna and the detection by the second antenna. Based on observations made in previous tests, cage changes including detections at both antennas within 3 s were assumed as safe.

2) On the basis of the dataset with safe cage changes, we now looked for two consecutive safe cage changes and subsequently examined all detections in between these two cage changes.

2a) If the two safe cage changes were impossible, e.g., the mouse changed from left to right and again from left to right, the detections in-between were examined. This leads to three possible outcomes:
First, if there was no detection at all between the two safe cage changes, both RFID antennas must have missed the mouse (Fig. 4.1 D).

Second, if there was only one detection between the two safe cage changes, one of the two antennas must have missed the mouse (Fig. 4.1 B or C). As we know that an undetected cage change must have happened between the two safe cage changes, we used the timestamp from the single detection and reconstructed the missing cage change. Since cage changes are usually fast, we decided to accept the introduced uncertainty of a few seconds.
Third, if there was more than one detection between the two safe cage changes, we looked for cage changes lasting longer than 3 s. If there was only one such cage change, it was regarded as true, most likely resulting from a B or C situation depicted in Fig 4.1.

2b) If the two safe cage changes were possible, we examined the detections in-between for occurrence of detections indicating additional cage changes (i.e., presence detection in the wrong cage).
These could be caused by, for example, two cage changes during which one of the antennas did not detect the mouse. E.g., a mouse moves from left to right cage and is detected by second antenna (situation C), moves back from right to left cage and is again detected by second antenna (situation C) (~~A1~~ $\rightarrow$ A2, ~~A2~~ $\rightarrow$ A1). Thus, these two cage changes lead to a detection first left and then right, which would resemble a cage change from left to right. As a result, presence would be assumed in the wrong place, not matching the detected cage changes before and after. This mismatching is a first criterion, when finding these situations. As an additional criterion, the detections by the two antennas (originally from two cage changes) have to be more than 3 s apart, so that the mouse had time to leave the tube between cage changes and before passing again through the antennae.

3) After looking at possible and impossible cage changes, we went through the whole data set of each mouse again, to find additional cage changes which might have not fallen into the previous categories. For example, if between added cage changes were additional detections in the other cage which were not explained by a cage change yet (see Fig. 4.2b), this would be detected now. To do so, we again examined the detections between the now secured cage changes: Were there more than two detections which indicated a cage change (= the mouse was detected by a different antenna then before)? If so, and the time passed between the detections was under 15 s, we assumed that this was also a real cage change, but one in which the mouse moved slower than usual. (This was caused, for example, by multiple mice in the tube, which blocked each other's way.) As a test, we then also included cage changes taking even longer than 15 s, and this also proved to be correct, when comparing them to the video recordings (see following section).

### 4.2.3 Edge Cases of the Evaluation

**Situation D two times in a row:** Although extremely rare, it is possible that a mouse is missed by both antennas when passing through the tube (situation D). In principle this could happen two times in a row leading to an undetectable error based on evaluation of the order of cage changes. E.g., a mouse moves from the left to the right cage, then two cage changes are missed, and the next seen cage change happened logically reasonable from the right to the left cage. However, we could show that situation D (both antennas were missed) is very unlikely and therefore, it is even more unlikely that this occurs two times in a row.

**Situation C followed by situation B:** When a mouse passes from one cage to the other and is not immediately detected by the antenna corresponding to the new cage (A1 $\rightarrow$ ~~A2~~), this error can be inferred from the next regular cage change (situation C). However, this correction is not possible when during the subsequent cage change the same antenna (now A1, formerly A2) does not detect the mouse (~~A1~~ $\rightarrow$ A2). In this case, the recorded data will provide no hint that the mouse has been in the other cage. However, we did not observe this at all during evaluation and thus deem this situation to be very unlikely.

