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Behavioural markers of stress during routine laboratory handling in mice

Basma A. Elshafey, Sameh G. Ramadan, Enas K. Aziz *

Department of Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Menoufia, Egypt.

***Correspondence: Enas K. Aziz, E-mail: enas.aziz@vet.usc.edu.eg**

Abstract

Background: Some environmental factors lead to physiological and behavioral changes in laboratory animals.

Methods: Twenty male mice and twenty handlers were used in this study. The handlers were given three minutes to pick up and handle a mouse from his cage. Handlers were divided into two groups based on their scores when handling mice: low- and high score groups. For two weeks, each mouse was handled every other day. Mice's behavioral reactions to handling, such as hyperactivity, escape, and elimination, were observed. After each handling, mice were placed in a light-dark box (LDB) to examine their anxiety-like behavior, and at the end of the experiment, they were placed in an elevated plus maze (EPM).

Results and conclusion: In contrast to high handling scores, the results showed that low handling scores increased the mice's hyperactivity, escape, and elimination responses; they also increased the latency of mouse to move from dark to light compartments in the LDB; and they decreased the number of entries and the amount of time spent in the open arms during the EPM test. It is concluded that the prolonged handling of mice during ordinary work had a negative impact on the health of the mice and enhanced their fearful and stressful responses.

Keywords: Handling, Anxiety, LDB, EPM, Mice

1. Introduction

There is compelling evidence that an animal's life in a lab, is stressful by nature (Balcombe JP, 2006). In addition, the experimental animals are subjected to a number of procedures that obviously have a negative impact on their welfare, including transit, restraint, gavage, blood draws, even routine handling and weighing, often poor living conditions, inadequate anesthesia, and unusually easy access to food and drink. Additionally, numerous natural behaviors and activities are not allowed in the lab, including foraging, hiding, nest-building, escape, and exploring. (Bailey, 2017). Numerous studies employ mice and rats as laboratory animals that are subjected to various treatments over weeks or months, including repeated researcher manipulations of the animals. Whenever, adaptation to handling could alter an animal's reactions to experimental techniques and impact experimental results (Corda et al., 1980).

The handling of laboratory animals during experiments is accepted as a source of unexplained variance both within and between animal research, as it influences both the behaviour and physiology of animals (Gouveia and Hurst, 2013). This is crucial because it is impossible to standardize exactly how long animals must be handled for routine maintenance and experimental manipulations between studies and because the status and responses of experimental animals may be greatly impacted by the researcher's skills. Improving the number of animals needed for trials is a key to improving the variability in the responses of research animals. (Festing., et al. 1998; Howard, 2002). Therefore, understanding how to lessen strong stress reactions to handling is a main concern that could affect a very huge number of research outcomes, beside influence on the expression of anxiety behaviour of mice that are kept within animal facilities (Gouveia and Hurst, 2013). Anxiety and behavioral responses to stress are typically related. The choice of handling technique is therefore expected to have a big impact on the welfare of lab mice (Turner, 2013). Elicitation of defensive behaviour is a core component of the stress response, as aggression. Beside, handling can suppress exploratory behaviour leading to impaired test performance (Wood et al., 2003; Schellinck, 2010).

The light-dark test and the elevated plus maze, have been used to study anxiety-like behavior in mice (Macriet et al., 2002). In both adult rodents and young animals, the light-dark test has been extensively utilized to measure anxiety-like behavior (Steinberg, 2008; Eaton et al., 2012). This test is based on an approach-avoidance conflict between exploration of novel environments and avoidance of open spaces (Crawley, 1985).

Among all the currently available animal models of anxiety, the elevated plus maze (EPM) test is one of the most widely used (Crawley, 2007). The EPM is a raised plus-shaped apparatus consisting of two open and two closed arms (Gurfein et al., 2012). Since both open and closed arms are thought to elicit the same exploratory drive, avoiding the open arms is assumed to be the

outcome of the induction of fearfulness in open and elevated regions (Rodgers and Dalvi, 1997; Komada et al., 2008).
Therefore, the purpose of this study was to look at the effects of handler handling skills on health and anxiety-like behavior of laboratory mice.

