Habituation time and other standardisation parameters for select behavioural tests in Flinders Sensitive Line rats

JE Pienaar
orcid.org/ 0000-0002-1254-633X

Dissertation submitted in fulfilment of the requirements for the degree Master of Science in Pharmacology at the North West University

Supervisor: Prof CB Brink
Co-supervisor: Prof L Brand
Assistant supervisor: Dr SF Steyn
Assistant supervisor: Dr MR Lekhooa

Graduation: July 2019
Student number: 24160970
SOLEMN DECLARATION

I, Johanna Elizabeth Pienaar, declare herewith that the dissertation entitled,

**Habituation time and other standardisation parameters for select behavioural tests in Flinders Sensitive Line rats**

which I herewith submit to the North-West University, Potchefstroom Campus, in partial fulfilment of the requirements for the degree *Magister Scientiae* in Pharmacology, is my own work and has not already been submitted to any other university.

I understand and accept that the copies that are submitted for examination are the property of the University.

Me JE Pienaar
Student

As supervisors, Prof CB Brink, and Prof L Brand, we confirm that the above statement by Me JE Pienaar is true and correct.

Prof CB Brink
Supervisor

Prof L Brand
Co-supervisor
ABSTRACT

With the aim of refining experimental procedures for animal behavioural tests commonly performed in our laboratory, this study aimed to provide empiric-evidence for specific aspects of the methodological approach of select behavioural tests and to provide direction for future research in this laboratory which may contribute to also facilitate more robust between-study comparisons. As such, the present investigation had two main objectives, viz. A) to study the effect of pre-test habituation on the performance of test subjects in a sequence of cognitive and behavioural tests performed on a single dark cycle in the same test subjects, i.e. 1) the novel object recognition test (nORT; declarative memory), the open field test (OFT; locomotor activity) and the forced swim test (FST; behavioural despair), and B) to measure how habituation will influence the treatment response of FRL and FSL rats subjected to the procedures highlighted in A to a positive control, i.e. imipramine, 10 mg/kg/day x 14 days. A secondary objective was to study the influence of pre-test habituation on the performance of test subjects if the same tests as applied in (A) were to be performed only one per day on successive days, albeit also in both treatment conditions. In this regard, for objective A, male FSL (n = 48) and FRL (n = 48) rats were employed, divided into the following groups (n = 12 per group): a) treatment naive (saline-treated) FRL rats, b) imipramine-treated FRL rats, c) treatment naive (saline-treated) FSL rats, and d) imipramine-treated FSL animals. Due to the fact that the secondary objectives could not have been completed, neither the outline for this objective, nor its results will be outlined here (see Annexure B).

In the nORT, declarative memory was not affected by the two different pre-test habituation protocols, indicating that the pre-test emotional state of the animal does not significantly alter their inherent, non-manipulated cognitive performance as assessed in terms of declarative memory. With regards to OFT behaviour, alterations in locomotor activity was only apparent after 60-min pre-test habituation, resulting in FSL rats covering less distance compared to their FRL counterparts. Non-habituated FRL and FSL rats covered similar distances during the 5-min test session. In the FST, male FSL rats displayed increased depressive-like behaviour and decreased escape-related behaviour (irrespective of pre-test habituation time) compared to FRL rats, underlining the face validity of the FSL model. Both strains displayed increased depressive-like behaviour when pre-test habituation was negated, with no significant alterations in active behaviours.

IMI-treated FSL rats displayed comparable cognitive and depressive-like behaviour to SAL treated counterparts in the nORT and FST, respectively. Further, IMI-treated FRL rats also displayed comparable cognitive function compared to SAL treated cohorts. However, IMI-treated
FRL rats presented with exaggerated depressive-like behaviour in the FST compared to SAL receiving FRL controls.

In conclusion, we have shown that the cognitive performance of both FRL and FSL rats in the nORT are robust enough to withstand varying pre-test circumstances, despite alterations in locomotion after 60-min pre-test habituation. Further, depressive-like behaviour is bolstered in both strains when tested directly after relocation, without significantly affecting active behaviours. Therefore, collectively viewed, we argue that in order not to misinterpret the behaviour of FSL animals in the FST based on findings from the OFT, albeit falsely so, both FRL and FSL animals should be subjected to both the OFT and the FST without prior habituation. Due to the confounding results from IMI receiving cohorts, the predictive validity of this model could not be re-affirmed. However, as our data contradicted the majority of previous reports, it is unlikely that these findings were borne from inherent confounds in the model. Nonetheless, valid conclusions can still be made based on the robust baseline face validity of the FSL model that has been affirmed in the present work.

**Key words**

Finders Sensitive Line (FSL), Flinders Resistant Line (FRL), major depressive disorder (MDD), pre-test habituation, forced swim test (FST), open field test (OFT), novel object recognition test (nORT)
CONGRESS PROCEEDINGS

Excerpts from this study were presented as follows (presenting author underlined):

Pre-test habituation time for select behavioural tests in rats

JE Pienaar, L Brand, SF Steyn, CB Brink

The results were presented as a podium presentation for the Young Pharmacologist competition of the First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018.

The abstract presented at the congress along with the certificate of attendance can be found in Annexure C at the end of the dissertation.
ACKNOWLEDGEMENTS

Dankbetuig

"Ek het tot die insig gekom dat daar vir 'n mens niks beter is nie as om vrolik te wees en die goeie van die lewe te geniet. Dat die mens kan eet en drink en onder al sy arbeid nog die goeie kan geniet, ook dit is 'n gawe van God."

Prediker 3:12-13

Alle eer kom die Here toe, wie my oorlaai met die goeie - sonder Hom is al die swoeg en sweet tevergeefs.

"It is not my ability, but my response to God’s ability, that counts".

Corrie ten Boom

Graag bedank ek sleutelpersone wat aandeel aan die projek het. Dankie aan elke navorser en akademikus wat insette gelewer het in die projek. Dit was 'n voorreg om deel te kon wees van so 'n dinamiese groep navorsers met soveel passie vir hulle werk. 'n Spesiale dankie ook aan Prof Brian Harvey vir die befondsing wat hierdie projek moontlik gemaak het. Antoinette, Kobus, en Cor, vir die versorging van die proefdiere, asook julle hulp, kundigheid en vriendelikheid. Prof Suria Ellis, by wie ek sommer baie informeel kon gaan aanklop vir hulp met die statistiese en krag-analise.

'n Nagraadse studie kan nie sonder 'n baie groot ondersteuningsnetwerk plaasvind nie. Spesifiek sonder ek my ouers (Anette, Johnnie en Kobus) uit. Dankie vir julle liefde, ondersteuning, en wanneer dit nodig was, hard moed in praat. Aan die res van my familie sê ek ook graag dankie vir julle belangstelling en ondersteuning. Ek is lief vir julle. Al my vriende (veral Momo, Elzette, Kenneth, William, my huismaat Jeanne, en Tannie Urs), dankie vir julle geduld en verstaan as ons nie by mekaar kon uitkom nie en ek julle bietjie afgeskeep het. Dankie vir julle vriendskap word innig waardeer. Spesial dankie aan Prof Linda, die matriarg van die gang, onder wie se leierskap niks Prof se aandag ontgel nie. Prof se streng tog sagmoedige leierskap het 'n verreikende invloed op my lewe..< My medenagraadse studente, Arina, Isma, Nadia, Juandré, Geoffrey, Khulekani, Mandi, Ané, Cailin, Johané, Heslie en Carmen. Dankie vir julle hulp ten spyte van besige programme en stres. Met al ons uiteenlopende persoonlikhede, het ek nog steeds vriendskap en kameraadskap by julle elkeen gevind. Dankie vir elke kuiers, veral dat ons so baie kon saam eet en lag! Dankie ook aan elkeen van julle wat die nagraadse kantoor gevul het met vriendelikheid en 'n gemaklike atmosfeer. Julie is na aan my hart.
Gedurende hierdie twee jaar is ek ongelooflik geseën met mense oor my pad wat diep spore trap. Laurika Rauch sing, “Die mense wat ek lief het kom groei op my soos mos...”. Dankie dat ek julle as my moskombersie kan saam dra.

❖ Dr Stephan Steyn en Dr De Wet Wolmarans.

“If your actions inspire others to dream more, do more, and become more, you are a leader”.

*John Quincy Adams*

“Influence is having people follow you because of what you represent”.

*Paul Larsen*

Julle wese getuig van gravitas, julle vrese net vir Hom, julle raad altyd van toepassing en julle inspraak monumentaal. ‘n Spesiale dankie vir julle tyd, moeite en menswees wat julle met my gedeel het, waarby ek kon leer en wat my inspireer.


Vir Arina

(17/01/1993 – 03/02/2019)

“I keenly feel how your life has forever changed the arc of my own...”


Hierdie verhandeling dra ek op aan jou in liefdevolle herinnering, want niks kon jou onderkry nie.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of co-variance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARRIVE</td>
<td>Animal Research: Reporting of In-Vivo Experiments</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropyl fluorophosphate</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>F344</td>
<td>Fischer rat</td>
</tr>
<tr>
<td>FRL</td>
<td>Flinders Resistant line</td>
</tr>
<tr>
<td>FSL</td>
<td>Flinders sensitive line</td>
</tr>
<tr>
<td>FST</td>
<td>Forced swim test</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
<td>α&lt;sub&gt;1&lt;/sub&gt; gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>GLP</td>
<td>Good laboratory practice</td>
</tr>
<tr>
<td>IMI</td>
<td>Imipramine</td>
</tr>
<tr>
<td>LEW</td>
<td>Lewis rat</td>
</tr>
<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>MD</td>
<td>Major Depression</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>nORT</td>
<td>Novel object recognition test</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive compulsive disorder</td>
</tr>
<tr>
<td>OFT</td>
<td>Open field test</td>
</tr>
<tr>
<td>PND</td>
<td>Post-natal day</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
</tr>
<tr>
<td>SAL</td>
<td>Saline</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneous-hypertensive rats</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin re-uptake inhibitor</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS

SOLEMN DECLARATION ................................................................................ II
ABSTRACT ....................................................................................................... IV
CONGRESS PROCEEDINGS ........................................................................... VI
ACKNOWLEDGEMENTS ............................................................................... VII
LIST OF ABBREVIATIONS ........................................................................ IX

CHAPTER 1: INTRODUCTION...................................................................... 16
1.1 Dissertation approach and layout ......................................................... 16
1.2 Problem statement ................................................................................. 17
1.3 Study hypothesis and objectives ......................................................... 21
1.4 Project layout ......................................................................................... 22
1.5 Compliance and ethical approval ......................................................... 23

CHAPTER 2: LITERATURE BACKGROUND .............................................. 24
2.1 Translational animal models in neuropsychiatric disorders ................. 24
2.2 Validity criteria for animal models ..................................................... 24
2.2.1 External Validity ............................................................................... 25
   Face validity ............................................................................................ 25
   Construct validity .................................................................................... 26
   Predictive validity .................................................................................. 28
2.2.2 Internal Validity ............................................................................... 29
   Reproducibility ..................................................................................... 29
   Standardisation ...................................................................................... 32
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.6</td>
<td>Discussion</td>
<td>60</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Conclusion</td>
<td>64</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Acknowledgements</td>
<td>64</td>
</tr>
<tr>
<td>3.2.9</td>
<td>References</td>
<td>65</td>
</tr>
<tr>
<td>CHAPTER 4: SUMMARY, CONCLUSION AND RECOMMENDATIONS</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>4.1</td>
<td>Summary and conclusion</td>
<td>72</td>
</tr>
<tr>
<td>4.2</td>
<td>Limitations and future directions</td>
<td>76</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>ANNEXURE A: ADDITIONAL RESULTS</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>ANNEXURE B: ADDITIONAL WORK BY THE CANDIDATE</td>
<td></td>
<td>109</td>
</tr>
<tr>
<td>ANNEXURE C: CONGRESS ABSTRACT AND CERTIFICATE</td>
<td></td>
<td>115</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2-1: Summary of possible confounding variables affecting test performance in tests of memory and cognition. Adapted from Paul et al. (2009). .......................... 37

Table 2-2: Brief listing of the most influential environmental factors for laboratory animals investigated over the past 50 years. Adapted from Izidio et al. (2005). .......................................................................................................................... 39

Table 2-3: Summary of 50 randomly selected studies from 2007 to 2017 displaying the habituation time employed before behavioural testing. FST, forced swim test; OFT, open field test; NORT, novel object recognition; EPM, elevated plus maze. ........................................................................................................ 42

Table 4-1: Summary of the treatment-naive results obtained for declarative memory (nORT), locomotion (OFT) and depressive-like behaviour (FST) in FRL and FSL rats that were either not habituated prior to testing (0 min) or habituated for 60 min before the onset of behavioural assessment (Chapter 3). ↔: no significant difference, ↑: significantly increased, ↓: significantly decreased, compared to the indicated control............................... 74
LIST OF FIGURES

Figure 1-1: Schematic layout of the primary objective. SAL: saline. IMI: imipramine. FRL: Flinders Resistant Line. FSL: Flinders Sensitive Line. nORT: novel object recognition test. OFT: open field test. FST: forced swim test. PND: post-natal day. ................................................................. 22

Figure 1-2: Floor plan of the GLP (good laboratory practice) area in which the experiments were conducted. Home cages were transported from the holding room to the testing room for the nORT and the OFT. After assessment in the OFT, the cages were transported to the adjacent FST room. nORT: novel object recognition test. OFT: open field test. FST: forced swim test. ................................................................. 23

Figure 2-1: A survey of 1,576 researchers on the irreproducibility of research findings gathered from an online questionnaire. The investigation revealed that more than 60% of researchers attribute irreproducibility of research findings to either pressure to publish or selective reporting (Baker, 2016). ................................................................. 31

Figure 1: Discrimination index of novel object exploration in FRL and FSL rats after 0-min or 60-min of pre-test habituation. n = 12 for all groups. Data represent mean ± SEM. 0-min: no pre-test habituation. 60-min: pre-test habituation of 60 min. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat. ................................................................. 57

Figure 2: The mean distance travelled in an open field by FRL and FSL rats after 0-min or 60-min of pre-test habituation. n = 12 for all groups. Data represent mean ± SEM. Statistical descriptors are reported in the text, with ** p ≤ 0.001. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat. ................................................................. 58
Figure 3: The effects of pre-test habituation on the FST behaviour of FRL and FSL rats. (A) time spent immobile; (B) time spent struggling\textsuperscript{a} and (C) time spent swimming after 0-min or 60-min of pre-test habituation. Data represent the mean ± SEM. Statistical descriptors are reported in the text. **** $p \leq 0.0001$. \textsuperscript{a}Outlier identified for FSL rats at 0-min pre-test habituation time but included in analysis. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat.
CHAPTER 1: INTRODUCTION

1.1 Dissertation approach and layout

This dissertation is presented in the article format for submission according to the postgraduate regulations approved by North-West University. Included in this format is:

- **Chapter 1** as an introductory chapter that provide the problem statement, hypothesis and research questions;

- **Chapter 2** containing a relevant literature overview;

- **Chapter 3** in which the key data is prepared as a concept article, as for submission to a peer-reviewed scientific journal;

- **Chapter 4** concludes the study findings with future recommendations.

- **Annexure A** contains data relevant to Chapter 3, but that could not, for the reasons explained, be included in the main manuscript.

- **Annexure B** contains work that formed part of the original investigation, but that could not have been completed by the time this dissertation has been submitted. Although included for the benefit of the reader, *Addendum B cannot be submitted for examination purposes*, given that the lack of all the relevant control and necessary comparative groups, are not included.

- Excerpts from the study were presented as a podium presentation (see Congress Proceedings). The abstract presented at the congress can be found in Annexure C, along with the certificate of attendance.
1.2 Problem statement

Valid translational animal models are needed to effectively study complex neuropsychiatric disorders such as major depressive disorder (MDD) in our endeavours to improve current pharmacological treatment strategies (Nestler & Hyman, 2010). Limitations to effectively model complex, uniquely human disorders of the brain hamper progress in pre-clinical animal research as it is often difficult to model symptomology such as e.g. sadness, guilt, suicidal ideation etc. (Nestler et al., 2002; Nestler & Hyman, 2010), or in the event that specific behavioural and neurobiological trait markers of the human condition can be measured in animals, assays are often inadequate or executed poorly (Fonio et al., 2012). Nevertheless, some success of animal models has been achieved when face-, predictive and construct validity for particular disease and treatments have been demonstrated (Fernando & Robbins, 2011; Nestler & Hyman, 2010). Still, recent focus on the lack of reproducibility in pre-clinical research seems to confirm the shortage of robust data attained from these animal models (Baker, 2016), mandating a re-evaluation of our current thinking and understanding of behavioural assaying in animal models. Initiatives such as Reproducibility 2020 prompted the global research community to pay attention to confounding factors in study design and laboratory protocols, in an attempt to improve credibility and reliability of pre-clinical findings (Freedman et al., 2017).

One of the factors that may affect performance in behavioural testing is the physiological and psychological state of animals, particularly following transport from their home cage environment to a testing room, even when transport is done between rooms in close proximity (e.g. across the hall) in the same facility, with little general disturbance, and with similar environmental condition regarding temperature, pressure, humidity, light and room interior finishing. In such instances habituation to the new environment is believed to reduce the effect of the transport and a new environment (see more in par. 2.7 in Chapter 2). Since we often perform a battery of tests per animal in one session, it may be important to understand whether, under our experimental conditions, habituation is important at all, and whether it may be possible to save time by totally omitting it.

The Flinders Sensitive Line (FSL) rat and its genetic control, the Flinders Resistant Line (FRL) rat, is a model commonly applied in MDD research (Overstreet, 1993; Overstreet et al., 2005), and in studies investigating treatment resistant depression (Brand & Harvey, 2017a; Brand & Harvey, 2017b). In our laboratory, it is therefore of particular importance to understand the effect of habituation time, and the need, or not, of habituation at all under our experimental conditions. FSL rats resemble the classic picture of depressed individuals in several respects as defined by the three most commonly applied validation criteria, i.e. face-, construct and predictive validity. The most prominent behavioural feature of the FSL rat is the exaggerated floating behaviour displayed in the forced swim test (FST), with just enough movement to keep the head above the
water, which is believed to mimic the behavioural despair often observed in clinical depression. When faced with the inescapability of the water-filled cylinder of the FST, FSL rats spontaneously adopt a characteristic immobile posture reminiscent of a depressive-like state (Overstreet, 1986; Overstreet, 2002; Overstreet et al., 1986) more readily and to a greater extent, compared to FRL animals. Furthermore, chronic, but not acute antidepressant treatment attenuates this depressive-like state in FSL rats (Castagne et al., 2010; Overstreet et al., 1995), more closely resembling the human scenario. The FST may be regarded as a highly stressful test (Connor et al., 1997), so that one may expect that the relative and potential effect of the pre-test conditions e.g. transportation and a novel environment, may not significantly affect the behaviour in this test.

