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CHARACTERIZATION OF BEHAVIORAL PROFILES FOR INBRED P AND NP AND CONGENIC P.NP AND NP.P RATS

For the degree of Master of Science

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CHARACTERIZATION OF BEHAVIORAL PROFILES FOR INBRED P AND NP AND CONGENIC P.NP AND NP.P RATS

A Thesis

Submitted to the Faculty

of

Purdue University

by

Meredith Jensen

In Partial Fulfillment of the

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of

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Indianapolis, Indiana

Dedicated to my husband, Kenneth Kragh Jensen, PhD, and our wonderful son, Oliver Poul Kragh Jensen without their love and support I would not have enjoyed so much success in this endeavor and they bring to me so much joy in this life. Also to my incredible parents, Robert and Jenise Bills, without their unconditional love and training in discipline I could not have come this far.

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TABLE OF CONTENTS

Page

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT ix

CHAPTER 1 INTRODUCTION 1

1.1 Introduction 1

1.2 P and NP Lines 4

1.3 Inbred & Congenic Strains 6

1.4 Chromosome 4 Quantitative Trait Locus 7

1.5 The Multivariate Concentric Square Field 9

1.6 Hypotheses 11

CHAPTER 2 METHOD 13

2.1 Method 13

2.2 Experiment 1 – Multivariate Concentric Square Field 14

2.3 Experiment 2 – Open Field 15

2.4 Experiment 3 – Elevated Plus Maze 16

2.5 Statistical Analysis 16

Page

2.5.1 Multivariate Concentric Square Field 17

2.5.2 Open Field and Elevated Plus Maze 18

CHAPTER 3 RESULTS 19

3.1 Experiment 1 – Multivariate Concentric Square Field 19

3.1.2 Inbred & congenic pairwise comparisons 19

3.1.3 Trend Analysis – Comparisons among inbred and congenic strains 21

3.1.4 Trend Analysis – Pairwise comparisons between inbred and congenic strains 21

3.1.5 Other behaviors 23

3.2 Experiment 2 – Open Field 34

3.2.1 Inbred & congenic pairwise comparisons 34

3.3 Experiment 3 – Elevated plus maze 36

3.4 PCA analysis 40

3.5 Shelter/Risk Index (Anxiety-like Behavior) 41

3.6 Slope/Bridge Interval (Impulsive-like Behavior) 42

CHAPTER 4 DISCUSSION 43

4.1 Behavioral profiles 44

4.1.1 iP and iNP 44

4.1.2 P.NP and NP.P 47

4.2 Quantitative trait locus 48

4.3 General discussion 49

Page

REFERENCES 52

APPENDICES

Appendix A.....57

Appendix B.....58

LIST OF TABLES

Table Page

Table 1 MCSF functional categories 11

Table 2 MCSF paramenter results of descriptive data with SEM 25

Table 3 Summary of between group differences for MCSF functional categories 30

Table 4 Descriptive data and SEM of other behaviors in the MCSF..... 33

Table 5 Descriptive data and SEM in the Open field 35

Table 6 Summary of between group differences in Open field..... 36

Table 7 Descriptive data and SEM in the Elevated plus maze 37

Table 8 Summary of between group differences in Elevated plus maze..... 39

LIST OF FIGURES

Figure Page

Figure 1 Alcohol consumption of inbred and congenic strains 8

Figure 2 Trend analysis results 23

Appendix Figure

Figure A.1 Multivariate Concentric Square Field (MCSF) 57

Figure B.1 PCA analysis iP and iNP 58

Figure B.2 PCA analysis iP and P.NP 59

Figure B.3 PCA analysis iNP and NP.P 60

ABSTRACT

Jensen, Meredith M.S., Purdue University, December 2011. Characterization of Behavioral Profiles for Inbred P and NP and Congenic P.NP and NP.P Rats. Major Professor: Nicholas Grahame.

Alcoholism inheritance rates have been estimated as high as 60% in a human population. Many significant features of alcohol dependence have been replicated in rodent animal models of alcoholism, however not in totality. These animal models include inbred preferring (iP) and nonpreferring (iNP) rat types. Congenic rats have been engineered from the iP and iNP strains whereby a P congenic rat has in its genome a well-chosen chromosomal portion taken from an NP rat (P.NP) and, reciprocally, an NP congenic rat has acquired the analogous DNA from a P rat (NP.P). In this case, a quantitative trait locus (QTL) from chromosome 4 is the donor genetic material for the congenic rats. It is of great interest to further study this chromosome 4 QTL because it has been found to control a significant portion of ethanol consumption behavior in iP and iNP rats. This study aimed to behaviorally profile the iP, iNP and reciprocal congenic rats. As a result of the behavioral profiling of these genetically related groups, some conclusions could be made regarding which behaviors appear to be controlled by the chromosome 4 donor DNA.

