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The Presence of a Shelter in an Open Field Test Has Differential Effects on the Behavior and Stress Response of Two Mouse Species

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ABSTRACT

The open field test (OFT) is frequently used in research to assess anxiety-like behavior and locomotor activity. Its simple design can lead to the misconception that it is a standardized procedure comparable between laboratories. However, some modifications in the setup can cause changes in behavior. Different species might also react differently to the modifications introduced. There is thus need for a better understanding of the impact of modifications and their value for the species in question. Here, we tested two closely related mouse species, *Mus musculus* and *Mus spretus*, in an OFT with and without the presence of a shelter. We assessed mouse exploratory behavior through the analysis of multiple behavioral traits, and stress response through the measurement of circulating cortisol levels. Both species had elevated cortisol levels during the OFT in contrast to control animals which were not exposed to the OFT. While the presence of a shelter in the OFT increased the exploratory behavior in both mouse species, *M. spretus*, but not *M. musculus*, showed a reduction in cortisol levels. Also, other measured behaviors show a rather proactive coping strategy of the commensal *M. musculus* in contrast to a reactive strategy of the non-commensal *M. spretus*. Our study revealed a strong species-specific influence of the OFT design on the resulting behavior and stress levels of mice, illustrating the importance of OFT designs to account for the characteristics of the species under study. The addition of a shelter might be considered to improve experimental results by promoting animal welfare.

1 | Introduction

The open field test (OFT) has been used since 1934 to analyze behavior (Hall 1934). The basic setup is simple, consisting of an open area surrounded by walls that prevent individuals from escaping. The test's framework is based on the conflict between exploring new environments and the fearful avoidance of exposed areas. This emotional stimulation is associated with

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Summary

- The presence of a shelter in the OFT can reduce anxiety and increase exploratory behavior in mice.
- The presence of a shelter does not necessarily reduce stress levels.
- Different rodent species exhibit different responses to the open field test.

physiological responses, measurable through defecation and urination, and ultimately influences behavior. Initially, the test was developed to measure shyness based on the number of droppings produced by the animal (Hall 1934). "Emotional animals" would defecate more, when placed in a strange environment. Over the years, the analysis of OFT trials was refined to accommodate the measurement of different traits, and both theoretical and practical principles were accounted for to produce accurate and reliable results (Gould, Dao, and Kovacsics 2009; Walsh and Cummins 1976). Arena features, such as size, shape, and the presence of a shelter, as well as the presence of objects have been shown to influence the output of the OFT (Yaski, Portugali, and Eilam 2011; Zimmermann et al. 2001). For mice, an arena size of at least 40×40 cm has been recommended (Gould, Dao, and Kovacsics 2009). The test has been used mostly in rodents (e.g. Prut and Belzung 2003), but it has also been successfully applied to other model species, for example, dogs (Wormald et al. 2016), cats (Siegford et al. 2003), mini pigs (Lind et al. 2005), fish (Ahmad and Richardson 2013), and birds (Pittet, Houdelier, and Lumineau 2014). The OFT is also of interest to behavioral ecologists analyzing the influence of different environmental factors on the activity and exploration of wild species to answer questions of ecological and evolutionary relevance (Soloaga et al. 2016; Stratton, Nolte, and Payseur 2021; Bednarz and Zwolak 2022; Ohmer et al. 2023; Iwińska et al. 2024). Exploratory behavior has been shown to be repeatable and heritable (Dingemanse et al. 2002; Rudeck et al. 2020) and correlate with behavioral, ecological, and life-history traits in the wild (Boon, Réale, and Boutin 2007; Bárdos, Török, and Nagy 2024).

Many small rodents, like mice, are prey species and have a gregarious lifestyle, inhabiting tunnel systems or small spaces inside natural vegetation or human-made structures. Consequently, the OFT represents two challenges for mice: being separated from the group and being exposed in an open area. The OFT is thus commonly used in laboratory mice, descendants of the wild house mouse (*Mus musculus*), to test anxiety or explorative behavior (Belzung 1999; Bourin et al. 2007). It has been discussed if the propensity for exploration can be really tested or if it is overshadowed by the influence of anxiety (Bourin et al. 2007; Rosso et al. 2022). Similarly, anxiety might not be easily assessed by the OFT in all cases (Carola et al. 2002; Prut and Belzung 2003). Some of these issues likely arise because of the connection between anxiety and the propensity to explore: an individual experiencing high levels of anxiety will in many cases explore less. However, a detailed analysis of the behavior of animals in the OFT allows to at least partially disentangle both behavioral tendencies. In this sense, animals expressing anxiety-like behavior are more likely to move near the walls of a testing arena to avoid being exposed in the center (Bailey and Crawley 2009). Exploratory behavior, in contrast, has been associated with the animals' response to novelty, expressed by the distance moved and the area covered inside the testing arena (Lipkind et al. 2004). An individual in an anxious state might cover less area in the center of the open field than an individual in a less anxious state. The added measurement of glucocorticoid hormones can further assist in separating the emotional state from the exploratory tendencies of the tested animal.

Nevertheless, if a behavioral test causes high anxiety in most or all participating individuals, individual differences in behaviors, such as the tendency to explore, will likely be obliterated. If the aim of a test is the analysis of individual exploratory drive, a test should thus not strongly increase the anxiety of the tested animals. A way to reduce anxiety in the OFT can be the provision of a shelter. Both in rats and voles, it has been shown that if a shelter is provided in an OFT, the animals readily use it (Eilam 2010; Welker 1959). However, to our knowledge, it has never been tested if the presence of a shelter in an OFT indeed decreases the stress response, expressed by the levels of glucocorticoid hormones, and how exploratory behavior changes accordingly.

In this study, we compared two OFT designs, one classic open, empty arena, and another offering a shelter in the center of the arena. We hypothesized that the presence of a shelter reduces the animal's stress levels, decreasing anxiety related behaviors and increasing exploratory effort. To test these hypotheses, we compared two wild mouse species with different ecological niches, the highly gregarious, commensal house mouse, *M. musculus*, and the less gregarious, noncommensal Algerian mouse, *Mus spretus*, in both versions of the OFT. The house mouse thrives in anthropogenic environments. They live in large family groups, which include several females and their offspring, which they raise in communal nesting, and can reach densities of up to 7 individuals per square meter in locations with ample food supply (Gray and Hurst 1997; König et al. 2012). The non-commensal Algerian mouse, distributed in the western Mediterranean region, inhabits a variety of open habitats, including shrublands, farmlands and young or open forests, but is not commonly found very close to or inside human structures (Gray et al. 1998). Males are territorial, with their territories overlapping with those of 2 to 7 females, resulting in lower population densities compared to *M. musculus* (Gray et al. 1998; Palomo, Justo, and Vargas 2009). Communal nesting is not known for this species. The strong social link found between a single female and male suggests a facultative monogamous mating system (Cassaing, Cervera, and Isaac 2009). Although these two mouse species occur sympatrically, they typically do not share habitats and are therefore considered to behave as if they were allopatric (Palomo, Justo, and Vargas 2009).

