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EVALUATION OF BEHAVIORAL RESPONSES TO RESTRAINT STRESS IN THE HOUSE MOUSE (*Mus musculus musculus*) OF WILD ORIGIN

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Abstract
Stress is a fundamental concept for many areas of animal research. In laboratory rodents, exposure to various stressors results in immediate and postponed behavioral changes that can be measured with standard tests. Less is known about how wild rodents that have not undergone domestication respond to stressors in laboratory settings. This study was aimed at evaluating behavioral responses to restraint stress in male and female wild-derived house mice (*Mus musculus musculus*). One week after the end of three daily restraint sessions, alterations in mouse behavior were assessed in two tests. In the open field test, stressed mice (n = 23) entered the unprotected central zone less frequently ($P < 0.05$) and showed increased duration of self-grooming ($P < 0.05$) compared to controls (n = 23), indicating elevated anxiety. In the forced swim test, most mice (44 out of 46) displayed episodes of behavioral despair, but the influence of stress was insignificant. To characterize baseline performance of wild-derived mice, their behavior was compared to that of a laboratory strain. As compared to C57BL/6 males (n = 10), *M. m. musculus* males (n = 14) showed significantly greater exploratory activity in the open field and longer latency of the first immobility episode in the forced swim test ($P < 0.001$). Overall, these findings confirm ecological validity of the widely used animal model of restraint stress and may serve as a basis for future studies.

Keywords: Animal models. Anxiety. Mice. Open Field Test. Reproducibility of Results.

1. Introduction

The concept of stress as an organism's natural response to environmental challenges is fundamental for many areas of animal research. Compared to laboratory mouse strains, the wild house mouse *Mus musculus* (*M. m.*) is rarely used in experimental laboratory studies. Nonetheless, recent evidence suggests that certain characteristics of wild mice, such as the preservation of the natural microbiota and an adequate immune response, more closely reflect physiology of humans than that of laboratory mouse strains (Rosshart et al. 2019; Oh and Rehermann 2021; Cardilli et al. 2023). Mice of wild origin also show more pronounced interindividual variation of observed traits (Augustsson and Meyerson 2004; Lecorps and Féron 2015), which is very similar to the variation seen in the global human population. As such, wild mice may be useful in the assessment of ecological validity of experimental animal models (Shultz et al. 2021; Shemesh and Chen 2023). In laboratory rodents, exposure to various stressful events typically provokes immediate and postponed changes in anxiety-like and/or depressive-like behaviors (Verbitsky et al. 2020; Molina et al. 2023). These alterations are commonly assessed under unconditioned laboratory paradigms using e.g. an open field, elevated plus-maze, light/dark exploration, forced swim, or tail suspension. Less is known about the behavioral stress responses of wild house mice. The literature suggests that test responses of wild mice may not be similar to those of laboratory strains, which have undergone domestication processes. For instance, in the elevated plus-maze, which is commonly employed to measure anxiety in rodents (Harro 2018), wild-derived mice display high reactivity in the form of increased escape motivation and an unusual preference for the open arms (Holmes et al. 2000). Similarly, wild-derived mice show more intense emotional flight-related behaviors as compared to laboratory mice when exposed to an experimental predatory threat (Hendrie et al. 1996; Blanchard et al. 1998). In this regard, an inescapable experimental setting, such as an open field surrounded with high walls, appears to be a better way to test anxiety. There is a considerable body of literature on exploratory and locomotor activities of wild-derived mice in various open-field settings (Augustsson et al. 2005; Vošlajerová Bímová et al. 2016; Frynta et al. 2018.; Geuther et al. 2021; Hiadlovská et al. 2021; Stratton et al. 2023; Bardos et al. 2024). Nevertheless, these research articles have barely addressed anxiety-related issues. In the meantime, the behavior of wild-derived mice in tests of depressive-like behavior, such as forced swim and tail suspension, has largely been unexplored. In one work, the absence of immobility bouts in swimming activity (characteristic of behavioral despair) of wild mice was reported as a side observation (Hiadlovská et al. 2014).

The present study was designed to fill the aforementioned gaps in the literature. The specific aim was to investigate delayed effects of short-term physical restraint stress on anxiety-related and depressive-like behaviors in wild-derived house mice (*M. m. musculus*). The restraint method was chosen for being the preferred means of stress application to rodents (Molina et al. 2023). An extended period of rest before testing (6 days) was implemented because of many reports of a postponed behavioral response to stress exposure in rodents: “stress incubation” (Gupta and Chattarji 2021; Lorsch et al. 2021).