When tested in an open field test (OFT), FSL rats show alterations in locomotor activity only after foot shock stress, indicating that at baseline these rats display comparable mobility with their FRL counterparts (Overstreet et al., 1986). Also, FSL rats seemingly model MDD without comorbid anxiety, as evinced by low levels of anxiety at a pre-pubertal age in the elevated plus maze (EPM) (Braw et al., 2006), which subsequently adjusts to a level akin to that observed in the FRL control group by adulthood. However, FSL rats present with anxiogenic behaviour in other tests of anxiety more resembling of complex behavioural and cognitive ability, e.g. the social interaction test and active avoidance test (Overstreet et al., 2004; Overstreet et al., 1990). Further, the broad cognitive function of the FSL rat appears to be comparable to that of the FRL control (Bushnell et al., 1995; Russell et al., 1982); however, recent literature identified deficits in emotional (Eriksson et al., 2012) and declarative memory (du Jardin et al., 2016; Gomez-Galan et al., 2016) in the novel object recognition test (nORT) in comparison with FRL rats, albeit after manipulation of their behaviour.

Valid animal models in neuropsychiatric research are not only reliant on appropriate species selection, but also on adequate behavioural tests (Homberg, 2013; Sousa et al., 2006). In the current investigation, we will afford attention to a number of cognitive and behavioural tests as they are commonly applied in our laboratory, i.e. the nORT, OFT and FST. The nORT is applied in rodent models as a test of working memory, attention and recognition (Goulart et al., 2010; Silvers et al., 2007). Normally, it assesses an animal's natural exploratory instinct without the animal being subjected to extra positive or negative reinforcement protocols or stress (Baxter, 2010). Control rats will theoretically spend more time investigating the novel object in the presence of a familiar object as recognition of novelty requires more cognitive skill compared to a test measuring only exploration of novel environments or objects (Silvers et al., 2007). That said, stress (such as induced by transportation and novel environments) may potentially have a significant confounding influence on memory performance (Conrad et al., 2004; Dominique et al., 2000; Roozendaal, 2002; Wolf, 2003). Nevertheless, this needs to be assessed.
In the OFT, the natural fear of a rodent for open spaces is utilised to test anxiety when these animals are forced into a large, open arena. Anxiety may also be triggered by isolating the rat from its social group during the test and not habituating the animal to such circumstances prior to the execution of the test (Prut & Belzung, 2003). Under such circumstances, rodents are regarded as less fearful (less anxious) when they spend increased time in the centre of the arena compared to preferring the periphery (Royce, 1977). The OFT is also employed to measure behaviour other than anxiety, including sedation or activity—the context of its application here—as most of the behaviours measured in the OFT are easily quantified and present with good face validity (Walsh & Cummins, 1976). However, locomotion may be influenced by several factors e.g. exploratory drive, motor output, and fear-related behaviour, which in turn is affected by experimental design and the pre-test environment (Rodgers, 2007).

The fundamental construct of the FST is based on the characteristic immobile posture a rat adopts after realizing the inescapability from the water-filled cylinder, anthropomorphically viewed as behavioural despair. Chronic, but not acute, administration of antidepressants has been shown to decrease the time an animal spends in this immobile posture (Castagne et al., 2010). Following its initial description, validation and characterization, the test was further refined by Detke et al. (1995) to distinguish between different active behaviours and their underlying neuropathophysiology. Changes in ‘climbing’ behaviour was found to be associated with noradrenergic mechanisms, whereas changes in ‘swimming’ behaviour was associated with serotonergic mechanisms (Cryan & Lucki, 2000; Detke et al., 1995; Hemby et al., 1997). Despite the FST being a significant stressor itself (Connor et al., 1997), several factors may influence behaviour in the test (Bogdanova et al., 2013). This is not surprising as certain stressors have been shown to increase the occurrence of depressive episodes (Wager-Smith & Markou, 2011) and possibly influence behaviour in the FST as well.

This being said, pre-clinical neuropsychiatric research is dependent on and limited to investigations of animal behaviour expressed in paradigms that at most suffice to provide an indirect perspective on the emotional state or cognitive ability of an animal (Ramos, 2008), i.e. interactions with non-reactive objects, location-specific movements in an open field arena, or forced exposure in a swim chamber. Therefore, with respect to the collective appraisal of both within- and between-laboratory findings, an important aspect of consideration is methodological congruence. In fact, while specific behavioural tests—in the case of the present work, the nORT, OFT and FST—are often applied to measure the same neuropsychiatric constructs, within- and between-laboratory results and conclusions can realistically only be compared and drawn if near analogous experimental conditions are applied. The studies of Tillmann and colleagues are the most recent example of between-study variation where both impairment (Tillmann et al., 2019) and improvement (Tillmann & Wegener, 2019) in object memory was reported in FSL rats when
similar test methodology was followed. These variations under similar test procedures suggest that external factors may play a significant contributing role to the incongruent results obtained between different investigations.

Data from literature points to the fact that test- and result reliability of stress-sensitive tests are influenced by seemingly minor laboratory procedures, with minor influences on stress-related biology regarded as the main causative factor (Balcombe et al., 2004). This notion was borne from reports of elevated endocrine, neurosympathetic and immunological measurements (Armario et al., 1986a; Armario et al., 1986b; Black et al., 1964; Dallmann et al., 2006; Gartner et al., 1980; Sharp et al., 2003; Sharp et al., 2002a; Sharp et al., 2002b; Tabata et al., 1998) after routine laboratory procedures (reviewed in Balcombe et al. (2004) and Castelhano-Carlos and Baumans (2009)). Therefore, it is suggested that routine laboratory procedures may be an important confounding factor to consider in any experimental design, especially in research pertaining to stress-sensitive neuropsychiatric disorders. It is however important to note the lack of behavioural investigations in this regard, as most, if not all, of the conclusions drawn from these studies are based on only physiological parameters without behavioural correlates.

Due to the time-consuming nature of neuropsychiatric experimental design in which several different behavioural tests are normally conducted singly on successive days, we have recently investigated the plausibility of conducting a sequence of tests on the same test subject on the same day (Mokoena et al., 2015). While results from this investigation indicated that test outcomes relating to said sequence are akin to that observed following the former approach, it is important to consider that this work did not address the contextual and indirect factors that may have influenced the pattern of findings reported. Therefore, considering the paucity of literature pertaining to behavioural test outcomes as they may be influenced by the pre-test environment, and to provide direction for future research in our laboratory, the current investigation is based on the following questions:

1. Will the behavioural test performance of FRL and FSL rats tested in a sequential battery of cognitive and behavioural assessments, i.e. the nORT, OFT and FST, differ as a function of pre-test habituation (Chapter 3)?
2. Will the effects of imipramine, a widely-applied positive control used for the treatment of depressive-like phenotypes, in the sequence of tests outlined in (1) be affected as a function of pre-test habituation (Annexure A)?
3. Does the effect of habituation differ when a sequence of tests is used as opposed to a single test exposure (Annexure B)?
Due to the lack of standardisation in terms of pre-test conditions that precede widely applied behavioural assessments in pre-clinical literature, the current study will explore the effect of two different pre-test habituation protocols on behavioural test outcomes in three cognitive and behavioural tests applied either in sequence on a single day, or as single tests on successive days, i.e. 1) the nORT, 2), the OFT and 3) the FST.

1.3 Study hypothesis and objectives

With respect to the present investigation in which the influence of pre-test habituation on test outcomes in a sequence of behavioural and cognitive tests as commonly applied in this laboratory is investigated, a stress sensitive, genetic animal model of depression, i.e. the FSL rat and its comparator control, the FRL rat will be used. Whether pre-test habituation will influence the results of behaviour in other animal models, may warrant further investigation.

The primary objective (A) of the current investigation is (1) to explore the effect of two different pre-test habituation protocols (0-min or 60-min) on behavioural test outcomes in a sequence of behavioural assessments as often applied in FRL and FSL rats, viz. 1) the nORT, 2), the OFT and 3) the FST and (2) to investigate how such behaviours are influenced by imipramine treatment (10 mg/kg/day, s.c.i).

In this study, it is hypothesised that behavioural testing outcomes will significantly differ between animals subjected to the different habituation protocols. In this regard and as this study will investigate treatment-naive, and non-manipulated behaviour, it is postulated that, in the absence of pre-test habituation (0-min), both FRL and FSL rats will present with impaired declarative memory in the nORT, reduced locomotion in the OFT and increased depressive-like behaviour in the FST. Our hypothesis is as such, as we expect transportation and handling immediately prior to behavioural testing to induce transient arousal and that such arousal may yield results akin to that of animals in a higher state of anxiety, compared to less-anxious controls. Importantly: The current investigation will not measure anxiety per se and will simply suffice as a putative proof-of-concept that, in terms of the exact underlying mechanisms that will supposedly be causative of the potential differences displayed here, will have to be elaborated on in future investigations.

The secondary objective (B) is to investigate (1) whether the effects of the same pre-test habituation protocols as observed in the sequence of tests (0 min vs 60 min) would yield different behavioural outcomes if the nORT, OFT and FST are applied as single tests on separate days vs. its application in the sequential battery test paradigm and (2) how such behaviour is again influenced by imipramine treatment.
Note: Not all of the necessary comparative groups, i.e. the nORT in both strains, as well as the OFT and FST in FRL rats, could have been completed due to practical constraints, nevertheless the data are still presented for the benefit of the reader in Addendum B (to be viewed as data from identified unsuccessful experiments, or as additional deficient data to be expanded on in prospective studies, and should not compromise the scientific integrity of the main study).

1.4 Project layout

This layout is provided for the primary objective only (Figure 1-1). However, the same procedure was followed for the secondary objective, albeit only applying one behavioural test per day.

In this project, FRL and FSL rats were transported in their home cages by the experimenter from the holding room to the testing room (Figure 1-2). Upon entry in the testing rooms, animals were either left to habituate in their home cages for 60-min before behavioural testing commenced, or directly tested after being transported. Please refer to Chapter 3 for a detailed explanation of the methods followed. The nORT and the OFT were performed in the same room, with the FST in an adjacent room. Thus, the rats were transported from the holding room to the nORT and OFT testing room, and only after the OFT has been completed, were they transported to the FST room (Figure 1-2). Between the nORT and the OFT, the rats in the 0-min group were only returned to their home cages while the arenas were being cleaned, while those in the 60-min group were again allowed 60 minutes to habituate in their home cages between the execution of the nORT and the OFT.

![Figure 1-1: Schematic layout of the primary objective. SAL: saline. IMI: imipramine. FRL: Flinders Resistant Line. FSL: Flinders Sensitive Line. nORT: novel object recognition test. OFT: open field test. FST: forced swim test. PND: post-natal day.](image-url)
Figure 1-2: Floor plan of the GLP (good laboratory practice) area in which the experiments were conducted. Home cages were transported from the holding room to the testing room for the nORT and the OFT. After assessment in the OFT, the cages were transported to the adjacent FST room. nORT: novel object recognition test. OFT: open field test. FST: forced swim test.

1.5 Compliance and ethical approval

The study was approved by the animal research ethics committee of North-West University, NWU-AnimCareREC (approval no. NWU-00283-17-A5). NWU-AnimCareREC is registered with the National Health Research Ethics Council (Reg. no. AREC-130913-015).

The student, Ms JE Pienaar, received the necessary training in animal ethics and animal handling, and was authorised to perform these procedures by the SAVC (auth. No. AL17/16481).

Animals were bred and housed at the Vivarium of the national Pre-Clinical Drug Development Platform, a joint venture between North-West University and the Department of Science and Technology, Potchefstroom campus (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019).
CHAPTER 2: LITERATURE BACKGROUND

2.1 Translational animal models in neuropsychiatric disorders

Research in neuropsychiatry remains problematic, since studying changes in the brain is far more complex than in other organs. Techniques available to study the human brain are either implemented post-mortem or in vivo using neuroimaging; however, both techniques have limitations and lack specificity (Krishnan & Nestler, 2008). Additionally, modelling uniquely human psychological symptomology in animals is also challenging due to the heterogeneity of symptoms and the inter-individual variability of neuropsychiatric disorders in human subjects (American Psychiatric Association, 2013). Furthermore, there are limitations in the extent to which symptoms can be modelled in animals. For example, abstract psychological symptoms such as sadness, guilt, suicidal ideation, hallucination, and delusion cannot convincingly be demonstrated in animals, and yet, all these symptoms are included in diagnostic criteria for depression in humans (American Psychiatric Association, 2013; Nestler & Hyman, 2010).

The term translational animal model is used to describe the ability of an animal model to represent a particular human condition so that it will allow us to test a specific hypothesis or to investigate drug action. In essence, translational models can be used to make deductions regarding the human condition from the findings obtained in the animal model (McGonigle, 2014). As such, a well-validated animal model allows for associations to be made between physiological and behavioural variations and emotionality, disease aetiology and treatment response (Bourin, 2015). These aspects are addressed within the areas of face (symptoms), construct (neurobiology) and predictive (treatment response) validity, respectively.

2.2 Validity criteria for animal models

Animal models of neuropsychiatric disorders should comply with multidimensional validity criteria as determined to be relevant for the human disorder. To this extent, two interdependent components to valid animal models also come into play, i.e. external- and internal validity. External validity pertains to the general applicability of the results to the target population (Belzung & Lemoine, 2011) or other environmental situations, populations and species (Wurbel, 2000). Internal validity considers the experimental design and its consistency which is dependent on factors such as reproducibility, randomization and test-control design (van der Staay et al., 2009). As these two components pertain to the current study, they will now be discussed in more detail.
2.2.1 External Validity

External validity in context on translational animal models of human disorders focuses primarily on face, construct (including aetiological validity) and predictive validity (Belzung & Lemoine, 2011; Nestler & Hyman, 2010). It is important to note that the initial concepts of these three types validity proposed by (Wilner et al, 1984), briefly reflected upon below, have since been adapted to offer more contemporary interpretations (Belzung & Lemoine, 2011; Nestler & Hyman, 2010; van der Staay, 2006) and therefore, the terms cannot be used interchangeably throughout the history of the literature.

Another important distinction to be understood is that of models of conditions and behavioural tests which are often erroneously used interchangeably (van der Staay, 2006). A model in the context of translational neuropsychiatric research refers to the specific organism used; together with the provoking intervention (naturally occurring behaviours, selective breeding, genetic or pharmacological manipulation *inter alia*) employed to induce a robust and repeatable behavioural phenotype. Following such an intervention, the induced behaviour (which is intended to mimic the behaviour and biopathology of a particular condition) is normally quantified by assessing the model animals against non-manipulated controls in behavioural tests. In the current investigation, a genetically manipulated line of behaviourally depressive-like rats, known as the Flinders Sensitive Line (FSL; see par 2.3) serves as the model of MDD, and their behaviours are assessed in a *behavioural test* known as the forced swim test (FST; *inter alia*). It is imperative that this distinction be understood, since the satisfactory validation of animal models is largely achieved by examining the data derived from behavioural tests; therefore, the attention afforded to methodological precision during behavioural testing, contributes markedly (albeit indirectly) to the dependability of translational neuroscience discoveries.

*Face validity*

Face validity pertains to the observable similarity between the behavioural phenotype observed in an animal model of a particular psychiatric condition and the clinical symptom profile typically seen in humans (Czeh *et al*., 2016); this is in addition to similarities pertaining to the relevant biomarkers (Nestler & Hyman, 2010). For example, the naturalistic, stereotypical, repeated vertical jumping and pattern running in deer mice mimics repetitive motor actions observed in patients suffering from OCD (Wolmarans *et al*., 2013). Also, reduced sucrose intake by depressive-like rodents seemingly resembles the anhedonia experienced by patients suffering from MDD or schizophrenia (Liu *et al*., 2018). Last, and with respect to the current investigation, inflated levels of immobile behaviour and reduced struggling behaviour demonstrated by the depressive-like cohort of FSL rats mimics the behavioural despair characteristic of patients with...
MDD (Overstreet et al., 2005). Another important aspect regarding face validity is the discernment between modelling the entire human condition, e.g. MDD, or only a single endophenotype i.e. a single characteristic symptom of a particular condition e.g. anhedonia as a symptom of MDD. Several limitations exist with the latter approach, as it is unlikely to model the entire human neuropsychiatric disorder and its behavioural features in an animal. Similarly, even single behavioural parameters cannot fully represent the human situation (Czeh et al., 2016). Consequently, there have been calls to minimize the anthropomorphism of observable depressive-like behaviours in rodent models of MDD, shifting focus to more robust, empirical measurements (see below; (Holmes, 2003)). This is because species present with their own specific evolved behaviours in response to particular situations (e.g. rodent avoidance of illuminated spaces) which may confound the translation of human to animal behaviour, and for this reason, face validity is generally considered the weakest of the three types of validity discussed here (van der Staay, 2006). For example, compulsive grooming to the point of self-mutilation in genetically altered mice has been linked to OCD-related behaviour. However, it may lack aetiological, cognitive and emotional construct since the behaviour likely stems from the specific genetic manipulation and not any particular affective disturbance (Nestler & Hyman, 2010). As a result, face validity reiterates the importance of behavioural testing to screen animal behaviour that can be translated to the phenotype observed in humans as closely as possible, but since the psychological state of animals cannot be inferred solely by the observations of these behaviours (Cryan & Holmes, 2005; Holmes, 2003), more emphasis should be placed on the other types of validity (van der Staay, 2006). However, given that the majority of models of mental disorders or tests measuring the endophenotypes of such conditions are founded in the measurement of observable behaviours, any methodological interferences such as transportation and handling stress (Balcombe et al., 2004; Castelhano-Carlos & Baumans, 2009) or a lack of sufficient habituation to experimental conditions (Gouveia & Hurst, 2017; Van der Zee et al., 2004) which theoretically may affect the affective state of the animals, can easily confound the face validity of a model/behavioural test and any deductions made thereof.