We analyzed classical behavioral measures for anxiety and exploratory activity and assessed the physiological stress response through the measurement of circulating cortisol levels measured in the serum. While corticosterone is considered the primary glucocorticoid in *M. musculus*, it has been suggested

that cortisol responds quicker to acute stressful situations than corticosterone (Gong et al. 2015). Studies analyzing glucocorticoids in *M. spretus* are limited (e.g. Da Silva Junior et al. 2012). However, given their high evolutionary proximity to *M. musculus* (Palomo, Justo, and Vargas 2009) and the high stability of the hypothalamus-pituitary-adrenal system across the evolutionary path (Denver 2009), *M. spretus* is often used as source of phenotypic variation to unravel complex traits (see Dejager, Libert, and Montagutelli 2009 for details). We expected mice of both species to reduce their stress response in the presence of a shelter. We further expected animals to change their exploratory strategy and their tendency for thigmotaxis (wall-seeking). Due to their regular use of highly variable human-made habitats, we expected the commensal species (*M. musculus*) to show a lower stress response and a higher exploratory drive than *M. spretus*.

2 | Material and Methods

2.1 | Ethical Statement

All procedures were approved by ORBEA, the animal welfare body of the Faculty of Sciences, University of Lisbon (Statement number 1/2018), following European guidelines (86/609/EEC). Capture, transport, and maintenance of the animals was authorized by the Portuguese competent authorities, ICNF (license number 428/2018). All experimental procedures were performed by experts in laboratory animal science (FELASA category C) accredited by the Portuguese Veterinary Authority. Body mass of mice was measured weekly, and no significant changes were observed.

2.2 | Animals

A total of 28 *Mus musculus* (Linnaeus, 1758; 13 females and 15 males) and 28 *M. spretus* (Lataste, 1883; 10 females and 18 males) were tested. *M. musculus* were captured in Lisbon (Portugal), in horse stables and animal food storage buildings, and *M. spretus* were captured approximately 30 km away, in natural areas in the Sintra-Cascais Natural Park. Individuals were trapped using Sherman live traps, baited with sardine paste. A ball of hydrophobic cotton was provided to increase thermal insulation inside traps. After capture, mice were transported to the animal facility at the Faculty of Sciences, University of Lisbon, and housed individually in Makrolon type III cages ($26.5 \times 42.5 \times 14\ cm^3$) with wood shavings as bedding. Cardboard tubes, cotton, hay, and tissue paper were provided as nesting materials and environmental enrichment. A standard maintenance diet (commercial rodent chow—A04, Safe, France) and tap water were provided *ad libitum*. Individuals were maintained under controlled laboratory conditions (20°C ± 2°C; 12 L:12D, lights on at 7:00 a.m.).

As our study species occur in sympatry within the study area, the taxonomical identity of individuals was confirmed through genetic analysis to avoid an erroneous species assignment. A small tail clip was excised from each mouse and stored in absolute ethanol at −20°C. Genomic DNA was extracted using a commercial kit (E.Z.N.A Tissue DNA Kit, Omega BIO-TEK) and kept at −20°C until further analysis. The mitochondrial D-loop was amplified by PCR (Polymerase Chain Reaction) using the primer pair L15774 (5′—CGC CTG TTT ATC AAA AAC AT—3′) and H2228 (5′—CTC CGG TTT GAA CTC AGA TC—3′) according to the protocol described in Gabriel, Mathias and Searle (2015). PCR products were purified with ExoSAP-IT (ThermoFisher Scientific) and sequenced commercially at STAB VIDA on an ABI Prism 3730xl DNA sequencer. Resulting sequences were submitted to the NCBI nucleotide collection for species identification using the BLAST algorithm.

2.3 | Experimental Procedure

Preceding experimental trials, all animals underwent an acclimatation period of 1 week that included habituation to human presence and handling (Figure 1). Each individual was removed from its home cage once a day and handled for a period of 10 to 15 min by the researcher that would later conduct the behavioral experiments.

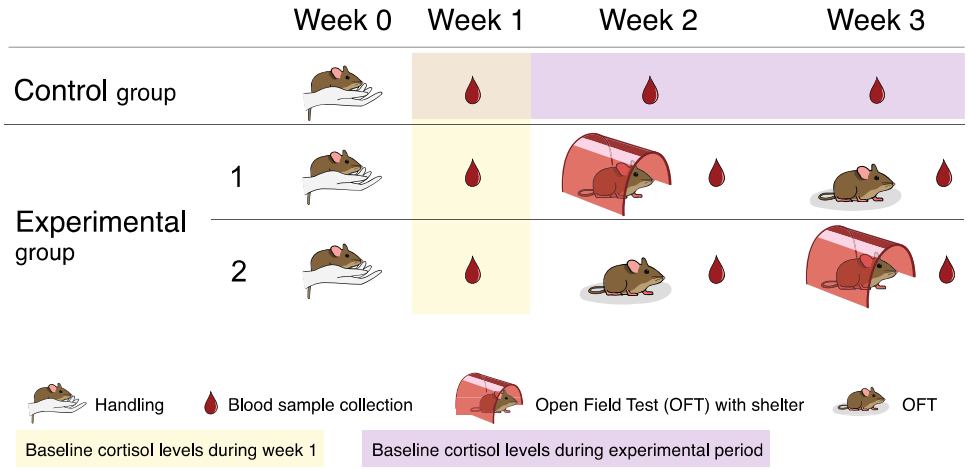

FIGURE 1 | Experimental scheme portraying the open field test (OFT), with and without a shelter, applied to wild house mice ($n = 28$) and wild Algerian mice ($n = 28$). The control group was subjected to a weekly blood collection to assess baseline levels of cortisol, as were all experimental groups during the first week. For the experimental groups, blood samples were collected on a weekly basis to quantify circulating cortisol levels immediately after behavioral tests were performed.

In the 3 weeks following the habituation period, mice were allocated to experimental groups to undergo hormonal and behavioral testing. For each species, mice were randomly assigned into one of three groups: Control group, Experimental group 1 and Experimental group 2 (see Figure 1). Mice in the Control group were used to assess hormonal levels over the experimental period in the absence of behavioral testing (treatment "no OFT"). Mice in the experimental groups were used to assess hormonal levels (weeks 1, 2, and 3) and behavior (weeks 2 and 3). Hormonal levels of week 1 served as baseline values for all groups. All mice in the experimental groups were subjected to two different versions of the OFT setup, one with a shelter (treatment "OFT with shelter"), and one without (treatment "OFT no shelter"). To avoid an effect due to treatment order, half of the individuals (Experimental group 1) were first subject to the OFT with a shelter, and the other half (Experimental group 2) were first subject to the OFT without a shelter.