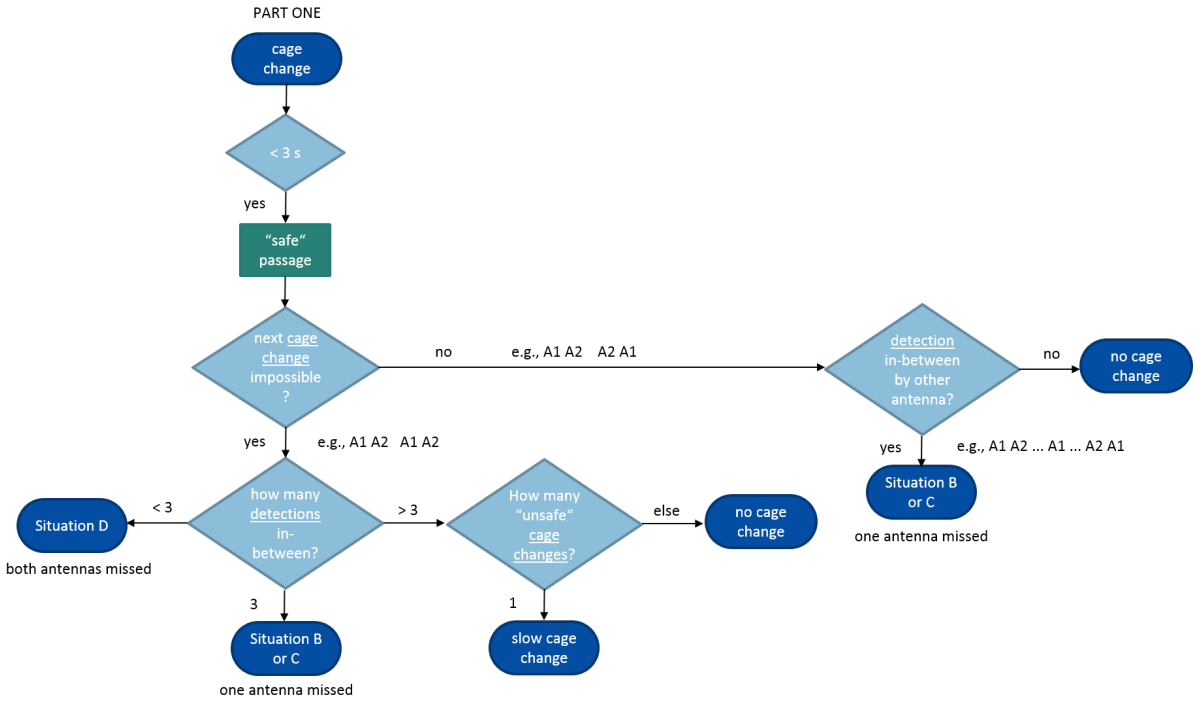
### 4.2.4 Customization of the Evaluation Script

Depending on the research question, the evaluation script can be freely customized. The script is well commented and easy to apply for anyone. See the script and our dataset (in the Supplements: https://zenodo.org/record/4650404) to try the evaluation first hand.
For setups in which the time to change cages for the animal is shorter or longer than the default of three seconds (e.g., if the distance between antennas is longer), the time for a safe cage change can easily be adjusted. If the absolute highest certainty for cage changes is needed, all detections which were deduced from missed antenna detections can be removed from the dataset (loss of 3.98% cage changes for the validation dataset of Experiment 1). There is no limit to the number of animals or duration of the experiment.

Table 4.1: Output from evaluation script of the validation experiment (Experiment 1). Detection and cage change are defined as described at the beginning in the glossary. Percentages are calculated as part of the total cage changes each mouse made.

| | Mouse Number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Total | Percentage |
| Error 1 | 0 | 0 | 4 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 7 | 0.095 |
| Error 2 | 24 | 14 | 10 | 11 | 13 | 22 | 13 | 15 | 9 | 12 | 8 | 11 | 162 | 2.195 |
| Error 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Error 4 | 8 | 0 | 8 | 12 | 9 | 13 | 6 | 2 | 7 | 34 | 3 | 23 | 125 | 1.693 |
| Error 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Events | 997 | 582 | 599 | 552 | 500 | 873 | 431 | 722 | 808 | 569 | 327 | 422 | 7382 | 100 |
| Transitions | 398 | 220 | 247 | 211 | 182 | 319 | 148 | 319 | 289 | 205 | 125 | 141 | 2804 | 37.984 |

(a)




(b)

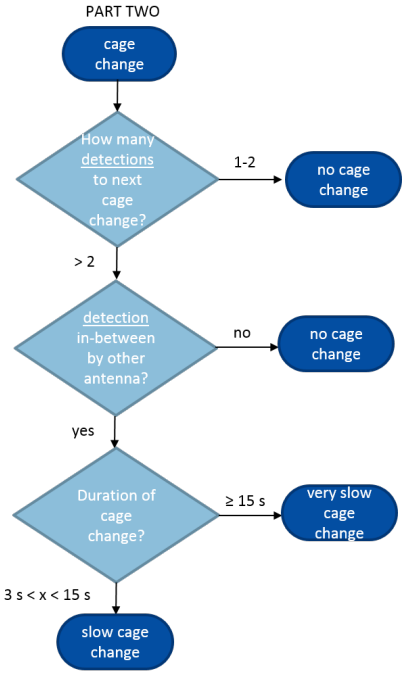



Figure 4.2: Logical reconstruction with the help of the R script. a) Part one: First, cage changes are identified and "safe" cage changes are defined based on their duration ($<$ 3 s between both antenna detections). Next, the cage changes are compared to the following cage change and depending on whether this sequence is possible or impossible, the detections between the two cage changes are analysed. b) Part two: The data set with safe cage changes is recalculated, including the reconstructed cage changes from part one. Then, the recorded data are examined for additional cage changes, indicated by a detection from the other antenna in-between.