**Construct validity**

This criterion states that the underlying biology in both animals and humans should be similar or at least comparable (Willner, 1984), e.g. the reduced serotonin (5-HT) synthesis observed in depressed humans is also observed in the FSL rat (Hasegawa et al., 2006). Due to the broad definition of construct validity, aetiological validity also forms part of this criterion, as the construct of a condition can not only be found in the biological nature and dynamics of the specific disorder, but also in the triggering external factors leading to the development and maintenance of disease and its dysfunctional neuro-regulatory systems (Belzung & Lemoine, 2011). As the exact
aetiology and pathophysiology of most neuropsychiatric disorders remains uncertain, most models do not, or only partially fulfil this criterion (Nestler & Hyman, 2010). Modelling by means of genetic alteration or inbreeding results in valuable hereditary behaviour abnormalities, but these techniques lack validity regarding aetiology, as genetically altered animals may present new behavioural responses as adaptive responses to the deleted/inserted gene (Nestler & Hyman, 2010). Nonetheless, models may also be regarded as valid if “stress vulnerable” animals are exposed to environmental factors to elicit disease-specific behaviour, even if congenital abnormalities are absent (Czéh et al., 2016), but again, these only partially fulfil the criteria. Construct validity therefore equally takes account of the similarity of the theoretical construct of, firstly the dysfunctional behaviour (developmental, cognitive, behavioural and/or physiological; Alonso et al., 2015; Belzung & Lemoine, 2011) in the clinical setting and in the model, and secondly the aetiology and development of the dysfunction as well as the correlation between the two constructs (Willner, 1994). In the broadest sense then, construct validity of psychiatric models employed in pre-clinical pharmacological studies can be established by demonstrating disruption in the typical function of entire neurotransmission systems as seen in particular human conditions (Albelda & Joel, 2012; Alonso et al., 2015; Cryan & Holmes, 2005). This is typically demonstrated by a positive response to effective pharmacological treatments (which overlaps somewhat with the third type of validity, i.e. predictive validity, see below), e.g. serotonergic interference in MDD (Pitchot et al., 2005; Svenningsson et al., 2006) or dopaminergic interference in movement disorders (Nespoli et al., 2018; Wylie et al., 2018). In line with this, certain depression-typical endophenotypical behaviours such as immobility in the FST respond to clinically effective drugs like imipramine (Cohn et al., 1996; Keller et al., 1998) over a wide range of doses (Castagne et al., 2010). Imipramine is a tricyclic antidepressant which inhibits serotonin and noradrenalin reuptake (Krishnan & Nestler, 2008) and which was employed in the current study. Considering the actions of imipramine in a number of bio-molecular locations, including being a potent noradrenaline reuptake inhibitor, and to a lesser extent inhibiting the reuptake of serotonin (5-HT) as well (Krishnan & Nestler, 2008), alongside its anticholinergic, antihistaminergic and α1-adrenergic receptor blocking effects (Nathan & Gorman, 2015), deductions about the particular neurotransmission systems involved, can be tenuous. However, considering more selective agents, e.g. the selective serotonin reuptake inhibitors (SSRIs; altering swimming behaviour in the modified FST protocol (Cryan & Lucki, 2000b)) and noradrenalin targeting drugs (decreased climbing behaviour; Cryan et al., 2002) are also effective in the FST (reviewed in Cryan et al., 2005), serotonergic and noradrenergic malfunction is indeed implied in the FSL model (Detke et al., 1995). Further strengthening of the construct would involve demonstration of abnormalities in specific receptors, transporters, brain regions/circuits, bio-molecular synthesis and catabolic enzymes known to be dysregulated in MDD (reviewed in Ferrari & Villa, 2017)).
Predictive validity

Predictive validity describes how accurately the animal model would predict how humans with the modelled disorder would respond to contextual influences such as treatment, be they surgical, pharmacological, behavioural, or any other type of ameliorating intervention (Nestler & Hyman, 2010; Willner & Mitchell, 2002). This means that an animal model with robust predictive validity would respond similarly to corresponding external influences that the human disorder would respond to and inversely, that it would not respond to external influences that the human correlate would not respond to. In context of the current study, predictive validity typically rests heavily on pharmacological human-animal correlations. It is therefore concerned with the therapeutic outcomes, e.g. a treatment which decreases symptomology in humans should also diminish symptoms in the animal (Albelda & Joel, 2012; Belzung & Lemoine, 2011; Willner & Mitchell, 2002), while conversely, clinically ineffective treatments should also be ineffective in animals. Further predictive validity can be demonstrated by mimicking human treatment modalities and subsequent treatment responses as closely as possible i.e. antidepressant or anti-obsessional responses only being established following chronic, but not acute treatment, similar to the clinical scenario observed in humans (Willner, 1984).

Therefore, it can be argued that the model should allow for accurate predictions to be made based on the measurable response to treatment as assessed by the performance of model animals e.g. the presently employed FSL rats in behavioural tests designed to test endophenotypical behaviours of the modelled condition (i.e. FST) (Belzung & Lemoine, 2011). This therefore renders the model an effective screen for effects of known and novel drugs. However, as most biological targets of “gold standard” drugs used in the treatment of neuropsychiatric disorders were discovered by chance (Malenka et al., 2009), current models validated by these drugs at best only represent models of the specific known mechanism of action, potentially limiting the model’s validity when evaluating novel compounds with novel, different mechanisms of actions (McGonigle, 2014), in which case there is a risk that the model may not accurately predict human response outside of the known paradigm.

In summary, the demonstration of a treatment response to imipramine treatment in the current study will demonstrate all to a certain extent three types of internal validity viz. changes to observable condition-specific endophenotypical behaviours (face), demonstration of serotonergic dysfunction (construct) and a positive response to an effective treatment (predictive).
2.2.2 Internal Validity

Validity has previously been defined as “… the agreement between a test score or measure and the quality it is believed to measure” (Kaplan & Saccuzzo, 2017). Therefore, the use of an animal model is not to demonstrate the actuality of the model, as the model in itself is not validated, but rather the data and consequent interpretations obtained from the model are validated to ensure accuracy and credibility (van der Staay et al., 2009). The confidence with which data can be interpreted is therefore not only dependent on an accurate theoretical, aetiological, and ethological framework required for a valid model, but is built on appropriate and adequate experimental design and data analysis, i.e. internal validity.

Generalization of a specific brain-behaviour relationship is dependent on different laboratories testing different animals under different conditions (Isaacson, 1971), resulting in heterogeneous outcomes and difficulty in comparing results. Nonetheless, for reliable inter-laboratory comparisons to be made, unambiguous measures should be employed that are resistant to experimental conditions (Kalueff & Tuohimaa, 2004), thus yielding a more robust assessment and thereby providing robust internal validity. Reliability and reproducibility are the foundation of internal validity, as valid results within the laboratory is required to produce reliable outcomes between laboratories. Good study design and strict control over confounding influential factors ensures exclusive change in the dependant variable as a result of the manipulation of the independent variable, and not due to other influencing factors (Guala, 2003), a key focus of the current investigation.

Reproducibility

According to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, pre-clinical pharmacological research should comply to a minimum standard for methodology, experimental design and data analysis to ensure that published findings are reproducible and reliable (Curtis et al., 2015). However, variations in standards of design and analysis, as well as the reporting thereof, still exist (Drucker, 2016). These guidelines have been implemented to varying degrees of commitment since 2015, although transparent reporting in pharmacological studies has been wanting. A lack of detail regarding experimental design, group sizes and importantly, animal welfare (which may also affect results), potentially diminishes confidence in research wishing to build on previous work (McGrath & Lilley, 2015). Unfortunately, research is plagued with errors of inappropriate and inadequate designs, lacking in power and validity, inappropriate and inadequate interpretations of the resulting statistical analysis and misconceptions as to the meaning of statistics and their practical implications. Therefore,
conscious changes in pre-clinical research approaches are necessary to address the issue of irreproducibility (McGrath & Lilley, 2015).

Valid statistical analyses are only attained from studies of which the experimental design and execution allow randomly determined results (Curtis et al., 2015). This reiterates the important of sound study design and subsequent statistical analysis that form the fundamentals from which reliable and reproducible outcomes may be obtained. Rigorous standardisation of the laboratory environment and experimental methodology is commonly implemented in an attempt to improve reproducibility (Baker, 2016). This has for example been shown in failed attempts to reproduce results in a widely excepted mouse model of familial amyotrophic lateral sclerosis, where the authors concluded that failure to reproduce the original study’s results in an adequately designed and powered repeat-study are attributed to uncontrolled confounding factors and type 1 errors (Scott et al., 2008).

Other factors influencing reproducibility have been identified (Figure 2-1). In a questionnaire completed by over a thousand researchers, more than 60% conceded that selective reporting and pressure to publish is the two main reasons for poor reproducibility (Baker, 2016). Funding is paramount in any research field, and thus the race to publish has left the door open for research to be consumed by bureaucracy and competition for grants and positions (Baker, 2016), resulting in negligence in control and oversight of experimental methodology and conditions e.g. vague or insufficient reporting of materials and methods (Prinz et al., 2011). These time-consuming distractions also keep experimenters from focusing on good research design and execution. Lack of mentoring was indicated by more than half of researchers as a significant contributor to poor reproducibility (Baker, 2016). This is problematic as the next generation of researchers are not properly groomed by their superiors in the proper application of the scientific method, contributing to the continuation of the irreproducibility cycle.
In an attempt to reproduce results from 67 different studies, primarily pre-clinical oncology studies, only 7% of the models could be reproduced when directly copied the experimental method according to the original published data. It was also reported that only up to 25% of published data were in line with in-house replication outcomes (Prinz et al., 2011). It should be kept in mind that results are not expected to be replicated precisely, however the concern is that not even the main concept or conclusion of earlier published data can often be reproduced (Begley & Ellis, 2012). Also, at least 50% of published data cannot be replicated in industrial laboratories to reach the same conclusion (Booth, 2011). Also, data published in prestigious journals and independent groups are included in these statistics, demonstrating that journal impact factor does not necessarily guarantee improved reproducibility (Mullard, 2011; Prinz et al., 2011).
Increased bias significantly reduces the probability of results being dependable (Ioannidis, 2005). As bias is usually not intentional, but rather a result of ignorance, researchers are sometimes unaware of the substantial influence bias may have on experimental outcomes (Rosenthal & Lawson, 1964). More specifically, confirmation bias occurs when researchers perform experiments and interpret resulting data to fit their preconceived ideas and hypotheses (Mynatt et al., 1977). This form of non-blinded experimentation is confounded in poor experimental design. Blinding is an essential element which should be built in from the start of the study design, eliminating bias from multiple sources (Curtis et al., 2015). Another form of bias may exist, pertaining to positive results being easier to publish. This extent of bias towards a preference for publishing positive results and whether constraints on publishing contradictory findings to previously published results in high impact journals, exist, remains to be investigated (Baker, 2016).

It is clear that a crisis regarding replicability exists and can be attributed to several factors. However, in this study, it is postulated that most of these factors are founded in inappropriate and inadequate experimental design or oversights of what might be considered ‘minor experimental details’. Experiments should be designed according to methodologically valid constructs to measure behaviour in similar contexts as observed in the clinical milieu, whilst also keeping the conditions between laboratories as similar as possible. In pre-clinical stress research, this is only possible when carefully considering all possible sources of stressful confounding influences also not inherent to the actual testing procedures (transport; treatment; habituation inter alia), as well as when it is appropriate to control for these stresses when considering the experimental aims.

**Standardisation**

Standardisation has been proposed as an attempt to increase reproducibility and inter-laboratory comparison (Crabbe et al., 1999; van der Staay & Steckler, 2002). When considering standardisation, it is important to keep in mind that knowledge is gained through experience and that the need for standardisation is only realized after several experiments have been performed under systematically varied conditions. Such studies have revealed diverging results, thereby prompting standardisation of e.g. housing conditions and circadian rhythms. The studies of Rudolf et al. (1999) and McKernan et al. (2000) are important examples of standardisation of experimental methodology and environment. Opposing results were found in their studies investigating desensitisation of the α1 gamma-aminobutyric acid (GABA\(_A\)) receptor subtype in response to diazepam. Comparing both the experimental design of these studies, significant differences in experimental methodologies were apparent. Locomotor activity was measured in a familiar environment (Rudolf et al., 1999) compared to measurement in a novel environment (McKernan et al., 2000), while ataxia was measured at different speeds (2 vs 18 revolutions per
In a repeat-study, when both research groups agreed on a similar experimental design under comparable conditions (locomotor activity in a novel environment and ataxia under identical conditions), similar effects regarding the mutation of the \( \alpha_1 \) GABA\(_A\) receptor subtype were found in both laboratories (Crestani et al., 2000). Conversely, despite rigorous attempts at environmental standardisation between three different laboratories, contrasting behavioural results were still found in different mice strains (Crabbe et al., 1999). The authors concluded that the major genetic interactions are robust enough to withstand inter-laboratory environmental differences. However, subtle effects may either go unnoticed or be incorrectly attributed to genetic manipulations. The expression of behavioural phenotypes may either be suppressed or promoted by different environments, or otherwise “drowned out” in the “noise” of uncontrolled environmental factors (Calisi & Bentley, 2009). Various fields of research, e.g. in neurochemical and neuroanatomical studies (Catalano et al., 1997; Moser, 1990), consider standardisation as an imperative aspect for credible outcomes, especially where reference data is concerned (Alemáan et al., 1998). Behavioural studies should be no different, with standardisation enabling comparisons within and between laboratories.

A major concern regarding standardisation is producing results that are idiosyncratic, i.e. the results obtained are only valid for the specific conditions it is tested in (Beynen et al., 2001). The risk is that a result produced under highly standardized conditions may be highly reproducible, but may only be valid under those circumstances, leading to poor generalization to other populations (external validity) and other situations – with resulting ethical issues as animal lives are at risk with minimal information gained (Wurbel, 2000). It is postulated that by systematically varying putative influential factors believed to modulate behavioural results, a better understanding of behavioural variation and generalization may be possible, as neither minimization through rigorous standardisation or allowing uncontrolled environmental influences alone are adequate (Paylor, 2009).

“Standardisation is the process of developing and covenanting technical standards and stipulating them in a document that establishes uniform specifications, criteria, methods, processes, and/or practices. Standardisation is essentially consensus-built, aiming at achieving, assuring, and maintaining a high level of repeatability, compatibility, and quality of an experiment, and enabling valid comparison between studies”.

(van der Staay et al., 2010)
2.3 The Flinders Sensitive Line rat, a genetic model of Major Depressive Disorder

A well validated translational animal model of major depressive disorder (MDD) specifically used in this study, is the previously mentioned FSL rat, and its behavioural and genetic control, the Flinders Resistant Line (FRL) rat. These rat lines were originally inbred from Sprague Dawley (SD) rats in an attempt to produce animals that are genetically resistant to diisopropyl fluorophosphate (DFP), an organophosphate anticholinesterase agent. However, the FSL rat proved to be more sensitive to DFP and subsequently more sensitive to cholinergic receptor agonists, as well as in expressing higher numbers of muscarinic receptors in several brain regions (Overstreet & Russell, 1982; Overstreet et al., 1984; Overstreet, 1986). During its development, it was found that these animals presented with profound depressive-like features, such as elevated rapid eye movement (REM) sleep as well as increased hormonal sensitivity to cholinergic agonists (Overstreet & Wegener, 2013). Earlier, Janowsky et al. (1994) suggested that depression is a state of cholinergic hyperactivity. Subsequently these animals have been shown to display characteristics that would render them with robust face, construct, and predictive validity for modelling MDD, such that the FSL rat has become a well-established genetic animal model of depression (Overstreet et al., 2005). In fact, the construct validity of FSL rats is aligned not only with the cholinergic super sensitivity hypothesis of MDD, but also with several other hypotheses of the biological basis of depression, including dysfunctional serotonergic (Hasegawa et al., 2006; Zangen et al., 1997), glutamatergic (Hascup et al., 2011; Kovačević et al., 2012), nitrergic (Wegener et al., 2010) and neurotrophic (Elfving et al., 2010a; Elfving et al., 2010b) systems. Importantly, response to an antidepressant in this model requires chronic treatment, similar to what is typically seen in the human illness (Overstreet & Wegener, 2013), adding to its predictive validity.

Both the FSL rat and depressed patients display elevated, cholinergic-mediated REM sleep (Benca et al., 1996), while their passive behaviour, i.e. increased immobility in the FST, resembles that seen in depressed patients after stress (Overstreet & Wegener, 2013). Such behavioural despair manifests spontaneously. Additionally, FSL rats seem to model depression without comorbidity of anxiety. Interestingly, in the elevated plus maze (EPM), FSL rats displayed decreased anxiety compared to FRL rats, and spent more time in the open arms (Abildgaard et al., 2011). The FSL model also complies with predictive validity in that most drugs that produce antidepressant effects in humans, produce antidepressant-like effects in FSL rats, while drugs not expected to have antidepressant-like effects in the clinic, have also failed in this regard. Also, chronic treatment of control FRL rats with antidepressants has consistently failed to improve hang-time in the FST, indicating that the antidepressant-like effects are only present in rats with innate exaggerated immobility (Overstreet & Wegener, 2013). However, several other drugs
including psychostimulants, atypical antipsychotics, anticholinergics etc. have been shown to influence immobility, thereby producing false positive results, reiterating the importance of good study design (Petit-Demouliere et al., 2005). These false positive results can be brought to light by selecting an appropriate animal strain, analysing active coping behaviours in adjunction to immobility, or accounting for changes in general locomotor activity (Castagné et al., 2006).

2.4 **The test battery as a robust behavioural analysis tool**

Recently the use of behavioural test batteries (sequences of cognitive and behavioural tests performed in the same test subject on a single day) in our laboratory have been investigated (Mokoena et al., 2015). The use of these batteries allows for more rapid testing which significantly reduce the time spent in the laboratory. Also, three other advantages have been proposed for the use of test batteries, i.e. 1) allows for correlation of phenotypes with possible corresponding or overlapping central nervous system (CNS) function, 2) reduces the number of animals required when several behavioural domains are tested in the same subject and 3) correctly delineating a broad range of behavioural phenotypes of relevance for e.g. depression (McIlwain et al., 2001). However, important to note is that the results in the investigation of Mokoena and colleagues did not address the contextual and indirect factors, such as training effect, test order and inter-test interval, all of which may have influenced the pattern of findings reported. The authors have highlighted that these indirect factors are indeed important for consideration. For instance, distance moved in the open field test was significantly decreased for battery tested subjects compared to subjects naïve to the test experience (McIlwain et al., 2001). Conversely, they found no influence of test order on distance moved seen from comparable locomotor activity when the OFT was either placed first, second, third or last in the test order (McIlwain et al., 2001). Important for this study is the inter-test interval. The original authors only investigated the shortening of the inter-test interval from 1 week between tests to 1-2 days between testing and concluded that an inter-test interval of as short as 1-2 days may be used without adversely affecting behaviour of the test subjects (Paylor et al., 2006). In the study of Mokoena and colleagues, an inter-test interval of 60-min was used (Mokoena et al., 2015).
2.5 Stress: influencing behavioural test performance

“Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals”.


An animal’s performance during testing is dependent on its behaviour, which is susceptible to modification by stressors. From literature it is clear that stress can modulate behaviour, especially in stress-sensitive neuropsychiatric research (Wahlsten, 2010). As such, behaviour of stressed animals compared to unstressed controls in the nORT, OFT and FST is an important consideration when attempting within and between study comparisons.