2.4 | Open Field Test

The Open Field arena was made of plastic-coated plywood (80 × 80 cm wide and 65 cm high). Depending on the OFT treatment, in the center of the arena was positioned either a $10 \times 10\ cm^2$ white opaque PVC flat square, from now on referred to as "shelter base" (treatment "OFT no shelter") or the shelter base coupled with a half tube-shaped shelter made from translucent red PVC (treatment "OFT with shelter"). A video camera (Canon Legria HF S200) was positioned above the arena to record the animals' behavior during trials. All trials were performed between 10 and 11 a.m., in a room illuminated with white light from above, as it is common in OFT. While mice are primarily active during dawn and dusk, they are also active during daytime especially in winter (Gray et al. 1998) and no differences in open field activity were found when testing house mice during the light or dark stages of the diurnal cycle (Beeler, Prendergast, and Zhuang 2006).

Each individual was transported from their home cage to the OFT arena either inside the shelter or on top of the shelter base, depending on the OFT treatment, in both cases covered with a black lid. At the beginning of each trial, the covered shelter/base containing the animal was placed in the center of the arena and the black lid was removed after 1 min. In the treatment "OFT no shelter," after removing the lid, the mouse was exposed to an open environment, while in the treatment "OFT with shelter," after removing the lid, the mouse was protected by the shelter. Trials began when the animal left the shelter/base and had a duration of 10 min, during which the animal could explore the arena freely. After each trial, the mouse was removed from the arena and returned to its home cage. The arena, base, shelter, and lid were cleaned with 70% ethanol to remove any chemical cues left by individuals during testing. During the trial, the experimenter was not in the room. All the individuals in the experimental groups were tested for both treatments, with a 1-week interval between tests.

2.5 | Behavior Analysis

OFT videos were analyzed using the video-tracking software ANY-MAZE (version 4.56 h Beta; Stoelting, USA) and the event-recorder software BORIS (Friard and Gamba 2016).

Videos were loaded into the video-tracking software and the whole area of the OFT arena was divided into three virtual zones: *Shelter/Base-zone* – the area of the shelter/base; *Wall-zone* – a 10 cm margin area along the walls; *Open-zone*–the remaining central area of the arena. After tracking was completed, the following variables were extracted for each trial: (i) total duration in the shelter/base area; (ii) percentage of total area explored; (iii) total distance traveled in the Wall-zone and in the Open-zone; (iv) average speed in the Wall- and Open-zones. To retrieve the area explored by each individual, we extracted the path traveled by each mouse. From these paths, considering a path width of 7 cm, the total area covered was calculated using ImageJ. We then determined the proportion of the area explored in relation to the total area of the arena. A path width of 7 cm was chosen on the assumption that mice can acquire tactile cues with their left and right-side vibrissae from at least 3.5 cm to each side of their nose (Ibrahim and Wright 1975).

With the event-recorder software BORIS (Friard and Gamba 2016), four additional behavioral variables were measured: (i) number of jumps, (ii) duration of grooming, (iii) duration of freezing, and (iv) number of rearing events.

2.6 | Blood Collection and Hormone Analysis

Three blood samples were collected from each individual, once a week for three consecutive weeks. Samples were collected at 10 a.m., for the Control group, and immediately after the OFT trial (thus between 10 and 11 a.m.) for mice in the experimental groups. Individuals were lightly anesthetised with inhaled isoflurane and blood was collected using the retro-orbital sinus technique, within 2–3 min maximum, either after retrieving the mice from the cage (Control group) or after the OFT trial was finished (Experimental groups; Romero and Reed 2005). To reduce potential health issues, blood samples were collected alternately from each eye, using only one eye per sampling session.

After collection, blood samples were left to clot for 1 h at room temperature and centrifuged for 20 min at 2000 g. Serum was then stored at −80°C until further analysis. Cortisol serum levels were measured using Immunoassay (EIA) commercial kits (BioVision ELIZA Kit–human/mouse/rat Cortisol).

2.7 | Statistics

All statistical tests were done in R (version 4.2.1) (R Development Core Team 2018). Different models were fitted, using the functions glm, lmer and glmer (package lme4). The fit of each model was checked through the inspection of residuals using QQ-plots and Kolmogorov–Smirnov tests, and the best fitting distributions were chosen accordingly. Some of the behavioral response variables were log- or sqrt-transformed to improve model fit (see respective model description for distributions and transformations). In all cases of multiple testing, resulting *p*-values were corrected using Tukey-adjustment. The hormonal data was analyzed by fitting linear models (glm and lmer) in three steps. The main models of each step had *Species* (2 levels: *M. musculus*, *M. spretus*) and *Sex* (2 levels: female, male) as

fixed factors, and *Cortisol concentration* (ng/ml) as a response variable. First step: to understand if cortisol levels changed over time in captivity, we compared the levels of cortisol of the Control group (no OFT, Figure 1) over the 3 weeks. We fitted a model with *Week* (3 levels) as an additional fixed factor, followed by pairwise comparisons between pairs of weeks. *Individual* was included as a random factor in this model, as individuals were measured repeatedly. Second step: to test if the three groups of mice (Control group, Experimental group 1 and Experimental group 2) belong to the same population, we compared the levels of cortisol from week 1 (baseline, no OFT; see Figure 1) among the three groups. We fitted a model with *Group* as additional fixed factor. Third step: to compare the levels of cortisol between the three treatments, no OFT, OFT with shelter and OFT without shelter, we fitted a model with *Treatment* (3 levels: no OFT, OFT with shelter, OFT no shelter) and the interaction of *treatment* and *Species* as additional fixed factors. We fitted two additional mixed models (one per species), each model having *Treatment* and *Sex* as fixed factors and *Individual* as random factor. For all models of the third step, we grouped the data of the two experimental groups, as we found no difference between the baseline levels of the three groups in step 2. While it would have been interesting to also analyze interactions between *Week* and *Sex* (step 1) and *Treatment* and *Sex* (step 2), our sample size did not allow for such complex models: an attempted inclusion of such interactive terms in the models showed no significant influence of the interactions and the resulting models had a worse fit than the original. This fit was assessed by analyzing the stability of the models' coefficients through a bootstrapping analysis (see Suporting Information S1 Material Model Comparison. xlsx for a description of this additional analysis).

