Behavioural Ethograms of Treatments-/Assays-Naïve Mice on a Novel Anxiety Multitest

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ABSTRACT

Anxiety sensitivity deficiencies of individual preclinical anxiety tests/models are a major contributory factor to the high anxiety drug discovery attrition rates and wide anxiety disorder therapeutic gap. Mitigating this factor calls for the refinement and modification of the exiting rodent anxiety tests. Use of test batteries (deploying multiple single anxiety tests at different times) and multi-testing i.e., quasi-simultaneous experimental anxiolytic screening of rodents on multiple test apparatuses physically joined together into composite units have been suggested to obliterate the idiosyncrasies, broaden the anxiety sensitivity of individual single anxiety tests – with the overall goal of improving their translational capacity. Previously, some rodent multi-tests have been invented – including the triple test consisting of light-dark (LDM), elevated plus (EPM), and open field (OFM) mazes but the ambiguity presented by the central (neutral) platform of the EPM and the monotonous nature of the OFM components often included in its design has constituted a significant drawback. These 2 maze components have been replaced in a recently invented Triple mouse anxiety test with an elevated zero maze (EZM) and a marble-burying maze (MBM), respectively. Thus, the aim of this study is to investigate the murine anxiety-inducing and anxiety sensitive property of a composite apparatus (Triple test) created by physically joining a unit each of LDM, EZM, and MBM, in that that order, via thorough fares in-between the units. This ethological evaluation was also concurrently carried out in standard single rodent anxiety tests i.e., elevated zero maze (EZMT), elevated plus maze (EPMT) and open field (OFT) tests for comparison. Groups of treatment-naive mice (n=12; equal sexes) were each exposed to Triple set 1 – with trials initiated from the LDM component (Appendix I), Triple test 2 – with trials initiated from the middle EZM component (Appendix II), Triple test 3 – with trials commenced from the MBM component (Appendix III), single EZMT, EPMT, and OFT with trials conducted adopting appropriate standard operating procedures. Trials lasted 20 minutes for the triple and 7 seven minutes for the single anxiety tests. Behavioural data were collated and analysed by descriptive statistics. Results were expressed as absolute counts, percentages or means ± S.E.M. Forty-eight (48) different primary and derived behaviours were obtained on the triple test (EZM component, 22; LDM and MBM components each, 13) (Tables 1, 2, & 3) compared with 22, 18, and 18 obtained on the single EZMT, EPMT, and OFT, respectively (Tables 3, 4, & 5). Walking, rearing/assisted rearing, and sniffing were the most frequent behaviours exhibited by the experimental subjects across all tests. These behaviours together with chewing, stretch-attend postures (SAP), grooming, and defecation/urination constituted 80-90% of all observed behaviours in each single or multi test. Marble-burying behaviour (MBE) was least frequent behaviour at 0.1 marble buried per mouse and only afforded by Triple test's MBM. Head dip (HD) was

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afforded only by the triple tests, single EPM and EZM. Significantly, central platform activities and data present in the EPMT were absent in the EZMT and Triple test trials. In both single and Triple tests, the experimental mice exhibited preferences for protected over unprotected portions of test devices (Table 6). Mice spent 47.5±4.2% in the dark (covered) compartment (DC.LDM) and 16.1±2.0% in the light (open) compartment (LC.LDM) of the LDM, 20.6±2.1% in closed (walled) (CS) and 4.4±0.6% in the open (unwalled) (OS) segments of the EZM, and 11.1±1.9% in the central (open) (CZ) and 39.0±5.4% in the peripheral (near-wall) (PZ) zones of the MBM components of Triple tests. Similarly, in the single tests, they spent 84.0±1.3% in closed and 15.9±1.3% in open segments of the EZM, 59.6±2.4% in closed, 17.2±1.7% in open, and 23.3±2.5 in the neutral central portions of the EPM, and 81.6±2.0% in CZ and 18.4±2.0% in PZ of the OFM (Table 6). Lastly, experimental mice exhibited differential test time distribution and exploratory activities on the Triple tests according to the component from which trials were initiated (Table 6). Mice initiated from the EZM (middle) component (Triple test 2) recorded a near even time and activity distribution of 38.4%, 33.6%, and 28% of the test duration spent in the Triple test's LDM, EZM, and MBM components, respectively, (Figure 1a) – with most of the animals reaching and exploring all the 3 components within 15 minutes of trial commencement. This pattern contrasts with trials initiated from either from the LDM component (Triple test 1) in which about 92.7, 5, and 2.3% of the test duration was spent by the mice on the LDM, EZM, and MBM components, respectively (Table 6). Additionally, only in half of the trials did the mice reach the second (EZM) component and only in 2 trials did they get to the third (MBM) component from the initiating maze (Data unshown). Triple test 3 trials also exhibited an uneven activity/time distribution – with experimental mice spending about 6.5, 9.5, and 84.0% of test durations on the LDM, EZM and MBM components of the composite anxiety test apparatus – with only 5 mice reaching the second (EZM) and 2 mice reaching the third (LDM) components from the starting MBM (Data unshown). In conclusion, the triple mouse test is sensitive to and capable of generating greater spectra of anxiety-related mouse behaviours than than any standard single anxiety assay. Findings also showed single acute trials initiated from the EZM component were associated with the most exploratory activity and even spread of the test duration by the mice investigated on the Triple anxiety test. It may be necessary to investigate the behavioural effect of anxiety-related drug treatments and repeated trials on mice exposed to this novel anxiety Multitest.