Declarative memory as tested in the nORT depends on several brain regions also implicated in the stress response, and as such it is not surprising that the stress response may influence declarative memory capabilities (LaBar & Cabeza, 2006; Roozendaal et al., 2006; Wolf, 2006). Different brain regions play an important part in the processing, storage and retrieval of memories, which are affected by acute stress resulting from increased glucocorticoids (GC) and/or sympathetic nervous system (SNS) activity (Diamond et al., 2007; Joëls et al., 2006; Roozendaal, 2002). Considering the effect of stress on memory, it has been shown that the effect is dependent on the memory phase. In this regard, consolidation of emotional memory appears to be enhanced by GC, while the retrieval of previously learned material may be impaired (Roozendaal et al., 2006; Wolf, 2006; Wolf, 2008). The direction of these effects results from GC activation in the amygdala and hippocampus. However, it has been shown that the effect of GC necessitates concurrent SNS activation in these brain regions (Abercrombie et al., 2006; de Quervain et al., 2007; Kuhlmann & Wolf, 2006a; Kuhlmann & Wolf, 2006b), resulting in declarative memory being dependent on the level of arousal/noradrenergic activity during testing. These alterations in declarative memory is a function of object recognition in the nORT. The nORT is divided into two sessions, first the sample session (acquisition) in which the rat is left to explore two identical objects placed in an open arena. In the second trial (retention) one of the sample objects is replaced with a novel object. Preferential exploration of the novel object is reflective of the successful recognition of the sample object and consequently intact declarative memory function (Ennaceur & Delacour, 1988). When a 4-hour inter-test interval was allowed between the acquisition and retention trial in the nORT, chronically stressed male rats spent equal amounts of time at both the familiar and the novel objects, indicative of a failure to recognise the familiar object (Bowman et al., 2003). In contrast, acute stress has been shown to facilitate memory via GC-dependent mechanisms. To which extent the seemingly ‘stressogenic’ construct investigated here, i.e. transportation and handling, will yield results that are comparable to this, is uncertain.
That said, several factors play an important role in memory and cognition (Table 2-1), significantly impacting test performance in these animals.

### Table 2-1: Summary of possible confounding variables affecting test performance in tests of memory and cognition. Adapted from Paul *et al.* (2009).

<table>
<thead>
<tr>
<th>Variables influencing animal performance in tests of memory and cognition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of maze</td>
<td>Stress</td>
</tr>
<tr>
<td>Selected protocol</td>
<td>Time and schedule of the study</td>
</tr>
<tr>
<td>Species and animal strain</td>
<td>Environmental cues</td>
</tr>
<tr>
<td>Animal gender</td>
<td>Animal age</td>
</tr>
<tr>
<td>Nutritional status of the animals</td>
<td>Drugs used</td>
</tr>
<tr>
<td>Potential infections</td>
<td>Stimuli driving behaviour: appetitive vs. aversive</td>
</tr>
</tbody>
</table>

The FST is commonly used to assess the potential depressogenic effects of stressors on rodents (Overstreet *et al.*, 2005; Rygula *et al.*, 2005) despite being a significant stressor itself (Paredes *et al.*, 2005; Vega-Rivera *et al.*, 2013), given that it induces stress-relevant neurochemical, endocrine and immunological alterations (Connor *et al.*, 1997). However, as the occurrence of depression may be enhanced by stressors (Kendler *et al.*, 2010; Wager-Smith & Markou, 2011), it is possible that stressors may exacerbate depressogenic effects in the FST. For example, chronic mild stress has been shown to increase active behaviours in the pre-swim session along with a greater decline in these behaviours from the pre-swim to the test session, which were more pronounced in male Long Evans rats compared to SD male and female rats (Bielajew *et al.*, 2002).

To which extent the nature and degree of the potential stressors applied here, i.e. transportation and handling prior to testing, will influence the assessments applied here, remains to be investigated. Suffice to say that the inconsistency of published results suggests that factors beyond the construct of the test itself play a significant confounding role, especially considering the large differences reported by different laboratories even in baseline immobility (Bogdanova *et al.*, 2013).

Therefore, we propose that since a behavioural test such as the FST, which is commonly used in the validation of animals models (such as the FSL rat) may subject to manipulation by a large variety of external factors including inter-trial periods, test interval days, test battery order, deliberate stressors, strain and species, it stands to reason that often overlooked factors such as habituation time and transport, handling and injections stressors may also contribute to idiosyncratic findings. In line with this, the current study has been conceptualised.
2.6 Stress: effect of laboratory environment on behaviour

“Care should be exercised in dismissing a procedure as non-stressful just because it is simple routine.”

Sharp et al. (2003)

Stress can broadly be classified into two distinct categories, physiological stress, and psychogenic stress. Physiological stress is regarded as a real threat to health, placing an immediate demand on the body for restorative responses, irrespective of conscious awareness or perception of the stressor e.g. injury with hypovolemia. Psychogenic stress on the other hand, relates to a perceived threat and relies on the perception of forthcoming anticipated aversive events, cognitive processing and interpretation of a stressor to assign a certain stressful experience (e.g. potential job loss), as a measure of its impact, which differs between individuals (Day, 2005; Morilak et al., 2005). The body responds to these demands by several processes and mediators in order to maintain homeostasis, influencing both physiology and behaviour (McEwen et al, 1998).

Another aspect in neuropsychiatry is the construct of eustress and distress. These are regarded as distinct from one another but are often generally defined under the collective term ‘stress’. The construct of eustress and distress is based on an individual’s perception of a stressor as either beneficial or harmful, which determines whether the stressor will be reacted to in a positive or negative manner (Selye, 1987). As opposed to traditional concepts of stress response as only reactionary (Selye, 1964), the construct of eustress and distress attributes the control of the response to the individual. In extension of this concept, an individual’s inability to adapt to or adequately respond to stressors/demands may lead to pathologic coping mechanisms, also referred to as allostasis, i.e. the ability to create “stability through change” (McEwen, 1998). The ability to alter one’s behaviour plays an important role in managing allostatic load (the collective burden of stress that accumulates over time due to deficient physiological and behavioural responses to stress), as adequate changes in behaviour may increase or decrease potential risk of harmful outcomes arising from stress (McEwen, 2000). In the context of this study, potential ‘stress’ will be related to any “external force or situation acting on the individual” and “deformations or changes produced in the individual as a result of those forces” (Kolt et al., 2003).

That said, behaviour is a complex, multidimensional, biological process primarily determined by two factors i.e. (a) the organism’s genotype (genes an individual was born with) and (b) environmental factors (i.e. all non-genetic influences) (Sousa et al., 2006; Ramos, 2008). In the pre-clinical setting, body activity and locomotion form the basis on which most behaviour is tested, since researchers are unable to develop a model measuring emotionality without non-emotional factors (i.e. motor activity), which is implicated as well (Ramos, 2008). Although behavioural
testing is generally a low-cost and straightforward method in comparison with other biological research, certain influencing factors should be carefully monitored and controlled to produce reliable and comparable outcomes, e.g. interpretation of data, standardisation of both husbandry and testing procedures as well as the training of examiners (Sousa et al., 2006). The environment is known to play an important role in modulating behaviour, and as such has been extensively researched. Table 2-2 provides a list of environmental factors studied, with references to relevant reports (as applicable to rodents, but with comparable impact on other animals).

Table 2-2: Brief listing of the most influential environmental factors for laboratory animals investigated over the past 50 years. Adapted from Izidio et al. (2005).

<table>
<thead>
<tr>
<th>Environmental factor in laboratory</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of testing</td>
<td>Gentsch et al. (1982)</td>
</tr>
<tr>
<td>Illumination level</td>
<td>Cardenas et al. (2001); Castelhano-Carlos and Baumans (2009)</td>
</tr>
<tr>
<td>Floor surface</td>
<td>Morato and Castrechini (1988)</td>
</tr>
<tr>
<td>Transportation</td>
<td>Morato and Brandão (1997)</td>
</tr>
<tr>
<td>Previous experience in the test apparatus</td>
<td>Bertoglio and Carobrez (2000); File et al. (1992); Treit et al. (1993)</td>
</tr>
<tr>
<td>Handling</td>
<td>Gartner et al. (1980); Henderson (1970) Brown and Martin (1974); De Boer et al. (1990)</td>
</tr>
<tr>
<td>Group housing</td>
<td>Maisonnette et al. (1993)</td>
</tr>
<tr>
<td>Experimenter</td>
<td>Chesler et al. (2002); Izidio et al. (2005)</td>
</tr>
<tr>
<td>Cage position in holding room</td>
<td>Exner and Clark (1993); Izidio et al. (2005)</td>
</tr>
<tr>
<td>Pre-testing arousal state</td>
<td>Izidio et al. (2005); Schmidtke and Heller (2004)</td>
</tr>
<tr>
<td>Blood collection and orogastric gavage</td>
<td>Balcombe et al. (2004)</td>
</tr>
<tr>
<td>Noise</td>
<td>Castelhano-Carlos and Baumans (2009)</td>
</tr>
<tr>
<td>Cage cleaning</td>
<td>Castelhano-Carlos and Baumans (2009)</td>
</tr>
<tr>
<td>In-house transportation</td>
<td>Castelhano-Carlos and Baumans (2009)</td>
</tr>
</tbody>
</table>

Inter-experimenter variability typically accounts for a high degree of inter-study variability. Another is cage position in the holding room, e.g. rats housed in the top shelves of housing racks have previously demonstrated reduced fearfulness and arousal state before testing (Izidio et al., 2005). Also, timing of testing may be affected by concurrent disturbance variables such as laboratory routine, handling, blood collection and oral gavage that may significantly increase heart rate and body temperature for up to 45 – 90 minutes, and which have also been accompanied by increased GC concentrations (Balcombe et al., 2004). Furthermore, light, noise, cage cleaning and in-house transport of laboratory animals induce an acute stress response altering physiological parameters (Castelhano-Carlos & Baumans, 2009).
In line with the research question investigated here, anxiety-related behaviour was hypothesised to vary depending on the arousal state of the test subjects immediately before testing (Izidio et al., 2005). Specifically, inbred Lewis (LEW) and spontaneously hypertensive rats (SHR) were removed from the cage rack and placed on a bench in the housing room. The rats were then classified into two groups, i.e. 1) aroused or 2) resting. Once allocated, a rat was taken from the home cage to be tested in the EPM in an adjacent room. Anxiety scores were significantly higher for pre-test aroused rats than their resting counterparts. It can therefore be expected that the arousal state of the test subjects before testing may vary within and between studies and laboratories. This led the authors to conclude that the behavioural state of the test subject prior to testing significantly influences test outcomes, ultimately contributing to decreased reliability and replicability. As these behavioural tests of anxiety consist of trials typically short in duration, variation in emotionality due to the pre-test circumstances may lead to testing the anxious state of the animal immediately prior to the session rather than the true psychological state of the subject, resulting in testing a temporary state rather than a permanent feature. With specific relation to the current investigation, the authors recommended that all animals should be aroused a few minutes before testing in the open field, EPM or black/white box tests to increase replicability of test outcomes (Izidio et al., 2005), which supports the idea of excluding habituation prior to these tests.

Another factor to consider in neuropsychiatric animal research is the routine handling of animals prior to and during experimentation, which is often regarded as inconsequential and incidental in nature but that has been shown to significantly influence the physiology and behaviour of laboratory animals (Balcombe et al., 2004; Deacon, 2006; Schellinck, 2010). In the study of Gouveia and colleagues (2017) the mayor influence of handling on behaviour of mice in subsequent testing was evinced, demonstrating the significant effect of these external stressors on performance of laboratory animals in behavioural tests (Gouveia & Hurst, 2017). In this regard, variation in handling techniques (comparing the traditional tail handling with handling tunnels or cupping with the hands) significantly altered exploratory behaviour between test subjects, causing impaired test performance within a single test modality. These findings further elaborate on existing data reporting the substantial impact of handling on anxiety-related behaviour in standardised tests, reducing replication success and direct comparisons within and between batches of animals, studies and laboratories (Ghosal et al., 2015; Gouveia & Hurst, 2013; Hurst & West, 2010). Even more important is that the background variation causes attention to be drawn from the task at hand, in other words, rather than measuring the performance the task was designed to measure, behavioural scores are resultant of inappropriate interaction with the test generating misleading data. Results from the investigation confirm that poor performance in the
behavioural test are associated with reduced exploration and a high degree of caution directly correlated with the aversiveness of the handling method (Gouveia & Hurst, 2017).

At this point, it is important to address the impact of habituation. It has been shown that repeated exposure to a stimulus leads to desensitisation of the response elicited by the original encounter, resulting in habituation (Grissom & Bhatnagar, 2009). To this end, laboratory rodents have shown no sign of habituation to handling, judged from a lack of changes in latency of heart rate to return to baseline over a course of 20 days of daily handling (Black et al., 1964). Also, it has been shown that the hormonal responses are dependent on the intensity of the handling stressor (Armario et al., 1986a; Armario et al., 1986b) which is founded in the physical manipulation of the animal (Sharp et al., 2003).

Part of animal testing encompasses the handling and moving of animals from the holding room to the testing area. Previously, such handling and transportation procedures have been associated with elevated serum levels of corticosterone and prolactin (Armario et al., 1986a; Gartner et al., 1980). Further, these elevations occurred within 8 minutes and lasted up to 60 minutes, with peak concentrations reached 15 minutes after initiation of handling (Gartner et al., 1980). Dallmann et al. (2006) also reported significantly elevated body temperature following transportation of F344 rats in their cages from the rack to a bench within the holding room, demonstrating core temperatures rising more than 0.5 °C for the following 120-min, with transportation through a noisy corridor to the testing room resulting in elevated body temperature by more than 0.5 °C for at least 60-min (Dallmann et al., 2006).

2.7 Habituation time after in-house transportation

A summary of literature investigated depicting methodologies of 50 randomly selected FST, EPM, nORT and OFT studies over the past decade (2007 – 2017) with regards to pre-test habituation is presented in Table 2-3. From the 50 studies investigated, only four specified pre-test habituation details (marked as blue in Table 2-3).
Table 2-3: Summary of 50 randomly selected studies from 2007 to 2017 displaying the habituation time employed before behavioural testing. FST, forced swim test; OFT, open field test; NORT, novel object recognition; EPM, elevated plus maze.

<table>
<thead>
<tr>
<th>Behavioural Test</th>
<th>Habituation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FST</td>
<td>Unspecified</td>
<td>Addy et al. (2015); Arunrut et al. (2009); Belozertseva et al. (2007); Craft et al. (2010); Desbonnet et al. (2008); Frankowska et al. (2007); Frankowska et al. (2010); Gamil et al. (2016); Hong et al. (2012); Isacson et al. (2011); Jennings et al. (2016); Kitamura et al. (2008); Koike and Chaki (2014); Kokras et al. (2017); McNamara et al. (2013); Miyake et al. (2014); Możdżeń et al. (2014); Possamai et al. (2015); Shishkina et al. (2010); Solomon et al. (2014); Swiergiel et al. (2007); Szewczyk et al. (2009); Tamano et al. (2009); Wu and Lin (2008); Wulsin et al. (2010); Yamada et al. (2013)</td>
</tr>
<tr>
<td>FST + OFT</td>
<td>Unspecified</td>
<td>Aisa et al. (2007); Citraro et al. (2015); Dang et al. (2009); Desikan et al. (2014); Fernandez-Guasti et al. (2017); Garcia et al. (2008); Gomez et al. (2014); Gutiérrez-García and Contreras (2009); Hamani et al. (2010); Jesse et al. (2008); Kawaura et al. (2015); Li et al. (2007); Marks et al. (2009); Podkowa et al. (2016); Turner et al. (2008); Xing et al. (2011); Yang et al. (2013)</td>
</tr>
<tr>
<td>FST + OFT</td>
<td>30-min</td>
<td>Xing et al. (2011)</td>
</tr>
<tr>
<td>FST + OFT + EPM</td>
<td>Unspecified (directly moved from OFT to EPM)</td>
<td>Qi et al. (2008)</td>
</tr>
<tr>
<td>FST + EPM</td>
<td>0-min</td>
<td>Chen et al. (2015)</td>
</tr>
<tr>
<td>FST + NORT</td>
<td>60-min</td>
<td>Borsoi et al. (2015); Pitychoutis et al. (2014)</td>
</tr>
</tbody>
</table>

Regarding the OFT, none of the studies indicated a pre-test habituation period. Taken together, habituation seems to involve either 0 or 60 minutes, with no extension reported beyond a 60-min period. However, the use of both a 30-min (Brand & Harvey, 2017b; Steyn et al., 2018) as well as a 60-min habituation period (Mokoena et al., 2015) has been reported in our laboratory. From Table 2-3 it is clear that for the past 10 years, no protocol has been established regarding the time needed for animals to habituate before behavioural testing, and whether pre-test habituation actually influences subsequent behavioural test outcomes.

2.8 Conclusion to Chapter 2

Taken together, the FSL rat is a robust model of MDD, exhibiting features of face-, construct- and predictive validity. However, despite the well-documented validity of this model, comparisons within and between laboratories as complicated by varying reports of even baseline cognition and emotionality. The laboratory environment is proposed to play a significant part in the lack of reliability and replicability in neuropsychiatric research, as seemingly minor practices inherently included in the pre-test circumstances are suggested to elicit stress-related changes, at least to some extent, in test subjects, confounding the outcomes measured by behavioural test performance. Considering the lack of standardisation of these practices in our laboratory, this
study will investigate the effect of pre-test habituation on the emotionality and cognition in the FSL rat model of depression as assessed in a sequence of tests in the same subjects on the same day. The main findings of this project can be found in Chapter 3 and Annexure A, along with the references at the end of the chapter. The summary and conclusion to the study as a whole can be found in Chapter 4, which also contains the practical recommendations deduced from this study. At the end of the dissertation, the bibliography containing references for the full dissertation is included, as well as additional data generated throughout the study, which is located in the annexures attached.
CHAPTER 3: ARTICLE MANUSCRIPT

This dissertation has been prepared in article format, as recognised by the North-West University. The main findings of this study are presented in the following article titled:

The effect of pre-test habituation in a sequence of cognitive and behavioural tests as applied in a genetic animal model of major depressive disorder

The article has been prepared according to the instructions to the authors of the journal, Behavioural Pharmacology. Therefore, the references pertaining to the article can be found at the end of the Chapter 3. However, to facilitate the reading process, tables and figures are included within the Results section, rather than at the end of the article as per the instructions of the journal.