2. Material and Methods

Animals

A total of 47 wild-derived house mice (27 males and 20 females of *M. m. musculus,* aged 3–4 months) were used. They were laboratory-bred first-generation offspring of 11 parental pairs that were trapped in Vladimir Oblast (Russia) in 2021 (coordinates: 56°21'N 41°21'E). The animals were kept under standard vivarium conditions at 21°C ± 2°C on a 14:10 light/dark cycle with lights on at 8 a.m. Dimensions of plastic cages for solitary housing were 26 × 17.5 × 13.5 cm (L × W × H). Feed (pelleted diet, oat grains, sunflower seeds; all supplied by Laboratorkorm, Russia) and water were available *ad libitum*. In addition, ten 4-month-old C57BL/6 male mice from a laboratory-bred population maintained under the same conditions were used. Mice were handled by means of ventilated 0.7 L plastic containers to reduce stress from contact with an experimenter’s hands.

House mice comprise three major subspecies, namely, *M. m. castaneus*, *M. m. domesticus*, and *M. m. musculus* (Fujiwara et al. 2022). Despite being genetic mosaics of these three main subspecies, the majority of laboratory mouse strains are more closely related to *M. m. domesticus*. In the wild, *M. m. musculus* is widespread from Central Europe to northern China, but its contribution to the genome of standard mouse strains is less than 10% (Yang et al. 2011). From this perspective, the subspecies that we chose can be considered less investigated in the laboratory.

Experimental design

Experimentally naive mice were randomly assigned either to a control group or to a stress group, and we avoided unequal distribution of siblings. Group sizes were determined based on previous behavioral studies (e.g., Augustsson and Meyerson 2004). Prior to the experiment, all mice were housed singly for at least 7 days. The stress group was subjected to repeated restraint stress for 3 consecutive days (days 1–3), while the control group remained undisturbed. After 6 days of rest (on day 10), mice were evaluated first in the open field test (OFT) and then 3 h later in the forced swim test (FST). All tests were conducted from 14 to 19 p.m. Body weights were measured on days 1, 3, and 10. C57BL/6 mice were subjected to the same testing procedures as the control group. Experiments were carried out in compliance with ARRIVE guidelines and EU Directive 2010/63/EU. All the procedures and study protocol were approved by the Ethics Committee of Severtsov Institute of Ecology and Evolution.

Restraint stress

Mice of the stress group were restrained for 1 h per day in well-ventilated 50 mL polypropylene Falcon tubes with additional stoppers inside to adjust for body length. The stress procedures were performed in a separate room from 11 a.m. to 12.30 p.m.

OFT

This test was administered to assess anxiety-related, exploratory, and locomotor behaviors in mice (Harro 2018). A test duration of 10 min was chosen to focus on the novelty reaction, which is generally sufficient to register key components of exploratory activity. The apparatus was designed as a white square arena with an inner size of 73 × 73 cm and 60-cm-high walls. The floor was divided into 16 equal squares, of which four squares in the middle constituted the central zone, and the remaining 12 defined the peripheral zone. Light intensity was 80 lux in the center and 50 lux in the corners. At the start, an experimenter placed a mouse in the center of the arena. The testing was video recorded. The arena was cleaned with a mild detergent and 70% ethanol to eliminate olfactory cues between subjects. Horizontal activity (the number of lines crossed), frequencies of rearing (total wall leanings and unsupported rearings), jumping, and defecation as well as total durations of self-grooming and freezing were quantified manually from the recordings. Videos were blind-coded before analysis. Time spent in the central zone (excluding the starting episode) and frequency of entries into the zone were determined with a trial version of ANY-maze Video Tracking System 7.08 (Stoelting Co).

FST

This test was utilized to assess depressive-like behavior in mice (Armario et al. 2021). A transparent cylindrical tank (50 cm in height and 20 cm in diameter) was filled with 25°C ± 1°C water to a depth of 15 cm. Water was changed after each mouse. At the start, a test subject was gently released into the center of the water surface, and then its behavior was recorded for 10 min. Total durations of climbing (escaping), swimming, and immobility behaviors as well as latency of the first immobility episode were scored in the videos. In terms of "climbing," highly mobile behavior with vertical movement of forepaws toward the walls was scored. "Swimming" was defined as active horizontal movement on the water surface. "Immobility" was defined as the lack of any movements other than those necessary to balance the body and keep the head above the water (Armario et al. 2021).