The behavioral data was analyzed by two sets of linear mixed-effects models. To understand behavioral differences between the two experimental treatments, we fitted six mixed models with *Treatment*, *Species*, *Sex* and the interaction of *Treatment* and *Species* as fixed factors and *Individual* and *Week* as random factors. The response variables were *Proportion of area covered* (lmer, inverse square root-transformed), *Duration inside shelter* (lmer, sqrt-transformed), *Number of jumps* (lmer), *Duration of grooming* (lmer, log-transformed), *Duration of freezing* (lmer, log-transformed), and *Number of rearing events* (lmer, log-transformed). To understand if the distance traveled and the speed of movement in both zones, "open zone" and "wall zone," differed between treatments, we fitted three mixed-effects models with *Zone* (2 levels: Open, Wall), *Treatment*, *Species*, *Sex* and the interaction of *Zone* and *Treatment* as fixed factors and *Individual* and *Week* as random factors. The response variables were *Distance* (calculated as distance moved per area of respective zone; lmer, sqrt-transformed) and *Speed* (calculated as distance moved per second inside each respective zone; glmer, inverse.gaussian). As in the hormonal analysis (step 3), we attempted an inclusion of the interactive term *Treatment***Sex* in the analysis of behavioral variables. Again, these models showed no significant influence of the interaction, and the resulting models had a worse fit than the original (see Supporting Information S1 Model Comparison. xlsx for a description of this additional analysis).

To analyze the influence of individuality on differences in hormonal levels and behavioral variables, we calculated repeatability for all variables using the function rpt (package rptR).

3 | Results

Genetical assignment confirmed morphological species identification made at the moment of capture, as *M. musculus* and *M. spretus*, with levels of sequence identity obtained in BLAST being > 99%.

Mice that did not participate in any OFT trials (Control group) decreased cortisol levels over time in the laboratory (Table 1, Figure 2). This decrease was significant between week 1 and both weeks 2 and 3, respectively, but not between weeks 2 and 3. Cortisol levels of the Control group did not differ between species or sexes. Individual repeatability of cortisol levels over weeks was low ($R = 0.127$, 95%-CI = [0, 0.574], $p = 0.350$).

Baseline levels of cortisol (week 1) did not differ significantly between the three groups, namely Control (no OFT), Experimental group 1 (first OFT with shelter) and Experimental

TABLE 1 | Results of mixed model and of paired comparisons testing the influence of *Week, Species, and Sex* on cortisol levels in mice, *Mus musculus* and *M. spretus*, of the control group. Effects considered significant are printed in bold.

Fixed factor	Estimate	SE	df	t	*p*
Intercept[a]	8.7100	1.8410	23.1740	4.7300	0.0001
Treatment−					
Week 2	**−6.6560**	**1.8530**	**22.9750**	**−3.5910**	**0.0015**
Week 3	**−9.7540**	**1.9530**	**24.6260**	**−4.9940**	**< 0.0001**
Species−*M. spretus*	−1.4540	1.8000	15.2970	−0.8080	0.4315
Sex−Male	3.7370	1.9580	17.1110	1.9080	0.0733
Contrast	**Estimate**	**SE**	**df**	**t**	***p***
Week 1 vs. Week 2	**6.6600**	**1.9000**	**21.6**	**3.5000**	**0.0056**
Week 1 vs. Week 3	**9.7500**	**2.0200**	**23.5**	**4.8340**	**0.0002**
Week 2 vs. Week 3	3.1000	1.9700	22.2	1.5720	0.2781

[a]Reference category; intercept represents the estimate for week 1 of females of the species *M. musculus*.

group 2 (first OFT without shelter) (Table S1, Figure S1, Supporting Information), nor between species or sexes.

Experiencing an OFT with or without a shelter increased the cortisol levels of mice (Table 2, Figure 3). This effect of treatment on cortisol levels showed an interaction with *Species*. In *M. musculus*, cortisol levels were higher in both versions of the OFT than when not experiencing an OFT (OFT with shelter vs. control: $p = 0.0003$, OFT without shelter vs. control: $p < 0.0001$; Table S2, Supporting Information). In *M. spretus*, cortisol levels were higher during the OFT without a shelter than both in the OFT with a shelter

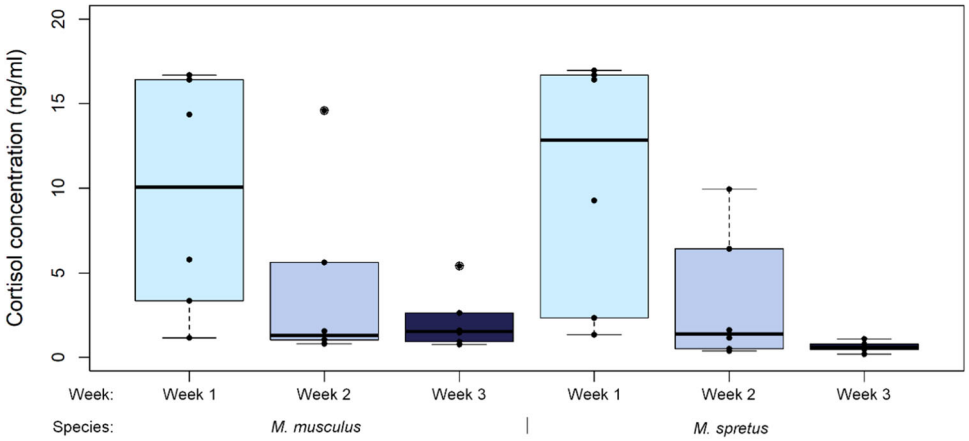

FIGURE 2 | Serum cortisol concentration (ng/ml) of mice of the Control group along time in the laboratory, separated by species, *Mus musculus* and *M. spretus*. Boxes represent the first, second and third quartiles and whiskers are 1.5 times the interquartile range; small dots are individual values; thick dots are outliers.

TABLE 2 | Results of mixed models testing the influence of *treatment, Species, and Sex* on serum cortisol levels of mice, *Mus musculus* and *M. spretus*, during the OFT trials. Effects considered significant are printed in bold.

Fixed factor	Estimate	SE	df	t	*p*
Intercept[a]	2.6396	1.2371	37.8445	2.1340	0.0394
Treatment−					
OFT with shelter	**10.0462**	**1.5929**	**42.8687**	**6.3070**	**< 0.0001**
OFT no shelter	**14.7557**	**1.8775**	**44.9011**	**7.8590**	**< 0.0001**
Species–*M. spretus*	−1.1888	1.5902	32.8422	−0.7480	0.4600
Sex–Male	0.6696	1.1135	40.2485	0.6010	0.5510
Treatment−OFT with shelter: Species-*M. spretus*	**−10.0570**	**2.3411**	**41.8995**	**−4.2960**	**0.0001**
Treatment−OFT no shelter: Species−*M. spretus*	−0.2048	2.5513	44.0971	−0.0800	0.9364

[a]Reference category; intercept represents the estimate for females of the control group of the species *M. musculus*.