Keywords: Swiss albino mice, ethological, Triple anxiety test, Elevated plus maze, Elevated zero maze, Marble-burying maze

INTRODUCTION

The need for greater translationality of preclinical anti-anxiety drug research findings to clinical applications on one hand, and the increasing ethical demands to minimize the number of experimental animal subjects in behavioural studies on the other, calls for the continued modifications and refinements of the existing animal anxiety assays. These modifications/refinements are envisaged to improve the external validity of their end-products by conferring on them the capacity to both exhibit greater anxiety sensitivity and to eliminate/minimize the anxiety sensitivity liabilities of the most frequently deployed single animal anxiety tests that could negatively impact research outcomes [1, 2, 3, 4].

Subjecting the experimental animals to combinations (batteries) of different anxiety tests was initially suggested in behavioural trials to enhance the broader detection of complex anxiety parameters, reduce the number of animals needed, and to mitigate the negative impact of the idiosyncrasies of the individual animal anxiety tests. Although the overall translational capacity was somewhat enhanced, however, battery experimental set-ups for behavioural studies were found to have certain drawbacks, including the risk of inter-test poor correlation, negative/positive temporal test performance biases, one-trial tolerance (OTT) phenomenon, and the cumbersomeness of re-testing experimental subjects essentially on different trial days

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[5, 6, 7, 8, 9, 10].

To overcome the afore-mentioned experimental challenges around the use of single tests and test batteries, near concurrent testing of laboratory animals on multiple (triple) anxiety assays was adopted to further improve the translational capacity of the preclinical behavioural studies [11]. Much as multi-testing has enhanced the translational and reproducibility of animal behavioural findings, there is still room for further refinement of the most outstanding of the rodent anxiety multi-tests. For instance, the open field and elevated plus components of the rodent anxiety triple test apparatus by Ramos et al., 2008 are inherently liable to OTT and interpretation ambiguity due to the central square of the elevated plus maze that could adversely affect the integrity of its behavioural outputs. An improvisation over this triple test which has its open field maze (OFM) replaced by a light-dark maze (LDM), and its elevated plus maze (EPM) by an elevated zero maze (EZM) was recently invented. This novel mouse anxiety triple test apparatus, dubbed U.ofA. Mouse Anxiety Multitest apparatus, consisting of LDM, EZM and marble-burying maze (MBM) is not only potentially bereft of the liabilities associated with the Ramos mouse anxiety triple test but also versatile – capable of generating broader spectra of rodent anxiety-related, panicogenic and obsessive-compulsive behaviours [12]. However, information regarding anxiety-related behavioural patterns of mice exposed to this novelty has not been documented. This study therefore seeks to observe the behavioural ethogram of treatments- and assay-naïve mice (equal sexes) exposed to this triple test compared to other classical rodent anxiety tests, on the first count. On the second count, to observe differences, if any, in the behavioural ethogram of experimental mice when their trials on the novel triple test were initiated from different components of the same multi-test apparatus. And thirdly to see if the novel mouse anxiety Multitest would be sensitive to broader spectrum of murine anxiety behaviours, including obsessive-compulsive behaviours.

MATERIALS & METHODS

Experimental Animals

One hundred (100) wild-types Swiss albino mice (*Mus musculus*) (equal sexes) were purchased from the National Institute of Pharmaceutical Research and Development (NIPRD) Idu, Abuja, Federal Capital Territory (FCT), in May 2023. They were kept in plastic cages in animal house of the Neuro-Behavioural & Stem cell lab of the Department of Pharmacology & Therapeutics, Faculty of Basic Clinical Sciences, College of Health Sciences, University of Abuja, Abuja, FCT, Nigeria under good laboratory practices with access to water, feed, and air *ad libitum*.

Behavioural Apparatuses & Room

A total of six (6) units of behavioural test devices were used in the study. These consisted of three (3) units of U.of A. mouse anxiety Multitest apparatus – consisting of an LDM, an EZM, and an MBM in that order, and 1 single unit each of an OFM, an EPM, and an EZM – all elevated to a uniform height of about 70 cm from the floor.