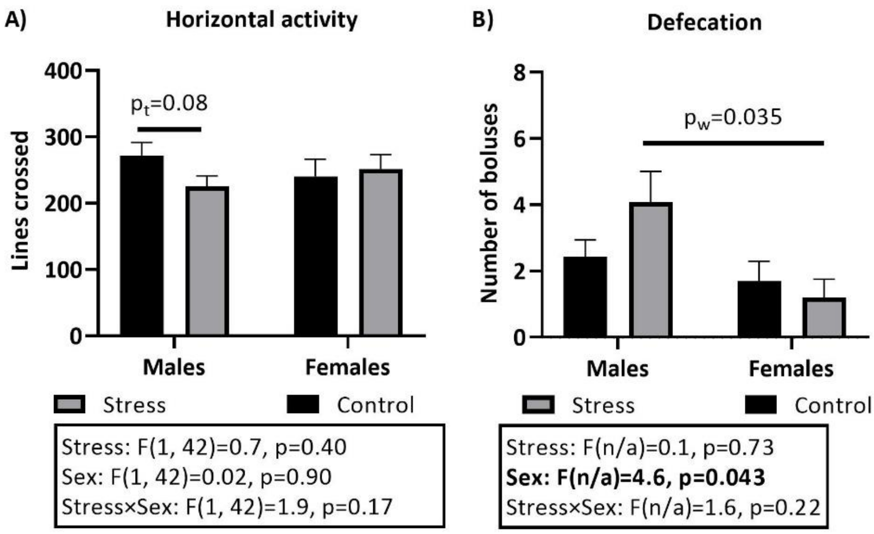
Data analysis

Statistical analysis was performed in STATISTICA 8.0 (StatSoft, Inc.) and RStudio 2023.03.0 Build 386 (Posit Software, PBC). OFT data (except for defecation) from one control male and one C57BL/6 male were excluded because the test subjects spent over 6 min without movement in the apparatus. FST data from one stressed female were missing because of a corrupted video file. Data on each behavior of wild-derived mice were tested for normality of the distribution (Shapiro–Wilk test) and for homoscedasticity (Barlett test). If the assumptions were met, then data were subjected to a two-way ANOVA with stress and sex as between-subject variables. The *t* test was performed for four types of planned comparisons (regardless of the significance of ANOVA results): control males vs. control females, stressed males vs. stressed females, control males vs. stressed males, and control females vs. stressed females. Nonetheless, for some variables (defecation, jumping, grooming, immobility in the OFT, and climbing in the FST), assumptions of normality were found to be severely violated. In such cases, robust two-way ANOVA for trimmed means was applied using the *t2way* function from the WRS2 R package (https://cran.r-project.org/web/packages/WRS2/vignettes/WRS2.pdf), and the nonparametric Mann–Whitney *U* test was carried out instead of the *t* test for the planned comparisons. Differences in body weight were examined by two-way repeated-measures ANOVA with stress and sex as between-subject variables and time as a within-subject variable. The Mann–Whitney *U* test was applied to compare behaviors between control males and C57BL/6 males. An α level of 0.05 was selected as a significance criterion for all statistical tests.

3. Results

Effects of restraint stress on OFT results

Data on open-field behaviors and results of statistical analyses are shown in Figure 1. Stressed mice entered the central zone of the open field significantly less frequently than the control group did (Figure 1H, ANOVA, $P < 0.05$). Stress exposure led to a marginally significant decrease in the time spent in the central area of the apparatus (Figure 1G, ANOVA, $P = 0.097$). Stressed mice also manifested significantly longer duration of grooming as compared to controls (Figure 1E, ANOVA, $P < 0.05$). Stress exposure had no significant influence on exploratory and locomotor activities (such as horizontal activity [Figure 1A], rearing [Figure 1C], and jumping [Figure 1D], ANOVA all *P*s > 0.1). On the other hand, the planned *t* tests detected marginally significantly lower horizontal activity in stressed males compared to control males (Figure 1A, $P = 0.08$ unadjusted for multiple comparisons). Total duration of immobility did not differ between groups (Figure 1F, ANOVA, $P = 0.43$). We found sex differences only for the number of defecation boluses (Figure 1B, ANOVA, $P < 0.05$), and the Mann–Whitney *U* test registered significantly greater defecation in stressed males than in stressed females ($P < 0.05$).