2. Materials and methods

The Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, University of Sadat City (protocol number VUSC-018-1-19), gave its approval for this work in accordance with national practice requirements.

2.1. Animals and experimental design

Twenty trained handlers, aged 19 to 20, and twenty male albino mice (5 to 6 weeks old) were employed in this experiment. By enabling them to pick up and handle a mouse from his home cage for three minutes during the light phase of the light-dark cycle, the handlers' abilities were assessed. Following is the handling technique: Firmly seize the mouse's tail at the base or in the middle. Put the mouse on the top of the wire cage. While holding the tail, firmly grip the loose skin with the thumb and first two fingers near the ears. The final two fingers are able to be used to grasp the tail (UNC-IACUC, 2017). Over the course of the five sessions, the mice were handled once every two days. According to the time it took to pick up and hold a mouse, the handling scores were described (Table 1). Handlers were divided into two groups based on their handling scores: the first group received scores between 0 and 2 (a poor score), and the second group received scores between 3 and 5 (a high score).
In each cage, mice were individually identified on the tail using coloring marking technique. Mice were kept on commercially balanced pellet food and unlimited access to clean water.

Table (1): Description of handling duration scoring method.

Score	Description
0	Pick up the mouse and hold it through more than180 seconds.
1	Pick up the mouse and hold it through 150–180 seconds.
2	Pick up the mouse and hold it through 120-150 seconds.
3	Pick up the mouse and hold it through 90-120 seconds.
4	Pick up the mouse and hold it through 60- 90 seconds.
5	Pick up the mouse and hold it through 30-60 seconds.

2.2. Mice responses

Mice's reactions to the handling procedure were seen. The mice's emotional state was revealed by signs of hyperactivity, escape, and elimination (urinating or defecating), among others.

2.3. Anxiety tests

2.3.1. Light-dark box (LDB) test

The mouse's exploratory actions in this test show how it balances risk avoidance with potential threats. The dark-light box, which measured 32 x 30 x 30 cm and had two equal sections, was used to test mice. The first part was painted black and had a black lid on top, while the second section was painted white and left uncovered. There was a tiny aperture 2.5 x 2.5 cm connecting the two sections. Each mouse was handled individually before being placed in the compartment, facing away from the aperture, and watched for three minutes. All four paws were extended, and the latency to cross to the light section was recorded (Marks et al., 2009; Huang et al., 2018).

2.3.2. Elevated Plus Maze (EPM) test

Mice were put to the test in an elevated plus maze the day following the final handling session. The unpainted plywood plus-maze device had two open arms that were 35 cm long by 5 cm wide and two closed arms that were 35 cm long by 5 cm wide and 15 cm high side walls that extended from a center square that was raised 40 cm above the floor. The mice were taken to the experimental room, each mouse was placed in the central square facing a closed arm and observed for 5 minutes (Marcondes et al., 2001). The following variables were assessed: number of entries into closed arms, the length of time spent in closed arms, numbers of entries into open arms, the length of time spent in open arms, number of visit to the end of an open arm, and latency until the first open-arm entry (time elapsed before mouse first entered an open arm). The apparatus was cleaned with 70% alcohol after each mouse was tested.

2.4. Statistical analysis:

The procedures in independent T tests in IBM SPSS statistics (SPSS for Windows, V 25.0; SPSS Inc., Chicago, IL, USA) were used to statistically compare all parameters between the two test groups. However, throughout the course of five sessions, the ANOVA test was employed to compare various parameters. The significant difference between the means of the treatments was ascertained using the Duncan Multi Range Test of significance. The Pearson correlation test was used to examine any associations between LDB test parameters and mouse emotional state. The data are all expressed as mean ± S.E. A level of significance of $P \leq 0.05$ or $P \leq 0.01$ was regarded as statistically significant.