# 5 Discussion

In the preceding sections, two approaches to preference tests for severity assessment were presented: an automatic RFID-based tracking system for home cage based preference tests (MoPSS) and two protocols for T-maze tests, which proved to be unsuitable for preference testing and obtained results in spontaneous alternating behaviour instead. As explained in the Introduction (Chapter 1), the overall motivation behind these experiments was to develop a method for severity assessment from an animal's perspective.

## 5.1 Developing Preference Tests for Refinement

The first and maybe greatest achievement is the development of the MoPSS. As discussed in detail in the Introduction of Chapter 4, until now home cage based preference tests were often limited by time-consuming manual video analysis or the high costs for automatic solutions. To our knowledge, the system we developed is the first system which is affordable, not based on proprietary software or proprietary equipment (except for the RFID readers), and thus, also easy to rebuild and/or adapt for similar research questions. We believe that this system will facilitate future home cage based preference tests to a great extent, especially when studying group housed mice (for a detailed discussion see Chapter 4, Discussion). In this manner, researchers can easily "ask" the mice which housing condition they prefer, and thus, refine the daily environment of the mice. This is important because to improve animal welfare not only experimental procedures should be considered but also the housing conditions during and between experiments (Lewejohann et al. 2020).
A first advance towards better housing conditions was already conducted in our research group: In an extensive study using the MoPSS, different enrichment items (including "active" and "passive" enrichment) were compared. In this study the good operability of the system and its reliable performance even over several months were confirmed (Hobbiesiefken et al., publication in preparation). Thus, the MoPSS has already been the key system in an extensive home cage based preference test and is suitable to find ways to improve the mice's housing conditions.
The second approach towards severity assessment from the mice's perspective was the T-maze preference test. For this, a working protocol was missing and we tested two different procedures. Neither did lead to the desired result: Mice did not make choices according to their preferences for food but instead alternated their choices. One possible explanation is that the mice did not show the expected behaviour because they were driven by another motivation than gaining food (e.g., search for an exit or shelter, as also discussed in the discussion of Chapter 3, publication 1). Motivation to gain food could be increased by food restriction – but this, on the other hand, would increase severity and is, therefore, not in the sense of our research. As a conclusion, we would advise other researchers against using the T-maze for preference tests because they

would have to reckon on extensive pre-testing to find a well-working protocol (for a detailed discussion see Chapter 3, Discussion).
Which leads us to the most important message of this dissertation: If we want to "ask" mice (or other animals) about their preference, we have to make sure we have a procedure that poses the question correctly. This includes (see also Kirkden and Pajor 2006):

1. The animals have to know what the options are. This means the mice have to know which options are provided, for example, different beddings (home cage based preference) or food (T-maze).

2. The animals have to know how it can gain access to these options. In the home cage based preference test, the mice have to be habituated to the tunnel connection (or the tunnel with barriers, as with the MoPSS) between the cages, and should not be afraid to move through this tunnel to the other cage. In the T-maze, for example, in experiment 2 of publication 1 (Chapter 3), the mice have to know that the millet can be found at a specific position (either left or right arm), and they have to experience whether the millet is gained by returning to the same arm or by visiting the other arm.

3. The animals have to be motivated to gain access to these options. In the home cage based preference test, mice should be motivated to explore the whole setup and experience both options. Otherwise we cannot be sure, if the mice actually preferred one option over the other. For the T-maze, another motivation might have been interfering with the motivation to gain food, for example, need for exploration or search for an exit.

## 5.2 Understanding Mice: Refinement of General Procedures

During the development of the preference tests, there were two factors which influenced the mice in their behaviour to a greater extent than expected. However, when handled correctly, these factors might help to "ask" the mice in a more efficient way. These two factors are habituation and motivation. To facilitate future research, I will give examples of the influence of these two factors from the development of preference tests, and explain the influence they had.
One of the most powerful tools – or greatest potential pitfalls – is habituation. In the description of procedures, this is sometimes treated with only one or two sentences (Shipton et al. 2014; Rakshasa and Tong 2020; Sharma et al. 2010a; Moy et al. 2008), sometimes not even mentioned at all (Hébert et al. 2017). However, it can make a large impact when, for how long and by which procedure mice are habituated. For example, for the T-maze, first we tested long voluntary habituation, arguing that this would be the most stress-free method. We allowed animals to explore the T-maze when they were highest motivated to do so. What we underestimated was the procedure of the T-maze test itself: taking the mouse out of the home cage, on a time set by the researchers not the mouse itself, and placing it first into an unfamiliar cage which then had a connection to the already familiar T-maze (but the T-maze itself lacked the connection to the home cage as it was the case during habituation).
If one considers all these changes compared to the habituation situation, this long voluntary habituation does not sound like habituation at all. Instead, for experiments like the T-maze daily short habituation trials seem to be more effective. In these trials a "dry run" is conducted: It imitates the procedure which will be used in the actual T-maze test later on, but it does not contain any of the to be tested cues and goods. In addition, only if the mouse is sufficiently habituated to