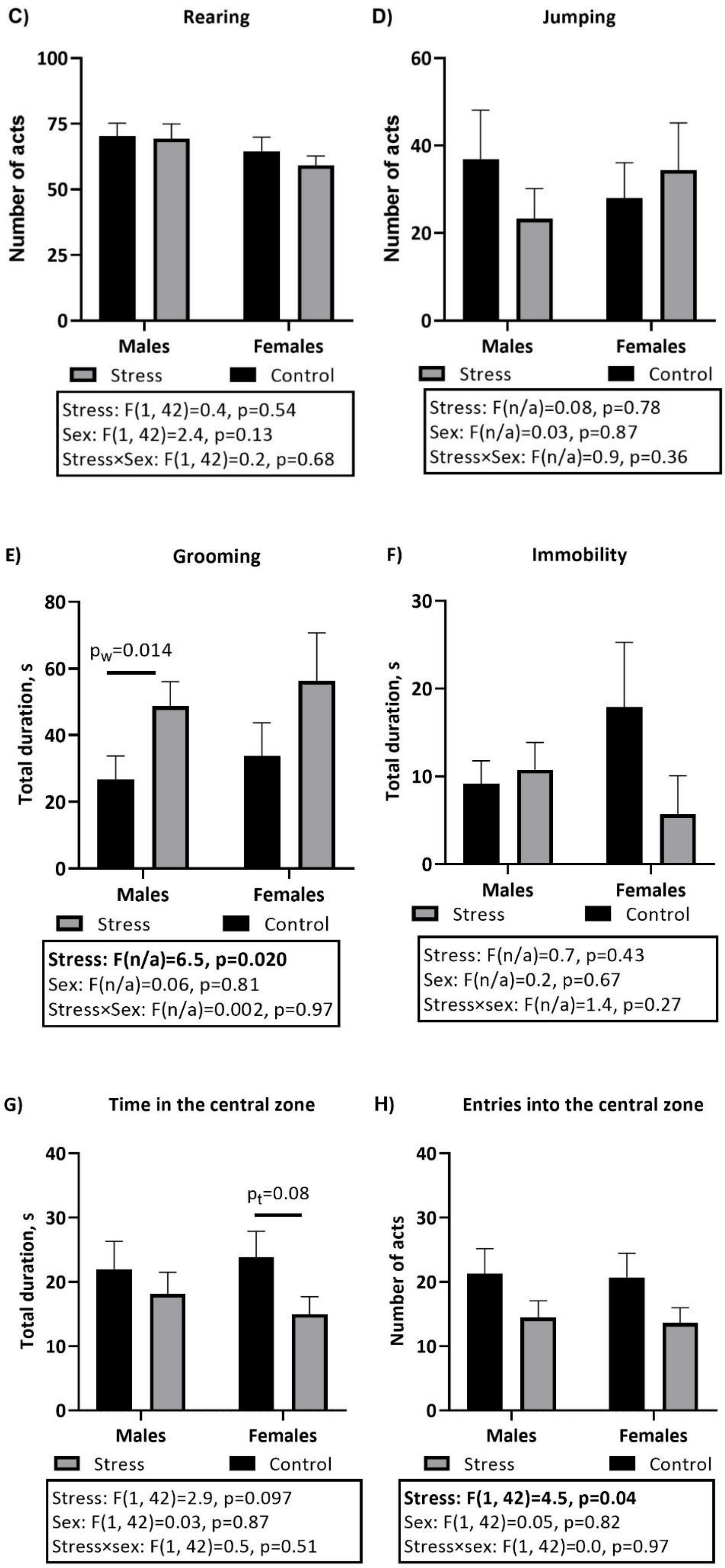


Figure 1. The impact of restraint stress on open-field behaviors of wild-derived *M. m. musculus*. A: Horizontal activity. B: Defecation. C: Rearing. D: Jumping. E: Grooming. F: Immobility. G: Time in the central zone. H: Entries into the central zone. Two-way ANOVA results are presented in frames, and statistically significant findings are underlined. Lines with P-values above bars indicate all the significant and marginally

significant differences (unadjusted for multiple comparisons) that were revealed with the planned tests listed in the Methods section. P_t denotes a *t*-test p-value, and p_w is a Mann–Whitney test p-value. The data are depicted as the mean + standard error of the mean.

Effects of restraint stress on FST results

Data from the mouse groups separated by sex are provided in Table 1. There were no significant effects of stress, of sex, or of stress-by-sex interactions on any of the behaviors evaluated in the FST (ANOVA, all *P*s > 0.1). The planned comparisons by Mann–Whitney or *t* tests did not reveal any significant differences in group means (all *P*s > 0.1).