This study primarily utilized the Multivariate Concentric Square Field apparatus (MCSF) to characterize behavioral profiles for the inbred and congenic rats. The Open field (OF) and Elevated plus maze (EPM) supported this effort. The MCSF is valuable in that it allows for the animals to interact within an environment that has ethological value. The 12 different zones that make up the field are characterized by some functional quality in terms of type and duration of behavior performed, etc. The behavioral data is aggregated and finally represented in terms of five functional categories, the elements of the behavioral profile: general activity, exploratory activity, risk assessment, risk taking, and shelter seeking. The study hypotheses were shaped by prior research suggesting that iPs should display lower general activity and risk taking strategy than iNPs in the MCSF. Inbred Ps should be more active in the OF and spend more time in the center of the EPM. Generally, it is expected that the iP QTL confer behavioral phenotypes to the iNP strain that deviate toward a "P" behavioral phenotype and reciprocally, the iNP QTL confer behavioral phenotypes to the iP strain that deviate toward an "NP" behavioral phenotype.

The results showed that iP rats performed more risk assessment and risk taking behavior and less shelter seeking and anxiety-like behavior than iNP rats. It followed that P.NP congenic rats significantly downgraded their risk assessment and risk taking behavior when compared to iP rats. This decrease can be attributed to the chromosome 4 QTL donated from the iNP breed. All together this study concludes that risk assessment and risk taking behavior in the iP rats is controlled by the same DNA region that, in part, determines voluntary intake of ethanol consumption. Further fine mapping of the QTL region should help in discovering if the same DNA sequences that influence ethanol intake also significantly influence risk behavior.

CHAPTER 1 INTRODUCTION

1.1 Introduction

In the study of high alcohol consuming populations significant genetic influences have been found. The heritability rates of the significant features of alcohol dependence are reported to be between 30-60% in humans (Heath et al., 1997; Hiroi & Agatsuma, 2005; Plomin, Owen, & McGuffin, 1994) and a highly significant quantitative trait locus has been found to control 1/3 of genetic variability in rats selected for alcohol preference (Carr et al., 1998). Alcohol dependence in humans is often comorbid with other psychiatric disorders and these behavioral features are highly correlated with two subtypes of alcohol dependence. Subtype 1 is associated with anxiety, depression and late onset and subtype 2 is associated with novelty seeking, impulsivity and early onset (Reese et al., 2010). Some behavioral features of alcohol dependence have been reproduced in rodent animal models selectively bred for high and low consumption of alcohol (Crabbe, Belknap, & Buck, 1994; T. K. Li, L. Lumeng, W. J. McBride, & J. M. Murphy, 1987). In order to sustain desired phenotypes for successive generations, selective breeding pressure can be applied to animal populations. This process increases the frequency of genes presumably related to the observed phenotype. The acquisition of all traits, in totality, that characterize alcohol dependence has not been accomplished in a single selected line (Crabbe & Phillips, 1998). Hypothetically, behavioral traits related to

the selected trait in high and low consumption are also divergent in their expression while other traits unrelated to the selected trait are similar between the two groups (Grahame, 2000). A recent study behaviorally profiled a multitude of rat lines selectively bred for high and low alcohol drinking and found that alcohol preferring rats used diverse behavioral strategies and concluded that heterogeneity is intrinsic to the alcohol preference phenotype and resembles heterogeneity observed in human alcoholics (Roman, E., Stewart, RB; Bertholomey, ML; Jensen, ML; Colombo, G; Hyytia, P; Badia-Elder, NE; Grahame, NJ; Li, TK; Lumeng, L, 2011). Identifying different behavioral features in selectively bred animal populations may provide clues to correlated traits that are either involved in the etiology of alcohol preference or, incidentally, genetically linked to genes responsible, in part, for alcohol preference. These correlated traits may or may not be directly related to the genesis of alcohol preference, nevertheless these traits or endophenotypes could be important antecedents to the development of alcohol preference. It's a goal in alcohol research to discover genetic components that account for the relationship between alcohol dependence and heritable traits. The multivariate concentric square field (MCSF)™ apparatus is useful to observe behavior toward this goal. The MCSF is a relatively new apparatus that takes into account individual rodent strategies with the goal of finding an overall pattern to the choices performed by the group as a whole on aspects of anxiety, shelter seeking, impulsivity, exploration, risk assessement, risk taking, and general activity. In the MCSF, the animals have the freedom to make choices in an arena that consists of several diverse zones and the exploratory strategy is scrutinized under the presumption that the strategy is controlled by the emotional and motivational "mental state" of the animal (Meyerson, Augustsson, Berg, & Roman, 2006).

