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When left is right: The effects of paw preference training on behaviour in mice

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ABSTRACT

Spontaneous limb preferences exist in numerous species. To investigate the underlying mechanisms of these preferences, different methods, such as training, have been developed to shift preferences artificially. However, studies that systematically examine the effects of shifting preferences on behaviour and physiology are largely missing. Therefore, the aim of this study was to assess the impact of shifting paw preferences via training on spontaneous home cage behaviour, as well as anxiety-like behaviour and exploratory locomotion (Elevated plus maze test, Dark light test, Open field test, Free exploration test), learning performance (Labyrinth-maze) and stress hormones (fecal corticosterone metabolites) in laboratory mice (*Mus musculus* f. *domestica*). For this, we assessed spontaneous paw preferences of C57BL/6J females ($N_{ambilateral}$ = 23, N_{left} = 23, N_{right} = 25). Subsequently, half of the individuals from each category were trained once a week for four weeks in a food-reaching task to use either their left or right paw, respectively, resulting in six groups: AL, AR, LL, LR, RL, RR. After training, a battery of behavioural tests was performed and spontaneous preferences were assessed again. Our results indicate that most mice were successfully trained and the effect of training was present days after training. However, a significant difference of preferences between RL and LL mice during training suggests a rather low training success of RL mice. Additionally, preferences of L mice differed from those of A and R mice after training, indicating differential long-term effects of training in these groups. Furthermore, left paw training led to higher levels of self-grooming, possibly as a displacement behaviour, and more time spent in the light compartment of the Dark light test. However, overall, there was no systematic influence of training on behavioural measures and stress hormones. Different explanations for this lack of influence, such as the link between training and hemispheric functioning or the intensity and ecological relevance of the training, are discussed.

1. Introduction

Behavioural asymmetry is a widespread phenomenon in the animal kingdom. A prominent example is the preferred use of one limb over the other. Such limb preferences can be found in vertebrate and even invertebrate species [1–3], with human handedness as the probably best-known case. Regarding the control of limb use, nerves from one side of the body are linked with the opposite cerebral hemisphere. Hence, depending on which hemisphere is predominantly active, the individual prefers the use of the contralateral limb [4]. Since the hemispheres control the performance of different behavioural patterns, limb preferences for specific behaviours may differ depending on which hemisphere is in control. For instance, it is hypothesized that the right hemisphere is involved in the control of avoidance, whereas the left hemisphere is involved in the control of approach behaviour [5]. Accordingly, due to the contralateral link between body and hemispheres, animals that prefer the left limb were found to show higher levels of avoidance behaviour when they encounter novel objects [6], whereas right limbed animals are faster to approach novel stimuli [6–8]. However, not only the direction, but also the strength of preferences has been associated with differences in the control of behaviours. It could be shown that lateralized individuals are, for example, better able to attend to two tasks simultaneously compared to non-lateralized (ambilateral) individuals [9,10]. This implicates a strong hemispheric specialization in

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lateralized individuals [11], which likely enables a simultaneous and independent processing of tasks in both hemispheres (e.g. searching for food in the left hemisphere, detecting predators in the right hemisphere) [12].

Besides the existence of spontaneous limb preferences, there are ways to artificially shift them. The consequences of and methods used for shifting preferences are highly diverse. In humans, for instance, short- and long-term change of hand use has been shown to affect the emotional state. More precisely, lefthanders that were forcefully retrained to use their right hand for writing showed higher incidences of stuttering, emotional problems and fears [13–15]. Furthermore, studies on short-term unilateral training in right-handers show that squeezing a ball with the left hand causes a more negative perception, judgement and feeling, whereas squeezing a ball with the right hand leads to a more positive perception, judgement and feeling [16,17], reviewed in [18]. In rodents, shifting paw preferences has been used to investigate the underlying mechanisms of paw preferences [19–32]. It was found that, for example, the increased use of one paw through training alters a cortical structure related to information storage in the hemisphere opposite to the trained paw [25]. Furthermore, a number of studies confirm that paw preferences can indeed be shifted to a certain degree [26–29]. Regarding the methods used for shifting preferences, they range from surgical interventions [19, 20, 22–24, 29, 30], to physical restraint [23, 31], to a modified environment to which the animals have to adapt [21, 25–29, 31, 32]. For the present study, we decided for the latter and customized the environment by presenting food in an offset position (also see [21, 25–29, 31, 32]), which is non-invasive and ecologically relevant. For this, a special testing chamber was designed according to the work by Collins [28]. In chambers with a so-called "world bias", a feeding tube could be presented either flush with the left (left biased world, or L-world) or the right (right biased world, R-world) wall, so that the animals were trained to use their left or right paw to reach for food.

While, so far, most studies using paw preference training in rodents have focused on the neuronal basis and the development of paw preferences [19–32], possible effects of paw preference training on behavioural and physiological measures have been largely neglected. Against this background, the aim of the present study was to systematically assess the impact of paw preference training on several behavioural parameters and stress hormone levels in laboratory mice using the abovementioned method. For this purpose, female C57BL/6J were initially tested for their spontaneous paw preference and categorized as ambilateral (A), left pawed (L) or right pawed (R). Although the inclusion of ambilateral animals is not yet a standard method (see [33–35]), it has been shown in different species that ambilateral animals differ from lateralized ones in terms of behaviour [9,10,36,37]. Therefore, we included them separately in a third category. Subsequently, mice were trained to either use their left (L) or right paw (R) to reach for food, resulting in six groups: AL, AR, LL, LR, RL and RR. Thereafter, the effects of paw preference training on spontaneous and anxiety-like behaviour, exploratory locomotion, learning performance and stress hormone levels (fecal corticosterone metabolites (FCMs)), were assessed. In line with previous findings from studies on paw preference in mice, we hypothesized that the distribution of spontaneously ambilateral (A), left pawed (L) and right pawed (R) mice does not deviate from chance [38]. Additionally, we expected to find a strong [28] and long-term influence of the world bias on paw preferences during and beyond the training. Lastly, we hypothesized the training to influence spontaneous and anxiety-like behaviour, exploratory locomotion and learning performance on the behavioural, and FCMs on the physiological level.

2. Material and methods

2.1. Animals and housing conditions

Subjects were 102 female C57BL/6J mice, purchased from Charles River Laboratories (Research Models and Services, Germany GmbH, Sulzfeld). Animals arrived on postnatal day (PND) 28 and were immediately marked with ear cuts to allow for individual identification. All mice were housed in pairs in transparent standard Makrolon cages type III (37 cm × 21 cm and 15 cm high) with wood shavings as bedding material (Tierwohl, J. Reckhorn GmbH & Co.KG, Rosenberg, Germany), enriched with a semitransparent red plastic house (Mouse House™, 11.1 cm × 11.1 cm and 5.5 cm high, Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany), a wooden stick (ca. 10 cm × 1.8 cm and 1.8 cm high) and a paper towel as nesting material. Food (Altromin 1324, Altromin GmbH, Lage, Germany) and tap water were provided ad libitum. Cages were changed and a new paper tissue was provided on a weekly basis, whereas the plastic houses and wooden sticks were renewed every 2 weeks. The housing room was kept at a reversed 12 h dark/light cycle with lights off at 1000 h, an ambient temperature of about 22 °C and a relative air humidity of about 50%.