Body weights

Initial body weights in the mouse groups were as follows (mean ± SD): control males: 15.45 ± 2.53 (n = 14), stressed males: 15.15 ± 1.77 (n = 13), control females: 13.32 ± 2.46 (n = 10), and stressed females: 12.78 ± 1.02 (n = 10). Animals were weighed again on Days 3 and 6 of the experiment. Analysis of the collected data by repeated-measures ANOVA did not reveal any significant effects of stress exposure or of stress-by-sex interactions on body weight [F(1, 43) = 0.4, P = 0.51, and F(1, 43) = 0.01, P = 0.92, respectively]. Females had significantly lower body weight than males did [ANOVA F(1, 43) = 11.6, P = 0.001].

The weight of C57BL/6 males was 28.79 ± 2.93 (n = 10).

Table 1. The influence of restraint stress on FST parameters in wild-derived *M. m. musculus*

	Males		**Females**	
	Control	**Stress**	**Control**	**Stress**
Number of mice	14	13	10	9
Latency of 1st immobility episode, s	243.64±136.18	250.92±112.20	310.90±186.04	254.44±142.92
Climbing, s	102.64±63.24	102.08±53.22	76.60±29.39	82.00±47.20
Swimming, s	433.50±63.46	451.92±66.98	474.60±54.04	458.33±42.93
Duration of immobility, s	63.86±54.69	46.00±54.08	48.80±48.72	59.67±53.54

Note. Values are presented as means ± standard deviations. Two-way ANOVAs with stress and sex as factors as well as planned tests did not detect any significant differences (all *Ps* > 0.1)

Comparison with behavior of C57BL/6 mice

To characterize baseline performance of wild-derived *M. m. musculus* mice in the OFT and FST, the behavior of control males was compared with that of C57BL/6 males. Detailed results and their statistical significance are given in Table 2.

Table 2. Behavioral characteristics of *M. m. musculus* compared to the C57BL/6 mouse strain

	***M. m. musculus* males**			**C57BL/6 males**			**p-value**
	n	**Mean**	**SD**	**n**	**Mean**	**SD**	
OFT							
Horizontal activity	13	271.62	71.52	9	120.89	42.91	<0.001***
Defecation	14	2.43	1.91	10	2.20	2.10	0.72
Rearing	13	70.31	17.87	9	38.67	12.94	0.0012**
Jumping	13	36.92	40.38	9	0.22	0.44	<0.001***
Grooming, s	13	26.77	25.33	9	45.44	24.49	0.035*
Immobility, s	13	9.15	9.45	9	0.33	1.00	<0.001***
Time in center, s	13	22.00	15.50	9	20.62	10.29	0.76

Entries into center	13	21.31	13.95	9	12.00	8.44	0.12
FST							
LP, s	14	243.64	136.18	9	105.22	50.89	<0.001***
Climbing, s	14	102.64	63.24	9	120.11	64.31	0.31
Swimming, s	14	433.50	63.46	9	431.33	66.34	0.64
Immobility, s	14	63.86	54.69	9	48.56	24.77	0.78

Note. LP: Latency of the first immobility episode in the FST. P-values indicate significance of differences between groups as determined by the Mann–Whitney *U* test. *P < 0.05, **P < 0.01, ***P < 0.001

4. Discussion

In this work, we assessed behavior of wild house mice in the OFT and in the FST after exposure to restraint stress. To the best of our knowledge, this is the first report of effects of restraint stress on wild-derived mice from the first generation of laboratory breeding. In the experiments, we used mice of both sexes, which allowed us to investigate sex differences.