the new environment, it will also consume the food to be tested (although there also seem to be differences between the types of food, as we could see in the pre-tests mentioned in Chapter 3. Thus, testing the preferences of mice requires "the right kind" of habituation. Otherwise everything unknown might interfere with the mice's behaviour. Without an effective habituation to the setup and the procedure, the mice might not even understand the options between which they are asked to choose. Without an effective habituation to the provided options, on the other hand, the measured preference might be influenced by its novelty. For instance, when comparing different housing conditions (publication 2, Chapter 4), it is important that the mice are already familiar with both. Otherwise they might spend most of their time in the condition which is more interesting at the beginning but not preferred in the long run.

The second important factor is motivation (for a detailed definition see Kirkden and Pajor 2006). As already explained above, in the T-maze the mice seemed to be motivated by something else than search for food (for a detailed discussion see discussion of Chapter 3, publication 1. In other words, instead of measuring the preference between two food options, we compared different motivations (similar two a "between-motivations appetence test", Kirkden and Pajor 2006) and found the motivation to feed to be less strong than some (unknown) other motivation which caused the mice to alternate. However, without several additional experiments, we can not ascertain which motivation this was.

Another good example of the importance of motivation (and the importance of understanding motivation) occurred during the development of the MoPSS for the home cage based preference tests: Before getting to the final setup design, I designed, build and tested several others to get the mice to slow down while moving through the connection tunnel between the cages: e.g., a see-saw in the middle of the tunnel, one-way flap doors or automatic doors. Although it is not the place to present data from these prototypes here, I would like to elaborate on some interesting observations because they might be helpful for future experiments.

At several points, I encountered the problem that mice seemed not enough "motivated" to explore the new setup. Some mice learned and habituated very fast to it but many mice did not change cages even after several days of presentation. One possible method to overcome this situation would have been to increase the motivation of the mice by providing food in one cage and water in the other cage. In general, if the passage from one cage to the other was not stressful (e.g., the usual tunnel), this splitting would result in more cage changes without further complications. However, if the passage from one cage to the other is stressful to the mice, the splitting would result in an even more stressful situation, as the mice would be forced to experience it frequently. Especially when developing new setups with unknown impact on the mice, this has to be considered very carefully.

In the example of the flap doors, I also tried a different approach. When mice did not move through the long flap doors, I replaced them by shorter flap doors (so that the flap did not fill the entire tunnel and was more like an obstacle reaching into the tunnel), and allowed mice to habituate to this setup. After this, I then moved on to the longer flap doors, and this time, mice learned to use them as well. Thus, beforehand I had implemented too much alteration too fast, or in other words: The mice's neophobia had been stronger than their motivation to explore. By reducing the hurdle, the motivation was again strong enough to learn the "rules" of the setup.

However, by the time all hesitant mice had learned to use the flap doors, some of the bolder mice already knew how to open the one-way flap doors the "wrong" way round, which interfered with our tracking setup. Thus, I added a second flap behind the first one to complicate using the flap doors for the "wrong" direction. To habituate them to the second flap, I had some habituation

steps with flaps of shortened length (similar to what I did for the first flap). Interestingly, however, cage change rates increased for several mice with the shortened double flap doors for several days, but then they dropped again. Some mice even stopped changing cages at all. Although I could not verify it, I believe that this decline of cage changes is an indicator that over a longer time, usage of the shortened double flap doors might be unpleasant for the mice; maybe due to a longer contact with their backs compared to the single flap doors. Thus, motivation – or a loss thereof – might itself work as an indicator of severity from the mice's perspective. A slightly similar approach is also used in severity assessment on the basis of motivation for voluntary wheel running (Häger et al. 2018).

## 5.3 Reproducibility: Refinement of Experiment Reporting

For an experiment to work (or for a mouse to understand the question that is asked), a lot of factors play a role. In the optimal case, all of them should be reported in the articles or protocols about the experiments. However, when researching for T-maze methods, it became clear that many factors are often not reported.

As our research focuses on refinement, it was very important to us to improve conditions for mice also outside the experiments (see also Lewejohann et al. 2020). For example, we used tunnel handling, and provided more bedding material (3-4 cm height) and enrichment (different types of nesting material, houses, handling tunnel, temporarily even running wheels) in the cages. We took care to also report all these details in our studies. However, in other studies this is often not the case. The type of handling is often not mentioned (which usually implies tail handling), although it increases anxiety-like behaviour (Gouveia and Hurst 2013, Hurst and West 2010) and can influence results (Gouveia and Hurst 2017). The type of bedding material is usually not mentioned, nor is the filling height, although the first can influence behaviour and even perception (Moehring et al. 2016), while the second influences the mice's physiology (Freymann et al. 2017).