The first (proximal) component of the triple test apparatus is a unit of an LDM made up of 60 cm long, 40 cm wide and 32 cm high 3 mm-thick plastic glass boxes partitioned into 40 x 40 cm white-painted open-roof proximal (light) and 20 x 40 cm black-painted closed-roof distal (dark) parts. This unit is placed level with a unit of an EZM with a central 5 x 6 cm aperture physically connecting lower parts of the outer wall of the LDM's dark segment to those of the outer wall of a closed segment of an EZM – which is the middle component of the composite device.

A unit of the EZM consists of 5 cm wide 0.75 inch thick circular polyvinyl cellulose runways with a 160 cm

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outer and 120 cm inner circumference. It has 2 segments each with 40 x 18 cm outer and 30 x 18 cm inner, black-painted plastic 2 mm-thick glass walls that are diametrically placed opposite each other. The walled and unwalled portions of EZM are the closed and open segments of the device. A 5 x 6 cm aperture centrally placed at the base of the outer wall of a closed segment physically connects with the lower parts of the proximal wall of the third component (MBM) of the triple test apparatus.

The MBM is a 40 x 40 x 32 cm box with walls made of 3 mm-thick, white-painted non-translucent plastic glass. A 6 x 5 cm aperture centrally placed at the base of the proximal wall of this unit physically connects with the outer wall of a closed segment of the EZM – the middle component of the Multitest apparatus. The floor of the MBM consists of a 1.5 cm-thick layer of wood shavings on which 8 broken peach-coloured marbles were spatially equally placed. Thus, the apertures between the 3 components and the uniform elevation platform supporting them afford free-flowing bi-directional movements of the experimental mice.

A single unit of the EZM is essentially the same as described for the EZM component of the triple test unit in shape and dimensions except that the single EZM unit does not have apertures in the segments. However, as a stand-alone test device, it is also supported on a 70 cm-high four-legged polyvinyl cellulose pipe platform.

The single open field maze (OFM) used in this study is a 40 x 40 x 32 cm box with walls made of 3 mm-thick, white-painted non-translucent plastic glass without any exits/apertures. The box is brightly lit, devoid of any item, and supported on a 70 cm-high four-legged polyvinyl cellulose pipe platform.

The single elevated plus maze (EPM) used comprises 2 closed (15-cm high walled) and 2 open (unwalled) segments on 4 horizontal 30-cm long 5-cm wide platforms supported on a 70 cm-high four-legged polyvinyl cellulose pipes. The segments are each 30 cm long and 5 cm wide and arranged so that similar segments are opposite to each other with all 4 segments having a 5 cm central neutral overlap (platform).

Experimental Procedure

The behavioural studies took place in the third week of July 2023 in the Neuro-Behavioural & Stem cell lab of the Department of Pharmacology & Therapeutics, Faculty of Basic Clinical Sciences, College of Health Sciences, University of Abuja, Abuja, FCT, Nigeria. The room illumination during testing was set at 60 lux from an overhead light source that was set at the same level as the cameras for the video recording of the activities of the experimental mice.

On the days of the experiments, groups (n=12) of both treatments- and assays-naive mice (equal sexes; 10-12 weeks old; 22.6±06) were brought in batches of 6 into the experimentation room for about 30 minutes before exposure to the assays. Before the commencement of every behavioural test and in-between consecutive tests, anxiety test apparatuses were cleaned with cotton wool soaked in 70 % ethanol and allowed to dry sufficiently before use.

Trials on the test devices were initiated by gently dropping a mouse from a batch at the centre of the light-open portion of the LDM component of Triple test 1 apparatus with the animal facing away from the LDM's aperture (appendix I), at the junction of open and closed segments of the EZM component of Triple test 2 apparatus (Appendix II) with the animal facing an open segment, at the centre of the MBM component of Triple test 3 device (Appendix III) with the mouse facing away from the exit, at the centre of a single OFM, at the single EZM open/closed segments' junction with the animal facing an open segment, or at the centre of a single EPM with the animal facing an open arm.

The mice were allowed to freely explore the test devices for 20 minutes for all tests though data were captured for first 7 minutes for the single test and for 20 minutes for the Multitest apparatuses. Observations

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were recorded by digital cameras and video files were stored for subsequent data retrieval and interpretation. Additionally, number of marbles buried, and defecations and urinations made by the subjects were manually collated from the test devices in-between trials. A marble was deemed as buried by a mouse if it was wholly, or more than 3/5 of its vertical length, was submerged beneath the MBM's wood shavings-laden floor.

Tabulated behavioural data were subsequently subjected to descriptive statistical analysis by the use of 2.0 IBM SPSS Statistics. Results were expressed percentages or means ± S.E.M.