3. Results

3.1. Mice responses:

In the current study, mice's behavioral reactions were negatively impacted by low handling scores compared to high handling scores. When handled for a longer period of time, mice displayed considerably higher hyperactivity, escape, and elimination behavior than when handled for a shorter period of time (Table 2).
Table (3) displayed the emotional state of the mice during the course of five sessions. The first session saw the highest levels of hyperactivity, escape, and elimination behavior in low score group, which then declined after that. The mice of high score group that were handled for a brief period of time exhibited no significant variations in their hyperactivity or escape across the five sessions. On the other hand, high handling score of the mice significantly decreased the elimination behaviour from the third session and disappeared in the last two sessions.

3.2. Anxiety-like behaviour

The mice's anxiety-related behavior was significantly impacted by the handling duration, whether it had a low or high score. In the LDB test, the latency for the mice to make the first cross to the light chamber was significantly delayed in the low handling score group compared to the high handling score group (Table 4).
When compared to mice handled for short duration and high score, mice exposed to long handling duration with low score displayed significant (P=0.01) increases in the number of entries and the time spent in the closed arms, along with a corresponding decrease in the number of entries (P=0.02), time spent in the open arms (P=0.01), and the number of visits to the end of the open arms (P=0.003).The latency to first entry into the open arm was also significantly (P=0.01) reduced in the high handling score group of mice compared to the low handling score group. (Table 4).
During five sessions of handling, the latency time to first cross to light chamber in the light-dark box test was significantly reduced from the first to fifth session in both low handling score (134.70±4.51, 133.50±2.18, 102.30±3.19, 74.50±3.71, 50.80±2.26 sec, p=0.01) and high handling score (54.30±3.42, 71.60±2.22,

66.60±3.76, 40.20±3.60, 39.10±2.91 sec, p=0.04) groups (Figure 1). Data from Table (5) denoted that, there is a positive correlation between handling duration and mice responses. When the handler take long time for mice handling, the latency for the mice to make the first cross to the light chamber of dark light box (r=0.512, P=0.01) and eliminative frequency (r= 0.802, P=0.01) were significantly increased. There were no significant correlation between handling duration, biting and escape frequency of mouse during handling.

Table (2): Effect of handling duration on the emotional status of the mice (Mean±SE)

Items	Groups		*P*-value
	Low	High	
	Emotional status (frequency)		
Hyperactivity	0.96±0.11	0.76±0.09	0.02
Escape	1.09±0.16	0.77±0.05	0.001
Elimination	1.33±0.22	0.88±0.11	0.003

Table (3): Effect of handling sessions on the emotional status of the mice (Mean±SE)

Emotional status	Handling sessions					*P*-value
	1st	2nd	3rd	4th	5th	
Hyperactivity (frequency)						
Low	$1.50±0.22^{a}$	$1.37±0.17^{ab}$	$1.00±0.09^{b}$	$1.00±0.01^{b}$	$1.00±0.01^{b}$	0.007
High	1.00±0.01	1.00±0.01	1.00±0.01	0.85±0.07	0.85±0.11	0.14
Escape (frequency)						
Low	$1.62±0.14^{a}$	$1.04±0.07^{b}$	$1.00±0.01^{b}$	$0.85±0.25^{b}$	$0.85±0.25^{b}$	0.01
High	1.00±0.01	1.00±0.01	0.85±0.01	0.85±0.11	0.85±0.07	0.33
Elimination (frequency)						
Low	$3.00±0.01^{a}$	$2.00±0.01^{ab}$	$1.00±0.01^{b}$	$1.00±0.01^{b}$	$1.00±0.01^{b}$	0.01
High	$1.00±0.01^{a}$	$1.00±0.01^{a}$	$0.75±0.13^{ab}$	$0.00±0.00^{b}$	$0.00±0.00^{b}$	0.05

a,b Mean values with different superscripts letters within the same group are significantly different.