These zones have particular attributes such as, an elevated platform, an incline toward a perceived danger zone, a dark, enclosed area, and areas that incite exploration. Behavior data for these zones are clustered according to how they correlate with one another. These behavior parameters are rank ordered across compared groups and nonparametric statistics analyze the data. The parameters, taken together, characterize some functional value and help define five functional categories. Further, the trend analysis is performed for clustered parameters and mean summed rankings are found for each functional category (general activity, exploratory activity, risk assessment, risk taking and shelter seeking). The multivariate concentric square field is advantageous in that it is useful in finding correlations between behavioral styles in the apparatus and genetic differences in selectively bred animals (Roman, Meyerson, Hyytia, & Nylander, 2007).

Congenic rat strains have been used to investigate the effect of genes on alcohol intake behavior. Specifically, congenics are vital in confirming quantitative trait loci (QTL), variable genetic characteristics that play a role in generation of phenotypic traits. Congenic animals are developed by taking a small region of DNA, whereupon lies a designated QTL, from a donor inbred strain and introgressing it onto a 'background' genome of another recipient inbred strain. This can be done reciprocally for two inbred strains thereby making it possible to study how the QTL from one strain differentially affects phenotypes of the opposite strain. The nomenclature for congenic strains is fashioned so that the background inbred strain name is denoted first followed by a period mark and the donor strain noted after the period. Reciprocal congenics for inbred alcohol preferring (iP) and nonpreferring (iNP) rats have been produced at Indiana University School of Medicine in Indianapolis and have been used to confirm a chromosome 4 QTL

that controls a fraction of the alcohol consumption behavior in iP and iNP rats. There is anticipation that this QTL contributes to other behavior traits concerning risk behavior, exploration, safety seeking, anxiety-like and impulsive-like behavior. In particular, the MCSF test may be capable of elucidating differences in behavioral strategy of iP and iNP and their reciprocal congenic strains on general activity, exploratory activity, risk assessment, risk taking and shelter seeking behavior.

In an effort to investigate the relationship between genes and behavior this study aimed to characterize behavioral profiles for iP and iNP rats and reciprocal congenic P.NP and NP.P rats. Behavior profiles were characterized using multivariate test approaches and multivariate data analysis techniques in order to examine the degree to which a chromosome 4 QTL is capable of determining patterns of behavior in iP and iNP rodents. The MCSF, open field (OF) and elevated plus maze (EPM) tests were used to evaluate group differences. The OF and EPM tests were used as comparison tools to previous research as well as a reference for data collected from the MCSF.

1.2 P and NP Lines

The P and NP lines were developed from a closed, outbred Wistar stock of rats which were bred according to criteria that bidirectionally selected for ethanol preference phenotypes. The method simply called for breeding pairs of animals that displayed extremes in voluntary ethanol consumption, effectively increasing the frequency of trait relevant alleles over time in the colony population. Breeders for the alcohol preferring phenotype must voluntarily consume greater than 5.0g/kg/day of a 10% ethanol solution with a 2:1 preference ratio (ethanol:water) and non-preferring breeders must voluntarily

consume less than 1.5g/kg/day with a 0.5:1.5 preference ratio (Li, Lumeng, McBride, & Murphy, 1987). The P and NP lines are well studied on many behaviors concerning alcohol dependence and important differences have been discovered. Considering that most research has been done using outbred P and NP rats their phenotypic differences are used to guide expectations for iP and iNP group differences. Therefore, a short synopsis of P and NP differences is provided in this section concerning ethanol consumption correlated traits that are related to the behavioral parameters evaluated in this study.

In the open field test P rats show greater activity than NPs and NP rats defecate more than Ps (Badishtov et al., 1995). Preferring rats showed greater behavioral activation than NPs in response to novel odors, however P and NP rats were not different in nosepoking in response to novel odors (Nowak et al., 2000). On three tests significant in identifying anxiety-like behavior, the passive avoidance paradigm, elevated plus maze, and slip funnel test, P rats displayed greater anxiety-like behavior than NPs (Salimov, McBride, Sinclair, Lumeng, & Li, 1996; Stewart, Gatto, Lumeng, Li, & Murphy, 1993). Problem drinking is often comorbid with anxiety and provides support for the hypothesis that high ethanol consumption results in self-medication in order to alleviate symptoms (Begleiter & Kissin, 1995) and research found anxiolytic effects in Sardinian P rats and P rats when alcohol was consumed voluntarily or administered via injection (Colombo et al., 1995; Stewart, et al., 1993) however there is evidence contradictory to this that shows no relationship between alcohol intake and anxiety-like behavior in P rats (Viglinskaya et al., 1995). As mentioned earlier, a recent study using outbred P and NP rats were characterized on the EPM and OF tests and for the first time on the MCSF (Roman et al., 2011). That study found that P rats have lower general activity and exploratory strategies

than NPs. Preferring rats showed more risk-taking behavior than NP rats but only when controlling for Ps lower activity, otherwise Ps showed less risk-taking strategy than NPs. Risk assessment and shelter seeking did not differ between the two groups. On the EPM test, P rats spent more time in the Center than NP rats and no other differences were found. On the OF test, P rats made more line crossings than NPs, which is consistent with previous studies (Badishtov, et al., 1995).