2.2. Ethics statement

All procedures complied with the regulations covering animal experimentation within Germany (Animal Welfare Act) and the EU (European Communities Council DIRECTIVE 2010/63/EU) and were approved by the local (Gesundheits-und Veterinäramt Münster, Nordrhein-Westfalen) and federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen "LANUV NRW", reference number 84–02.04.2018. A236).

2.3. Experimental design

The experiment consisted of three consecutive phases to assess the effects of paw preference training on a variety of outcome measures. Firstly, in the pre-training phase, spontaneous paw preferences of mice were assessed. In a second phase, animals were trained to either use their left or right paw in a food-reaching task (training phase). Lastly, in the post-training phase, possible effects of this paw preference training on anxiety-like behaviour, exploratory locomotion and learning performance was assessed and spontaneous paw preferences were measured a final time (see Fig. 1). Since the spontaneous paw preference test and training are challenging, not all mice met our defined success criteria, which led to different sample sizes between groups and measurements taken, see Table 1.

In the pre-training phase, mice were habituated to the testing box (PND 38, see Fig. 2), subsequently tested for their spontaneous paw preference (PND 43 ± 1) and categorized into left pawed (L), ambilateral (A) or right pawed (R) (see section "2.4.1 Data preparation" for definitions). Because not all animals participated successfully, the test was repeated on PND 50 ± 1 with mice that did not reach the success criterion in the first round. All other animals were control handled. Home cage behaviour (HCB, PND 34–37) and fecal corticosterone metabolites (FCMs, PND 41) were assessed before the training started to get basal values (see section "2.3.3 Behavioural measurements and FCMs" for more details).

In the 4-week lasting training phase, mice were assigned to either a left (L) paw training or a right (R) paw training routine, where the animals were trained to either use their left or right paw for retrieving food. Each mouse was trained once a week, hence four times in total. Mice were allocated to the training routines depending on their spontaneous paw preference (see Table 1.), resulting in the following six groups: AL, AR, LL, LR, RL, RR. Regarding the terminology, we use the expression "retraining" for a training, where the direction of the training, i.e. world bias, does not match the spontaneous paw preference (e.g. groups AL, AR, LR and RL). The term "training" is used for a situation where forced limb use is applied, but the initial preference is either not known or not relevant to the specific context.

In the post-training phase, animals were tested in a number of behavioural tests (see section "2.3.3.1 Battery of behavioural tests") over a duration of 3 weeks (PND 77–94). Additionally, HCB (PND

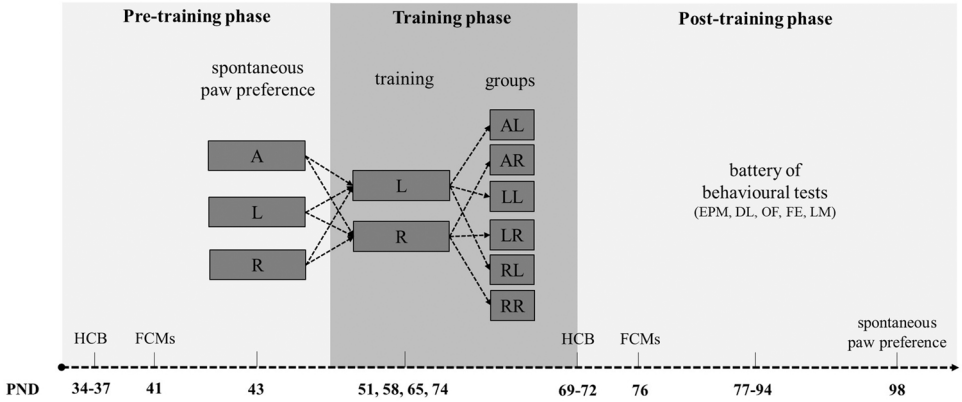

Fig. 1. Experimental design. Mice were tested for their spontaneous paw preferences (ambilateral (A), left (L) or right (R)) and allocated to either a left (L) or right (R) paw training. Afterwards, a battery of behavioural tests was conducted to assess their anxiety-like behaviour, exploratory locomotion and learning performance. Home cage behaviour (HCB) and fecal corticosterone metabolites (FCMs) were assessed in the pre- and post-training phase. Spontaneous paw preference was tested one final time in the post-training phase. Postnatal days (PND) indicate the approximate age of the animals at the respective time points. Please see sections below for more detailed age specifications.

Table 1
Sample sizes for each treatment group for spontaneous paw preference test in the pre-training phase, training, behavioural tests and spontaneous paw preference test in the post-training phase. Sample sizes reflect the number of mice that successfully passed the respective test or training and were included in the statistical analysis. Please refer to section "2.4.1 Data preparation" for more details on success criteria. A = ambilateral, L = left pawed, R = right pawed. _L = left paw training, _R = right paw training.

Spontaneous paw preferences	**A**		**L**		**R**		**Total N**	**Reduction according to success criteria**[a]
$N_{start\ of\ the\ experiment}$							102	
$N_{spontaneous\ paw\ preference\ -\ pre\text{-}training}$	23		23		25		71	-31^{sc1a}
Groups	**AL**	**AR**	**LL**	**LR**	**RL**	**RR**	**Total N**	
$N_{training}$	11	9	10	10	12	12	64	$-7^{sc1a\ \&\ 2}$
$N_{behavioural\ tests}$	11	9	10	9	6	12	57	$-7^{sc1a,\ 2\ \&\ 3}$
$N_{spontaneoous\ paw\ preference\ -\ post\text{-}training}$	9	6	8	7	9	11	50	$-14^{sc1a,\ 1b\ \&\ 2}$

[a] **success criterion 1a (sc1a)**: at least 50 reaches for food in the spontaneous paw preference test pre-training; **success criterion 1b (sc1b)**: at least 50 reaches for food in the spontaneous paw preference test post-training; **success criterion 2 (sc2)**: at least 50 reaches for food during the whole four days of training; **success criterion 3 (sc3)**: a significant z-Score in the direction that was trained, i.e. if the animal reached successfully significantly more often with the paw that matched the world bias compared to the non-matching paw