The specific instructions and guidelines for submitting an article to the journal of Behavioural Pharmacology be found at:

http://edmgr.ovid.com/bpharm/accounts/ifauth.htm
AUTHOR CONTRIBUTIONS AND CONSENT

<table>
<thead>
<tr>
<th>Author</th>
<th>Contributions</th>
<th>Consent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johanna E Pienaar</td>
<td>Carried out all experimental procedures and contributed to the statistical analyses. Prepared the first draft as well as the final version of the manuscript.</td>
<td><img src="signature1.png" alt="Signature" /></td>
</tr>
<tr>
<td>Linda Brand</td>
<td>Contributed to the design of the study and proofing of the manuscript.</td>
<td><img src="signature2.png" alt="Signature" /></td>
</tr>
<tr>
<td>Stephanus F Steyn</td>
<td>Oversaw experimental work. Conducted the statistical analyses. Contributed to the initial and final versions of the manuscript.</td>
<td><img src="signature3.png" alt="Signature" /></td>
</tr>
<tr>
<td>Christiaan B Brink</td>
<td>As supervisor, planned and designed the study. Also designated as corresponding author. Contributed to the proofing of the manuscript.</td>
<td><img src="signature4.png" alt="Signature" /></td>
</tr>
</tbody>
</table>

# I declare that I have approved the article and that my role in the study was as indicated above. I hereby give my consent that the article may be submitted as part of the thesis of Johanna E Pienaar.
**Title**

*The effect of pre-test habituation in a sequential battery of cognitive and behavioural tests, as applied in a genetic animal model of major depressive disorder*

**Short title**

Pre-test habituation in an animal model of depression

**Author names**

Johanna E. Pienaar^a, Linda Brand^a, Stephanus F. Steyn^a, Christiaan B. Brink^a

**Author affiliations**

^aCentre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North West-University, South Africa

**Corresponding Author**

Prof. Christiaan B. Brink

Tiaan. Brink@nwu.ac.za

Internal box 16, Division of Pharmacology, North-West University, Potchefstroom, 2520, South Africa. Tel: (+27) 18 299 2234. Fax: (+27) 18 299 2225.

**Conflicts of interest**

None declared

**Funding**

Academic reserve funds of Prof Brian H Harvey, North-West University.
3.1 Abstract

Objectives

The present work investigated the influence of a 60-min habituation period vs. no habituation on test outcomes prior to behavioural assessments in a sequential battery of behavioural tests as conducted in a genetic rodent model of major depressive disorder (MDD), i.e. Flinders Sensitive Line (FSL) rats vs. their behavioural control, i.e. Flinders Resistant Line (FRL) rats.

Methods

Male FRL and FSL rats (n = 12 per cohort per habituation protocol) were subjected to a chronological sequence of behavioural tests that consisted of firstly the novel object recognition test (nORT; declarative memory), thereafter a habituated open field test (OFT; general locomotor behaviour), and lastly the forced swim test (FST; depressive-like behaviour). Before the onset of experiments, rats were transported from the holding room, to the first experimental room and subsequently subjected to the series of tests, either immediately or after 60 minutes habituation in the test room.

Results

Pre-test habituation had no significant effect on declarative memory in FRL and FSL animals, irrespective of the behavioural test protocol followed. However, the locomotor activity of FSL, but not FRL animals was reduced following 60 minutes of pre-test habituation, as compared to no habituation. Without habituation prior to the FST, both FRL and FSL rats displayed a greater degree of depressive-like behaviour as evinced by an increased time spent immobile vs their habituated counterparts, whereas changes in active coping behaviours, i.e. struggling and swimming, did not significantly differ. Further, with respect to the depressive-like behaviour, FSL rats displayed a greater degree of immobility and reduced struggling behaviour, compared to FRL control subjects irrespective of the habituation protocol followed.

Conclusions

Although habituation following in-house transportation does not significantly alter performance in memory-related tasks, habituation may affect locomotor activity in the FSL rat, which may complicate the interpretation of immobility in the FST, where higher locomotor activity in non-habituated animals may reduce immobility in the FST, leading to wrong interpretation of the latter as reduced depressive-like behaviour (i.e. false negative results). Therefore, conclusions drawn from the FST may provide greater insight into depressive-like behaviour in the absence of pre-
OFT and -FST habituation. Lastly, our results affirm the face validity of the FSL rat as a genetic animal model of MDD.

**Keywords**

In-house transportation; Pre-test habituation; Flinders Sensitive Line (FSL) rat; major depressive disorder (MDD)
3.2 Manuscript

3.2.1 Introduction

The Flinders Sensitive Line (FSL) rat is a widely used genetic model of major depressive disorder (MDD) (Overstreet et al., 2005) and is commonly applied in our laboratory (Badenhorst et al., 2017; Mokoena et al., 2015; Mouton et al., 2016; Steyn et al., 2018; Uys et al., 2017), including in a translational animal model of treatment resistant depression (Brand & Harvey, 2017).

The FSL rat represents a well described genetic animal model of MDD, presenting with demonstrable face validity in that FSL rats display a higher degree of immobility in the forced swim test (FST), a validated screening test for depressive-like behaviour, elevated rapid eye movement (REM) sleep with reduced REM sleep latency, reduced appetite and inflated passive stress coping; for a detailed review of the FSL rat, see Overstreet et al. (2005); Overstreet and Wegener (2013). Further, FSL rats do not present with broad impairments in cognitive functioning. However, in light of the fact that patients diagnosed with MDD often present with symptoms related to cognitive disability, e.g. indecisiveness in tasks dependent on set-shifting performance (American Psychiatric Association, 2013; Matsuo et al., 2007; Vythilingam et al., 2004), it is noteworthy that a number of previous investigations identified deficits in declarative memory in FSL, compared to FRL rats as highlighted by the novel object recognition test (nORT) (du Jardin et al., 2016; Gomez-Galan et al., 2013; Oberholzer et al., 2018; Tillmann & Wegener, 2019; Uys et al., 2017). Further, although FSL rats have not been shown to be overly anxious in the open field test (OFT) (Overstreet et al., 1995; Schiller et al., 1991), they are known to be stress sensitive and have displayed inflated anxiety in more robust, and in itself even anxiogenic tests, i.e. the social interaction test (Overstreet et al., 2004) and the elevated plus maze (Abildgaard et al., 2011). That said, the most prominent phenotypical feature of the FSL rat is its characteristic passive stress coping response as evinced by an exaggerated degree of immobility when exposed in the FST (Castagne et al., 2010; Overstreet et al., 1995; Overstreet, 1986). Furthermore, the said spontaneously enhanced immobility is moderated by chronic, but not sub-chronic or acute antidepressant treatment (Overstreet, 2002; Overstreet & Wegener, 2013), findings that are generally viewed to be supportive of the predictive validity of the model. In line with its response to antidepressant treatment, the construct validity of the FSL model is also founded on neurobiological alterations similar to that observed in clinical depression, viz. altered serotonergic (Overstreet, 2002; Shayit et al., 2003; Zangen et al., 2001; Zangen et al., 2002), dopaminergic (Meyer et al., 2002; Meyer et al., 2001; Schwartz et al., 2003), and noradrenergic (Landau et al., 2015; Zangen et al., 2001) neurotransmission.
Pre-clinical neuropsychiatric research is dependent on and limited to investigations of animal behaviour expressed in paradigms that at most suffice to provide an indirect perspective on the emotional state or cognitive ability of an animal (Ramos, 2008), i.e. interactions with non-reactive objects, location-specific movements in an open field arena, or forced exposure in a swim chamber. Therefore, with respect to the collective appraisal of both within- and between-laboratory findings, an important aspect of consideration is methodological congruence. In fact, while specific behavioural tests—in the case of the present work, the nORT, OFT and FST—are often applied to measure the same neuropsychiatric constructs, within- and between-laboratory results and conclusions can realistically only be compared and drawn if near analogous experimental conditions are applied.

In this regard, one often neglected factor for consideration during animal experimentation is the prevailing pre-test circumstances that may have a significant impact on the behavioural baseline (Balcombe et al., 2004; Castelhano-Carlos & Baumans, 2009). In fact, several factors that are incidentally incorporated into behavioural study design, i.e. pre-test in-house transportation, human handling prior to and during experiments, and between-test cleaning of apparatus, may alter the performance of animals during testing (Hurst & West, 2010). In other words, it is likely that experiments applying similar behavioural assessment methodologies, may yield incomparable results not because of explicit experimental confounds per se, but as a result of differences in the circumstantial factors associated with the execution of such tests. One factor that is especially important in experiments designed to investigate neuropsychiatric constructs in animals, is changes in the baseline, pre-test stress- and anxiety level of the test subject (Gouveia & Hurst, 2017; Sousa et al., 2006). In this regard, acute stress can be regarded as rapid changes in the homeostasis of the experimental subjects that are intrinsically associated with the activation of the central stress response; this in turn must be regarded as a confound in the interpretation of behavioural data (Gouveia & Hurst, 2017). Indeed, the behaviours measured in the nORT, OFT and FST are all related to cognitive processes that are modulated and governed by the same neurotransmission systems as those implicated in the neuropsychological stress response (Gartner et al., 1980; Kemeny, 2003). This is especially true for the FSL rat which was bred to demonstrate cholinergic super-sensitivity, a neurobiological correlate of reduced stress tolerance (Markou et al., 1994; Pucilowski et al., 1990; Pucilowski & Overstreet, 1993).

Due to the time-consuming nature of an experimental design in which several different behavioural tests are conducted on subsequent days, we have recently investigated the plausibility of conducting a sequential battery of tests on the same test subject on the same day (Mokoena et al., 2015). While results from this investigation indicated that test outcomes relating to said sequence are akin to that observed following the former approach, it is important to
consider that this work did not address the contextual and indirect factors that may have influenced the pattern of findings reported. Therefore, considering the literature reviewed above and to provide direction for future research in this and other laboratories, which may contribute to facilitating more robust between-study comparisons, the current investigation explored the effect of two different pre-test habituation protocols, i.e. either no habituation or 60-min habituation, on behavioural test outcomes in a sequence of behavioural assessments as often applied in FRL and FSL rats, viz. 1) the nORT, 2), the OFT and 3) the FST.

3.2.2 Materials and Methods

3.2.2.1 Animals

Male Flinders Resistant Line (FRL; \(n = 24\)) and Flinders Sensitive Line (FSL; \(n = 24\)) rats were bred and housed at the Animal Research Centre (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-Clinical Drug Development Platform (PCDDP), North-West University. The original rat colony was obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA.

All experiments were approved by the AnimCare Animal Research Ethics Committee (NHREC reg. no. AREC-130913-015) of the North-West University (ethics approval no. NWU-00283-17-A5). Considering the primary aim of this investigation, experiments were only preformed in male FRL and FSL rats. Female rats were excluded to avoid hormonal influences of the oestrous cycle on test outcomes, to ensure a more homogenous sample in terms of behavioural performance as well as to avoid the inclusion of excessive animal numbers. The animals were weaned on post-natal day 21 (PND21) and divided into their respective cages \((n = 3 \pm 1)\). Cage density variation was not expected to influence behaviour (Armario et al., 1984; Bean et al., 2008; Latane et al., 1970) as all animals were at least pair housed (Stevens et al., 1997). Animals remained housed as such in identical polypropylene cages (380mm x 380mm x 230mm), with free access to food and tap water. Corncob bedding was replaced on a weekly basis. A 12:12 hour light-dark cycle (lights on 06:00 to 18:00) was maintained along with controlled positive air pressure at an air change rate (ACR) of 18 times/hour (HVAC system using HEPA filters), temperature (21 ± 2°C) and humidity (± 55%). They remained housed as such from PND21 until the conclusion of all the behavioural experiments on PND60. To simulate a 14-day period of injection stress which normally forms part of experiments in this laboratory (Badenhorst et al., 2017; Schoeman et al., 2017; Steyn et al., 2018), rats were weighed and injected subcutaneously with 0.9% saline (0.1ml/kg) daily from PND46 to PND59. Behavioural testing was performed on PND60 with all subjects having been euthanized on PND61. The health and general well-being of the animals were continually monitored throughout the study using daily monitoring sheets.
3.2.2.2 Pre-test habituation protocols

FRL and FSL rats were randomly divided into a 0-min or a 60-min pre-test habituation experimental group (n = 12 rats per group per behavioural cohort), respectively. All animals were handled daily during injection procedures from PND46 – PND59 (lifted by placing the hand of the researcher around the trunk of the animal while holding on lightly, i.e. not applying excessive force, for 3 – 5 seconds). On PND60, animals were transported in their home cages on a trolley from the holding room and placed on a workbench in the first testing room, i.e. the room in which the nORT and the OFT have been conducted. The location of the bench in terms of spatial orientation remained the same for all groups with no differences in temperature, lighting or sound being incorporated. Then, depending on the habituation protocol followed (see below), behavioural testing commenced either immediately or after 60 minutes following the relocation of the home cages from the trolley to the workbench. Unlimited access to food and water was maintained throughout the 60-min period. Importantly, the nORT and OFT were done in the same arenas under dim red light (40 lux), while the FST was conducted under bright white light (300 lux) in an adjacent room. The following procedures have been followed for the respective habituation protocols.

- Animals in the 0-min group were, following relocation of the home cages to the workbench, immediately assessed in the nORT. Following the completion of the nORT, they were relocated to their home cages, allowed to remain there only for the time that was necessary to clean and reset the nORT setup for use in the OFT, and promptly reintroduced to the testing arenas; each to the same testing cage as was used for the nORT. Following the completion of the OFT, they were once again reintroduced to their home cages, placed on the trolley and transferred to the adjacent room in which the FST has been conducted. Here, they were immediately introduced to the swim cylinders.

- Animals in the 60-min group were allowed to remain in the home cages for 60 minutes following the relocation of the home cages to the workbench (therefore prior to the nORT) and again before the OFT. Further, following the same transport procedures as described above, they were again allowed to remain in the home cages on the trolley in the adjacent FST room, before being introduced to the swim cylinders.
3.2.3 Behavioural Tests

As rodents are nocturnal animals, all procedures, including the transport and relocation of animals from their holding room, commenced only 60 minutes after the onset of the dark cycle to allow for initial foraging. Behaviour in the nORT and OFT were digitally recorded and analysed with Noldus Ethovision XT14 software (Noldus\textsuperscript{®} Information Technologies, Wageningen, The Netherlands. The FST was digitally recorded with immobility, swimming and struggling (or climbing) manually scored with previously validated (Badenhorst et al., 2017) continuous timer software (FST Scoreboard, version 2.0; NWU).

3.2.3.1 Novel Object Recognition Test (nORT)

The nORT measures declarative memory bias and is based on the natural exploratory instinct of rodents to explore novel objects without prior preconditioning (Ennaceur & Delacour, 1988). The setup used in the current investigation consisted of a roofless open field square box constructed from black opaque Plexiglas\textsuperscript{®} (100cm x 100cm x 50cm). No food or bedding was placed in the testing arenas. The test comprised three phases. As novel object recognition as highlighted under normal behavioural circumstances has been the focus of this assessment, all phases of the nORT was executed under dim red light (40 lux) to prevent the possible anxiogenic effects of white light exposure on exploration bias (Chaouloff et al., 1997). The testing phases consisted of 1) habituation (rats were allowed to explore the arena in the absence of any object for 10 minutes on PND59, 24 hours prior to phases 2 and 3; no habituation protocol was followed on this day — animals were immediately introduced to the testing arenas following relocation to the experimental room; 2) acquisition of object memory during a 5-min exploration session in the presence of two identical objects (objects A) that constituted the first behavioural procedure on PND60; and 3) a 5-min retention trial 90 minutes after the acquisition trial in which one of the familiar objects (A) was replaced with a novel object (B) to measure memory bias and novel object recognition. All animals were left in their home cages during the 90-min interval that separated the acquisition and retention trials. Therefore, with respect to the nORT, the different habituation protocols only applied with respect to the pre-acquisition trial period. The A- (familiar) objects were purple ceramic owls (5 x 5.5cm), while the B- (novel) objects were yellow rubber ducks (7.5 x 9.5cm). Following the completion of the third phase (retention trial), arenas were cleaned with normal saline solution and the pre-OFT habituation protocols detailed in paragraph 1.2.2 followed.

Object exploration was regarded as the time a subject spent (in seconds) to position its nose toward and within 5-cm of the object, as well as sniffing, touching, and ambulating in the proximity of the object (also within a 5-cm radius). Importantly, climbing and sitting on the object was not regarded as exploration (Antunes & Biala, 2012). Memory retention bias in terms of the familiar
object is represented as the *Discrimination Index* (DI), where the total time exploring the novel object in relation to the familiar object is divided by the total exploration time of both the novel and familiar object \( DI = \frac{(T_n - T_f)}{(T_n + T_f)} \). On a scale from -1 to +1, a negative value indicates more time spent exploring the familiar object, while a positive value indicates more time spent exploring the novel object. A value of zero or near to zero indicates no particular preference for either object (Ennaceur & Delacour, 1988).

### 3.2.3.2 Open Field Test (OFT)

The OFT, when conducted following habituation in the test arena as applied in this investigation, is used to measure general locomotor activity (Prut & Belzung, 2003). To control for the possible coincidental effects of drug administration, albeit not introduced in this investigation, on the general swimming ability of test subjects, knowledge pertaining to its inherent locomotor ability as measured by the total distance travelled in an open field arena, is a pivotal factor for consideration when evaluating FST data. The OFT therefore always precedes the FST. Briefly, rats were introduced in the centre of the arena at the beginning of the test, and left to explore for 5 minutes, also under dim red light (40 lux). After each session, arenas were cleaned with normal saline. The total distance travelled (cm) during the 5-min session was calculated and used as a broad index of locomotor ability. Importantly, due to the fact that all subjects were habituated to the test arenas prior to the first exposure in the nORT and OFT, we do not regard behaviours otherwise related to anxiety-related manifestations, i.e. time spent in the centre and corner zones, as an accurate measure of anxiety-related behaviour within the current framework. Indeed, robust anxiety-related measurements can, in our view — which is supported by others (Stanford, 2007) — only be made under non-habituated circumstances. Therefore, it was decided not to use the current open field results to draw conclusions with respect to anxiety-like behaviours.

### 3.2.3.3 Forced Swim Test (FST)

The FST, first conceptualised by Porsolt *et al.* (1977), is based on the anthropomorphic measuring of immobility in a forced, inescapable swim scenario as a behavioural correlate of despair which, if attenuated by antidepressant administration is regarded as a robust marker for depressive-like behaviour (Castagne *et al.*, 2010). Later, the FST has been modified and validated to be reflective also of struggling and swimming behaviour that have been shown to discriminate between noradrenergic and serotonergic involvement, respectively (Detke & Lucki, 1996; Detke *et al.*, 1995). In terms of the behaviour of the FSL rat in the FST, subjects are spontaneously significantly more immobile than their FRL controls without being subjected to pre-test swim stress 24 hours prior to the actual trial. The FSL rat has therefore been proposed as a model that not
only demonstrates a genetic predisposition for MDD, but in which the depressive phenotype is triggered by stressful circumstances (Overstreet & Wegener, 2013).