RESULTS

Forty-eight (48) different primary and derived behaviours were obtained on the triple test (EZM component, 22; LDM and MBM components each, 13) (Tables 1, 2, & 3) compared with 22, 18, and 18 obtained on the single EZMT, EPMT, and OFT, respectively (Tables 3, 4, & 5). Walking, rearing/assisted rearing, and sniffing were the most frequent behaviours exhibited by the experimental subjects across all tests. These behaviours together with chewing, stretch-attend postures (SAP), grooming, and defecation/urination constituted 80-90% of all observed behaviours in each single or multi test. Marble-burying behaviour (MBE) was least frequent behaviour at 0.1 marble buried per mouse and only afforded by Triple test's MBM. Head dip (HD) was afforded only by the triple tests, single EPM and EZM. Significantly, central platform activities and data present in the EPMT were absent in the EZMT and Triple test trials. In both single and Triple tests, the experimental mice exhibited preferences for protected over unprotected portions of test devices (Table 6). Mice spent 47.5±4.2% in the dark (covered) compartment (DC.LDM) and 16.1±2.0% in the light (open) compartment (LC.LDM) of the LDM, 20.6±2.1% in closed (walled) (CS) and 4.4±0.6% in the open (unwalled) (OS) segments of the EZM, and 11.1±1.9% in the central (open) (CZ) and 39.0±5.4% in the peripheral (near-wall) (PZ) zones of the MBM components of Triple tests. Similarly, in the single tests, they spent 84.0±1.3% in closed and 15.9±1.3% in open segments of the EZM, 59.6±2.4% in closed, 17.2±1.7% in open, and 23.3±2.5 in the neutral central portions of the EPM, and 81.6±2.0% in CZ and 18.4±2.0% in PZ of the OFM (Table 6). Lastly, experimental mice exhibited differential test time distribution and exploratory activities on the Triple tests according to the component from which trials were initiated (Figure 1). Mice initiated from the EZM (middle) component (Triple test 2) recorded a near even time and activity distribution of 38.4%, 33.6%, and 28% of the test duration spent in the Triple test's LDM, EZM, and MBM components, respectively, (Figure 1a) – with most of the animals reaching and exploring all the 3 components within 15 minutes of trial commencement. This pattern contrasts with Triple test 1 trial in which the mice spent about 92.7, 5, and 2.3% of the test duration on the LDM, EZM, and MBM components, respectively (Figure 1). Additionally, only in half of the trials did the mice reach the second (EZM) component and only in 2 trials did they get to the third (MBM) component from the initiating maze (Data unshown). Mice exposed to Triple test 3 trials also exhibited an uneven activity/time distribution – spending about 6.5, 9.5, and 84.0% of test durations on the LDM, EZM and MBM components of the composite anxiety test apparatus – with only 5 mice reaching the second (EZM) and 2 mice reaching the third (LDM) components from the starting MBM (Data unshown).

Table 1: Behavioural ethogram of the mouse in the LDM component of the Triple test

Behavioural parameter	Description
Walking (Wa)	The mouse walks freely about sometimes sniffing
Protected/Unprotected Rearing /Assisted Rearing (p-/u-Re/ARe)	The mouse sits erect within the peripheral/central zones of the LDM's light compartment on the rear limbs/tail with arms in the air/or against the wall
Light compartment of LDM re-entries (LC.LDMRs)	The mouse returns to the light compartment of the LDM after an initial exit

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Protected/Unprotected defecation/Urination (p-Def/Uri/u-Def/Uri)	The mouse passes discrete faecal and/or urine droppings on the floors of the light/dark compartments of the LDM
Distance travelled (D)	The total distance the mouse travels in the LDM in a trial
Immobile sniffing (IS)	The mouse stands erect immobile on its paws while sniffing
Protected/Unprotected Stretch-attend posture (p-SAP/u-SAP)	Number of SAP made with most of the mouse's body completely within the dark/light compartment of the LDM
Body/Face/Front/Hind paw grooming (BG/FaG/FPG/HPG)	The mouse combs the body/face/front/hind paws by the fast movement of its incissors or hands
Chewing (CH)	The mouse makes rapid horizontal jaw movements
Immobility (IM)	The mouse stands erect and fixed to a spot