In addition, although there are multiple studies which emphasize the positive effect of environmental enrichment (overview: Olsson and Dahlborn 2002; stereotypic behaviour: Gross et al. 2012; Würbel 2001; Olsson and Sherwin 2006; alopecia: Bechard et al. 2011; learning and memory: Tang et al. 2001; van Praag et al. 2000; immune system: Benaroya-Milshtein et al. 2004; Kingston and Hoffman-Goetz 1996; stress and anxiety-like behaviour: Bailoo et al. 2018a; Olsson and Sherwin 2006), it has not become common yet. In addition, also studies which use enrichment do not report what kind of enrichment they use (Gui et al. 2021). Incredibly, there are many publications which do not even report how many mice are kept in a cage (Lione et al. 1999; Granholm et al. 2000; Locurto et al. 2002; Guariglia and Chadman 2013; Correa et al. 2015), although this can have effect on behaviour (aggression in male mice: Bailoo et al. 2018b; anxiety-like behaviour: Davidson et al. 2007).

Another example of poor reporting policies is barbering. It is a frequent issue with mice, especially with the strain C57BL/6J (Garner et al. 2004), and it must be suspected that a large portion of barbered C57BL/6J mice is used in published experiments. Barbering is a model for a disorder (trichotillomania). Whisker-loss might cause altered behaviour especially in experiments in which whiskers are expected to play a major role, such as novel object recognition, marble burying and the open field test (Haridas et al. 2018; Tur and Belozertseva 2018). On the other hand, mice which barber show no difference in learning ability itself, with the exception of

an extra dimensional shift task (Garner et al. 2011). In some of our studies, we used barbered mice because we argued that the tasks that were required should not have been influenced by whisker-loss. However, we considered it of high importance to explicitly state this information in the articles. Only by providing this sort of detailed information it will be possible to explain any potentially contradictory findings in future studies instead of just contributing to the reproducibility crisis (Baker 2016; Percie du Sert et al. 2020).
In summary, if we want to increase reproducibility of experiments, we have to improve our reporting policy first. However, this is also necessary if we want to improve experiment conditions for mice: A comparison of experiments - and especially an assessment of the severity of experiments - will not be possible, if the reporting of procedures is vague or incomplete. In this case, we will not be able to determine which factors influence the mice or which increase severity and which do not.
As a consequence, we took special care to report our experiments as detailed as possible, providing additional information as well as the original data sets in the supplementary materials (Chapter 3, Appendix and section 4) and the pre-registration, wherever possible (T-maze test doi: 10.17590/asr.0000213).

## 5.4 Limitations

In the presented studies, we focused on C57BL/6J mice because this is the mouse strain most commonly used, and therefore, the results would have a greater impact for the laboratory mice. However, it is known that mouse strains differ in their physiology as well as in their behaviour (anxiety-like and social behaviours: An et al. 2011; spatial discrimination task: Crusio et al. 1990; Gerlai 1998; pain: Rudeck et al. 2020; Smith 2019). Thus, repeating the studies with mice of a different strain might lead to different results (e.g., regarding the alternating behaviour in the T-maze or the velocity in the MoPSS).
In addition, we used female mice only because they show less aggression in groups than male mice. This was necessary for our experiments, e.g., the home cage based preference test, for which we also wanted to investigate the performance of the MoPSS with large groups. As there are also large differences between male and female mice (sex effect in anxiety-like and social behaviours: An et al. 2011; appetitive learning: Mishima et al. 1986; pain and analgesia: Smith 2019), results might not be transferable to male mice.
Nevertheless, we believe we gained important insights which can be used to generate hypotheses for future research with other strains and male mice.

## 5.5 Conclusion and Outlook

To refine experimental procedures (including housing conditions) to reduce severity, we need to develop tests which reflect the mice's perspective on severity. One possible approach are preference tests like the T-maze, the home cage based preference test and the conditioned place preference test.
Here, we developed an automatic RFID based tracking system which will facilitate the conduction and analysis of home cage based preference tests. It is low-cost, open-source (except for the RFID readers), and thus, easy to rebuild for other laboratories. The tracking system was already used in our research group to compare different enrichment items. In the future, it will