Briefly, the FST has been performed as previously described in our laboratory (Steyn et al., 2018). On the day of testing (PND60), the apparatus consisting of four identical cylinders (60cm x 25cm) was filled with water (25 ± 1 °C) up to a depth of 30 cm. Four rats were analysed simultaneously. Each swim session was conducted under white light during the dark cycle. Sessions were recorded by means of a digital video camera mounted across from and in-line with the vertical middle of the swim tanks. The behaviour during 5 minutes of swimming was scored manually from the recordings. Immobility was regarded as floating without active movement, except those movements needed to keep the head above the water line. Struggling was considered as escape-related upward-directed movements of the forepaws while swimming was regarded as the horizontal movements throughout the cylinder while also moving from one quadrant into another. Diving was not included in the analyses (Cryan et al., 2005).

Importantly, as FRL control animals are normally more active in the FST, they are usually subjected to a 15-min pre-test swim session 24 hours prior to actual FST (Overstreet et al., 2005). This is usually necessary to highlight the immobile trait in FRL animals during the FST which have not been shown to respond to antidepressant treatment and thereby sufficing as an appropriate non-depressive behavioural control that enables the researcher to distinguish between despair related and non-despair-related immobility. However, due to the fact that the current study explored the influence of pre-test habituation on test outcomes, it was decided not to expose FRL animals to a pre-swim, as this would have introduced an additional experimental confound in terms of study design. Further, the purpose of this investigation was related to test outcomes following different pre-test habituation protocols, and not to divulge antidepressant action.

3.2.4 Statistical Analysis

Group sizes were based on previous work done in our laboratory which achieved statistically significant results and factor interactions using similar statistical analyses (Schoeman et al., 2017; Steyn et al., 2018). Normality of distribution and homogeneity of variance was determined by the Shapiro-Wilk and Levene’s tests, respectively. Instances where these assumptions were not true are reported in the text. All data sets were analysed for outliers using the Grubb’s test, with significance set at $\alpha = 0.05$. Data points identified as outliers were not removed as data analyses results were comparable with/without the inclusion of outliers (data not shown). Thus, group sizes for all data sets are $n = 12$, with outliers indicated in the figure legends where applicable (Kilkenny et al., 2010; McGrath & Lilley, 2015). Univariate analyses were performed regardless of normality
of distribution and homogeneity of variance results, seeing as these analyses are considered to be insensitive to such variance (Maxwell & Delaney, 2003; Nimon, 2012).

Normal two-way analysis of variance (ANOVA) analyses were performed on behavioural parameters as measured in the nORT (DI) and OFT (distance moved) to determine if two-way interactions existed between strain (FSL and FRL) and pre-test habituation time (0-min and 60-min) (Laerd Statistics, 2015). Because FST data are dependent on the general locomotor ability of the test subjects, the measured parameters of the FST were analysed with a two-way ANCOVA (analysis of co-variance) applying the mean distance moved of the OFT as a co-variate. Regardless of whether statistically significant interactions existed, the main effects of pre-test habituation time and strain were analysed and followed up with pairwise comparisons of the treatment groups using the Bonferroni post-hoc test (Howell, 2009; Laerd Statistics, 2018). In all instances, a 5% confidence limit for error was accepted as statistically significant ($p \leq 0.05$) for all analyses and data is reported with a 95% confidence interval (CI) of the mean difference.

Further, all statistical analyses were followed up by the calculation of the Cohen’s $d$ value (with a 95 % CI of the effect magnitude), in order to establish the effect magnitude between different groups (Lakens, 2013). This is in line with statistical reporting guidelines (Cumming et al., 2007; Wilkinson, 1999) and indicates strong trends by ruling out Type I (false positive) or Type II (false negative) errors (Ellis, 2010), especially where assumptions of homogeneity of variance were not true (Nimon, 2012). Only large ($d \geq 0.8$) and very large ($d \geq 1.3$) effect sizes were accepted as significant (Cohen, 1988; Sullivan & Feinn, 2012).

IBM SPSS® Statistics version 25 was used for statistical analysis of the data while GraphPad Prism® version 8.0 was used for the graphical representation of the results.
3.2.5 Results

3.2.5.1 nORT

![Graph showing discrimination index of novel object exploration in FRL and FSL rats after 0-min or 60-min of pre-test habituation.](image)

Figure 2: Discrimination index of novel object exploration in FRL and FSL rats after 0-min or 60-min of pre-test habituation. \( n = 12 \) for all groups. Data represent mean ± SEM. 0-min: no pre-test habituation. 60-min: pre-test habituation of 60 minutes. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat.

Figure 2: No statistically significant two-way interaction was demonstrated between strain and pre-test habituation time \( (F_{1,44} = 0.34, p = 0.560) \), nor any statistically significant main effects of either strain or pre-test habituation time. As such, pairwise comparisons of the means did not reveal any significant differences in the discrimination index of either habituated or non-habituated animals, irrespective of strain.
3.2.5.2 OFT

Figure 3: The mean distance travelled in an open field by FRL and FSL rats after 0-min or 60-min of pre-test habituation. $n = 12$ for all groups. Data represent mean ± SEM. Statistical descriptors are reported in the text, with ** $p \leq 0.001$. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat.

Figure 3: Strain and pre-test habituation time interacted significantly with respect to the total distance travelled in the open field ($F_{1,44} = 10.91$, $p = 0.002$). Further, pairwise comparisons revealed a significant difference in distance moved between strains only following 60 minutes of pre-test habituation (FRL vs FSL: $3010.25 \pm 408.70$ cm vs. $1954.39 \pm 943.19$ cm; 95% CI, 214.75 to 1897.56 cm; $p = 0.0071$; $d = 1.52$).
3.2.5.3 FST

Figure 4: The effects of pre-test habituation on the FST behaviour of FRL and FSL rats. (A) time spent immobile; (B) time spent struggling$^a$ and (C) time spent swimming after 0-min or 60-min of pre-test habituation. Data represent the mean ± SEM. Statistical descriptors are reported in the text. $^{****} p \leq 0.0001$. $^a$Outlier identified for FSL rats at 0-min pre-test habituation time but included in analysis. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat.
Figure 4A: Although no significant two-way interaction between strain and habituation time has been demonstrated with respect to the mean time spent immobile ($F_{1,44} = 0.03$, $p = 0.873$), statistically significant main effects were demonstrated for both strain ($F_{1,44} = 160.47$, $p \leq 0.0001$) and habituation protocol ($F_{1,44} = 6.54$, $p = 0.014$), taking into account that the total distance moved in the preceding open field was applied as a covariant to the data. FSL animals were more immobile than their FRL counterparts (95% CI, 94.36 to 130.09 s), irrespective of habituation protocol, while animals in the 0-min habituation group, were more immobile than those of the 60-min habituation group (95% CI, 4.80 to 40.52 s), irrespective of behavioural cohort. Further, post-hoc analysis revealed that in both the 0-min and 60-min groups, FSL rats were significantly more immobile compared to the FRL controls (0-min: $171.90 \pm 26.59$ s vs $61.10 \pm 42.13$ s; 95% CI, 76.19 to 145.43 s; $p \leq 0.0001$; $d = 3.29$; 60-min: $150.70 \pm 21.18$ s vs $37.02 \pm 28.94$ s; 95% CI, 79.04 to 148.27 s; $p \leq 0.0001$; $d = 4.68$).

Figure 4B: Again, no statistically significant two-way interaction between strain and habituation time has been demonstrated with respect to the mean time spent struggling ($F_{1,44} = 0.25$, $p = 0.620$). However, here, a statistically significant main effect of strain was identified ($F_{1,44} = 87.84$, $p \leq 0.0001$). Indeed, FSL animals in both the 0-min and 60-min groups spent significantly less time struggling, compared to their respective FRL control subjects (0-min: $41.81 \pm 20.13$ vs. 177.10 $\pm 67.05$ s; 95% CI, 81.74 to 188.84 s; $p \leq 0.0001$; $d = 0.86$; 60-min: $63.62 \pm 17.30$ vs. 185.24 $\pm 61.78$ s; 95% CI, 68.10 to 175.17 s; $p \leq 0.0001$, $d = 2.80$), also after controlling for the total distance moved in the preceding open field.

Figure 4C: In the case of swimming behaviour, neither a statistically significant two-way interaction between strain and habituation time ($F_{1,44} = 1.07$, $p = 0.306$), nor a simple main effect of either strain or habituation time, was identified.

3.2.6 Discussion

The main findings of the current investigation that studied the effect of different pre-test habituation protocols on test outcomes in a sequential battery of behavioural and cognitive tests, i.e. the nORT, OFT, and FST, can be summarized as follows: 1) declarative memory processing in the FRL and FSL strains seems to be insensitive to the effects of pre-test habituation, 2) pre-test habituation differentially affects activity of FRL and FSL rats in the OFT, with 60 minutes habituation rendering FRL rats significantly more active than FSL rats, but with no differences between FRL and FSL rats in non-habituated animals, 3) pre-test habituation had a significantly abrogating effect on the time spent immobile in the FST in both the FRL and FSL strains, and 4) compared to FRL animals, FSL rats present with marked depressive-like behaviour as evinced by their increased time spent immobile, irrespective of the habituation protocol followed.
Neuropsychiatric studies using animals are complex and time-consuming and usually necessitates the use of large numbers of animals allocated to different experimental groups to control for inter-individual differences and miscellaneous factors that may affect test outcomes, e.g. test-related anxiety. Further, several factors that are incidentally incorporated into behavioural study design, i.e. pre-test in-house transportation, human handling prior to and during experiments, and between-test cleaning of apparatus, may also alter the performance of animals during testing (Hurst & West, 2010). As such, within- and between laboratory findings from a singular test performed within the same experimental framework, may yield incomparable results not because of experimental differences per se, but as a result of differences in the abovementioned factors before or during the execution of such tests. Therefore, in an attempt to reduce the time spent on animal experimentation and to negate the risk of between-day and between-researcher variance, we have recently investigated the potential usefulness of combining a sequence of behavioural assessments in one animal over the course of a single day (Mokoena et al., 2015). However, one often neglected factor for consideration is handling-dependent changes in the baseline pre-test stress- and anxiety level of the test subjects (Gouveia & Hurst, 2017; Sousa et al., 2006). Indeed, especially with respect to the FSL rat, which was initially bred to demonstrate cholinergic super-sensitivity, a neurobiological correlate of reduced stress tolerance (Markou et al., 1994; Pucilowski et al., 1990; Pucilowski & Overstreet, 1993), such potential confounds need careful consideration. As such, the current investigation explored the effect of two different pre-test habituation protocols on behavioural test outcomes in a sequence of behavioural assessments as often applied in FRL and FSL rats, viz. 1) the nORT, 2), the OFT and 3) the FST, where one group of animals was subjected to testing in each of the tests in immediate succession (0-min), while the other group were left to habituate in their home cages in the rooms of investigation for 60 minutes preceding each test. The sequence in which such test batteries is devised, is based on previous tests performed in our laboratory, beginning with the nORT and OFT, and ending with the stressful FST (Connor et al., 1997; Mokoena et al., 2015).

In the nORT (Figure 2), we found no demonstrable differences in the object memory of FRL and FSL rats, or between animals of the same strain that were subjected to the different habituation protocols. Concerning the analogous performance of FRL and FSL animals in the nORT, irrespective of habituation protocol, our findings are in line with literature in as far as ambiguous performance of the FSL strain in the nORT is concerned. In fact, while some investigations demonstrated superior declarative memory performance in treatment-naive FRL compared to FSL rats (Abildgaard et al., 2011), other investigations only report reduced novel object exploration in following significant external intervention (Kosari et al., 2012; Mokoena et al., 2015). In other words, while stress-sensitive FSL rats may potentially present with declarative memory
impairment following external intervention, i.e. diet alteration (Kosari et al., 2012), we found no evidence to suggest that, as a strain, they perform worse than experimentally naive FRL rats. This is in line with previous conclusions (Overstreet et al., 2005; Overstreet & Wegener, 2013).

In the current study, treatment-naïve FSL rats displayed similar locomotor activity in the OFT, to that of FRL controls when a 0-min pre-test habituation time model was implemented (Figure 3). However, following 60-min pre-test habituation, FSL rats displayed significantly reduced general locomotor activity compared to FRL rats. Importantly, animals of both rat strains, irrespective of habituation protocol, were habituated in the open field arenas the day prior to testing. Therefore, as novelty-induced anxiety and escape-related movement can arguably be ruled out, our findings would therefore indicate that FSL animals present with an exploration drive akin to that of FRL animals, but that such behaviour abates over time compared to the consistent locomotion of their FRL counterparts. That the locomotor activity of FRL animals did not decrease over time, also imply that as pre-test to the FST, both FRL and FSL animals should be assessed in terms of general motor ability immediately, instead of following a habituation phase. Indeed, if the latter approach is followed, the data presented here indicates that the time spent being immobile by FSL rats in the FST, may be misinterpreted based on findings from the OFT measuring locomotor activity, and not psychomotor activity as intended by the FST. Moreover, inherent locomotor ability is rarely interpreted by itself and is in general regarded as a covariate in the analysis of findings related to tests which may be dependent on adequate locomotor capability, e.g. the FST.

Our findings with respect to the FST are not only congruent with literature (Overstreet et al., 1995; Overstreet, 2002; Overstreet et al., 2005; Overstreet et al., 1986; Overstreet et al., 2004), but also highlight key aspects of the methodological approach. First, while FSL animals spent more time being immobile (Figure 4A) and less time struggling (Figure 4B), animals of both behavioural cohorts seemed more sensitive to a non-habituated scenario. Irrespective of strain, all animals in the current investigation were significantly more immobile after being introduced to the FST immediately following transportation, compared to their 60-min habituated conspecifics. These findings held true, even after controlling for the significant difference observed in the locomotor activity of FRL and FSL rats after 60 minutes of habituation. Interestingly, the same trend was not demonstrated in terms of struggling (Figure 4B), in which case, based on the immobility data, it could have been expected that animals in the 0-min groups, irrespective of strain, would have demonstrated reduced struggling behaviour. However, no significant difference in this regard has been identified. Here, instead of habituation time playing a role as it did in the immobility scores, the effect of strain was dominant in that FSL animals struggled less than FRL animals, irrespective of habituation protocol. While we can only speculate as to what the reasons for this observation may be, it suffices to say that in terms of highlighting depressive-like behaviour in the FST,
animals should be subjected to the swim chambers immediately after being transported. In fact, it is possible that transportation stress may contribute to the magnitude of stress-sensitive depressive-like behaviour in the FSL rat. Although significant differences with respect to the immobility scores of FRL and FSL animals were demonstrated at both 0 and 60 minutes of habituation, it must also be interpreted in terms of the fact that FRL animals were not subjected to a pre-FST swim stressor in this investigation. This is generally applied to highlight a treatment-insensitive immobile trait, or in other words, an immobility picture reflective of coping strategies, rather than a depressive-like phenotype. That FSL animals do not have to be subjected to a pre-FST swim stressor to demonstrate a depressive-like phenotype in the FST (du Jardin et al., 2016), would also be supportive of the current view that in order to fully highlight and elucidate antidepressant action in the FST, both FRL—after being subjected to a pre-FST swim stressor—and FSL animals, should be introduced to swim chambers without prior habituation.

With respect to the significant difference shown between the struggling behaviour of FSL compared to FRL animals, while swimming behaviour was near similar, irrespective of the habituation protocol implemented, we can only speculate that FRL and FSL animals present with unique underlying noradrenergic, but not serotonergic, stress-coping mechanisms (see paragraph 3.2.3.3). This finding is especially interesting as the FSL strain has previously been shown to be hyper-responsive in terms of NA secretion under stressful circumstances compared to FRL controls (Landau et al., 2015). Further, FSL rats demonstrate reduced limbic serotonin synthesis compared to FRL rats (Hasegawa et al., 2006). It can only be speculated that instead of directly translating struggling and swimming behaviour to noradrenergic and serotonergic release per se, behaviours in the FST as elicited under treatment-naive circumstances, are more reflective of different noradrenalin-dependent coping strategies in the two strains, rather than being representative of serotonergic involvement. Although both noradrenalin and serotonin reuptake inhibitors are effective in improving depressive-like behaviour in the FST (Overstreet & Wegener, 2013), such responses can arguably be related to a supraphysiological increase in said neurotransmitter systems that may exert a unique effect in the neurobiology of treated rats, compared to their regulatory involvement under treatment-naive circumstances. Nevertheless, the findings reported here, confirms the FSL genetic rat line as a valid and useful framework in which to elucidate the etiopathology of and novel treatments for MDD.
3.2.7 Conclusion

In conclusion, this work demonstrated that pre-test habituation may have a significant impact on several key outcomes of behavioural assessments. We showed that while the treatment-naive cognitive performance of the FRL and FSL rat lines as assessed in the nORT is similar, habituation before the onset of the nORT will neither improve, nor exacerbate the declarative memory recall of either line. However, we also demonstrated that FSL, but not FRL animals become less active in an open field if they have been habituated in the testing room before the onset of experiments. Furthermore, depression-related scores in the FST were found to be more robust if animals are introduced to the swim chambers immediately following their relocation there. In order to prevent interpreting the exacerbated depressive-like behaviour of both strains in the FST as being potentially negative, albeit falsely so, subjection of both strains to the OFT and the FST without prior habituation, allows for more robust results. Further, our data are congruent with literature in supporting the FSL rat line as a valid, useful and robust animal model of MDD.

3.2.8 Acknowledgements

This work was financially supported by Prof BH Harvey from personal academic reserve funds.
3.2.9 References


Lakens, D. 2013. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. Frontiers in psychology, 4:863.


CHAPTER 4: SUMMARY, CONCLUSION AND RECOMMENDATIONS

4.1 Summary and conclusion

Animal experimentation with regards to neuropsychiatric studies is complex and time-consuming and usually necessitates the use of large numbers of animals allocated to different experimental groups to control for the possible coincidental effects of miscellaneous aspects on test outcomes, e.g. preceding behavioural test-related anxiety. Further, several factors that are incidentally incorporated into behavioural study design, i.e. pre-test in-house transportation, human handling prior to and during experiments, and between-test cleaning of apparatus, may also alter the performance of animals during testing (Hurst & West, 2010).