Table 2: Behavioural ethogram of the mouse in both the single and Multitest EZM

Behavioural parameter	Description
Closed segment entries (CSE)	The mouse walks into the closed segment of the EZM
Open segment re-entries (OSR)	Mouse returns to an EZM open segment after the initial exit
Protected/Unprotected Chewing (p-CH/u-CH)	The mouse makes rapid horizontal jaw movements in closed/open segments
Unprotected/Protected stretch-attend posture (u-SAP/p-SAP)	The mouse stretches & keeps its trunk low to the floor of the apparatus within the closed/open segment,
Open/closed segment walking (OS-Wa/CS-Wa)	The mouse walks freely within the open/closed segments of the EZM
Protected/Unprotected head dip (u-HD/p-HD)	The mouse dips its head below an open segment floor with its body or the hind limbs within/outside the closed segments
Protected/Unprotected Immobile Sniffing (u-ImSn/p-ImSn)	The mouse sniffs its environment standing within a closed segment/standing within the open segment
Unprotected/Protected Rearing/assisted Rearing (u-/p-Re/ARe)	Mouse maintains an erect/vertical posture wholly within a closed segment on the hindlimbs/tail with forearms freely in the air/held against a wall of the closed/open segment
Unprotected/Protected Defecation (u-Def/Ur./p-Def/Uri.)	The mouse produces faecal/urine excrements on the open/closed segments
Unprotected/Protected Body/Face/Front/Hind/Paw Grooming (u-/p-BG/FG/FPG/HPG)	The mouse combs the body/face/front/hind paws by the fast movement of its incisors/ hands in the open/closed segments
Unprotected/ Protected Immobility (u-Imm/p-Imm)	The mouse maintains an immobile posture in the EZM open segments
Percent open segment time (%OST)	Fraction of test duration spent by mice in EZM open segments
Closed segment entries/re-entries (CSEs/CSREs)	The mouse enters/re-enters EZM closed segments
Open segment re-entries (OSREs)	The mouse re-enters EZM open segments after the initial exit

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Table 3: Behavioural ethogram of the mouse in the MBM component of the Triple test

Behavioural parameter	Description
Protected/Unprotected Immobile Sniffing (u-ImSn/p-ImSn)	The mouse sniffs its environment standing within the peripheral/central portions of MBM
Protected/Unprotected Rearing/assisted Rearing/Cl (p-Re/ARe/u-Re/ARe)	The mouse stands/sits on the hind paws/tail with forearms hanging freely in the air/held against a wall of/attempts to climb the walls of the maze
Marble burying event (MBE)	A marble is said to have been buried when it is completely submerged in, or 3/5 of it vertically submerged below the height of, the 2-cm thick wood shavings in the MBM floor.
MBM re-entries (MBMRs)	Total returns to the MBM after an initial exit
Peripheral/central zone Walking (pz-Wa/cz-Wa)	The mouse walks around within the peripheral/central portions of the wood shavings-covered floor of the MBM
Body/Face/Front/Hind Paw grooming (BG/FG/FPG/HPG)	The mouse combs the body/face/front/hind paws by the fast movement of its incissors or hands
Protected/Unprotected Chewing (p-CH/u-CH)	The mouse makes rapid horizontal jaw movements in the peripheral/central portions of the MBM floor
Percent marble-burying maze peripheral/central zone time (%MBT-PZT/%MBT-CZT)	Fraction of the MBMT test duration time spent in the peripheral/central zones of the maze

Table 4: Behavioural ethogram of the mouse in the single Elevated Plus Maze (EPM)

Behavioural parameter	Description
Percent open arm time (%OAT)	Percent of test time spent in the open arms of the EPM
Open/closed segment re-entries (OARs/CARs)	The mouse returns to the open/closed segments after an initialexit
Open/closed segment walking (OA-Wa/CA-Wa)	The mouse walks around the EPM open/closed segmentssniffing or not sniffing its environment
Protected/Unprotected Rearing/assistedRearing (p-Re/Are/u-Re/ARe)	The mouse stands erect/vertical on its hind paws and/or sits onthe hind paws/tail with forearms hanging freely in the air/heldagainst a wall of the maze
Unprotected/ Protected Sniffing events (u-Sni/p-Sni & or cp-Sni)	A sniffing event occurs when the mouse sniffs an object inEPM open/closed segments & or central platform
Unprotected/Protected Immobility (u-Im/p-Imm & or cp-Imm)	The mouse assumes/maintains a fixed posture in open/closedarms or central platform
Distance travelled (D)	Total distance the mouse travels in the maze during the test
Body/Face/Front/Hind paw grooming(BG/FG/FPG/HPG)	The mouse combs the body/face/front/hind paws by the fastmovement of its incissors or hands
Protected/Unprotected Chewing (p-CH/u-CH & or cp-CH)	The mouse makes rapid horizontal jaw movements inclosed/open arms and/or central platform
Unprotected/ Protected Stretch-AttendPosture (SAP)	Mouse stretches & keeps trunk close to the floor of EPMopen/closed segments and/or central platform

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Protected/Unprotected Defecation/Urination (p-/u- Def/Ur.)	The mouse produces faecal & urinary excrements on the EPM's closed/open segments