Table (4): Effect of the handling score on the mice anxiety (means±SE)

Anxiety-like behaviour	Groups		*P*-value
	Low	High	
Light-dark box (LDB)			
Latency to first cross to the light chamber (sec)	99.16±2.25	54.36±2.65	0.02
EPM			
Number of entries into closed arms	4.25±0.47	2.25±0.25	0.01
The length of time spent in closed arms (sec)	140.75±2.15	17.00±2.12	0.01
Numbers of entries into open arms	2.00±0.40	3.50±0.28	0.02
The length of time spent in open arms (sec)	68.00±4.77	141.50±4.99	0.01
Number of visit to the end of an open arm	0.00±0.00	2.50±0.28	0.003
Latency to first entry into open-arm (sec)	95.75±3.51	32.00±3.31	0.01

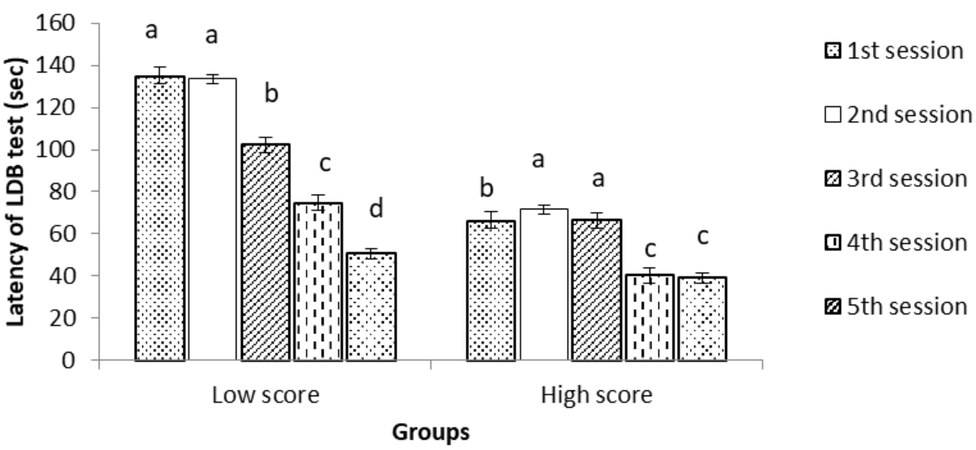

Figure (1): Effect of the handling sessions on the latency to first cross to the light chamber in the LDB test in the mice.

Table (5): Correlation coefficient between handling duration and mice responses.

Items	Dark light box	Biting frequency	Escape frequency	Elimination frequency
Handling duration	0.512^{**}	0.021	0.019	0.802^{**}
Dark light box		0.005	0.184	0.613^{*}
Biting frequency			0.248	0.425
Escape frequency				0.055

*(P<0.05)   ** (P<0.01).

4. Discussion

Mice are the most popular utilized animals in research. Despite this, people find it harder to relate to mice than to more familiar companion animals. Mice frequently exhibit severe handling stress and anxiety in reaction to capture because of their small size, which puts them vulnerable to predation or other injury. Mice are sensitive to various forms of handling and the operator's presence (Akatsu et al., 2015). Nevertheless, mice adapt readily to human contact if handled correctly. As a result, an animal's daily routine now requires care and handling (Claxton, 2011). Persons that perform care-related responsibilities and interact with laboratory animals should be considered a part of the animal’s social experience to decrease stress and aggression in the mice. In the present study, the mice's emotional status was negatively affected by increasing duration of handling, with significant aversion and increased hyperactivity (try to bite), escape, and elimination during handling when compared to mice that pick up and handled through 30 – 120 seconds. Grabbing mice by their tails causes aversion and distress. Although this strategy is still extensively employed, it appears to induce inherent fear of being captured in mice, which they do not easily to adapt (Hurst and West, 2010; Gouveia and Hurst, 2013). Handling methods can have assessable effects on operator scores of anxieties and fear-related responses of the animals therefore, non-tail handling methods make the mice easier to handle and showed lower levels of overt behaviours including struggling, vocalization and aversion on release when using the modified technique as tunnels or cupping (Davies et al., 2022).