Stressful events and environments are associated with the development of anxiety disorders in humans. Comparable results have been obtained in laboratory rodent models. The OFT is often employed to measure anxiety as well as locomotor and exploratory activities in mice. The proportion of exploration of the unprotected central area in the OFT is conventionally viewed as a measure of anxious behavior in rodents, and mice that spend less time in the center are considered less bold and more anxious (Harro et al. 2018). In our study, stressed mice showed a significantly reduced number of entries into the central area as compared to the controls, indicating an increase in anxiety (Figure 1H). We observed a similar marginally significant effect of restraint stress on the amount of time spent in the central area (Figure 1G). Some studies suggest that in laboratory rodents, restraint stress elicits changes in locomotor activity resulting primarily in an activity decrease (Molina et al. 2023). The present results did not confirm these observations in the entire sample of wild-derived mice. Meanwhile, a significantly lower number of center entries with unaltered locomotor activity are consistent with increased anxiety in the OFT, thereby confirming the specificity of the effect. Self-grooming displacement activity is another pattern of behavior that may contribute to poststress adaptation in animals. Various stressors have been reported to elicit repetitive self-grooming behavior in rodents (Liu et al. 2021; Zhang et al. 2023). Accordingly, restraint stress in our study led to significantly greater total duration of grooming in mice of wild origin (Figure 1E). In the OFT, significant sex differences were detectable only for the number of defecation boluses, with males defecating more than females. These results point to higher emotional reactivity in males compared to females; this greater reactivity was presumably triggered by previous stress exposure. The absence of evidence for sex differences in exploratory and locomotor activities in this test is consistent with previously reported observations about wild-derived house mice (Vošlajerová Bímová et al. 2016; Frynta et al. 2018).

Prolonged stress exposure in laboratory rodents often causes behavioral changes that resemble human depressive states. The total duration of immobility in the FST is commonly used to assess behavioral despair or depressive-like behavior in rodents. To our knowledge, behavior of mice of wild origin in the FST has not been described before. Hiadlovská et al. (2014) tested the behavior of wild-derived mice in the Morris water task and noted that there was no “motionless floating” upon being placed in water. In our study, in the FST lasting for 10 min, immobility episodes were registered in 44 out of 46 test subjects (96%). The discrepancy between the studies may be attributed to different sizes of the experimental setups as well as to prolonged latency of the first immobility episode and the additive stress load from the prior OFT exposure. In the FST, we noticed several behavioral patterns in wild mice that are similar to those registered in laboratory rodents, e.g., climbing, swimming, and immobility episodes (Table 1). Nevertheless, the results of our experiments indicate that restraint stress did not significantly shift the FST parameters. Thus, depressive-like behavior was not significantly affected, which may be partially due to the short duration of the stress that we applied. In this test, as compared to males, females tended to be less active, spending less time climbing and more time swimming, but these differences did not reach statistical significance.

In laboratory rodents, restraint stress may affect body weight and food intake, thereby commonly causing weight loss (Molina et al. 2023). This was not observed in our study probably owing to mild stress intensity or relatively low initial body weights. Males were significantly heavier than females of the same age, consistently with the sexual size dimorphism detected in house mice derived from free-living populations (Carlitz et al. 2019).

The findings of the present work may be limited to the source natural population from which test subjects originated. To characterize baseline performance of wild-derived *M. m. musculus* mice, we compared the behavior of control males to that of males of the C57BL/6 inbred laboratory strain in the two tests (Table 2). Overall, wild-derived males manifested significantly elevated exploratory and locomotor activities in the OFT, whereas in the FST, they showed significantly longer latency of the first immobility episode. Because of the latter finding, we would recommend that the typical 6 min duration of the FST be extended to 10–15 min for wild-derived mice. Besides, due to the ability of wild mice to jump high, it is necessary to use an open-field setup with greater wall height. On the other hand, key measures of anxiety-like and depressive-like states, such as entries into the center zone in the OFT and duration of immobility in the FST, did not differ significantly between the wild-derived and laboratory mice.

5. Conclusions

Our results indicate that in wild-derived house mice, short-term restraint stress can lead to significant enhancement of anxiety-like behavior in the OFT without affecting overall locomotor activity. Meanwhile, we did not observe significant effects of the stress exposure on depressive-like behavior in the FST or on body weight in these mice. Our findings may be limited by several factors such as the source population, the relatively small sample size, and the chosen experimental time frame. Nonetheless, there were no significant differences between the wild-derived males and laboratory C57BL/6 males in key measures of anxiety and depressive-like behavior as assessed in the standard tests. Thus, by testing the behavior of wild-derived mice, we obtained confirmation of ecological validity of the restraint stress model in laboratory rodents. Our findings also extend knowledge about adaptive behavioral responses in the subspecies *M. m. musculus* in particular and in wild house mice in general. Our data can serve as a basis for the design of more extensive research in the future.

Authors' Contributions: The author confirms sole responsibility for study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

Conflicts of Interest: The author has no relevant financial or non-financial interests to disclose.

Ethics Approval: All procedures were approved by the Ethics Committee of Severtsov Institute of Ecology and Evolution (protocol No. 59 from 28.01.2022).

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