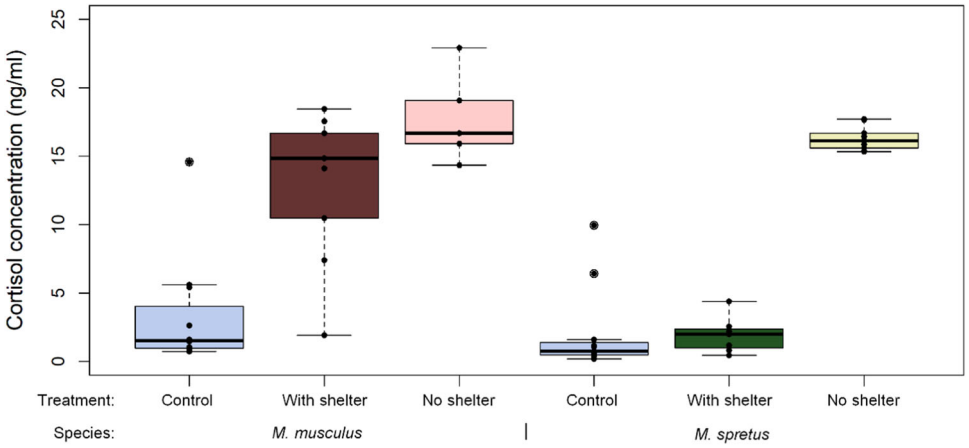

FIGURE 3 | Serum cortisol concentration (ng/ml) of mice of the control group and the two experimental groups comparing the values of no OFT, OFT with shelter and OFT without shelter, separated by species, *Mus musculus* and *M. spretus*. Boxes represent the first, second and third quartiles and whiskers are 1.5 times the interquartile range; small dots are individual values; thick dots are outliers.

and when not experiencing an OFT (OFT without shelter vs. OFT without shelter: $p < 0.0001$, OFT without shelter vs. control: $p < 0.0001$; Table S2, Supporting Information). Cortisol levels did not differ between sexes in both species of mice. Individual repeatability of cortisol levels between treatments was low (R = 0.224, 95%-CI = [0, 0.742], $p = 0.175$).

The presence of a shelter in the OFT influenced the behavior of the two mouse species: when a shelter was present, mice spent

TABLE 3 | Results of mixed models testing the influence of *treatment, Species, and Sex* of mice, *Mus musculus* and *M. spretus*, on behavioral variables. Effects considered significant are printed in bold.

Variable	Fixed factor	Estimate	SE	df	z/t	*p*
Duration in shelter/base	Intercept[a]	4.4054	0.1711	—	25.7430	0.0000
	Treatment—OFT no shelter	**−0.9103**	**0.2159**	**—**	**−4.2170**	**0.0000**
	Species—*M. spretus*	**−0.4555**	**0.2211**	**—**	**−2.0600**	**0.0394**
	Sex—Male	0.0849	0.1646	—	0.5160	0.6061
	Treatment—OFT no shelter: Species—*M. spretus*	0.4449	0.3017	—	1.4750	0.1402
Area covered	Intercept[a]	1.7184	0.2908	—	5.9090	0.0000
	Treatment—OFT no shelter	**−0.4251**	**0.2068**	**—**	**−2.0550**	**0.0398**
	Species—*M. spretus*	0.5328	0.3179	—	1.6760	0.0937
	Sex—Male	0.5294	0.2733	—	1.9370	0.0528
	Treatment—OFT no shelter: Species—*M. spretus*	0.6225	0.3391	—	1.8360	0.0664
Number of jumps	Intercept[a]	5.2725	0.7744	11.7825	6.8090	0.0000
	Treatment—OFT no shelter	−0.5209	0.4451	28.7290	−1.1700	0.2520
	Species—*M. spretus*	**−3.8193**	**0.8344**	**39.2377**	**−4.5770**	**0.0000**
	Sex—Male	1.1248	0.7761	30.2551	1.4490	0.1580
	Treatment—OFT no shelter: Species—*M. spretus*	0.8398	0.6196	28.6844	1.3550	0.1860
Duration of grooming	Intercept[a]	1.8604	0.3326	3.6228	5.5940	0.0067
	Treatment—OFT no shelter	−0.0706	0.2212	27.4634	−0.3190	0.7520
	Species—*M. spretus*	**0.6939**	**0.3028**	**46.7562**	**2.2920**	**0.0265**
	Sex—Male	0.0568	0.2615	28.4577	0.2170	0.8295
	Treatment—OFT no shelter: Species—*M. spretus*	0.2862	0.3080	27.4032	0.9290	0.3609
Duration of freezing	Intercept[a]	2.6642	0.7143	13.1096	3.7300	0.0025
	Treatment—OFT no shelter	**1.9831**	**0.9107**	**58.0474**	**2.1780**	**0.0335**
	Species—*M. spretus*	0.2718	0.8964	58.0000	0.3030	0.7628
	Sex—Male	−1.1007	0.6377	58.0015	−1.7260	0.0897
	Treatment—OFT no shelter: Species—*M. spretus*	−1.7667	1.2685	58.0858	−1.3930	0.1690
Number of rearing events	Intercept[a]	6.8593	0.6549	3.0436	10.4740	0.0017
	Treatment—OFT no shelter	−0.0265	0.4152	21.5798	−0.0640	0.9498
	Species—*M. spretus*	0.0827	0.5686	43.0195	0.1450	0.8851
	Sex—Male	−0.2272	0.4913	22.9587	−0.4620	0.6481
	Treatment—OFT no shelter: Species—*M. spretus*	0.3624	0.5782	21.5234	0.6270	0.5374

Distributions and–if applicable–transformations for each variable: area covered: gaussian, inverse sqrt-transformed; duration inside shelter: gaussian, sqrt-transformed; number of jumps: gaussian; duration of grooming: gaussian, log-transformed; duration of freezing: gaussian, log-transformed; number of rearing events: gaussian, log-transformed.

[a]Reference category; intercept represents the estimate for females of the species *M. musculus*.

more time in the shelter/base zone, covered a larger area and spent less time freezing than in the absence of a shelter (Table 3, Figure 4). The two species also differed in some behaviors: *M. musculus* spent more time in the shelter/base zone, jumped more and groomed less than *M. spretus* (Table 3, Figure 4). Neither *Treatment* nor *Species* had an influence on the number of rearing events. *Sex* had no influence on any of the measured variables.

Mice covered a larger distance moving along the wall than in the open area (Table 4, Figure 5). This difference was stronger when a shelter was not present (Table 4, Figure 5). Mice moved slower along the wall than in the open area (Table 4, Figure 5). Neither *Species* nor *Sex* had an influence on the distance or speed of movement.