hopefully be used by other research groups as well, and in this manner, find ways to improve housing conditions of laboratory mice, for example with regard to specific types of enrichment.
In addition, we investigated the usability of the T-maze for preference tests and found that the T-maze is unsuitable for this research question, at least with the protocols we used (which refrained from food-restriction to keep severity of the preference test itself as low as possible). This finding also emphasises that we are still not able to fully understand the mice's behaviour. Thus, it would be presumptuous for us to assume in the mice's stead what a mouse would or would not prefer with regard to experimental or housing conditions. Instead, we need to find effective methods to include the mice's perspective.
One additional approach is the conditioned place preference test which might even allow to compare experimental procedures with regard to their severity from the mice's perspective. As mentioned in the Introduction (Chapter 1), development of a suitable protocol to use this preference test for severity assessment is still ongoing as reproduction of existing protocols was not successful so far. It is possible that again the factors habituation and motivation are not handled effectively and / or reporting is insufficient. Nevertheless, we have not yet relinquished the hopes of finding an effective protocol for this preference test.
During the experiments it became clear that the factors habituation and motivation play a major role and should not be underestimated during test planning. In addition, all external factors which could influence the mice's behaviour should be reported in full detail, to allow study replication and provide potential explanations in case the results might differ.
Thus, although this task is challenging, we are still in need to develop further efficient methods to "ask" mice for their preference and assess severity from their perspective and refine animal experiments.

# 6 Summary

## Choice tests as a means for severity assessment from an animal's point of view

To refine experimental procedures (including housing conditions) and reduce severity for laboratory animals, we need to integrate the animal's perspective. This dissertation focuses on mice and the development of tests, which investigate the preferences of mice.

**In Chapter 2,** a literature review, potential approaches towards severity assessment from an animal's perspective are summarised. This includes preference tests (or choice tests) like the T-maze preference test, the home cage based preference test and the conditioned place preference test. These tests differ strongly in their conduction and can be used to assess different aspects of the experimental or housing procedures. However, they all share that options are compared typically pair-wise, leaving the mice a simultaneous, dichotomous choice. For example, the T-maze test can be used to compare different food items as a reward. Here, the mice is placed in the start arm of a T-shaped maze and can choose between the two goal arms, which each contain one of the options (in this case food). The home cage based preference test, on the other hand, can be used to investigate the mice's preference for specific housing conditions by connecting two cages with different housing conditions (e.g., different bedding materials) and measuring the time the mice spent in each cage.

**In Chapter 3,** the usability of the T-maze for preference test was investigated by development of a working protocol to compare different food rewards. However, instead of displaying preference behaviour for the food rewards, the mice alternated their choices. This was even the case when comparing reward vs. no reward (i.e., similar to a simple learning test). Thus, we have to conclude that the T-maze is unsuitable for this research question, at least with the protocols used here (without food-restriction to keep severity of the preference test itself as low as possible).

**In Chapter 4,** the home cage based preference test is thematised. Conduction of this test is usually time-consuming (when using video analysis) or cost-intensive (when using commercially available tracking software). Thus, we developed the Mouse Position Surveillance System (MoPSS), an automatic RFID based tracking system which facilitates the conduction and analysis of home cage based preference tests. It is low-cost, open-source (except for the RFID readers), accurate even for fast moving mice and easy to rebuilt, which ensures that other research groups can adopt this method for their experiments. The system will hopefully help to assess the preferences of mice, and on that basis, also to improve housing conditions of laboratory mice in the future.

**In Chapter 5,** the main findings of the publications presented in the previous chapters are summarised. Especially two main factors observed during the experiments are highlighted: habituation and motivation. An effective habituation is the necessary foundation to make sure, the animals will actually behave according to their preference of the presented options. Otherwise other motivations (e.g., exploration or fear) might interfere with the preference test. In addition,

reporting procedures in full detail in publications is mandatory to find influencing factors for preference.
In conclusion, developing tests to assess the mice's preference is challenging but nonetheless important if we want to include their perspective to improve experimental and housing procedures.

# 7 Zusammenfassung

### Belastungseinschätzung aus Tiersicht mit Hilfe von Wahlversuchen

Um die experimentellen Verfahren (einschließlich der Haltungsbedingungen) für Labortiere zu verbessern und ihre Belastung zu reduzieren, muss die Sichtweise der Tiere einbezogen werden. In dieser Dissertation geht es dabei speziell um das Labortier Maus und um die Entwicklung von Tests zur Bestimmung ihrer Präferenzen.