1.3 Inbred & Congenic Strains

Following 30 generations of bidirectional selection of the P and NP lines, inbreeding was commenced for the two lines without regard to selection criteria until all individuals were genetically identical and fixed at approximately 99.8% of all loci (Carr, et al., 1998). Inbred strains are maintained by breeding brother-sister pairs for 20 consecutive generations until all heterogeneity is lost (Grahame, 2000). Upon reaching the 19th inbred generation mean drinking scores of the iP and iNP strains were 6.7g/kg/day and 0.53g/kg/day, respectively (Carr, et al., 1998).

Congenic strains can assist researchers in studying QTL's, utilizing mapped genetic markers, and ultimately identifying genes. There are two reciprocal congenic strains used in this study where a chromosome region of target DNA from chromosome 4, donor DNA from either the iP or iNP line, is introgressed onto the recipient background genome. This was done reciprocally for both inbred P and NP strains so that the QTL from either strain was fitted on the background genome of the opposing strain. The congenic strains are developed using a marker assisted method through a series of 10 backcrosses of an inbred donor strain onto an inbred recipient strain followed by an

intercross in order to ensure homozygosity (Carr et al., 2006). Donor gene markers for a particular chromosomal region of interest are selected for at each generation of breeding (Lagrange & Fournie, 2009). The congenics in this study are of the same pedigree as the iP and iNP strains used in this study. A detailed methodology of the creation of the congenic animals has been described previously (Carr, et al., 2006). Ultimately stable P.NP and NP.P congenic strains have been developed and maintained. These congenics have established the chromosome 4 QTL as a significant underlying element in the divergence of drinking scores and are expected to help explain variability in behavioral endophenotypes that correlate with alcohol drinking observed in iP and iNP strains.

1.4 Chromosome 4 Quantitative Trait Locus

A highly significant QTL on chromosome 4 was discovered when F2 offspring from an F1(iP X iNP) cross were examined. The estimated 22 cM QTL region in the rat is approximately syntenic to chromosome 6 in mice and to several chromosome regions in humans including 7, 4 and 2 (Carr et al., 2006). Resultant data indicated its large contribution to the divergent drinking behavior of the iP and iNP strains (Carr, et al., 1998). Following discovery of the QTL, researchers created the first cohort of congenics predicting that the congenic strains voluntary alcohol consumption would deviate from their respective background strain consumption scores toward the mean drinking score typical of the opposite, donor strain, in other words, the QTL should systematically reduce or increase alcohol drinking. Researchers found that the donor QTL region did, in fact, decrease the magnitude in divergent consumption differences. The statistically significant differences from that study are illustrated in Figure 1. One of three congenic

NP.P strains and three of four P.NP strains differed significantly in their drinking compared to their respective inbred strains. The data illustrates the expected potentiation in alcohol consumption in NP.P congenic rats while P.NPs showed the expected reduction in consumption.