Individual repeatability of behavioral parameters was significant for all but two parameters (time in shelter, freezing; Table 5).

4 | Discussion

4.1 | A Shelter in the OFT Increases Exploratory Activity of Mice

When mice in our study, *Mus musculus* and *M. spretus*, had the opportunity to retreat to a shelter during the OFT test, both of them used this opportunity, regardless of the species, just as expected. However, despite spending more time inside the shelter, mice also covered a larger proportion of the area of the arena, performed less wall-seeking (thigmotaxis) and had fewer freezing events. Both wall-seeking and freezing are typically interpreted as anxiety-related behaviors in small mammals and are connected to increased levels of stress response (Bailey and Crawley 2009; Belzung 1999; Bourin et al. 2007). Wall-seeking is a natural behavior of mice and other agoraphobic small mammals to reduce the perceived or actual risk of being exposed.

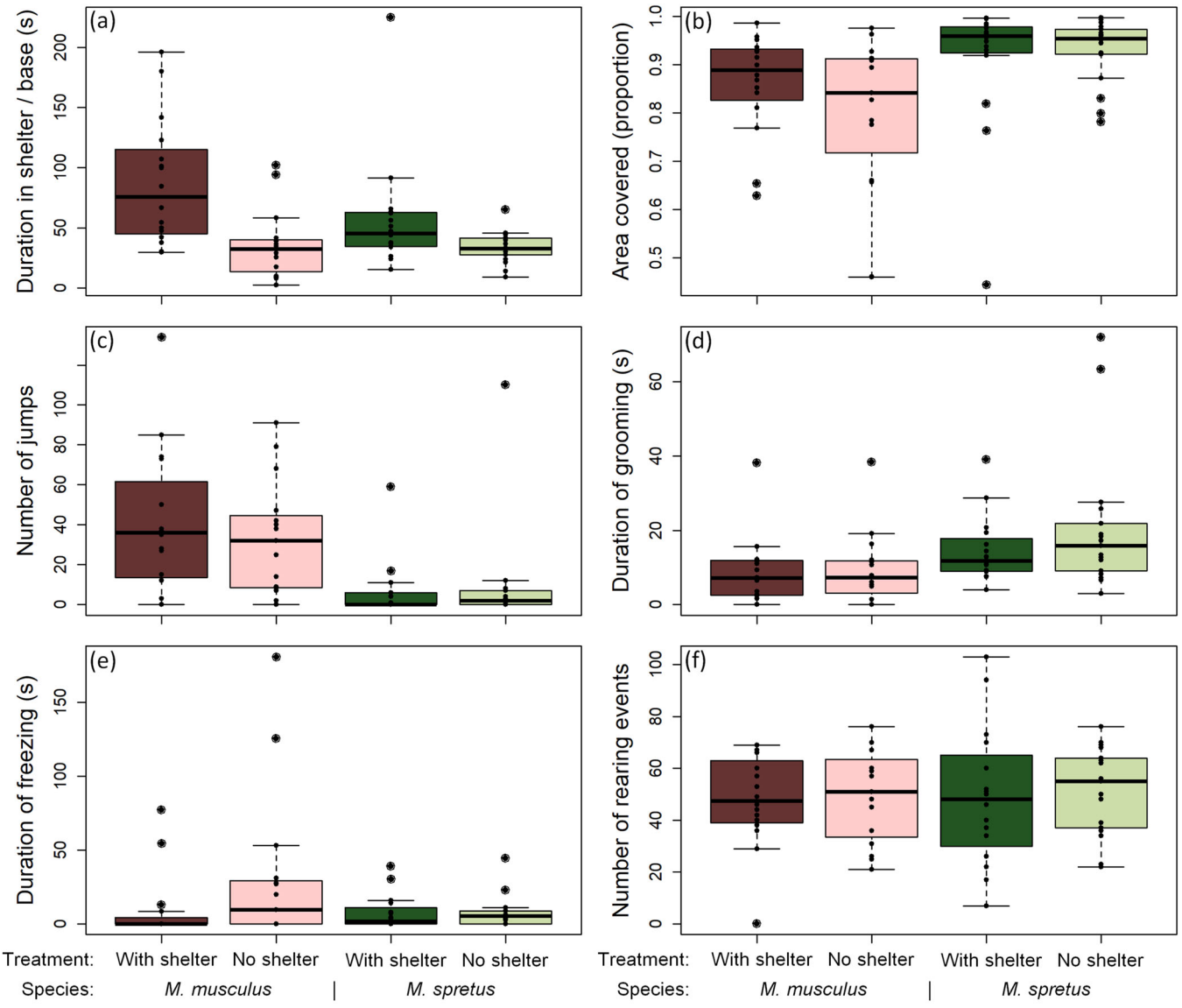

FIGURE 4 | Influence of the presence of a shelter in the OFT on six behavioral parameters, separated by species, *Mus musculus* and *M. spretus*. Behaviors shown are (a) duration in the shelter/base zone, (b) area covered, (c) number of jumps, (d) duration of grooming, (e) duration of freezing, and (f) number of rearing events. Boxes represent the first, second and third quartiles and whiskers are 1.5 times the interquartile range; small dots are individual values; thick dots are outliers.

TABLE 4 | Results of mixed models testing the influence of species, sex, and OFT treatment and OFT area on distance and speed of mice, *Mus musculus* and *M. spretus*. Effects considered significant are printed in bold.

Variable	Fixed factor	Estimate	SE	df	t/z	*p*
Distance	Intercept[a]	6.1452	0.6750	3.1448	9.1040	0.0023
	Zone−Wall	**2.7753**	**0.2719**	**91.3554**	**10.2050**	**0.0000**
	Treatment−OFT no shelter	**−0.6189**	**0.2737**	**91.9895**	**−2.2620**	**0.0261**
	Species−*M. spretus*	0.2301	0.5124	30.2907	0.4490	0.6566
	Sex−Male	0.9112	0.5123	30.2830	1.7790	0.0853
	Zone−Wall: Treatment−OFT no shelter	**1.3064**	**0.3846**	**91.3554**	**3.3970**	**0.0010**
Speed	Intercept[a]	−2.0526	0.1108	—	−18.5290	0.0000
	Zone−Wall	**−0.5930**	**0.1222**	**—**	**−4.8530**	**0.0000**
	Treatment−OFT no shelter	0.0709	0.1414	—	0.5020	0.6159
	Species−*M. spretus*	−0.0530	0.0833	—	−0.6360	0.5247
	Sex−Male	0.1509	0.0842	—	1.7920	0.0731
	Zone−Wall: Treatment−OFT no shelter	−0.1488	0.1753	—	−0.8490	0.3960

[a]Reference category; intercept represents the estimate for females of *M. musculus*.
Distributions and–if applicable–transformations for each variable: Distance: linear, sqrt-transformed; Speed: inverse. gaussian.