**In Kapitel 2** sind mögliche Ansätze für eine Belastungseinschätzung aus Tiersicht zusammengefasst. Dazu gehören Präferenztests bzw. Wahlversuche wie der T-Maze Wahlversuch, der Heimatkäfig basierten Wahlversuch und der Conditioned Place Preference Test (konditionierte Ortspräferenz). Diese Versuche unterscheiden sich stark in ihrer Durchführung und können verwendet werden, um unterschiedliche Aspekte der experimentellen Methoden oder Tierhaltung zu beurteilen. Allen ist jedoch gemeinsam, dass die Optionen typischerweise paarweise verglichen werden, sodass die Mäuse eine gleichzeitige, dichotome Wahlmöglichkeit gegeben wird. Zum Beispiel lassen sich mit dem T-Maze Test verschiedene Futterbelohnungen vergleichen. Hierfür wird die Maus an den Anfang eines T-förmigen Labyrinthes gesetzt und kann zwischen den zwei Zielarmen wählen, die jeweils eine der Optionen (in diesem Fall Futter) enthalten. Der Heimatkäfig basierte Präferenztest wiederum eignet sich dafür, die Präferenz der Mäuse im Bezug auf bestimmte Haltungsbedingungen zu untersuchen. Dafür werden zwei Käfige mit unterschiedlichen Haltungsbedingungen (z.B. unterschiedliches Einstreu) miteinander verbunden und es wird die Zeit erfasst, die die Mäuse in jedem Käfig verbringen.

**In Kapitel 3** wird die Eignung des T-Mazes für Präferenztest untersucht. Dafür sollte ein funktionierendes Protokoll entwickelt werden, mit dem verschiedene Futterbelohnungen verglichen werden können. Anstatt jedoch ein Präferenzverhalten gegenüber dem Futter zu zeigen, besuchten die Mäuse abwechselnd beide Labyrinth-Arme. Dies war auch der Fall, wenn keine Belohnung mit Belohnung verglichen wurde (ähnlich zu einem einfachen Lerntest). Daher liegt der Schluss nahe, dass das T-Maze für diese Fragestellung ungeeignet ist, zumindest mit den hier verwendeten Protokollen (d.h. ohne Futterrestriktion, um die Belastung durch den Präferenztests selbst so gering wie möglich zu halten).

**In Kapitel 4** wird der Heimatkäfig basierte Präferenztest thematisiert. Die Durchführung dieses Tests ist üblicherweise zeitaufwändig (bei Video-Auswertung) oder kostenintensiv (bei der Benutzung von kommerziell erhältlicher Tracking-Software). Aus diesem Grund haben wir das Mouse Position Surveillance System (MoPSS) entwickelt, ein eigenes, RFID-basiertes Tracking-System, das die Durchführung und Auswertung von Heimatkäfig basierten Präferenztests erleichtert. Es ist kostengünstig, quellenoffen (außer die RFID-Lesegeräte), akkurat (sogar für Mäuse bei hoher Laufgeschwindigkeit) und leicht nachzubauen, damit andere Forschergruppen es auch für ihre Versuche verwenden können. Dieses System wird hoffentlich dazu beitragen, die Präferenzen von Mäusen zu ermitteln und auf dieser Grundlage dann die Haltungsbedin-

gungen von Labormäusen zu verbessern.

**In Kapitel 5** werden die Hauptaussagen der wissenschaftlichen Publikationen der vorangegangenen Kapitel zusammengefasst. Besonders zwei Faktoren, die während der Experimente beobachtet wurden, werden hier hervorgehoben: Habituation und Motivation. Eine effektive Habituation ist die notwendige Basis, um sicherzustellen, dass die Tiere sich tatsächlich entsprechend ihrer Präferenz verhalten werden. Andernfalls können andere Motivationen (beispielsweise Explorationsdrang oder Angst) den Präferenztest beeinflussen. Darüber hinaus ist es wichtig, in den wissenschaftlichen Artikeln sämtliche Methoden detailliert zu berichten, um mögliche Einflussfaktoren auf die Präferenz finden zu können.

Insgesamt stellt die Entwicklung von Tests zur Präferenzbestimmung von Mäusen zwar eine Herausforderung dar, ist aber trotzdem sehr wichtig, wenn wir die Mausperspektive in die Verbesserung von Experimenten und Haltungsbedingungen einbeziehen wollen.

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# 9 Appendix

## 9.1 Author's contribution

### Publication "Severity Assessment from an Animal's Point of View" (Chapter 2)

**Anne Habedank:** conceptualisation, literature research, writing of the original draft (section Preference tests, Introduction, Conclusion), review and editing (all sections)

**Co-authors:** conceptualisation, literature research, writing of the original draft (section Tests on emotional valence, Introduction, Conclusion), review and editing (all sections), funding acquisition, supervision

### Publication "O mouse, where art thou? The Mouse Position Surveillance System (MoPSS) - an RFID based tracking system" (Chapter 4)

**Anne Habedank:** conceptualisation, literature research, development of technical equipment, development of study design, experimental planning, validation of the method, conduction and analysis of experiments, care taking of the animals, writing of the original draft (all sections except Electronics and Software), review and editing (all sections), correspondence with the reviewers

**Co-authors:** conceptualisation, development of technical equipment, involvement in the validation of the method, writing of the original draft (sections Electronics and Software), review and editing, funding acquisition, supervision