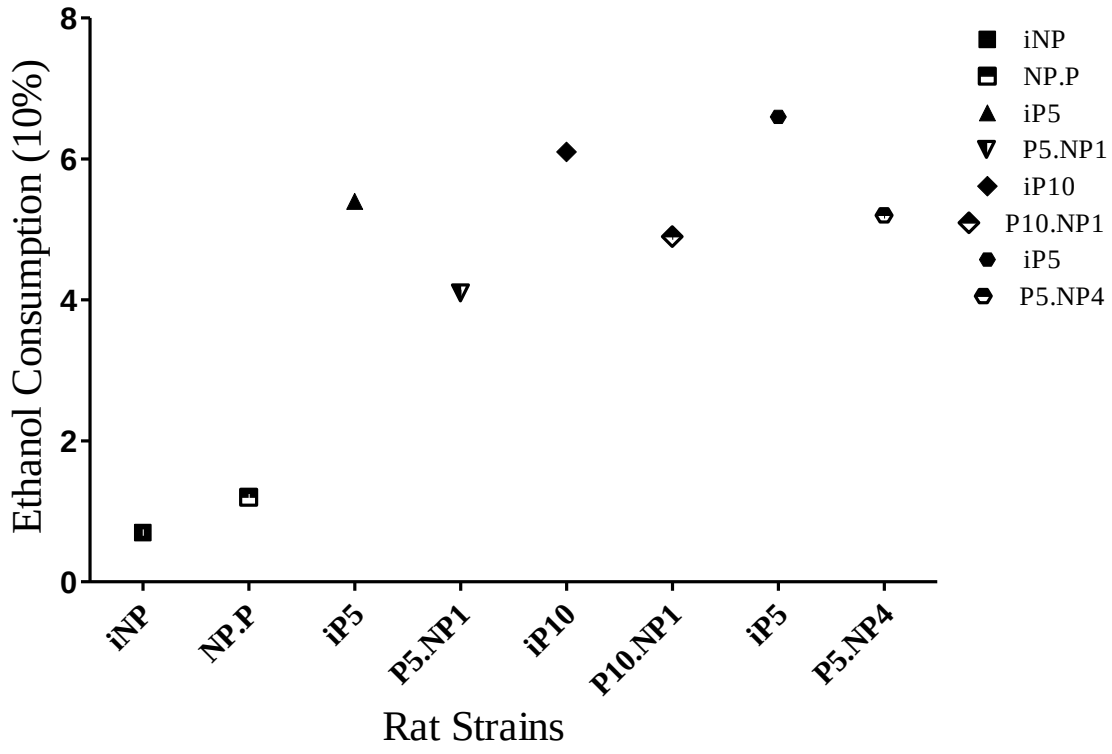

Figure 1

Alcohol consumption of inbred and congenic strains (Carr et al., 2006).

The Y-axis is a scale of consumption of a 10% ethanol solution and the X-axis shows the groups that displayed significant differences between the congenic and background strains as analyzed with T-tests. The rat strain names can identify 3 different

components first, the background strain, second the donor strain and third, numeration identifies the specific inbred strain used to create the congenic. The independent inbred strains iP5, iP10, iNP1 and iNP4 were used to create the congenic strains (Carr et al., 2006).

Within the chromosome 4 QTL lies DNA that encodes for neuropeptide Y (NPY). NPY is a 36 amino acid neuropeptide that is distributed throughout the brain as well as in the peripheral nervous system and research has shown its involvement in such behaviors such as anxiety, food and alcohol consumption and response to stressful stimuli (Bannon et al., 2000) (Badia-Elder, Gilpin, & Stewart, 2007). Inbred P rats have overall lower expression of NPY in the brain than iNPs (Kimpel et al., 2007) and NPY has been shown to decrease intake of ethanol when administered intracranially in P rats (Badia-Elder et al., 2001). Furthermore, recent gene expression research has found that NP.P congenic strains have demonstrated reversal of attributes in NPY distribution in the brain, similarly as was found in the case of alcohol consumption. The iP QTL confers lower NPY expression in amygdala, hippocampus, caudate putamen, nucleus accumbens, and frontal cortex in P.NP congenics (Spence, Liang, Habegger, & Carr, 2005).

1.5 The Multivariate Concentric Square Field

The MCSF was the leading apparatus used to observe animal behavior in this study. The MCSF is relatively new to behavioral analysis of rodents as it relates to traits significant to alcoholism and therefore additional information concerning the MCSF is provided in this section. A more detailed description and history can be found elsewhere (Meyerson, et al., 2006).

The apparatus was designed with consideration to ethology and was meant to imitate the characteristics of a natural environment in which the animal could choose freely in how it explored. The MCSF apparatus (Appendix A) adapted for rats has 10 zones: Center, Center Circle, South Corridor, North Corridor, West Corridor, dark corner room (DCR), Hurdle, Slope, Bridge Entrance, Bridge. Previous research helped to verify the perceived safe or risk zones. When pups from a lactating dam were placed on the Bridge area following the pups were retrieved and immediately taken to the DCR area, therefore the DCR was established as a safe zone. Alternatively, when pups were placed in the DCR the dam did not relocate the pups to any other zone. In another experiment food deprived male rats were observed as they hoarded food pellets. Food pellets were placed in the Bridge area and males retrieved the pellets and transported them to the DCR. Again, the pellets were placed in the DCR and no males moved the pellets from the DCR. Additionally consumption of the food pellets never occurred on the Bridge, but some males did eat in the DCR. Differences in pup retrieval and food pellet hoarding from the Bridge versus DCR were statistically significant, hence safe and risk zones were verified (Meyerson, et al., 2006).

Behavioral parameter data are collected from each zone and, in brief, consists of: frequency of visits (Freq), duration of visit (Dur), time spent per visit (Dur/Freq) and latency to visit (Lat). Related data parameters are clustered to form a functional category that capture the nature of the behavior and/or provide additional face validity to the variable (Roman & Colombo, 2009). The nature of the zones help to define functional categories used to characterize rodent behavior.