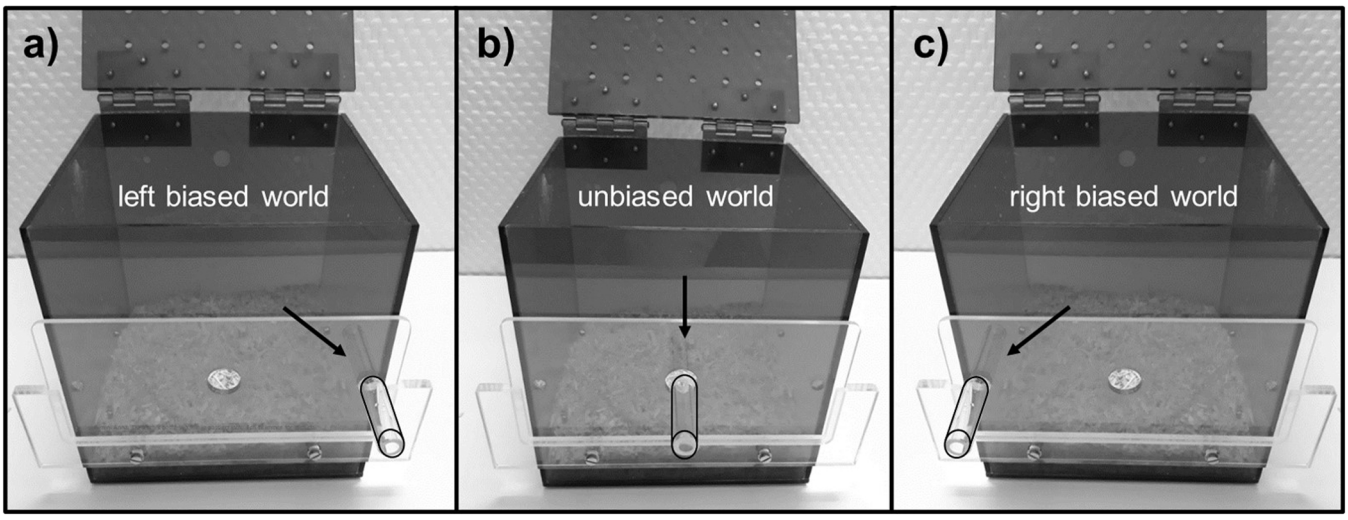

Fig. 2. Testing/training box. Depending on the usage of the testing box, the feeding tube can be attached a) flush with the left wall for a left paw training (left biased world), b) equidistant from the two side walls for assessing spontaneous paw preferences, or c) flush with the right wall for a right paw training (right biased world).

69–72) and FCMs (PND 76) were assessed a second time to test for possible effects of the training. Finally, the spontaneous paw preference of mice that completed the training was assessed one last time to test for its stability (PND 98). Throughout the experiment, individual body weights of mice were monitored on a weekly basis on PND's 34, 41, 48, 55, 62, 69, 76, 83, 90 and 97.

2.3.1. Spontaneous paw preference test

Spontaneous paw preference of mice was assessed using a modified version of the Collins' paw preference test [39]. We built a semi-transparent red plastic box (14 cm × 14 cm and 14 cm high), with a cylindrical, removable feeding tube (3.5 cm length, 6 mm inner diameter). Depending on the usage of the testing box, the feeding tube could be attached to the front wall in either an equidistant position from the two side walls (spontaneous paw preference test (Fig. 2b) or flush with

the left wall (left paw training, Fig. 2a)) or flush with the right wall (right paw training, Fig. 2c), at a height of 7 cm above ground (see Supplementary Fig. F1). The test was preceded by a short, one-day habituation phase, where the animals were individually placed in the test box for 15 min. After 15 min, mice were brought back to their home cages. To familiarize them with the food that was provided in the feeding tube during testing, mice received small amounts of dissolved baby oat flakes directly after the habituation in their home cages. In preparation for the test, the food in the animals' home cages was removed. After 5 min of habituation in the test box, the feeding tube was attached in the middle position (Fig. 2b) and animals were required to perform a reaching task to retrieve mash-like food (dissolved baby oat flakes) from the tube. After the tube was attached, a camera (SONY HDR-XC6, night shot mode) recorded the paw reaches for 15 min. Thereafter, the mice were placed back in their home cages and recordings were used to assess paw preference performance. We used the freeware behaviour coding program Solomon Coder (version: beta 19.08.02, solomon.andraspeter.com) to analyze the recordings. After coding 50 reaches, the analysis was terminated, e.g. [38–41].

For the spontaneous paw preference test at the end of the post-training phase, no prior habituation was needed since the animals were already habituated to the testing box.

2.3.2. Paw preference training

The training was conducted using the same testing box that was used for the spontaneous paw preference test (see section "2.3.1 Spontaneous paw preference test" above). Here, however, the feeding tube was attached flush with the left wall of the box for a left paw training, also referred to as a left world bias or L-world, (Fig. 2a) or flush with the right wall of the box for a right paw training, referred to as a right world bias or R-world (Fig. 2c) [28,32]. The smaller reaching angle that was created through this lateral position of the feeding tube enforced animals to use the paw matching with the world bias and made it more difficult for them to retrieve food with the incorrect paw. For the training, mice were placed in the testing box and after 5 min of habituation, the feeding tube was attached and a camera (SONY HDR-XC6, night shot mode) recorded the paw reaches for 15 min. Thereafter, the mice were placed back in their home cages and recordings were used to assess the success of paw preference training. We used the freeware behaviour coding program Solomon Coder (version: beta 19.08.02, solomon.andraspeter.com) to analyze the recordings. Here, the whole 15 min were analyzed. In a previous study, the number of training reaches and the elapsed time since training were shown to affect paw preference performance. More precisely, between 20 and 100 training reaches seemed to be optimal for affecting paw preferences and 7 days after training, the preference for the trained paw reached a maximum [32]. Hence, we conducted the training four times in total to increase the number of training reaches and at a time point (approx. after 7 days), where memory consolidation of past training reaches appears to be optimal [32]. Hence, the training phase lasted for four weeks, with one training per week on PND's 51 ± 1/53, 58/59, 56/66 and 74/75, resulting in 4 × 15 min training time per animal.

2.3.3. Behavioural measurements and FCMs

To gain a comprehensive picture of the effects of paw preference training on the animals' behaviour and stress hormone levels, several distinct measures were taken. After the training, anxiety-like behaviour and exploratory locomotion was investigated using several well-established tests, such as the Elevated plus maze test (EPM, PND 77 ± 1), Dark light test (DL, PND 79 ± 1), Open field test (OF, PND 84 ± 1) and Free exploration test (FE, PND 87 ± 1). Finally, learning performance was tested in the Labyrinth-maze (LM, PND 93 ± 1). Home cage behaviour (HCB) and fecal corticosterone metabolites (FCMs) were measured and observed once before and once after the training phase. Additionally, body weights were repeatedly measured during the experiment.

2.3.3.1. Battery of behavioural tests. Mice were tested during their active phase of the day, starting at 1315 h ± 30 min and ending at 1630 h ± 60 min. The testing order was pseudorandomized. After each mouse, test apparatuses were cleaned with 70% ethanol. For all tests, they were transported to a separate testing room in a darkened transport box (EPM, DL, OF) or in their home cage covered with a black cloth (FE, LM). The animal's movements were recorded by a webcam (Webcam Pro 9000, Logitech, Europe S.A., Lausanne, Switzerland) and automatically analyzed by the video-tracking system ANY-maze (Version 5.33, Stoelting Co., Wood Dale, USA) for the EPM, DL, OF and FE. Measures for the LM were taken via live observations by an experienced observer (B.S.). All test apparatuses were built by the institute's own workshop according to the specifications in [39, 42–44] and/or companies retailing (animal) behaviour observation soft- and hardware.

2.3.3.1.1. Elevated plus maze test. The Elevated plus maze (EPM) consisted of a wooden plus-formed apparatus with four arms (30 cm × 5 cm each) and a central square (5 cm × 5 cm), elevated 50 cm above the floor. While two opposing arms were enclosed by a wall of 20 cm height, the open arms only had a small barrier of 0.4 cm to prevent the mice from falling off the apparatus. All surfaces were light-gray PVC. The illumination level was set to 25 lux in the center. After transportation to the testing room and one minute in the transportation box, mice were placed on the apparatus with their head facing towards the closed arm of the apparatus pointing away from the experimenter. After starting the tracking software, the mice were allowed to explore the apparatus for 5 min, while the experimenter left the room. The time spent on the open arms compared to the total time spent on open and closed arms and the number of entries to the open arms compared to the total number of entries to open and closed arms were used to assess anxiety-like behaviour. Exploratory locomotion was assessed by comparing between the total number of arm entries.