Table 5: Behavioural ethogram of the mouse in the single Open Field Maze (OFM)

Behavioural parameter	Description
Percent centre zone time (%CZT)	Percentage of the test time spent in the OFM central zone.
Centre zone re-entries	The mouse returns to the OFM central zone after an initial exit
Peripheral/central zone walking (pz-Wa/cz-Wa)	The mouse walks around the OFM sniffing or not sniffing within OFM peripheral/central zones
Protected/Unprotected Rearing/assisted Rearing/Cl (p-Re/Are/Cl/u- Re/Are/Cl)	The mouse stands erect/vertical on its hind paws and/or sits on the hind paws/tail with forearms hanging freely in the air/held against a wall of the maze in OFM peripheral/central zones
Unprotected/ Protected Sniffing events (u-Sni/p-Sni & or cp-Sni)	A sniffing event occurs when the mouse sniffs an object in OFM peripheral/central zones
Distance travelled (D)	The total distance the mouse travels in within the maze
Body/Front/Hind paw grooming	The mouse combs the body/face/front/hind paws by the fast movement of its incissors or hands
Protected/Unprotected Chewing (p-CH/u-CH)	The mouse makes rapid horizontal jaw movements in OFM peripheral/central zones
Unprotected/Protected Immobility (u-Im/p-Imm & or cp-Imm)	The mouse assumes/maintains a fixed posture in OFM peripheral/central zones
Unprotected/ Protected Stretch-Attend Posture (SAP)	Mouse stretches and keeps trunk close to the floor of the OFM peripheral/central zones
Protected/Unprotected Defecation/Urination (p-/u-Def/Ur.)	The mouse produces faecal & urinary excrements on the floor of OFM's peripheral/central zones

Table 6: Percent Time Distribution of Experimental Mice in the Unprotected and Protected Portions of the Test Apparatuses

Test apparatus		% Time in unprotected portions	% Time in protected portions	% Time in neutral central platform
Multitest	LDM	16.1±2.0	47.5±4.2	Absent
	EZM	4.4±0.6	20.6±2.1	Absent
	MBM	11.1±1.9	39.0±5.4	Absent
Single EZM		15.9±1.3	84.0±1.3	Absent
Single EPM		17.2±1.7	59.6±2.4	23.3±2.5
Single OFM		18.4±2.0	81.6±2.0	Absent

LDM, light-dark maze; EZM, elevated zero maze; MBM, marble-burying maze; EPM, elevated plus maze; OFM, open field maze.

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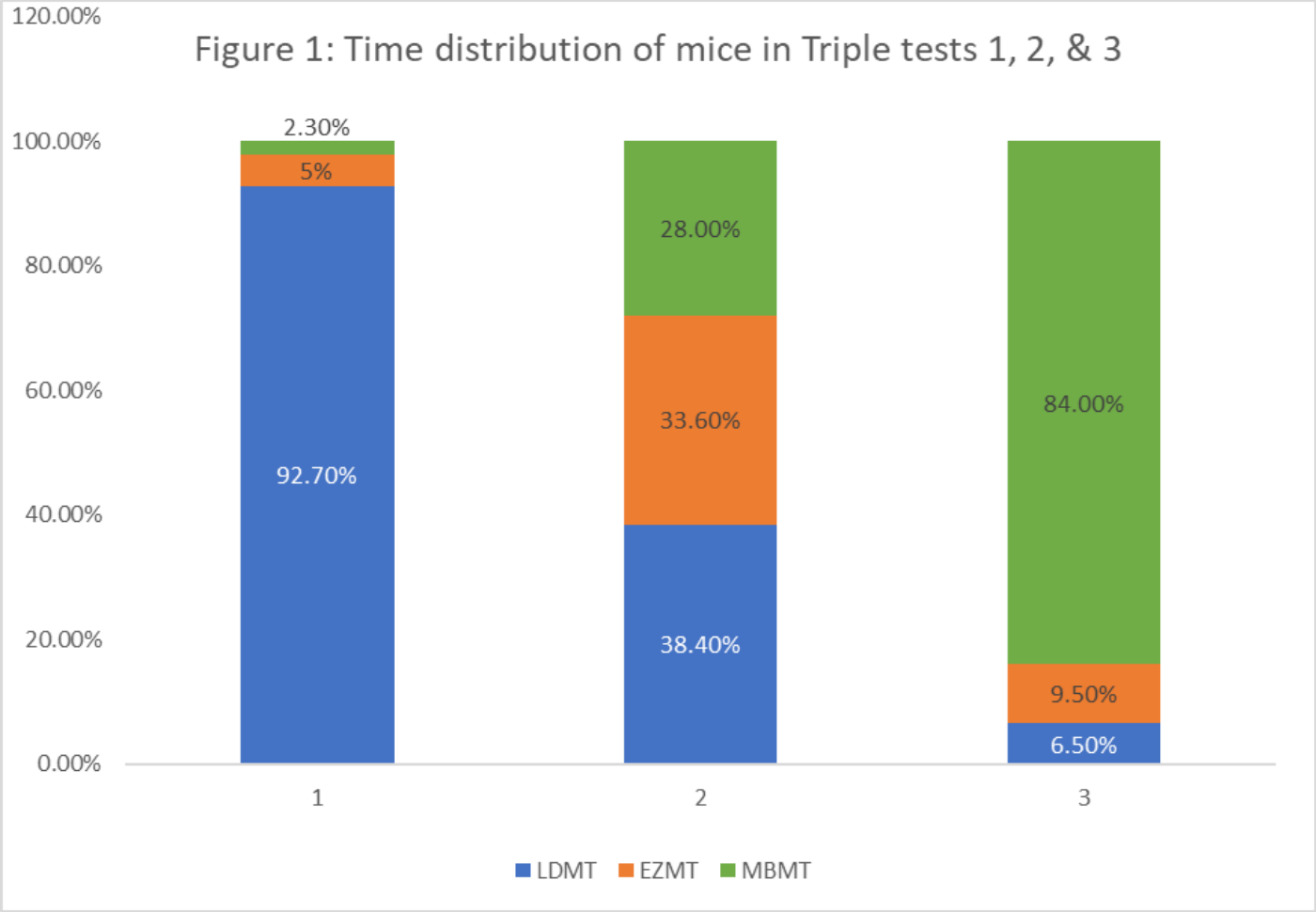