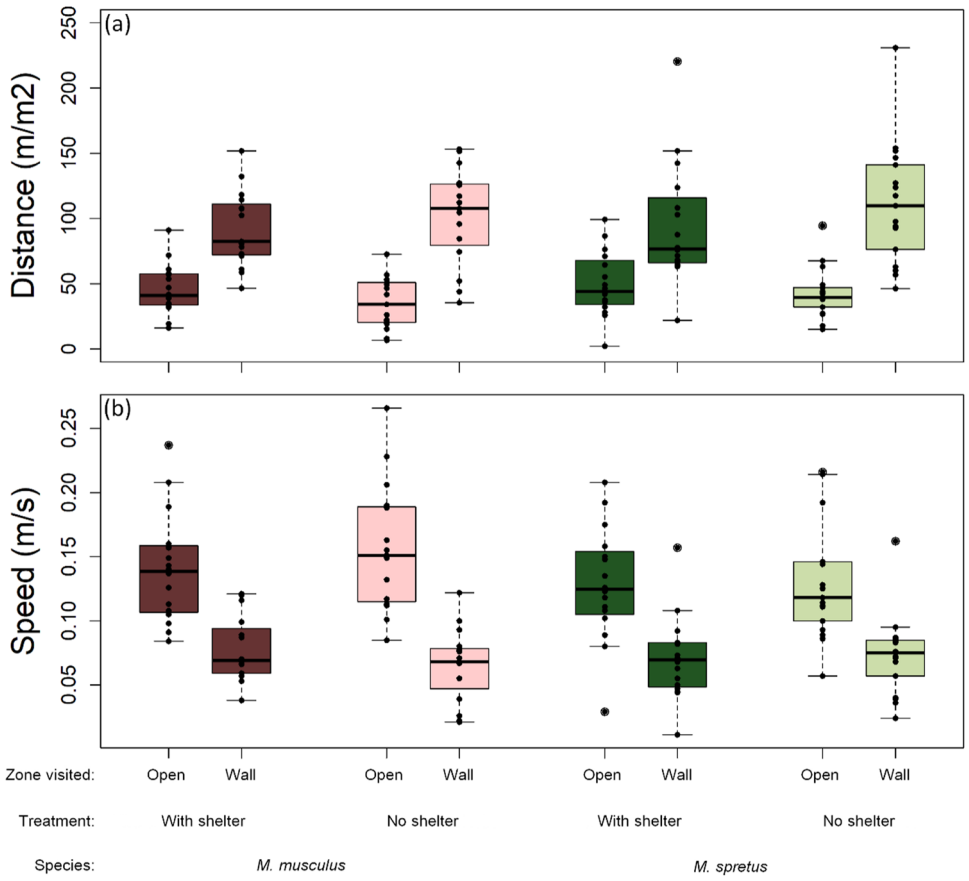

FIGURE 5 | Influence of the presence of a shelter in the OFT on the (a) distance covered and (b) speed of mice, *Mus musculus* and *M. spretus*, along the wall and inside the open space of the OFT arena, separated by species. Boxes represent the first, second and third quartiles and whiskers are 1.5 times the interquartile range; small dots are individual values; thick dots are outliers.

While the mice in our study moved preferably along the walls rather than within the open space of the OFT in both treatments, this difference was less pronounced when a shelter was present. Instead, mice spent more time inside or close to the shelter. This follows one of the basic assumptions of the Open Field Test: animals establish a home-base, soon after being introduced to the open field (Clark, Hamilton, and Whishaw 2006; Eilam and Golani 1989). In the presence of a shelter, the

TABLE 5 | Results of repeatability analysis of hormonal and behavioral variables of mice, *Mus musculus* and *M. spretus*. Repeatabilities considered significant are printed in bold.

Variable	R	95%-CI	*p*
Time in shelter	0.007	[0.000, 0.349]	0.485
Area covered	**0.406**	**[0.091, 0.676]**	**0.007**
Jumps	**0.701**	**[0.473, 0.852]**	**< 0.001**
Grooming	**0.433**	**[0.124, 0.684]**	**0.004**
Freezing	0.000	[0.000, 0.328]	1.000
Rearing	**0.422**	**[0.115, 0.697]**	**0.021**
Distance	**0.522**	**[0.265, 0.716]**	**< 0.001**
Speed	**0.396**	**[0.192, 0.560]**	**< 0.001**

shelter itself can be used as the home-base, while in its absence, mice will likely choose a corner or edge of the open field. We acknowledge that positing the shelter in the center of the arena may have generated confounding results between shelter seeking and exploring the open area, however, it allowed a clear distinction between wall-seeking and shelter-seeking.

By covering a larger proportion of the arena when a shelter was present, mice had the chance to learn more about this novel environment. We thus conclude that both mouse species increased their exploration efforts in the presence of a shelter. By using the total area covered as opposed to general movement or activity, we aimed to distinguish exploratory behavior from pure activity or attempts to escape (Welker 1959). Exploration of novel surroundings is an important trait of animals. It is not directly connected with the search for food, a shelter or a partner, but is still considered highly adaptive, as it enables animals to gain knowledge of their surroundings, which can ultimately increase their fitness (Renner 1990). Exploration results from the interaction of several intrinsic factors such as motivation, motor capacity and cognitive aspects (Matzel et al. 2006), with partially opposing drives: the drive to explore the novel space (risk-prone behavior) and the anxiety of exposure leading to seeking of shelter (risk-averse behavior). Only if anxiety is low enough, can the drive to explore overpower any hesitation, allowing the animal to start exploring the environment. The OFT is a novel and stressful situation for mice and an increased stress response can be expected. In our study, all mice exposed to the OFT had a higher stress response than the mice of the control group, which stayed inside their familiar home cages. It has been shown multiple times that mice and rats reduce exploratory activity after exposure to stress (e.g. Arnsten, Berridge, and Segal 1985; Berridge 1987; Britton et al. 1982; but see Matzel et al. 2006 who did not find such a correlation).