As such, the present investigation originally had two broad objectives specific to the conditions and needs of our laboratory. A) to study the effect of pre-test habituation on the performance of test subjects in a sequence of cognitive and behavioural tests performed consecutively on a single session during the dark cycle in the same test subjects, i.e. the novel object recognition test (nORT), the open field test (OFT) and the forced swim test (FST), and then to assess this in both treatment-naive animals and post-treatment (imipramine, 10 mg/kg/day x 14 days), and B) to study the influence of pre-test habituation on the performance of animals if the same tests as applied in (A) were to be performed only one per day on successive days, albeit also in both treatment conditions. Due to a number of practical constraints, only objective A could have been completed (Chapter 3 and Annexure A), whereas the data for objective B were incomplete and the results therefore inconclusive (Annexure B). Hence, the bulk of the conclusions in this chapter will focus on objective A, i.e. the work disseminated in Chapter 3 and Annexure A, while the work presented in Annexure B, will briefly be summarized in paragraph 4.2, with shortcomings pointed out and recommendations be made for prospective studies.

* * *

The current investigation concerning the possible effects of pre-test habituation on the test performance of Flinders Resistant Line (FRL) and Flinders Sensitive Line (FSL) rats in a sequential battery of three behavioural tests that are commonly applied in our laboratory, has provided valuable insight into the importance of the pre-test environment, in particular the role of habituation following transportation, on the behaviour of test subjects. While specific behavioural tests — in the case of the present work, the nORT, OFT and FST — are often applied to measure the same test-specific neuropsychiatric constructs, within- and between-laboratory results and conclusions can only be compared and drawn if near analogous experimental conditions are applied, highlighting the importance of methodological congruence. In this study we have shown
that a common, seemingly insignificant laboratory practice, namely habituation following transportation, can influence the behaviour of laboratory animals in subsequent testing and that careful consideration of these should be incorporated into the study design and laboratory protocols to ensure reliability of within- and between-laboratory comparisons.

Whereas the main results from the behavioural tests performed on male FRL and FSL rats that were subjected to two different pre-test habituation protocols (0-min or 60-min) before and during the execution of a sequential battery of behavioural tests are summarised in Table 4-1, the following main findings have been made:

1. FSL rats displayed similar declarative memory to their FRL counterparts, irrespective of the pre-test habituation protocol employed. Despite the lack of strain difference between the FRL and FSL rats, the different pre-test habituation time protocols also had no significant effect on declarative memory within each strain (Chapter 3).

2. Differences in locomotor activity between FRL and FSL rats were only evinced after 60-min of pre-test habituation, where FRL rats demonstrated significantly higher locomotor ability (Chapter 3).

3. In the absence of prior habituation (0-min), both the FRL and FSL strains displayed significantly increased depressive-like behaviour, compared to their habituated within-strain counterparts (60-min) (Chapter 3).

4. Compared to the FRL strain, FSL rats displayed increased depressive-like behaviour and decreased active escape-related behaviour in the FST. These results corroborate the face-validity of the FSL rat (Overstreet et al., 2005; Overstreet & Wegener, 2013) (Chapter 3).

5. Imipramine failed to elicit an anti-depressant-like response in FSL rats and induced a depressive-like phenotype in FRL rats (Annexure A). As these results are not congruent with literature, it will be subject to further investigation.
Table 4-1: Summary of the treatment-naive results obtained for declarative memory (nORT), locomotion (OFT) and depressive-like behaviour (FST) in FRL and FSL rats at that were either not habituated prior to testing (0-min) or habituated for 60 minutes before the onset of behavioural assessment (Chapter 3). ↔: no significant difference, ↑: significantly increased, ↓: significantly decreased, compared to the indicated control.

<table>
<thead>
<tr>
<th>Behavioural Test</th>
<th>60-min (FSL vs FRL)</th>
<th>0-min (FSL vs FRL)</th>
<th>0-min FRL (vs 60-min FRL)</th>
<th>0-min FSL (vs 60-min FSL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nORT (declarative memory)</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>OFT (locomotor activity)</td>
<td>↓</td>
<td>↔</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>FST Immobility (depressive-like behaviour)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Struggling</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Swimming</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
</tbody>
</table>

**The two pre-test habituation protocols employed in this investigation had a significant impact on the performance of both FRL and FSL animals in the OFT and FST, but not in the nORT. In terms of general locomotor activity, FRL and FSL animals demonstrated opposite trends in terms of their response in the OFT. More specifically, following 60 minutes of habituation in the OFT, FRL animals presented with a higher degree of locomotion, while FSL animals showed a reduced level of activity. In this regard, it would be valuable that future investigations apply the locomotor behaviour of non-habituated FRL and FSL rats as a covariate in the analysis of FST data, as the locomotor behaviour of both strains after having been allowed to habituate before assessment in the OFT, may result in the misinterpretation of findings in the FST. Indeed, a lack of depressive-like response in FSL rats subjected to the FST, may be interpreted as a false negative, if compared with findings from the OFT after 60 minutes of habituation.**

**This work also confirmed the FSL rat as a robust and reliable genetic animal model of depressive-like behaviour that is founded in robust face validity as evinced by the characteristic state of behavioural despair in the FST, compared to their ‘non-diseased’ FRL counterparts. Moreover, the characteristic depressive-like behaviour of FSL rats persisted when the pre-test conditions were modified by the two habituation protocols investigated in this study. That said, both strains were indeed more sensitive to a non-habituated circumstance, displaying a greater degree of depressive-like behaviour when tested directly after transportation to the FST testing room as opposed to only being tested after a 60-min habituation period. Moreover, active escape-directed**
behaviours (i.e. struggling and swimming) did not demonstrate similar trends as seen in the depressive-like behaviour.

This work found no indication of cognitive impairment related to declarative memory in FSL compared to FRL control animals. Although our findings are in-line with initial reports indicating no definitive evidence of cognitive disturbances at baseline compared to FRL controls (Bushnell et al., 1995; Overstreet et al., 2005), more recent evidence indicated both declarative- (Gomez-Galan et al., 2013) and emotional memory deficits (Eriksson et al., 2012) in FSL rats compared to Sprague Dawley (SD) and FRL controls, respectively. We therefore postulate that the lack of congruence on the declarative memory function of the FSL rat at baseline may be attributed, amongst others, to differences in test procedures, viz. behavioural or pharmacological manipulation prior to testing (Gómez-Galán et al., 2013) or scoring methodology (Abildgaard et al., 2011) and the selection of appropriate behavioural controls. Nonetheless, the current investigation demonstrated that the inherent cognitive ability of the FRL and FSL strains are not influenced by pre-test habituation circumstances and that such animals may in future be assessed directly following in-house transportation.

In conclusion, the present study highlighted the important effect of laboratory environment and pre-test conditions in pre-clinical neuropsychiatric research by demonstrating that common laboratory practice such as in-house transportation, handling of test subjects prior and during experimentation and pre-test habituation significantly influences behavioural test outcomes in some, but not all, tests. In this regard we showed that while the treatment-naive cognitive performance of the FRL and FSL rat lines as assessed in the nORT is similar and insensitive to the effects of in-house transportation, FSL, but not FRL animals become less active in an open field if they have been habituated in the testing room before the onset of experiments. Further, depression-related scores in the FST were found to be more robust if animals were introduced to the swim chambers immediately following relocation. Therefore, collectively viewed, we argue that in order not to misinterpret the behaviour of FSL animals in the FST based on findings from the OFT, albeit falsely so, both FRL and FSL animals should be subjected to both the OFT and the FST without prior habituation.
4.2 Limitations and future directions

Although the current investigation has highlighted a number of valuable and important aspects of the FRL and FSL rat, numerous shortcomings and questions arose from the current data that should be addressed in future studies. In short, they can be summarised as follows:

First, this work failed to display the predictive validity of the FSL rat in the FST (Annexure A). Imipramine (IMI) administration did not attenuate the depressive-like behaviour (i.e. time spent immobile) in the FST in FSL rats and even exacerbate the immobility of FRL rats in the FST. Our findings are perplexing as numerous previous reports, not only from our own laboratory, have shown a significant reduction in immobility at IMI doses similar to the dose used in this study (Brand & Harvey, 2017a; Brand & Harvey, 2017b; Fenton et al., 2015; Wainwright et al., 2016; Wróbel et al., 2014). While the exact reasons for the findings presented here remains highly speculative, we hypothesize that while the FSL model held true with respect to its face resemblance of depressive-like behaviour, methodological confounds may possibly be to blame. Indeed, it is likely that 1) inflammation induced by the adverse skin reactions observed with IMI administration may have contributed to the findings presented here as the absorption of IMI might have been altered by the tissue condition of the skin (Ballard, 1968); 2) injection stress associated with IMI specifically might have been severe enough to counteract the positive pharmacological effect of IMI; or 3) as the drug was not re-tested after its release in 2013, gradual deterioration might have influenced its efficacy and integrity. As a result, the data from the positive IMI control group may not be credible, and as such, no reliable conclusions can be made in this regard. Hence, these findings are presented in Annexure A and must be subjected to future investigation.

* * *

Second, the behavioural effects observed in this investigation are solely based on the inherent phenotypical expression of FRL and FSL rats subjected to assessment without it being correlated with potential markers of neurochemical change. Again, while practical and time-related constraints prevented such analyses, the lack of specifically monoaminergic analysis in blood or brain tissue may be regarded as a considerable limitation as it prevents robust conclusive delineation of the mechanisms specifically underlying swimming and struggling behaviour in the FST (Cryan et al., 2005; Detke & Lucki, 1996; Detke et al., 1995; Lucki, 1997).

* * *

Third, excluding female counterparts in this investigation is a significant limitation. Traditionally, behavioural pharmacology relies on validation studies that only employ male subjects. This due to concerns about the influence of hormonal fluctuations during the female oestrus cycle (Simpson
et al., 2012) on behavioural test outcomes. As the female oestrus cycle have been shown to substantially influence assessment outcomes, the addition of female cohorts would have dramatically increased the number of animals used which is currently limited by the strict ethical framework within pre-clinical animal research. Even though the investigation into the effect of pre-test habituation in female FRL and FSL rats is an interesting avenue for future investigations, the investigation into gender difference does not fall within the primary aim of this investigation, which was to provide insight into the pre-test conditions in our laboratory.

* * *

Fourth and last, that we could not complete phase B of this investigation (see Annexure B; assessment of FRL and FSL rats in a single test per day, subjected to both habituation protocols and treated with both placebo and IMI), is unfortunate. Although the sequential test paradigm is often used in our laboratory (Mokoena et al., 2015; Mouton et al., 2016), projects also employ designs where only a single test is executed on a specific day (Moller et al., 2013; Oberholzer et al., 2018). Completion of this phase of the investigation would potentially have highlighted important aspects for consideration to be taken into account when methodologies are designed.

* * *

The current investigation provided valuable insight into the coincidental effect of pre-test circumstance on behavioural test performance of FRL and FSL rats in our laboratory, and as a result lead us to the conclusion that these rats should not be habituated prior to nORT, OFT and FST. Expanding this work to normal rat strains i.e. Sprague Dawley rats or even other rodents such as mice, may be an interesting avenue to explore for future investigations, adding to the body of research needed in the attempt to standardise and refine our experimental procedures, ultimately facilitating robust and reliable within- and between laboratory comparisons.


Frankowska, M., Golda, A., Wydra, K., Gruca, P., Papp, M. & Filip, M. 2010. Effects of imipramine or GABA B receptor ligands on the immobility, swimming and climbing in the forced swim test in


Lucki, I. 1997. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behavioural pharmacology*.


ANNEXURE A: ADDITIONAL RESULTS

A.1 Introduction

The primary objective of the work presented in this dissertation was to investigate the possible effects of pre-test habituation on outcomes following the assessment of Flinders Resistant Line (FRL) and Flinders Sensitive Line (FSL) rats in a sequence of behavioural tests as commonly applied in our own laboratory, viz. 1) the novel object recognition test (nORT), 2) the open field test (OFT), and 3) the forced swim test (FST) (Chapter 3). However, we also aimed to investigate this question within the same experimental paradigm, but in FRL and FSL rats pre-treated for 14-days with imipramine (10 mg/kg/day), a tricyclic antidepressant commonly applied as a positive control in the genetic FSL model of major depressive disorder (MDD). As such, this data was originally intended for inclusion in Chapter 3; however, as will be explained below, imipramine treatment failed to elicit the same, well-known and widely published (Castagne et al., 2010; Chen et al., 2010; Gillman, 2007; Overstreet et al., 1995; Tillmann et al., 2019) anti-depressant-like response within the context of the current investigation. Therefore, while these findings will have to be expounded and investigated in future investigations, it could for understandable reasons not be included in the main manuscript (Chapter 3). Nevertheless, this annexure will provide a brief summary of the methods followed, results obtained and a discussion of findings within the context of this investigation and in light of available literature.

A.2 Materials and Methods

With respect to the methods pertaining to this annexure, the reader is referred to the ‘Materials and Methods’ section of Chapter 3. While all procedures have been carried out exactly as has been reported in Chapter 3, this phase of the study involved two separate groups of FRL and FSL rats, respectively (n = 12) per group. However, instead of being injected with normal saline from PND46 – PND59, animals included in these groups, received imipramine (10 mg/kg/day) every day from PND 46 – PND59 injected subcutaneously at a volume of 1 ml/kg (Brand & Harvey, 2017a; Fenton et al., 2015; Wainwright et al., 2016; Wróbel et al., 2014).

A.2.1 Statistical Analysis

Due to treatment being introduced as third variable in the experimental analysis, the data presented here have been analysed by means of an ordinary three-way, instead of two-way, analysis of variance (ANOVA). All other statistical procedures, post-hoc tests and levels of significance have remained unchanged.
A.3 Results

A.3.1 Novel object recognition

Figure A-1: Discrimination index of novel object exploration in saline vs. imipramine-treated FRL and FSL rats after 0-min or 60-min of pre-test habituation. n = 12 for all groups. Data represent mean ± SEM. SAL: saline. IMI: imipramine. 0-min: no pre-test habituation. 60-min: pre-test habituation of 60 min. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat.

Figure A-1: No statistically significant three-way interaction existed between strain, treatment and time ($F_{1, 88} = 1.365, p = 0.246$), nor any significant two-way interactions.

A.3.2 OFT

Locomotor activity

The table below summarizes the distance moved in the OFT for the various experimental paradigms.
### Table A-1: Summary of the distance moved (cm) in the OFT by saline vs. imipramine-treated animals of both strains after 0-min or 60-min pre-test habituation. Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>0 min</th>
<th>60 min</th>
<th>Difference (0-min vs 60-min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distance moved (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRL</td>
<td>SAL 2348.16 ± 669.15</td>
<td>3010.52 ± 408.70</td>
<td>p = 0.006 (irrespective of treatment)</td>
</tr>
<tr>
<td></td>
<td>IMI 2099.66 ± 691.28</td>
<td>2719.84 ± 418.14</td>
<td></td>
</tr>
<tr>
<td>FSL</td>
<td>SAL 2714.53 ± 849.45</td>
<td>1954.36 ± 943.19</td>
<td>p = 0.013 (irrespective of treatment)</td>
</tr>
<tr>
<td></td>
<td>IMI 2425.32 ± 1035.07</td>
<td>2030.76 ± 1019.90</td>
<td></td>
</tr>
</tbody>
</table>

*Table A-1:* No statistically significant three-way interaction existed between strain, treatment and time (F₁,₈₈ = 0.40, p = 0.529) for mean distance moved in the OFT. However, a statistically significant two-way interaction existed between strain and pre-test habituation time (F₁,₈₈ = 14.28, p ≤ 0.0001), so that FSL rats, irrespective of treatment group, traveled, on average, 872.15 cm (95% CI, 419.52 to 2325.71 cm) less than FRL rats after 60-min of pre-test habituation. Furthermore, pairwise comparisons revealed that after a 60-min pre-test habituation time, FSL rats covered 577.36 cm (95% CI, 124.27 to 1030.45 cm) less than FSL rats that were not subjected to pre-test habituation (p = 0.013), whereas FRL rats were more mobile after a 60-min pre-test habituation period, compared to FRL rats not subjected to pre-test habituation (p = 0.006), irrespective of treatment.
B.3.2 The forced swim test

Figure A-2: The effects of pre-test habituation on the FST behaviour of saline- vs. imipramine-treated FRL and FSL rats. (A) time spent immobile; (B) time spent struggling and (C) time spent swimming after 0-min or 60-min of pre-test habituation. **** $p \leq 0.0001$. Data represent the mean ± SEM. SAL: saline. IMI: imipramine. FRL: Flinders Resistant Line rat. FSL: Flinders Sensitive Line rat.
Additional Results

Figure A-2A: No statistically significant three-way interaction between existed between strain, treatment and pre-test habituation time ($F_{1, 88} = 0.19, p = 0.663$) for mean immobility time in the FST. However, a statistically significant two-way interaction was identified between strain and treatment ($F_{1, 88} = 4.40, p = 0.039$), so that saline-treated FSL rats were 112.23 s (95% CI, 95.45 to 129.02 s) more immobile than saline-treated FRL rats ($p \leq 0.0001$), irrespective of pre-test habituation time. Conversely, imipramine-treated FSL rats were 87.17 s (95% CI, 70.39 to 103.95 s) more immobile than imipramine-treated FRL rats ($p \leq 0.0001$), irrespective of pre-test habituation time. Furthermore, imipramine-treated FRL rats also spent 34.13 s more (95% CI, 17.35 to 50.92 s) immobile when compared to saline-treated FRL rats ($p \leq 0.0005$), irrespective of pre-test habituation time.

Figure A-2B: Neither a significant three-way interaction between strain, treatment and pre-test habituation time ($F_{1, 88} = 0.01, p = 0.930$), nor any significant two-way interactions between any of the three variables existed for mean time spent struggling data in the FST. However, a statistically significant main effect of strain ($F_{1, 88} = 157.00, p \leq 0.0005$), putatively supports the immobility data. In this regard, FRL rats spent 112.1 s (95% CI, 94.3 to 129.9 s) more time struggling, in relation to FSL rats, irrespective of both treatment and pre-test habituation time. Importantly, this effect is only considered supportive and to be confirmed in prospective studies.

Figure A-2C: Also, with respect to the time spent swimming, no statistically significant three-way interaction between strain, treatment and pre-test habituation time ($F_{1, 88} = 0.02, p = 0.878$), nor any significant two-way interactions were identified.

A.4 Discussion

The main findings of this phase of the study, aimed at investigating the effect of pre-test habituation on the behavioural response of FRL and FSL rats in a sequence of behavioural tests in response to imipramine as a positive control, were:

1. Pre-test habituation did not affect declarative memory capacity in either FRL or FSL rats, irrespective of subjects being treated with imipramine or saline;

2. Imipramine treatment had no significant effect on general locomotor activity. Still, pre-test habituation protocol differences significantly altered the locomotor activity of FSL and FRL rats differently. Overall FSL rats display reduced locomotor activity when compared to FRL rats, following a 60-min pre-test habituation protocol.
Additional Results

3. Pre-test habituation protocol differences had no effect on depressive-like behaviour in either FSL or FRL rats. Imipramine failed to elicit an anti-depressant-like response in FSL animals, and while the FRL vs FSL behaviour in saline treated rats is congruent with literature, the introduction of imipramine as a positive control, was not successful. In fact, imipramine seemed to have induced a higher degree of depressive-like behaviour in the non-diseased FRL animals, while not affecting the behaviour of the depressive-like FSL animals.