To ensure a quick and secure restraint, both the handler and the mice must be properly positioned. Animals must feel safe and remain entirely immobile to avoid struggling, which can cause anxiety and injury to the animal or the handler (Hubrecht and Kirkwood, 2010). Unsuccessful attempts to trap a mouse can increase its anxiousness and make it more elusive, making the handler impatient and frustrated (Fawcett, 2012). This may explain why the mice in the low score handling group were more prone to hyperactivity, escape, and elimination, when being handled than those in the high score handling group. These findings are in agreement with those of Balcombe et al., (2004) and Bailey, (2017), who discovered that the threat of human interaction causes stress and anxiety, which has a detrimental influence on animal welfare and makes managing animals that might bite or escape more difficult.

The emotional status of the mice, including hyperactivity, escape, and elimination behaviours, was significantly higher in the first session compared to the subsequent four sessions in the low score group. In the high score group, conversely, no significant alterations in hyperactivity or escape behaviour were seen during the five sessions, despite the fact that the first session had the highest numerical values. In the high score group, however, eliminative behaviour was much higher in the first and second sessions than in the other sessions.

These results may be attributed to improving the handling skills of the handlers through the five sessions because frequent handling leads to increased ease of handling among laboratory animals and reduced emotional status in response to human contact (Buerge and Weiss, 2004; Deacon, 2006). Therefore, these data suggest that repeated handling may be needed to habituate animals to researchers. The present results agree with Hubrecht and Kirkwood, (2010) who reported that mice become less fearful after multiple handling sessions but continue to show signs of escape. The elevated plus maze test and the light-dark box were used to explore anxiety-like behaviour in mice. In the light-dark box, the latency for the mice to make the first movement to the aversive, brightly lighted chamber was significantly delayed in the low score handling group as compared to those in the high score handling group. As a result, mice that handled for long duration took longer to walk to the light box, indicating anxiogenic behavior (Huang et al., 2018).

In the EPM test, anxiety was manifested by a reluctance to visit the open arms, with the number of entries and total time spent on the open arms being lower among mice in the low score handling group. The mice in the high score handling group, on the other hand, showed significantly more open arm exploration and less latency to first open arm entries than those in the low score handling group. Therefore, avoidance of open arms may be attributed to the induction of higher levels of anxiety (Rodgers and Dalvi, 1997). It has been hypothesized that mice in the low score handling group's aversion to entering and exploring the open arms is due to their fear of open and elevated places. As a result, our findings are in accordance with Gouveia and Hurst, (2017), who found that reducing stress and anxiety in mice during handling process could improve animal performance in behavioural tests by reducing or removing unnecessary anxiety. In the LDB test, the latency to first cross to the light chamber was considerably higher in the first three sessions in both groups than in the last two sessions. These findings may be explained by the mice's increased fear and anxiety during the first trials of handling during interactions with either low or high score. These findings are in agreement with those of Costa et al., (2012), who found that repeated handling reduced anxiety in rats.

In general, mice handling for long duration may cause anxiety or fear in the mice, which can result in crushing (Ness and Gebhart, 1990) and a negative sensory and emotional experience. According to Price BB, (2002) aversive or unpleasant pain is essentially determined by its duration and intensity, causing anxiety and/or fear. Therefore, limiting the duration spent picking up the mice are critical in lowering stress and suffering. It's also critical to utilize appropriate and skilled handling to ensure that mice accept or actively seek human contact. Health and well-being of laboratory animals are very critical for the accuracy of the results obtained from any experiment.

Conclusion

It was concluded that people who take long duration to handle the mice causing the mice to exhibit higher emotional status and anxiety-like behaviour that affecting results of experiments. Furthermore, repeated handling helped to tame the mouse and improve the handler's skill. Handling duration scoring can be used to evaluate skill of laboratory animal technicians. Additionally, familiarity with handling and nonthreatening handlers is often beneficial in reducing mouse distress, and discomfort.

Conflict of interest

The authors declare that they have no conflict of interest.

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Authors' contribution

Conceptualization, B.A.E, S.G.R, E.K.A; Sample collection, E.K.A; Diagnosis, B.A.E, S.G.R, E.K.A; Methodology, investigation, and data curation, B.A.E, S.G.R, E.K.A collected literature then drafting the manuscript in consultation with E.K.A, B,A.E; Review and editing, B.A.E, S.G.R, E.K.A All authors read and approved the final manuscript.

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