Table 1 MCSF functional categories

Functional Category	Clustered parameters
General Activity	TotAct, Freq TotCorr, Dur/Freq TotCorr, Freq Center
Exploratory Activity	Dur TotCorr, Dur Center, Dur Hurdle, Nosepoke, Rearing
Risk Assessment	Dur/Freq Slope, Dur/Freq Bridge Entrance, SAP to Center, SAP to Slope, Freq Slope, Freq Bridge Entrance
Risk Taking	Freq Bridge, Dur Bridge, Dur/Freq Bridge, Freq CentCir, Dur CentCir, Dur/Freq CentCir
Shelter Seeking	Freq DCR, Dur DCR, Dur/Freq DCR

Clustered parameters used in trend analysis and their corresponding functional category (Roman, et al., 2011)

1.6 Hypotheses

Based off prior work that used the MCSF to assess outbred P and NP rats it is expected that the iP group will show lower general activity and lower risk taking strategies than iNPs (Roman, et al., 2011). Also, based off the same work in the MCSF, iP rats should make fewer visits to the Center zone than iNP and more visits to Slope, Bridge entrance and Bridge zones than iNP (Roman, et al., 2011). No differences are expected to be found for exploratory behavior, risk assessment and shelter seeking. It is expected that iP rats display more activity in the open field with more line crossings and spend more time in Center of EPM than iNP, which are related to activity and risk

assessment, respectively. Prior research has shown outbred P rats to be more active than NPs in the open field (Roman, et al., 2011) (Badishtov, et al., 1995). Previous research has not been able to replicate early demonstrations of Ps spending less time in open arms of the elevated plus maze and no differences have been observed in number of total arm entries (Roman, et al., 2011) (Viglinskaya, et al., 1995); hence no differences are expected in these respects. Additionally, this study should replicate previous work that showed Ps have lower body weight compared to NPs (Alam et al., 2005). Pairwise comparisons were analyzed between the congenic strains and their corresponding inbred strains as well as between the two inbred strains. The predicted outcomes for this study were guided primarily by previous behavioral research on outbred P and NP rats since a greater body of research has been performed for these groups than for iPs and iNPs.

In general, it is expected that the iP QTL confer behavioral phenotypes to the iNP strain that deviate toward a "P" behavioral phenotype and reciprocally, the iNP QTL confer behavioral phenotypes to the iP strain that deviate toward an "NP" behavioral phenotype. To this effect, by observing its inbred counterpart, the direction of a behavior for a congenic group can be anticipated, with the understanding a prediction can come to fruition only if there is some significant genetic element within the QTL that indeed controls the behavior. It is also acknowledged that the battery of behaviors tested in this study may not be exhaustive of the behaviors affected by the QTL. This study was able to (a) provide a behavioral profile for iP, iNP, P.NP and NP.P. and (b) provide evidence for the chromosome 4 QTL as a major element in determining differences between iP and iNP selected breeds as tested using the MCSF and other supportive tests.

CHAPTER 2 METHOD

2.1 Method

Alcohol naïve, 24 week old adult, male rats were used including four groups: iP (n=12), iNP (n=12), P.NP (n=11), and NP.P (n=11), all bred at Indiana University School of Medicine in Indianapolis. Animals were housed in a temperature and humidity controlled vivarium with a reversed 12-hour light/dark cycle (lights out at 10am). Animals were housed in pairs, except for 2 unmatched single rats, one from each congenic group, in acrylic cages (45x23x20cm) containing wood-chip bedding material. Animal sustenance consisted of ad libitum access to standard pellet chow and water. All research protocols were approved by the IUPUI School of Science Institutional Care and Use Committee and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (Academies, 2003).

The animals habituated for at least two weeks to housing conditions and were handled for 3 days prior to experimentation where they were weighed and transported in buckets to and from homecage. On habituation days and subsequent test days, the animals were transported in their homecage from the vivarium to a holding area then transported to the weighing scale (only on habituation day) or actual test apparatus via buckets.

The animals were tested during their nocturnal active phase thus, efforts were made to keep the holding area dimly lit in order to cause the least disturbance to their circadian system. The facilitator for each behavioral test remained constant throughout the experiment. In all behavioral trials the facilitator recorded the testing period by video camera/monitor setup and waited outside testing area. The animal groups on each apparatus were tested on a counterbalanced schedule. A previous pilot study indicated that behavioral results from the MCSF apparatus were variable based on the prior testing experience of the animals therefore naïve animals were tested on this apparatus first, the OF second, and the EPM last (Augustsson, 2004).