As alluded to before, the current investigation explored the effect of two different pre-test habituation protocols on behavioural test outcomes in a sequence of behavioural assessments as often applied in FRL and FSL rats, viz. 1) the nORT, 2), the OFT and 3) the FST, where one group of animals was subjected to testing in each of the tests in immediate succession (0-min), while the other group were left to habituate in their home cages in the rooms of investigation for 60 minutes prior to each test (Chapter 3). However, we also attempted to investigate this study question in FRL and FSL rats treated with imipramine, a conventional monoaminergic tricyclic antidepressant often used as a positive control in pre-clinical rodent research, most notably so also in the FSL genetic model of MDD. Indeed, its efficacy has been well documented (Arroll et al., 2016; Brand & Harvey, 2017a; Brand & Harvey, 2017b; Castagne et al., 2010; Oberholzer et al., 2018; Slattery & Cryan, 2012; Tillmann et al., 2019; Zhao et al., 2015).

With respect to the nORT (Figure A-1), the findings presented here are in line with that reported in Chapter 3, i.e. that no differences in declarative memory between FRL and FSL rats were observed. However, although it seems that imipramine failed to elicit an adaptive response in the declarative memory of both rat strains, this must be considered with caution as imipramine also failed to elicit the expected, much-desired, and well-documented response in the FST. As such, the possibility of imipramine influencing the cognitive ability of FRL and FSL rats, cannot be excluded.

Again, the same trend as has been reported in Chapter 3, has been shown for imipramine-treated FRL and FSL animals; pre-test habituation seemed to increase the total distance travelled in FRL rats, while reducing it in FSL rats (Table A-1). Importantly, locomotor activity is generally introduced as a co-variate in the analysis of findings from other behavioural tests, e.g. the FST, and should therefore not be interpreted as a stand-alone parameter. Further, it must be reiterated that no clear reports exist that pertain to baseline differences in the general locomotor ability of FSL and FRL rats (Fischer et al., 2012; Strenn et al., 2015; Tillmann et al., 2017) — likely due to the effect of pre-test habituation time reported here. Again, imipramine treatment failed to elicit an effect on the general locomotor behaviour of FSL and FRL animals when compared to the effect of normal saline treatment. Although this would be regarded as the desired response when
analysing the effect of imipramine treatment on depressive-like behaviour in the FST, this result must again be interpreted with great consideration for reasons alluded to above.

With regards to the behaviour of FRL and FSL animals in the FST and the effect that imipramine had on coping behaviour, the following can be concluded. First, imipramine treatment elicited a significant depressive-like response in FRL animals. Second, imipramine failed to elicit an anti-depressant-like response in FSL animals, irrespective of the habituation protocol followed. Third, while the face validity of the genetic FSL model of MDD seems to hold true, our findings with respect to the response of both FRL and FSL animals to imipramine intervention clearly needs further investigation. In fact, imipramine was not expected to modulate the behaviour of FRL rats (Overstreet et al., 1995). Further, a significant cause for concern is that the model does not display the predictive validity as reported in other numerous studies (reviewed in (Overstreet et al., 2005; Overstreet & Wegener, 2013) and that it can therefore not be regarded as an accurate framework in which to assess anti-depressant action, albeit within the context of this investigation only. Hence, keeping the intent of the original larger investigation in mind, we could not apply the model to assess the effect of pre-test habituation on the behaviour of FRL and FSL animals subjected to different treatment protocols.

Although the exact cause for our findings reported here remains uncertain, a number of aspects that could have contributed to these results, may be considered for future elucidation. First, injection stress per se can most likely be excluded as a contributing factor as all animals in the current investigation were injected, either with normal saline or with imipramine. Although the behaviour displayed by imipramine-treated FSL rats in this study would under different circumstances have been reminiscent of treatment-resistant depressive-like behaviour previously investigated in our laboratory (Brand & Harvey, 2017a; Brand & Harvey, 2017b), this conclusion cannot be drawn as 1) the FSL rats were not subjected to severe stressors as has been reported previously, and 2) imipramine induced a depressive-like phenotype in the FRL control.

This leaves a more likely explanation for the data reported here. The lack of support for the predictive validity of the FSL model in this investigation must not be attributed to a lack of integrity of the model itself, but rather to possible experimental design flaws unknowingly introduced during the processes of drug preparation and administration. During the sub-chronic drug treatment regimen, both FRL and FSL rats that received IMI-injections presented with adverse skin reactions. More specifically, the area around the injection site formed a hard, red crust with black discolouration several days after the injection. No similar reports could be found from literature; however, the following explanations for this response can be weighed:
Additional Results

- On consultation with the supervising veterinarian, it was suggested that the likely cause of this reaction was skin cell necrosis due to either a local irritant effect of the chemicals used to constitute the imipramine solution, or an acidic pH of the injected solution, despite the solution having a pH within range of the allowed pH for subcutaneous injections (pH 4.5 to 8) (Waynforth & Flecknell, 1992; Diehl et al., 2001; Morton et al., 2001; Shimizu, 2004).

- The batch applied was released in 2013. Thus, at the time of the investigation, it was the 5th year following the release of the specific batch, which required its re-testing for efficacy. This has not been done. As such, it may be possible that the integrity of the compound may have been influenced during storage.

- We applied subcutaneous, as opposed to intraperitoneal injection. Administration of imipramine is usually performed via intraperitoneal injection (Abbasi-Maleki et al., 2017; Bagot et al., 2017; O’Leary et al., 2016; Srikumar et al., 2017; Zhao et al., 2015); however, subcutaneous imipramine injection has also been employed in our laboratory (Brand & Harvey, 2017a). It may be possible that changing the route of administration to the less stressful subcutaneous route allowed for adverse skin reaction not necessarily encountered with i.p. administration.

In this regard and following the observation of said skin reactions, a serious adverse event report was submitted to the AnimCare Research Ethics Committee. Consequently, an amendment to the initial injection protocol was made allowing for alternating injection sites for each injection, starting at the rear of the animal and gradually moving up the body with each successive injection. This did not seem to have the desired effect.

A.5 Conclusion

The present work failed to illustrate the predictive validity of the model as sub-chronic imipramine treatment failed to attenuate depressive-like behaviour of FSL rats in the FST and elicited a depressive-like response in FRL controls. Therefore, no concrete conclusions in terms of the influence of habituation on behavioural test outcomes in treated vs. non-treated FRL and FSL animals can be drawn from the findings. This clearly warrants further investigation. Nonetheless, despite the adverse behaviours observed in the imipramine-treatment animals, the different strains still displayed behaviours coherent with that expected from the model, signifying the face validity of the model.
ANNEXURE B: ADDITIONAL WORK BY THE CANDIDATE

Important: The work contained in Annexure B is to be viewed as data from identified unsuccessful experiments, or as additional deficient data to be expanded on in prospective studies and should not compromise the scientific integrity of the main study. Although included in the originally conceptualized investigation, this section of the study could, for a number of practical reasons, not be completed in full and can hence not be assessed with respect to scientific integrity. Further, while every effort has been made to execute this phase of the investigation in a scientifically appropriate manner, assessment of the necessary control groups has not been completed. As such, scientific conclusions cannot be drawn from the data reported here. That said, to provide the reader with a broader overview of the original intent, we include this work as a reference background. This phase of the study will be completed in follow-up investigations.

B.1 Introduction

With this study, we not only intended to investigate the influence of pre-test habituation on test outcomes with respect to sequential testing in a specific subject over the course of a single dark cycle in both treatment-naive (Chapter 3) and antidepressant (imipramine)-treated (Annexure A) FRL and FSL rats, but also in rats subjected to a single behavioural test session on a specific day, i.e. where either the novel object recognition test (nORT), open field test (OFT) or forced swim test (FST) was conducted on a specific day. Although the sequential test paradigm is often used in our laboratory (Mokoena et al., 2015; Mouton et al., 2016), projects also employ study designs in which only a single test is executed on a specific day (Moller et al., 2013; Oberholzer et al., 2018). Hence, the objective was also to explore the effect of pre-test habituation on the performance of FRL and FSL rats exposed to either only the nORT, OFT or FST. As alluded to earlier, our laboratory has previously shown that a sequence of behavioural tests performed on a single day on a single subject, does not affect test outcomes if performed in order of potential anxiogenic intensity beginning with the least anxiogenic, i.e. 1) the nORT, 2) the OFT and 3) the FST (Mokoena et al., 2015). However, caution is warranted in considering this a standardised paradigm as the aim of the referenced investigation was not to standardise the sequential test paradigm in our laboratory, but simply putting it forward as a potential realistic, and time-saving alternative. In this regard, the aim of the current investigation was to compare the effects of methodological manipulation, viz. pre-test habituation, on test outcomes when performed sequentially or as single tests, respectively. In Chapter 3 and Annexure A, pre-test habituation in a sequence of tests was explored in an animal model of major depressive disorder (MDD), i.e. the Flinders Sensitive Line (FSL) rat, a commonly applied animal model in our laboratory. In this annexure, the investigation into pre-test habituation in a single test paradigm is presented.
However, due to time and practical constraints, only FSL, and not FRL control animals also, could have been assessed by the time at which this dissertation had to be submitted for examination. Further, this phase of the investigation also did not involve assessments of novel object recognition. As such, the lack of including FRL control groups and the omission of the nORT, are regarded as significant constraints as the conclusions drawn from this experiment is limited in terms of comparative analyses against the test results presented in Chapter 3 and Annexure A. However, the findings reported in this annexure form a basis for future studies.

**B.2 Materials and Methods**

Apart from this experiment only including FSL animals, all methods and procedures, including both habituation protocols, have been performed as explained in Chapter 3. However, whereas the behavioural experiments described in Chapter 3 were performed sequentially in the same subject during a single dark cycle, the behavioural assessments described in this annexure has each been performed on separate, but successive days and only included the OFT and the FST. Therefore, rats were only transported to the respective testing rooms, and not to other test rooms also, on any specific assessment day.

**B 2.1 Statistical Analysis**

As strain was excluded as a variable, only pre-test habituation protocol and treatment was analysed as between-subject factors. The OFT data presented in this annexure have therefore been analysed by means of an ordinary two-way analysis of variance (ANOVA). The FST data have been analysed using two-way analysis of co-variance with the mean distance travelled by the respective groups in the OFT applied as co-variate. All other statistical procedures, post-hoc tests and levels of significance have remained unchanged.
B.3 Results

B.3.1 Open Field Test

Figure B-1: The mean distance travelled in an open field by FSL rats receiving normal saline after 0-min ($n = 12$) or 60-min ($n = 11$) of pre-test habituation compared to imipramine-treated rats after 0-min ($n = 12$) or 60-min ($n = 11$) of pre-test habituation, respectively. Data represent mean ± SEM. **SAL**: saline. **IMI**: imipramine. *Smaller group sizes due to lower than expected birth rate at the specific date.

*Figure B-1*: No statistically significant two-way interaction was demonstrated between pre-test habituation protocol and treatment ($F_{1,42} = 0.27$, $p = 0.607$) with respect to the mean distance travelled in the OFT. However, a statistically significant main effect of treatment was identified ($F_{1,42} = 5.97$, $p = 0.019$), so that imipramine treated male FSL rats travelled, on average, 467.51 cm (95% CI, 81.41 to 853.60 cm) less than their saline treated counterparts (3153.74 ± 102.57 vs 3625.56 ± 159.56 cm), irrespective of the pre-test habituation protocol followed.
B.3.2 Forced Swim Test

Figure B-2: The effects of pre-test habituation time and treatment on the FST behaviour of normal saline and imipramine treated FSL rats. (A) time spent immobile; (B) time spent struggling and (C) time spent swimming after 0-min or 60-min of pre-test habituation. Data represent the mean ± SEM. Statistical descriptors are reported in the text. 0-min groups $n = 12$; 60-min groups $n = 11$ (smaller sample number due to reduced birth rates at time of investigation.)
**Figure B2A:** No statistically significant two-way interaction was demonstrated between pre-test habituation protocol and treatment with respect to the time spent immobile in the FST ($F_{1,43} = 2.02, p = 0.1627$). Further, neither habituation protocol nor treatment exerted any significant main effect on the results presented here.

**Figure B2B:** Again, no statistically significant two-way interaction between pre-test habituation protocol and treatment was reported with respect to the time spent struggling in the FST ($F_{1,43} = 0.60, p = 0.4426$). However, pre-test habituation protocol had a significant main effect on struggling behaviour in the FST ($F_{1,43} = 6.58, p = 0.0139$). Specifically, male FSL rats that were not habituated in the testing room prior to the FST, spent, on average, 20.98 s (95% CI, 4.48 to 37.50 s) more struggling when compared to their habituated counterparts (77.89 ± 27.36 vs 57.09 ± 27.82 s), irrespective of treatment.

**Figure B2C:** Neither a significant two-way interaction between pre-test habituation protocol and treatment ($F_{1,43} = 1.03, p = 0.3161$), nor a significant main effect of either variable was identified for the swimming behaviour data reported here.

**B.4 Discussion**

The main findings of this reported study phase can be summarized as follows:

1) Imipramine-treated FSL rats moved significantly less than their saline-treated counterparts, irrespective of the habituation protocol followed.

2) Saline and imipramine treated FSL rats displayed comparable immobility in the FST, irrespective of the pre-test habituation protocol followed.

3) Struggling in the FST was only influenced by the pre-test habituation protocol, irrespective of treatment. Specifically, non-habituated FSL rats struggled significantly more compared to their habituated counterparts.

In an attempt to optimise behavioural testing of animals in neuropsychiatric research, test batteries have been developed in our own (Mokoena et al., 2015) as well as other laboratories (Crawley & Paylor, 1997; McIlwain et al., 2001; Paylor et al., 2006). This allows for several tests to be performed on the same test subjects on a single testing day. However, depending on the specific objectives of each study, several study designs apply single test exposures on successive days (Eriksson et al., 2012; Moller et al., 2013; Mouton et al., 2016; Wegener et al., 2012). However, the question remains whether in-house transportation as affected by different pre-test habituation protocols would have different effects when applied on single- or multiple tests per day. To this extent, the aim of this experiment was to explore the effect of pre-test habituation on behaviour in the OFT and FST as applied as single tests per day. While the original intent was
to compare all three of the behavioural assessments reported in Chapter 3 and Annexure A in single format and as a function of treatment also in this phase of the study, practical constraints prevented the completion of this work, which unfortunately nullifies comparative conclusions between the two separate phases of this work. As such, the data presented in Annexure B, suffice only as preliminary results that must be elaborated on in future investigations.

In brief, we found that locomotor activity of male FSL rats were significantly reduced by imipramine treatment, regardless of the pre-test habituation protocol employed (Figure B-1, main effect of treatment). This finding can possibly be ascribed to the negative results obtained with the imipramine treatment employed in the current investigation (see Annexure A) and must therefore be the subject of future investigation.

In the FST, depressive-like behaviour was not influenced by either the treatment or the pre-test habituation protocol (Figure B-2A). This finding is also congruent with the lack of an imipramine-induced anti-depressant-like effect in the FST, performed as part of the sequence of assessments, elaborated on in Annexure A. This result is therefore, given the data presented in Annexure A, not surprising, but warrants further investigation. With regards to struggling, the exclusion of habituation prior to testing resulted in increased struggling behaviour in both treatment cohorts (Figure B-2B, main effect of pre-test habituation). As this finding is the opposite of what was reported when the FST formed part of a larger sequence of tests, it will have to be appraised within the larger scope of findings that will be delivered once the complete project has come to a satisfactory close.

B.5 Conclusion

The work presented in this annexure warrants further investigation as it was confounded by incomplete execution and the use of imipramine that has not delivered the desired positive response in the FST. Indeed, the behaviour of saline-treated FSL animals as observed in single-test-per-day assessments of the OFT and FST still needs to be validated against FRL control animals.
ANNEXURE C: CONGRESS ABSTRACT AND CERTIFICATE

In this annexure the abstract presented at the First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018, is shown. Excerpts from this study were presented as follows:

**Pre-test habituation time for select behavioural tests in rats**

Johanna E Pienaar¹, Stephanus F. Steyn¹, Christiaan B Brink*

¹Centre of Excellence for Pharmaceutical Sciences, School of Pharmacy, Faculty of Health Sciences, North-West University, Potchefstroom, North West, South Africa

*Corresponding author: E-mail address: Tiaan.Brink@nwu.ac.za (C.B. Brink)

**Study Objectives:** Translational animal models are useful tools to study psychiatric disorders, including major depressive disorder (MDD). However, inter-laboratory variability and seemingly minor differences in methodology, including conditions for pre-test environmental habituation, is common. In the current study we investigated the impact of pre-test habituation time of 0 minutes versus the standard 60 minutes on rodents in a battery of behavioural tests following room transferal.

**Methods:** Flinders Sensitive Line (FSL – genetic animal model of depression) and Flinders Resistant line (FRL – normal behaviour) rats were transported in their home cages from the holding room to the experimental room and exposed to behavioural testing directly after transportation (0 min) or after a habituation time of 60 min. Behavioural testing was divided into two groups, 1) a single behavioural test or 2) a battery of behavioural tests including the novel object recognition test (NORT), open field test (OFT) and the forced swim test (FST). Radiotelemetry measurements were also recorded for 2 hours post-transportation for blood pressure, heart rate and body temperature.

**Results:** Pre-test habituation time had no influence on the immobility time (depressive-like behaviour) of FSL rats in both a single or battery test exposure. Struggling (noradrenergic neurotransmission), but not swimming (serotonergic neurotransmission), was increased at 0 min vs. 60 min in a single exposure but decreased in the battery testing. Neither anxiety-like behaviours in the OFT, nor novel object exploration in the NORT, were affected by habituation time. Mean blood pressure and body temperature did not significantly differ at 1 min vs. 60 min, but heart rate was increased at 1 min vs 60 min.

**Conclusion:** Acute stress evoked by transportation between rooms does not significantly influence physiological markers in addition to the majority of behaviour in subsequent testing, except for noradrenergic driven behaviours, thereby negating the need for pre-test habituation.
This serves to confirm that

Johanna Elizabeth Pienaar

attended the

First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018

at

Spier Conference Centre, Stellenbosch, and Cape Town, South Africa

Prof Helmuth Reuter
Chair: Organising committee