2.3.3.1.2. Dark light test. The Dark light test (DL; [42]) apparatus consisted of a modified Makrolon type III cage (37 cm × 21 cm and 15 cm high). The dark compartment, one third of the cage, was painted black and covered with an opaque lid and separated from the light compartment with an opaque partition wall including a sliding door. The illumination level in the light compartment was set to about 40 lux. After transportation to the testing room, mice were placed in the dark compartment and spent one minute therein to acclimatize. Then, the sliding door was opened, the tracking software was started and the experimenter left the room. The animals could freely explore the apparatus for 5 min. To assess anxiety-like behaviour, the time spent in the light compartment and the latency to the first entry to the light compartment were used. The number of entries to the light compartment were used to assess the exploratory locomotion.

2.3.3.1.3. Open field test. The Open field test (OF; [43,44]) apparatus consisted of a square arena (80 cm × 80 cm) made out of white coated plywood that was surrounded by white walls (42 cm high). The illumination level was set to about 35 lux in the center. After transportation to the testing room and one minute of acclimatization in the transportation box, mice were placed in the apparatus with their head facing towards the left lower corner. After starting the tracking software, the mice were allowed to explore the apparatus for 5 min, while the experimenter left the room. Measures taken were the time spent in the center (defined as the area being located at least 20 cm distant from the walls) and the number of center entries (anxiety-like behaviour). Exploratory locomotion was assessed by using the total distance travelled.

2.3.3.1.4. Free exploration test. In contrast to the before mentioned test, in the Free exploration test (FE; [45,46]), mice could freely choose to either stay in their home cages or to explore a new environment. The apparatus consisted of a square arena (80 cm × 80 cm) made out of white coated plywood that was surrounded by white walls (35 cm high). The illumination level was set to about 40 lux in the center. The home cages could be connected to the apparatus via a Plexiglas tunnel and an opening in one wall (11 cm × 15 cm). After transportation to the testing

room in the animal's home cage, mice were placed in the transport box for one minute. During this time, the empty home cage could be attached to the apparatus. Then, mice were placed back in their home cages and a sliding door was opened, so that the animals could freely explore the arena for 15 min. After starting the tracking software, the experimenter left the room. To assess anxiety-like behaviour, the latency to the first entry to the arena, as well as the total number of entries and the time spent in the arena were used. The total distance travelled was used to assess exploratory locomotion.

2.3.3.1.5. Labyrinth-maze. The labyrinth-maze (LM) apparatus consisted of a white platform (40 cm × 24 cm) with several transparent acrylic glass walls (15 cm), partly with passageways to form a labyrinth [47]. The exit of the labyrinth led to the animal's home cage, which was connected via a short tunnel (8 cm). After transportation to the testing room in the animal's home cage, mice were placed in the transport box for one minute. During this time, the empty home cage could be attached to the apparatus. Thereafter, mice were placed in the start position of the LM, allowing them to freely explore the apparatus and to find the exit within 5 min maximum. After having solved the task by reaching the home cage, the mouse had a 5 min break in its home cage, while the LM was thoroughly cleaned with 70% ethanol. Subsequently, the mouse was again placed in the start position to perform a second trial for 5 min maximum. The parameters measured were the time needed to exit the LM and the number of mistakes. A mistake was defined when a mouse either took a wrong passageway or when it took a correct passageway but went the same way back again. For analyzing the learning performance of mice, we analyzed the percentage difference between the first and the second trial via dividing the difference of the first and the second trial by their mean.

2.3.3.2. Home cage behaviour. Regarding home cage behaviour, we analyzed general activity, maintenance and exploratory behaviour. Behaviours were observed in the animals' housing room once in the pre-training phase and once during the transition from the training to the post-training phase. Each animal was observed 40 times on both of these occasions, i.e. 80 times in total. Observations were made using instantaneous and one-zero sampling with observation intervals lasting 20 s for each mouse (focal animal sampling) [48]. At the beginning of each interval, the activity at this time point (scan) was recorded using instantaneous sampling. During the observation interval, one-zero sampling was applied to record various home cage behaviours (Table 2). Animals were observed in always the same order. Observations took place in the dark phase under red light conditions and were conducted by an experienced observer (B.S.). For the statistical analysis, the percentage of scans/intervals, in which a behaviour was observed, was calculated. For this, we corrected for the number of scans/intervals where the animal was not visible. Definitions of behaviours were based on previous publications [49–52] and the website mousebehavior.org), see Table 2. Because stereotypic and social behaviours occurred very rarely, they were excluded from statistical analysis.

Table 2
Definitions of home cage behaviours, based on previous publications ([49–52] and the website mousebehavior.org).

Behaviour	Definition
Activity – *Instantaneous sampling at the beginning of each observation interval*	
Active	The mouse is active when it shows any kind of motion, except for tiny whisker, ear or tail movements.
Inactive	The mouse is inactive when it is not active.
Home cage behaviours - *One-zero sampling during 20 s observation interval*	
Maintenance	
Feeding	A mouse ingests food.
Drinking	A mouse nibbles at a water bottle.
Self-grooming	A mouse scratches, grooms or licks its own body.
Exploration	
General locomotion	Sum of all locomotor activities, leading to spatial dislocation, except for climbing, digging, social interactions and feeding with displacement involved.
Climbing	The mouse does not touch the ground with any paws and holds to the cage lid.
Digging	A mouse moves substrate by a series of fast alternating forepaw movements with its snout lowered into the substrate.

We additionally recorded stereotypic and social behaviours. Because they occurred very rarely, we excluded them from the statistical analysis.

2.3.3.3. Fecal corticosterone metabolites (FCMs). The stress hormone level of mice was monitored non-invasively by measuring fecal corticosterone metabolites (FCMs) [53,54]. Always starting at 1015 h, animals were placed individually in Makrolon cages type III equipped with a thin layer of wood shavings and the standard housing enrichment (see section "2.1 Animals and housing conditions"). After 3 h, each mouse was transferred back to its home cage. Subsequently, all feces were collected and frozen at −20 °C. Samples were dried and homogenized, and aliquots of 0.05 g were extracted with 1 ml of 80% methanol. For the analysis of the samples, a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay was used, which was established and successfully validated to measure FCMs in mice (see [53,55]) to evaluate the activity of the hypothalamic-pituitary-adrenocortical axis (for a review see [56]). EIA sensitivity was 1.7 ng/0.05 g and the intra- and inter-assay coefficients of variation were below 10% and 12%, respectively.

2.4. Statistics

2.4.1. Data preparation

To evaluate whether a mouse was lateralized (L, R) or not (A), a binomial Z-score was calculated based on the number of right-paw reaches, using the following formula:

$$z - \text{Score} = \frac{r - \frac{N}{2}}{\sqrt{N * p * q}},$$

where r is the number of right paw reaches, N the total number of right and left paw reaches, and p = q = 0.5. In accordance with the literature, we defined mice with Z-scores higher than 1.96 as having a right paw preference (R), those having a Z-score lower than − 1.96 as having a left paw preference (L) and those having a Z-score in between as ambilateral (A) (e.g. [38,57,58]). Additionally, for each animal, a laterality index (LI = (frequency of right paw reaches - frequency of left paw reaches)/total number of reaches) was calculated to evaluate the direction of paw preferences (e.g. [38,58,59]). Laterality indices were then used to analyze the effects of the training.