1, 2, & 3, Triple tests in which mouse trials were initiated from LDM, EZM, & MBM, respectively.

LDMT, time spent in the light-dark maze component of the triple test apparatus.

EZMT, time spent in the elevated zero maze component of the triple test apparatus.

MBMT, time spent in the marble-burying maze component of the triple test apparatus.

DISCUSSION

The outcome of this study indicates our triple test is capable of engendering and detecting close to fifty different primary and secondary anxiety behaviours in the experimental mice exposed to it (Tables 1-5). Like the triple rodent anxiety test by Ramos et al., 2008 (11) after which the current test device is fashioned, the rich behavioural repertoire observed on this novel Multitest which is more than double that generated by any of the standard single murine anxiety tests in the same trials is a justification for its invention (Tables 1-5). This is because the broad natural murine activity spectra generated by treatments-/assays-naïve mice on the novel Multitest, under the current investigation, can be aggregated into specific behavioural patterns to both characterize the new anxiety Multitest itself and to serve as our Departmental/Laboratory data benchmark for future behavioural studies/trials. Again, the two triple murine anxiety tests share similar

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potential attributes of environmental friendliness, operational simplicity and cost-effectiveness, and overall improved translationality. However, this study may the first report to characterize a triple mouse anxiety test made up of an LDM, EZM, and MBM deployed in preclinical anxiety research. The double substitution of EPM and OFM, respectively, in the Triple test by Ramos by EZM and MBM in our invention, will have the potential of broadening its anxiety-related senstivity as well as engender reproducibility of behavioural outputs due to the elimination of ambiguous data from the EPM's central platform in our Triple test. Our finding of mouse marble-burying activities, observed only on some of the new Triple test trials, in addition to other anxiety traits – including protected space preferences – detected alike by both single and Triple tests indicates our innovation may be sensitive to murine anxiety, compulsive-obsessive and panicogenic behaviours.

A curious finding of ours is the variation in the exploratory activities of the mouse groups depending on the constituent mazes from which their trials were initiated. Mice initiated from the middle (EZM) of the Triple mouse anxiety apparatus (Triple test 2) (Appendix II) exhibited the ambulatory and exploratory activities – reaching the remaining two components within 15 minutes of trial commencements and having repeated voyages while the trials lasted. This contrasts sharply with the mouse groups initiated from the LDM (Triple test 1) (Appendix I) and MBM (Triple test 3) (Appendix III) components, respectively, which exhibited limited ambulation and exploration with less than half of them neither venturing out of their starting mazes nor reaching out to the other components of the Triple test apparatus. In our view, the 'potent protection' offered by the dark (opaque) compartment of the light-dark maze (LDM) – the initiating maze in Triple test 1 unto this group of mice might be responsible for their comparatively low ambulatory/exploratory activities as the perceived 'safety' of this covered and darkened environment might make them feel so much 'safe' there and so much 'vulnerable' elsewhere. But for the Triple test 3 mice, their reluctance to explore and ambulate is thought to be due to the physical semblance between the floor wood shavings of their home cages and the MBM (the trials-initiating maze for Triple test 3 mice). Thus, this group of mice might just simply feel 'safe' and 'at home,' and not feel the need to venture anything outside of the 'homely' initiating maze. However, if these explanations would account for the restricted locomotory/exploratory activities of these two groups of mice, why did the highlighted factors of LDM and MBM not have similar stuck on effect on the mice in Triple test 2, even after repeated exposure? Could the maiden assay experience of Triple test 2 mice on EZM component of triple test apparatus have an anxiolytic effect on them, thus allaying their anxiety in both LDM and MBM components? This and more questions, perhaps, will be answered by controlled repeated daily trials of groups of mice on the same sets of triple sets to detect any temporal variation from the data garnered from the 1st sets of trials on the same novel composite murine test device.