### Publication "Alternate without alternative: Neither preference nor learning explains behaviour of C57BL/6J mice in the T-maze" (Chapter 3)

**Anne Habedank:** conceptualisation, literature research, development of study design, experimental planning, conduction and analysis of experiments, care taking of the animals, writing of the original draft, review and editing, correspondence with the reviewers

**Co-authors:** conceptualisation, review and editing, funding acquisition, supervision

## 9.2 Publication Index

### 9.2.1 Original Articles

Habedank A, Urmersbach B, Kahnau P, Lewejohann L:
O mouse, where art thou? The Mouse Position Surveillance System (MoPSS) - an RFID based tracking system.
Preprint on bioRxiv available, doi: 10.1101/2020.11.13.37971
Accepted for publication by Behavior Research Methods, 2021

Habedank A, Kahnau P, Lewejohann L:
Alternate without alternative: Neither preference nor simple learning behaviour shown by C57BL/6J mice in the T-maze.
Preprint available on bioRxiv, doi: 10.1101/2020.11.11.377788
Published in Behaviour, 2021
doi: 10.1163/1568539X-bja10085

### 9.2.2 Meta-Analyses and Systematic Reviews

Van der Mierden S, Leenaars C, Boyle E, Ripoli F, Gass P, Durst M, Goerlich-Jansson V, Jirkof P, Keubler L, Talbot S, Habedank A, Lewejohann L, Tolba R, Bleich A (2020):
Corticosterone: measuring stress in animals, a mapping review and metaanalysis.
ALTEX - Alternatives to animal experimentation, 00. doi: 10.14573/altex.2004221

Leenaars C, van der Mierden S, Durst M, Goerlich-Jansson V, Ripoli F, Keubler L, Talbot S, Boyle E, Habedank A, Jirkof P, Lewejohann L, Gass P, Tolba R, Bleich A (2019):
Measurement of corticosterone in mice: a protocol for a mapping review.
Laboratory Animals. 02367721986849.
doi: 10.1177/0023677219868499.

### 9.2.3 Reviews

Kahnau P, Habedank A, Diederich K, Lewejohann L (2020):
Behavioral Methods for Severity Assessment.
Animals, 10(7), 1136.
doi: 10.3390/ani10071136

Habedank A*, Kahnau P*, Diederich K, Lewejohann L (2018):
Severity assessment from an animal's point of view.
Berliner und Münchener tierärztliche Wochenschrift.
doi: 10.2376/0005-9366-18007.
(*These authors contributed equally.)

### 9.2.4 Talks

Habedank A, Kahnau P, Lewejohann L (2020):
Alternativloses Alternieren: Das T-Maze ist ungeeignet für Präferenz- und Lernversuche mit

C57BL/6J Mäusen.
GV-SOLAS Tagung, online
Abtract in: Program Book of the "58. Wissenschaftliche Tagung der Gesellschaft für Versuchstierkunde GV-SOLAS und 19. Fortbildungsveranstaltung der IGTP"

Habedank A, Kahnau P, Lewejohann L (2020):
Wo ist meine Maus? Das Mouse Position Surveillance System (MoPSS).
GV-SOLAS Seminar, Berlin

Habedank A, Urmersbach B, Kahnau P, Lewejohann L (2020):
The Mouse Position Surveillance System (MoPSS): an RFID based tracking system for home cage based choice tests.
BB3R Seminar, Dahlem Research School Graduate Studies Biomedical Sciences

Habedank A, Kahnau P, Lewejohann L (2019):
Choice tests a s a means for severity assessment from an animal's point of view.
PreDocSymposium, German Federal Institute for Risk Assessment (BfR)

Habedank A, Kahnau P, Lewejohann L (2018):
Choice tests a s a means for severity assessment from an animal's point of view.
Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences", Freie Universität Berlin

Habedank A, Kahnau P, Lewejohann L (2018):
Choice tests as a means for severity assessment from an animal's point of view.
BB3R Seminar, Dahlem Research School Graduate Studies Biomedical Sciences

### 9.2.5 Posters

Habedank A, Urmersbach B, Kahnau P, Lewejohann L (2020):
The Mouse Positioning Surveillance System (MoPSS): an RFID based tracking system for home cage based choice tests.
Tagung der Ethologischen Gesellschaft, Tübingen
Abstract in Program Book

Habedank A, Kahnau P, Lewejohann L (2019):
Development of an automatic tracking system for home cage base choice tests.
Tagung der Ethologischen Gesellschaft, Hannover
Abstract in Program Book

Habedank A, Kahnau P, Lewejohann L (2019):
Development of an automatic tracking system for home cage base choice tests.
PreDocSymposium, German Federal Institute for Risk Assessment (BfR)

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## 9.5 Conflict of Interest

The author declares no competing interests.

## 9.6 Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 14.10.2021 Anne Jaap