For the analysis of the data from the spontaneous paw preference test in the pre- and the post-training phase, we included animals that met our defined success criterion (sc1a & b) of 50 reaches for food. For the analysis of the data from the training, we summed up all successful feeding tube entries made by the animals during the whole training phase. By our definition, an animal passed the training if it met our success criterion (sc2) of at least 50 reaches during the whole four days of training. Furthermore, we defined the training as successful (sc3), when an animal had a significant z-Score in the direction that was trained, i.e. if the animal reached successfully significantly more often with the paw that matched the world bias compared to the non-matching paw. Following this definition, the training was not successful for seven animals. Therefore, these individuals were excluded from the analysis where we assessed the effects of training on spontaneous and anxiety-like behaviour, exploratory locomotion, learning performance and FCMs and body weights (see Table 1).

2.4.2. Data analysis

Data was analyzed using the statistical software R (R Core Team, 2020, Version 4.0.3) and R Studio (RStudio Team, 2020, Version 1.3.1093).

Total sample size of 102 animal was calculated using the "samplesize_mixed" function from the sjstats package (Version 0.18.1) in R. Note

that the actual sample sizes were reduced to unexpected low participation rates of mice in the first spontaneous paw preference test.

In cases where we calculated linear mixed effects models, we graphically examined their residuals for normality and homoscedasticity. Furthermore, we tested for normal distribution with the Shapiro-Wilk test and applied Bartlett's test for normally distributed and Levene's and Fligner-Killeen test for not normally distributed data to check for homogeneity of variances between groups. We transformed raw data to meet the model assumption of normally distributed model residuals (see Supplementary Tab. A1 and A2 for detailed information). If interactions and main effects were significant, Tukey HSD post hoc comparisons were conducted. Differences were considered significant at $P \leq 0.05$. Partial eta squared (η^2p) was calculated as a measure of the magnitude of the reported effects [60].

2.4.2.1. Validation of training. We calculated a chi-square test with the number of spontaneously left pawed, ambilateral and right pawed mice to assess whether paw preferences deviate from a random distribution.

We used a linear mixed model with fixed between-subject factors "spontaneous paw preference" (three levels: L, A, R), "training" (two levels: L, R) and their interaction, as well as the random between-subject factor "batch" (two levels: 1, 2) to assess the effects of spontaneous paw preference and training on paw preferences (measured as laterality index (LI)) during training.

2.4.2.2. Long-term impact of training. To assess the effect of spontaneous paw preferences and paw preference training on the spontaneous paw preferences measured three weeks after the last training session, we used the same model as before, with "spontaneous paw preference" and "training" as fixed between-subject factors and random between-subject factor "batch". However, instead of the laterality index (LI) during training, we here used the LI from the spontaneous paw preference test in the post-training phase as dependent variable. Additionally, we calculated a Spearman's rank correlation to test for the overall association of paw preferences between the two time points. Here, we used a non-parametric test because we did not achieve normal distributions for the two parameters using transformations.

2.4.2.3. Effects of training on behaviour, FCMs and body weights. Two different linear mixed models were fitted to analyze the effects of spontaneous paw preference and paw preference training on various readout measures. More precisely, for variables that were measured only once (i.e. all behavioural tests), we used the same linear mixed model as in the section "2.4.2.2 Long-term impact of training" above with fixed between-subject factors "spontaneous paw preference" (three levels: L, A, R), "training" (two levels: L, R) and their interaction, as well as the random between-subject factor "batch" (two levels: 1, 2). Likewise, we used a linear mixed model for repeated measures with fixed between-subject factors "spontaneous paw preference" (three levels: L, A, R), "training" (two levels: L, R) and "time" (two levels: pre-training, post-training) and their interaction, as well as the random between-subject factor "batch" (two levels: 1, 2) and "animal ID" (N = 57) for dependent variables that were measured repeatedly (i.e. HCB, FCMs and body weights). The between-subject factor "time" is coded as a factor, denoting if the measure was taken in the pre- or post-training phase.

3. Results

3.1. Validation of training

102 female C57BL/6J mice entered the experiment. 70% of the animals (71 mice) reached the minimum number of 50 reaches in the spontaneous paw preference test. The distribution of ambilateral (A), left pawed (L) and right pawed (R) and mice did not deviate significantly from chance (A = 23, L = 23, R = 25; $\chi^2_2 = 0.11$, P = 0.945).

Subsequently, they were trained to use either their left (L) or right (R) paw to reach for food. The majority of mice adapted to the training world and shifted their use of paw according to the world bias (see Fig. 3). However, 17% of mice did not adjust to the training world. More precisely, one left pawed mouse did not adjust to the right and six right pawed mice did not adjust to the left biased world.

Accordingly, the main effect of training ($F_{(1,\ 58)} = 326.227$, $P < 0.001$) was found to be significant, indicating a successful training. Furthermore, spontaneous paw preference ($F_{(2,\ 58)} = 9.047$, $P < 0.001$) and the interaction of training and spontaneous paw preferences ($F_{(2,\ 58)} = 3.364$, $P = 0.041$) significantly affected paw preferences during training. Post hoc between-group comparisons revealed significant differences between all R vs. L trained groups (see Supplementary Tab. A1), showing that the training was successful for all groups. Additionally, the RL group significantly differed from the LL (P = 0.002), indicating that in the RL group overall, retraining was not successful (Fig. 3).

3.2. Long-term impact of training

Spontaneous paw preferences were assessed one final time around three weeks after the last training session in an unbiased world. Overall, paw preferences during the training were strongly correlated with paw preferences three weeks after the training (Spearman's rank correlation: $r_S = 0.770$, N = 49, $P < 0.001$).

Comparing the different groups, spontaneous paw preference ($F_{(2,\ 41.575)} = 13.187$, $P < 0.001$, Fig. 4) and training ($F_{(1,\ 43.569)} = 22.521$, $P < 0.001$, Fig. 4) had significant main effects on the laterality index (LI) in the final spontaneous paw preference test, suggesting that the effect of training was still present, independent of spontaneous paw preferences. Post hoc tests revealed a significant difference between spontaneously left pawed and ambilateral (P = 0.002, Fig. 4) and spontaneously left and right pawed mice ($P < 0.001$, Fig. 4), indicating that spontaneously left pawed mice, compared to ambilateral or right pawed mice, had a stronger tendency to shift back to their initial preference.

3.3. Effects of training on behaviour, FCMs and body weights

3.3.1. Spontaneous and anxiety-like behaviour, exploratory locomotion and learning performance

Regarding spontaneous behaviours observed in the home cage, no interaction of spontaneous paw preference, training and time was found. However, the maintenance behaviour "self-grooming" was affected by training, with left paw trained mice grooming themselves more often than right trained animals. Additionally, time had a significant main effect on the maintenance behaviours "feeding" and "self-grooming", as well as on "general locomotion". More precisely, mice groomed themselves less, but ate more in the pre- compared to the post-training phase. Furthermore, mice moved around in the cage more often in the post-, compared to the pre-training phase (for statistical details see Table 3).