2.2 Experiment 1 – Multivariate Concentric Square Field

The MCSF apparatus (Appendix A) adapted for rats has 10 zones: Center, Center Circle, South Corridor, North Corridor, West Corridor, DCR, Hurdle, Slope, Bridge Entrance and Bridge. The apparatus is 100x100cm and has an open center area of 70x70 cm and located within this larger Center is a smaller Center Circle area that is 25 cm in diameter. This open center area has walls that are 25cm high. All corridors can be accessed from the Center zone. Off of the 3 main corridors is the DCR, a slightly elevated area with a hole board meant to incite exploration (Hurdle) and a entry to inclined, Slope zone that leads to the Bridge entrance and elevated Bridge area. Much of the apparatus areas are lit by low lighting (10-20 lux), the DCR with very low to no light (<1 lux), the Slope zone with moderately low light (<30 lux) while the Bridge area is highly illuminated (600-650 lux). The DCR is accessible only by the South Corridor. The Hurdle connects both the West and North corridors. The North corridor also gives

access to the Slope zone at the opposite end from the Hurdle. The Slope gives access to the Bridge Entrance and ultimately the Bridge area, which has a metal, grated floor (Roman & Colombo, 2009).

At the start of the test the animals are positioned facing the Center wall shared with the Bridge zone and the test session is 20 minutes. Each zone was scored on frequency of visits (FRQ), duration (DUR), time spent per visit (DUR/FRQ), and latency (LAT). Number of stretched attend posture behavior (SAP) to Center and Slope, rearing, grooming, fecal boli and urination output was scored. Bridge/Slope interval measured how long it takes for animals to enter the Bridge relative to first entering the Slope zone and is an index for impulsive-like behavior. Shelter/Risk interval is calculated to reveal differences in time spent in DCR versus the Bridge, in relation to the total time spent in the two zones and is a measure of anxiety-like behavior. Performance on Bridge and Center Circle area provides information on risk-taking behavior. SAP and performance on Slope and Bridge Entrance provides information on risk assessment. (Roman & Colombo, 2009)

2.3 Experiment 2 – Open Field

The apparatus is a square open field (90X90 cm with upright panels (30cm) enclosing the field). White lines divide the open field into a matrix of smaller squares (15X15 cm). At the start of the test the animals were positioned 15 cm away from a wall. The test session is 10 minutes long. The test was performed in dim lighting. The dependent variables analyzed: number of visits to Periphery (PeripheryFREQ), time spent in Periphery (PeripheryDUR), time spent per visit to Periphery (PeripheryDUR/FREQ),

number of visits to Center (CenterFREQ), time spent in Center (CenterDUR), time spent per visit to Center (CenterDUR/FREQ), frequency of Line Crossings (CrossingsFREQ), fecal boli output and urine output.

2.4 Experiment 3 – Elevated Plus Maze

The apparatus consists of two open arms (50x10cm) that sit at right angles to two enclosed arms. The enclosed arms have upright panels (50x10x50). The apparatus is elevated 90 cm from the floor. At the start of the test animals were positioned in the center where the four arms intersect. The test session is 5 minutes long. The test was performed in dim lighting. The dependent variables analyzed: number of visits to Center (CenterFreq), time spent in Center (CenterDur), time spent per visit to Center (CenterDurFreq), number of visits to Open Arms (OpenFreq), time spent in Open Arms (OpenDur), time spent per visit to Open Arms (OpenDurFreq), number of visits to Closed Arms (ClosedFreq), time spent in Closed Arms (ClosedDur), time spent per visit to Closed Arms (ClosedDurFreq), fecal boli output and urine output.

2.5 Statistical Analysis

Data were manually scored using the Observer 8.0 Noldus information technology software (Wageningen, Netherlands). Data were analyzed using SPSS 16.0 statistical package. An alpha level of 0.05 was used for omnibus statistical tests. The pairwise comparisons made in this study were iP vs iNP, iP vs P.NP, and iNP vs NP.P; Bonferonni adjustment was applied to relevant posthoc comparisons which set the critical value to $\alpha = 0.0167$ (0.05 divided by 3 (# of comparisons) equals 0.0167).

Shapiro-Wilk's W-test was used to test for normal distribution of data. Analysis of variance (ANOVA) was used to analyze normally distributed data and Tukey HSD posthoc test was used. Body weight data were normally distributed. MCSF data was analyzed using Mann Whitney U or Kruskal-Wallis H nonparametric rank ordered statistics. Trend analysis sum ranked clustered behavioral parameters for each genetic strain and was used to find statistical differences in behavioral strategy in the MCSF. Principal components analysis took into account all behavior in the MCSF zones for paired groups and illustrated the extent of similarities and differences in performance in MCSF and, in general, described their overall relationship.

Due to an odd number of animals in the two congenic groups there were two individuals single housed and consequently excluded from all behavioral analyses, previous research shows that social housing conditions can affect behavioral test outcomes (Andrade & Guimaraes, 2003). Nevertheless, those two individuals were included in the body weight analysis. Also, an NP.P rat was injured in the MCSF apparatus to the extent that it affected its performance during the test therefore data collected from this individual were excluded from the MCSF analysis.