There are some limitations to this study. One, most of the behavioural data were manually captured; a more automated data harvesting would limit bias from human errors. Secondly, data loss on the activities of the experimental subjects within the opaque (non-transparent) dark compartment of the LDM could be minimized if the Triple test apparatus could be equipped with a laser beam technology that would enable visualization even in the dark environment.

In conclusion, this novel triple test comprising of an LDM, EZM, and MBM is sensitive to and capable of inducing broad anxiety-related spectra, including panicogenic and compulsive-obsessive behaviours in mice. Secondly, based on the optimal ambulatory/exploratory indices recorded on the Triple 2 test, it is hereby proposed that acute one-off trials should be initiated from the EZM component of the Triple test apparatus. Finally, there may be need to determine the anxiety-related behaviours of treated and untreated mice subjected to daily repeated exposure to this novel Triple test.

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APPENDIX I

Triple mouse anxiety test 1: each trial was initiated from the LDM component of the Multitest apparatus

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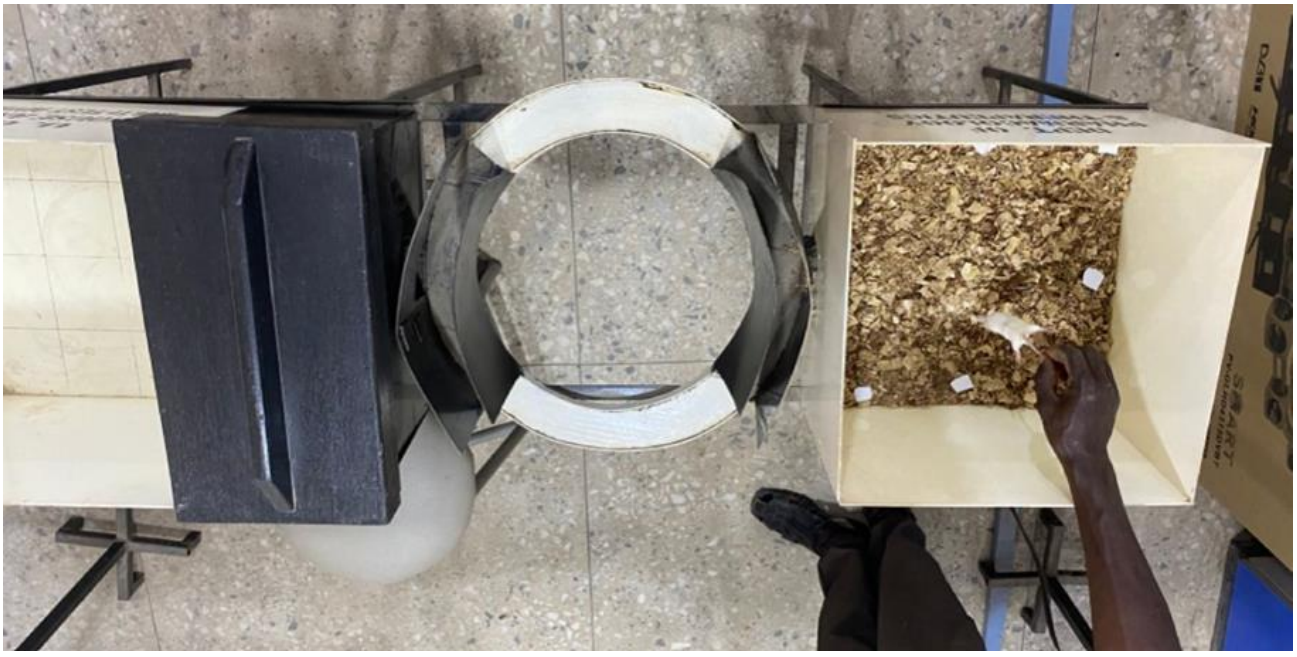
APPENDIX II

Triple mouse anxiety test 2: each trial was initiated from the EZM component of the Multitest apparatus

APPENDIX III



Triple mouse anxiety test 3: each trial was initiated from the MBM component of the Multitest apparatus