Regarding anxiety-like behaviour, exploratory locomotion and learning performance, a battery of different tests was conducted (EPM, DL, OF, FE, LM). Concerning anxiety-like behaviour, the amount of time spent in the light compartment of the DL test was affected by the training. More precisely, mice that experienced a left paw training spent more time in the light compartment compared to right trained animals ($F_{(1,\ 50.006)} = 4.103$, P = 0.048, Fig. 5a). Regarding exploratory locomotion, the amount of entries into the light compartment of the DL test was affected by spontaneous paw preference. In more detail, post hoc analysis showed that mice without a spontaneous paw preference (A) entered the light compartment more often than left pawed ($P < 0.001$, Fig. 5b). Learning performance, as well as all other parameters on anxiety-like behaviour and exploratory locomotion were not affected by the spontaneous paw preference nor by training or by an interaction of both (Fig. 6, see Supplementary Tab. A3 for data overview).

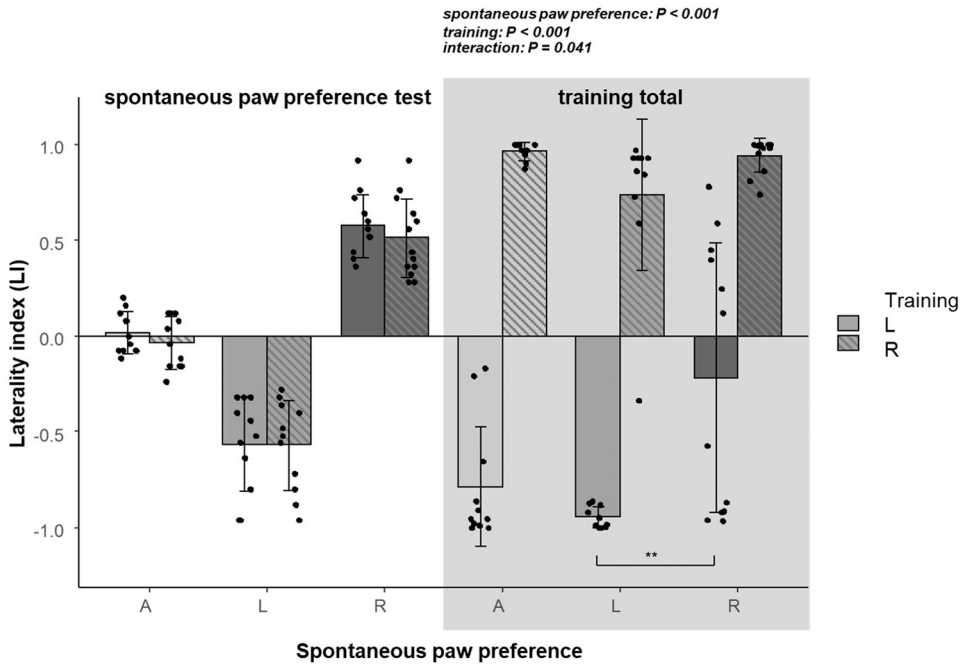

Fig. 3. Comparison of the laterality indices (LI) from the spontaneous paw preference test and training total. Mice were tested for their spontaneous paw preference (A, L or R) and trained to either use their left (L) or right (R) paw in a food-reaching task, resulting in the following six treatment groups: AL, AR, LL, LR, RL, RR. Statistics: Linear mixed model, post hoc Tukey HSD; Sample sizes: Spontaneous paw preference test – AL = 12, AR = 11, LL = 11, LR = 12, RL = 12, RR = 13; Training total – AL = 11, AR = 9, LL = 10, LR = 10, RL = 12, RR = 12. Data is presented as means ± SD. * * P ≤ 0.01. P-values of main effects are given as text. Relevant, significant post hoc effects are shown graphically. A significant interaction of spontaneous paw preference and training was found. Note that the statistical specifications refer only to the training total LI's (highlighted in grey). Please refer to section "3.1 Validation of training" for more information about statistical differences.

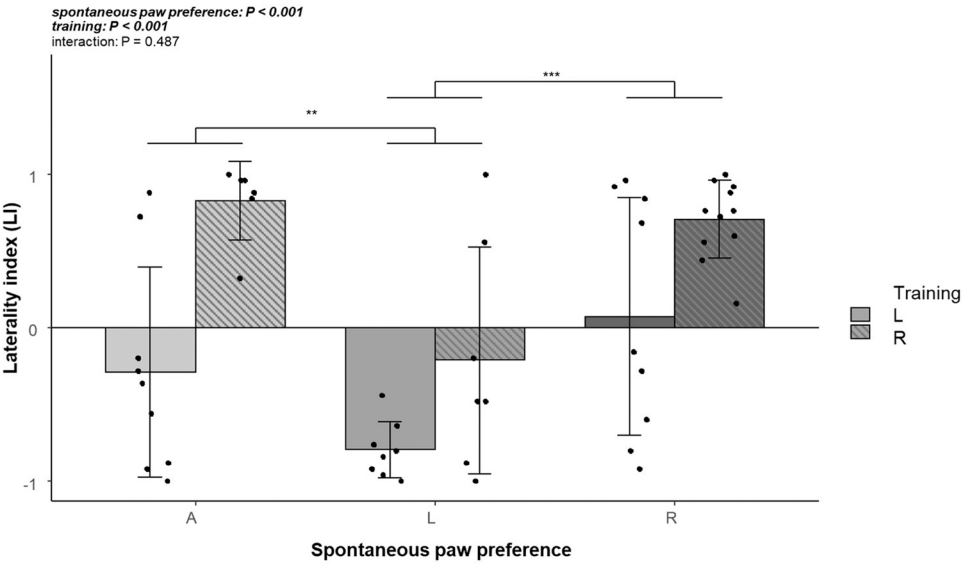

Fig. 4. Laterality indices (LI) of the spontaneous paw preference test in the post-training phase. Mice were tested for their spontaneous paw preference (A, L or R) and trained to either use their left (L) or right (R) paw in a food-reaching task, resulting in the following six treatment groups: AL, AR, LL, LR, RL, RR. Statistics: Linear mixed model, post hoc Tukey HSD; Sample sizes: AL = 9, AR = 6, LL = 8, LR = 7, RL = 9, RR = 11. Data is presented as means ± SD. * * ≤ 0.01, * ** P ≤ 0.001. P-values of main effects are given as text. Relevant, significant post hoc effects are shown graphically. Spontaneous paw preference and training significantly affected the laterality index of the spontaneous paw preference test in the post-training phase. Please refer to section "3.2 Long-term impact of training" for more information about statistical differences.

Table 3
Statistical details of home cage behaviours.