4.2 | A Shelter in the OFT Reduces Stress Levels in *M. Spretus* But Not *M. Musculus*

A safe place like a shelter could potentially reduce the anxiety-related stress response even during an unknown situation, which in turn could lead to increased exploratory activity. This seems to have been the case for *M. spretus*. While both species in our study explored more when a shelter was present, only *M. spretus* also had a lower stress response. *M. musculus*, on the other hand, had an elevated stress response in both versions of the OFT, with and without a shelter. Also in other behaviors, *M. musculus* revealed a more proactive mechanism of coping with the OFT. While *M. spretus* performed more grooming behavior, *M. musculus* jumped more and spent more time in the shelter/base zone. The latter seems contradictive but can likely be explained by a particular behavior of *M. musculus*. We observed several times that *M. musculus* individuals used the shelter as a vantage point, standing on top of it, rearing or even jumping from this elevated position, which did not occur in *M. spretus*. Jumping behavior has been associated with the spatial complexity of the environment (Marashi et al. 2003), which is in line with the commensal lifestyle of *M. musculus* and its use of human-made habitats. In these habitats, house mice often use vertical structures like walls and elevated places, such as the wooden beams inside sheds or houses, when exploring the environment (Frynta et al. 2018). A commensal lifestyle, close to humans, might also confront house mice with a more variable and changing environment. Changes in the environment can lead to short events of stress. As opposed to chronic or long-lasting stress, which can be harmful for health, short term stress can activate physiological systems preparing the animal for the appropriate behavioral reaction, that is, fight-or-flight (Bonier et al. 2009; Dhabhar 2014). In humans, acute stress has been even shown to improve decision-making (Shields et al. 2016). It is thus possible that *M. musculus* have an advantage of having elevated cortisol levels in a range of different situations, following a more proactive style of coping with stressful events. *M. spretus*, in contrast, has been described as rather docile compared to other species of the genus *Mus*, with a less aggressive and rather ritualized and olfactory-based territory defense (Hurst et al. 1996; Hurst et al. 1997; Palomo, Justo, and Vargas 2009). Fitting to this lifestyle, *M. spretus* engaged more in grooming, a behavior indicative of stress and anxiety (Kalueff and Tuohimaa 2005; Smolinsky et al. 2009), likely using it as a reactive stress-coping mechanism.

Another possible explanation for the presence of a shelter reducing stress levels in *M. spretus* but not in *M. musculus* might be the highly gregarious life-style of the latter. House mice live in social groups typically composed by a dominant male, several females, their offspring and subordinate individuals of both sexes. Several females may breed within the group and cooperate in the care of their own young and those of other females (König 2012). The Algerian mouse, on the other hand, seems to form smaller social groups, dominant males only overlapping their territory with those of a few females (Gray et al. 1998; Palomo, Justo, and Vargas 2009). All mice used in our study were housed individually during the course of the experiment. Although care was taken to minimize the effect of partial isolation (i.e., physical isolation) and laboratory confinement through environmental enrichment measures, we cannot disregard it may have induced some stress in individuals. As such, it is possible that the house mice used in our trials were experiencing an overall higher level of stress from being separated from conspecifics, felt even when the shelter was present during the OFT trials.

Most of the analyzed behaviors had a moderate (area covered, grooming, rearing, speed: $R = 0.3 - 0.5$) to high (jumps, distance

moved: R > 0.5) individual repeatability. Those values align with the results of a meta-analysis, in which repeatability had an overall average of 0.37, depending on the behavior measured (Bell, Hankison, and Laskowski 2009). Only time in the shelter and freezing were not repeatable, likely caused by potential ambiguities in the analysis of those behaviors. Both time in shelter and freezing might consist of more than just one behavior: Animals can visit a shelter with different motivations (e.g. to rest or to hide), and also stop moving for different reasons (e.g. out of fear or to focus their attention on something close by). In a smaller set-up, such fine differences in behavior might be distinguishable, but our design prevents the discrimination of such nuances, making them easily confounded. Levels of cortisol were not repeatable (R = 0.1 – 0.2). Again, this fits the results of a meta-analysis finding that repeatability of baseline glucocorticoid levels are, in comparison to stress induced or long-term measures, rather low (R = 0.18; Taff, Schoenle, and Vitousek 2018).

4.3 | Stress Levels of Mice Decreased Over Time in the Laboratory

When wild small mammals are brought to the laboratory, they experience a period of adjustment to the novel conditions. During this time, their levels of stress hormones decrease gradually (Carrilho et al. 2024). As we worked with wild caught mice, we allowed them a period of 2 weeks before starting behavioral experiments. Nevertheless, we made control measurements of circulating cortisol in animals not tested in the OFT. Those measurements confirm our decision regarding the waiting time period, as stress response decreased significantly between the measurements done 1 week and 2 weeks after trapping. This decrease was remarkably similar between species.

Additionally, the adjustment period not only led to a general reduction in the levels of stress hormones, but also to a decrease of their variability. Several variables influence the physiological responses of animals. Environmental factors such as temperature, humidity and light are variable but predictable following seasonal or circadian patterns (Kronfeld-Schor and Dayan 2008; Nelson, Demas, and Klein 1998). Other stressors, like predation risk and encounters with conspecifics are less predictable, thus the assessment of cortisol in wild animals can report high levels of variability depending on the time and duration of the events experienced before capture (Eccard and Ylönen 2003; Wirsing et al. 2021). This might explain the higher variability of cortisol levels in our study after the first week in the laboratory, and its considerable reduction after 2 weeks in captivity. Moreover, the genetic background of the animals is unknown; thus we cannot exclude that different characteristics along the hypothalamus-pituitary-adrenal axis are responsible for the variability of the stress response observed, for example, differences in glucocorticoid receptors (Wüst et al. 2005).

Carrilho et al. (2024) also found that, for the wood mouse, *Apodemus sylvaticus*, the adjustment to laboratory conditions is characterized by both a decrease in stress response and individual variability therein, lasting about 2 weeks. The recommendation for an acclimatation period after the transport of mice to the laboratory (Lee, David, and Huerkamp 2020; Obernier and Baldwin 2006) seems to be appropriate for a range of wild mouse species, not only to lower their stress levels but also to reduce the intra-group variation prior to any experimental procedures are applied to the animals.

5 | Conclusions

Our results contribute to the discussion of the validity of the OFT to measure anxiety and exploration (Rosso et al. 2022). While some laboratories use the OFT rather to test exploration, others use it to test anxiety (Bourin et al. 2007). As we showed here, these behaviors might be disentangled in some but not all study species. Increased levels of anxiety might have been preventing exploration in *M. spretus*, as seen by the increased drive for exploration in the presence of a shelter. In *M. musculus*, however, exploration and anxiety could not be separated, as animals kept an elevated stress response, even when increasing their exploration efforts. While most lab mouse strains have their origin in the species *M. musculus*, we cannot draw conclusions on the stress response of laboratory mice between open field tests with or without a shelter. Through breeding efforts, different lab strains might have developed distinct physiological and behavioral strategies than their wild counterparts. It is thus important for researchers to consider the biology of their study species or the background of their strain to decide, depending on their research question, if the addition of a shelter is advisable or not. While in most cases researchers might wish to test animals with low levels of stress, some studies might require a full physiological response of animals, even including the induction of stress-like behaviors. In such experiments, the use of a shelter is not advisable as it might prevent the exhibition of a full behavior repertoire.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Data is provided by author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.