HCB	Training		Time		Effect of training	Effect of time
	L	R	pre-training	post-training		
Self-grooming [%]	16.6 ± 0.8	13.2 ± 0.7	13.2 ± 0.7	16.4 ± 0.8	$F_{(1,\ 50.016)} = 18.256$; **P = 0.006**	$F_{(1,\ 51)} = 7.30$; **P = 0.009**
Feeding [%]	28.9 ± 1.4	29.1 ± 1.2	32.3 ± 1.4	25.8 ± 1.2	$F_{(1,\ 50.006)} = 0.013$; P = 0.911	$F_{(1,\ 51)} = 14.478$; **P < 0.001**
General locomotion [%]	21.3 ± 1.1	22.6 ± 1.2	20.4 ± 1.2	23.6 ± 1.1	$F_{(1,\ 51)} = 0.405$; P = 0.527	$F_{(1,\ 51)} = 8.155$; **P = 0.006**

Means ± SEM of selected home cage behaviours of mice that either received a left (L) or right (R) paw training measured before (pre-) or after (post-training) the training phase. Bold numbers: P ≤ 0.05

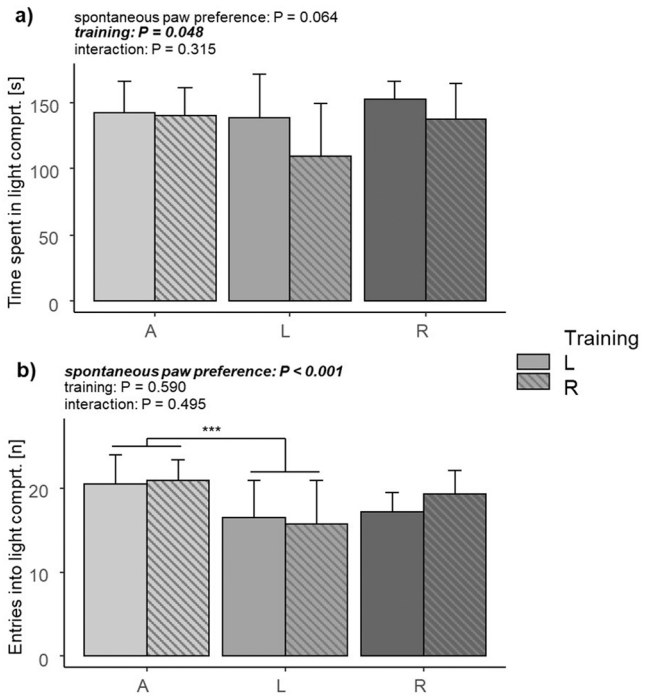

Fig. 5. Dark light (DL) test. Mice were tested for their spontaneous paw preference (A, L or R) and trained to either use their left (L) or right (R) paw in a food-reaching task, resulting in the following six treatment groups: AL, AR, LL, LR, RL, RR. a) Dark ight test: time spent in light compartment. b) Dark light test: entries into light compartment. Statistics: Linear mixed model, post hoc Tukey HSD; Sample sizes: AL = 11, AR = 9, LL = 10, LR = 9, RL = 6, RR = 12. Data is presented as means ± SD. * ** P ≤ 0.001. P-values of main effects are given as text. Significant post hoc effects are shown graphically. Training affected the time spent in the light compartment of the Dark light test. Spontaneous paw preference affected the number of entries into the light compartment.

3.3.2. Fecal corticosterone metabolites (FCMs) and body weights

Fecal corticosterone metabolites (FCMs) were assessed directly before and after the training phase and body weights were measured repeatedly on a weekly basis. For FCMs, the model revealed significant differences between some of the groups before the training started. Namely, mice from the LR group (234.4 ng/0.05 g ± 19.3) had significantly higher corticosterone metabolite concentrations in their feces compared to mice from the AL (165.3 ng/0.05 g ± 8.8; P = 0.005), LL (165.7 ng/0.05 g ± 15.1; P = 0.007) and RR group (175.4 ng/0.05 g ± 12.8; P = 0.023). Due to that fact, we calculated an additional linear mixed effects model to test if *relative* concentrations of FCMs are affected by the treatment. But, neither spontaneous paw preference or training, nor an interaction of both influenced relative FCMs (see Supplementary Tab. A2). Apart from this, a significant main effect of time (pre-training: 182.5 ng/0.05 g ± 7.0; post-training: 78.5 ng/0.05 g ± 2.3; $F_{(1, 51)} = 254.642$, P < 0.001) reflected a general decrease of FCMs over time. Similarly, only time significantly affected body weights (pre-training: 16.5 g ± 0.1; post-training: 20.7 g ± 0.1; $F_{(1, 165)} = 2154.047$, P < 0.001), reflecting a general weight gain over the period of the training phase.

4. Discussion

Since previous studies have mainly used paw preference training to investigate the neuronal basis and the development of paw preferences, the current study examined possible effects of paw preference training on various behavioural measures and FCMs. More precisely, the aim was to investigate the impact of paw preference training on spontaneous and anxiety-like behaviour, exploratory locomotion and learning performance, as well as stress hormone levels in female mice. The training was successful for most animals and influenced their paw preferences persistently. However, the effect of training on various behavioural measures and FCMs was limited to two out of 23 parameters.

4.1. Validation and long-term impact of training

4.1.1. Validation of training

We expected mice to adjust their paw use during training to the world bias. Indeed, this was the case for 83% of mice. However, 17% of animals retained their initial preferences and used the paw opposite to the world bias during training. Similar numbers of "untrainable" individuals were found by Collins [28]. Possibly, not all mice adjusted to the world bias because some mice might have already had a strong preference before the training started. Since paw preferences develop in dependence of previous choices [32,61], the paw preference test preceding the training might have formed and reinforced preferences to a point where they could no longer be influenced by the subsequent training.

Interestingly, on a descriptive level, ambilateral mice adjusted their paw use during training to the world bias more readily than lateralized ones. More precisely, all ambilateral mice (AL and AR) used the paw matching the world bias, whereas only 50% of right pawed (RL) and 90% of left pawed mice (LR) adjusted correctly to the world bias. Likely, this is caused by the fact that lateralized individuals have to give up their spontaneous preference and adopt a new one, whereas ambilateral mice simply have to reinforce the use of a paw they have already used before. This indicated difference in paw use plasticity between ambilateral and lateralized animals complements investigations showing that these groups also differ in terms of other aspects on the behavioural level, such as noise phobia [36], foraging success [37] and dual task performance [9,10]. Surprisingly, despite the evidence, the use of ambilateral individuals as a third group is not yet a standard method in studies on paw preferences (discussed in [35]).

Notably, the unsuccessful retraining of right, but not left pawed mice, might be due to functional hemispheric differences. Indeed, the left hemisphere controls routine behaviours [62,63] and the right hemisphere is involved in the detection and analysis of novelty [61,62]. Furthermore, the retrieval of short- and long term memory seems to be a lateralized process [64]. Regarding routine behaviours, the preferred use of the opposite right body part might generally be linked to routine formation. Accordingly, right pawed mice might express stronger routines, i.e. be more rigid in their preferences and therefore less trainable, compared to left pawed mice. With regards to the detection of novelty, during training, mice experienced a novel situation as they were forced to find an alternative strategy to reach the food reward. Hence, left pawed mice (right hemisphere dominance) might have had an advantage in adjusting their behaviour to the new environment over right pawed ones due to the specialization of the right hemisphere for detecting and analyzing novelty. Lastly, the lateralization of short- and long-term memory might also explain differences in paw use adjustment in left and right pawed mice since learning is involved in the acquisition of a new preference.

4.1.2. Long-term impact of training

We hypothesized the training to influence paw preferences in the long-term. Thus, we expected to find an effect of the world bias on spontaneous paw preferences measured three weeks after the training. Overall, we can conclude that training affected paw preferences for a certain period of time because 81% of mice showed preferences consistent with the ones learned at least for three weeks beyond the training phase. However, in line with studies in rats (e.g. [29,31]), 19% of mice showed preferences inconsistent with the ones learned three weeks after training.

The fact that left pawed mice differed significantly from right pawed

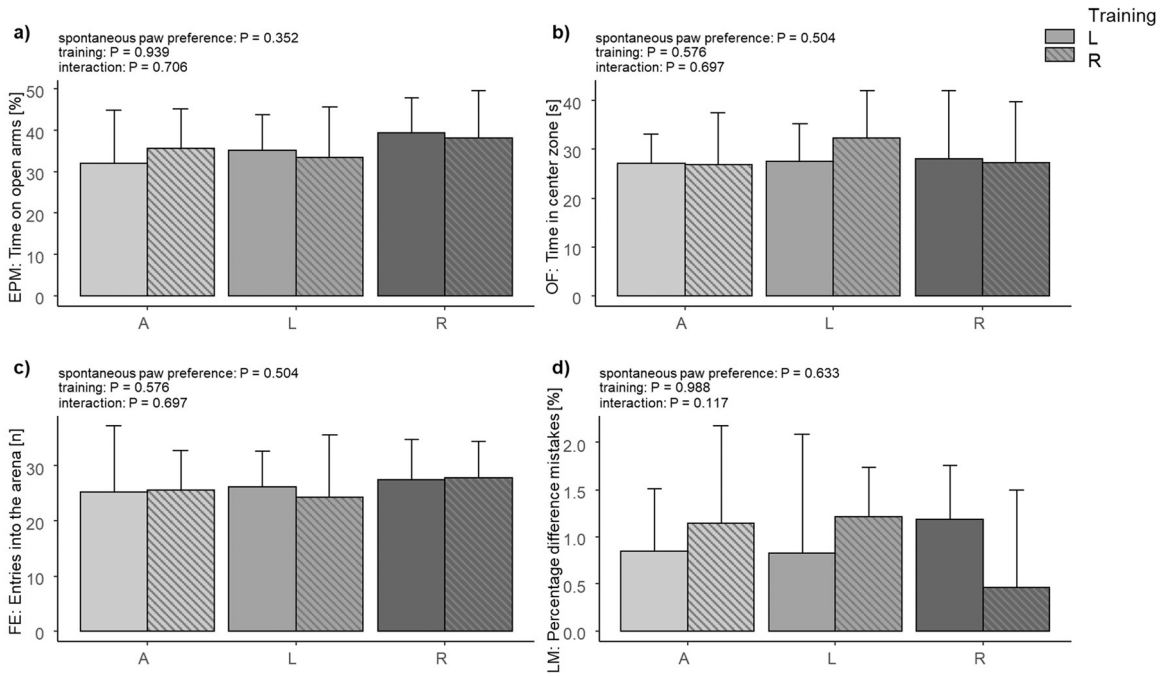

Fig. 6. Behavioural tests on anxiety-like behaviour, exploratory locomotion and learning performance. Mice were tested for their spontaneous paw preference (A, L or R) and trained to either use their left (L) or right (R) paw in a food-reaching task, resulting in the following six treatment groups: AL, AR, LL, LR, RL, RR. a) Elevated plus maze test (EPM): Relative time spent on open arms. b) Open field test (OF): Time spent in the central zone. c) Free exploration test (FE): Total entries into the arena. d) Labyrinth-maze (LM): Percentage differences of mistakes between first and second round. Statistics: Linear mixed model; Sample sizes: AL = 11, AR = 9, LL = 10, LR = 9, RL = 6, RR = 12. Data is presented as means ± SD. P-values of main effects are given as text.

and ambilateral ones in their paw preferences three weeks after the training might suggest that they had a stronger tendency to shift back to their initial preference compared to the other two groups. Possibly, similar to the resistance to paw preference training, this might be also due to the importance of the left hemisphere for learned routine behaviours [62,63]. More precisely, the rather weak routines in left pawed mice could explain why mice from the LR group did not show preferences consistent with the ones learned during training beyond the training phase.

4.2. *Effects of training on behaviour and FCMs and body weights*

We expected to find a systematic influence of paw preference training on various behavioural measures and FCMs. Out of 24 parameters investigated in total, paw preference training affected one measure of spontaneous behaviour (self-grooming) and one measure of anxiety-like behaviour (time spent in the light compartment of the DL test). In addition to the effect of training, we investigated the effects of spontaneous paw preferences on behaviour and FCMs. In this respect, we found one parameter of exploratory locomotion to be influenced (entries into the light compartment of the DL test). Thus, although a few parameters were affected, we assume that there is neither a systematic influence of paw preference training nor a specific influence of spontaneous paw preferences on various behavioural parameters and FCMs.

With regards to the significant effects we found, functional hemispheric differences might account for differences in self-grooming rates. More precisely, self-grooming can be performed in the form of a displacement behaviour [65] and as such may be associated with stress (e.g. [66]). Since the right hemisphere is involved in stress regulation [63], left paw training might lead to higher levels of self-grooming due to its potential to affect right hemispheric activity. Lower levels of anxiety-like behaviour in left paw trained mice and lower levels of exploratory locomotion in spontaneous L, compared to A mice, however, contradict previous findings. More precisely, it has been shown on one hand, that left paw preference is linked to higher levels of anxiety-like behaviour and behavioural despair [67–70]. On the other hand, spontaneous ambilaterality (A) has been previously linked to risk-averseness and cautiousness [71–73]. Against this background, it is difficult to assess how the observed effects come about and what significance the training has, e.g. as a possible therapeutic method.

Regarding the lack of a systematic influence of paw preference training on behavioural measures, FCMs and body weights, there are three different possibilities on a mechanistic level. First, the training possibly did not affect the underlying neuronal mechanism of paw use (i.e. hemispheric functioning [4]) and thus, did not lead to changes in the observed traits. Second, assuming that hemispheric functioning can be affected by paw preference training, the intensity of training might have been critical. Indeed, here, a non-invasive, ecologically relevant method, namely presenting food in an offset-position (see also [21, 25–29, 31, 32]) was used to manipulate paw preferences. However, this rather natural method could have been too low in intensity to cause a change in hemispheric functioning. Lastly, changes in phenotypic traits may manifest with a time lag [74,75]. Hence, a change of hemispheric functioning could become apparent only after a substantial lag-time.

From an ecological perspective, mice live in a constantly changing social and physical environment [76]. Therefore, phenotypic adaptations in response to subtle changes might become maladaptive rather quickly. Instead, it may be advantageous for individuals to adapt only in response to strong, reliable environmental cues [74]. To the extent paw preference training was conducted in the current study, its ecological relevance might have been limited, thus not leading to an adaptation in hemispheric functioning, behaviour and/or physiology.

As an outlook, follow-up studies might include control groups with untrained, control-handled spontaneously left, ambilateral and right pawed mice in order to assess the effects of paw preference training per se. This can be of particular interest since it has been shown that repeated cognitive stimulation might affect experimental subjects in regards to cognitive performance [77,78] and anxiety-like behaviour and stress hormone levels [79,80].

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CRediT authorship contribution statement

S.K., N.S., and S.H.R. conceived the study. S.K., B.S., N.S., and S.H.R. designed the experiments. S.H.R. supervised the project. B.S. carried out the experiments. R.P. determined and analysed the hormonal data. B.S. conducted the statistical analysis of the data and wrote the initial draft of the manuscript. All authors critically revised the manuscript for important intellectual content.

Conflict of interest

The authors have no potential competing interests to report.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2022.113929.

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