2.5.1 Multivariate Concentric Square Field

At the present time there is no inter-observer reliability coefficient to report, however, informally, following training for manual scoring, research personnel made several test trials on previous behavioral data with the goal of reproducing the record of scores. The same person scored all behavior data. Behavior was scored as a visit if both of the animal's hind legs crossed into the zone.

Mann-Whitney U analysis analyzed parameter data. Trend analysis analyzed clustered parameter data to elucidate behavioral strategy. Multiple comparisons was controlled for with a Bonferroni adjustment. Kruskal-Wallis H was used when comparing more than two groups. Principal components analysis (PCA) was used as a supportive analysis to reveal any overall relationships or similar behavioral patterns between pairwise groups. If an animal did not visit a zone or did not perform a scored behavior then that data point was not included in the analysis ie. not scored as zero but as a missing value. Fisher's exact test (2-tailed) analyzed significant difference in behaviors performed or not performed and zones entered or not entered (Occurrence or OCC).

2.5.2 Open Field and Elevated Plus Maze

ANOVA was used to analyze data followed by Least Significant Differences (LSD) posthoc analysis. Bonferroni adjustment for multiple comparisons was used.

CHAPTER 3 RESULTS

3.1 Experiment 1 – Multivariate Concentric Square Field

This section includes outcomes for behavioral parameters as well as behavioral strategy results as evaluated by the trend analysis and is illustrated in Figure 2. There are tables that display descriptive data with mean and SEM values in Table 2. A summary of significant group differences for all behavioral parameters is shown in Table 3. Other behaviors scored in the MCSF are shown in Table 4. Mann-Whitney U analyzed parameter data for significant differences. Trend Analysis results was analyzed using Kruskall-Wallis test. There was no statistical difference in occurrence (OCC) for any of the groups.

3.1.2 Inbred & congenic pairwise comparisons

3.1.2.1 iP and iNP

Inbred P rats took significantly more visits and more time on the Slope (p = .003, .010, respectively) Bridge Entrance (p = .012, .017, respectively), and Bridge (p = .004, p < .000, respectively) zones than iNP rats, in accordance with previous research (Roman, et al., 2011). Shelter seeking, iNP's spent more time seeking shelter, but not to a statistically significant extent, U = 37.0, p = .045. Regarding performance in the DCR, iNP's appeared to spend more time in the DCR than iP's (p = .045).

3.1.2.2 iP and P.NP

Inbred P's chose significantly more risky behavior patterns by taking more visits to Slope (p = .009), Bridge Entrance (p = .017), and spending more time in Slope (p = .002) and Bridge (p = .002) zone than P.NP's. These results are in agreement with the behavioral trend forecast by the iP and iNP outcomes on these parameters.

Indeed these results suggest that "P" genome animals participate in a behavioral strategy differently from their "NP" counterparts and can be interpreted as risk-taking. It may be that since iP's take part in more risk taking behavior, their risk assessment scores are higher too. In all differences found between iP and P.NP the direction of the behavior could be predicted by differences found between iP and iNP, therefore it is the case that the QTL plays some role in controlling these behaviors. In other words, insertion of the "NP" QTL onto a recipient, "P" background seemed to significantly shift the behavior away from iP profile toward a less risk-taking strategy of an NP profile and to an extent that can actually be captured in quantitative terms.

3.1.2.3 iNP and NP.P

NP.P's tended to visit and spend less time in DCR (p = .058, .129) than iNP's and visited the Center (p = .111) zone more than iNP's. NP.P's did visit Center Circle significantly more times than iNP's (p = .007). Although differences were often not found to an extent that was statistically significant trends for these behavioral parameters could be predicted by the iP and iNP outcomes.

3.1.3 Trend Analysis – Comparisons among inbred and congenic strains

No significant differences were found for general activity H = 1.156, p = .764 and exploratory activity H = .352, p = .950. Differences were found for Risk assessment H = 11.740, p = .008 and Risk taking H = 12.947, p = .005. A trend toward significance was found for Shelter seeking, H = 5.88, p = .118.

3.1.4 Trend Analysis – Pairwise comparisons between inbred and congenic strains

3.1.4.1 iP and iNP

No significant differences found for general activity, U = 38.0, p = .277. No significant differences found for exploratory activity, U = 50.0, p = .808. Significant differences were found for Risk assessment, U = 30.5, p = .017 where iP's showed greater risk assessment than iNP's. Significant differences were found for risk taking, U = 15.5, p < .000, where iP's also displayed greater risk taking than iNP's.

3.1.4.2 iP and P.NP

No differences found for General activity, U = 53.0, p = .674. No differences found for exploratory activity, U = 58.0, p = .923. Significant differences were found for Risk assessment, U = 12.5, p = .001. Significant differences found for Risk taking, U = 22, p = .011 with iP scoring higher than iNP's. No differences shown for Shelter seeking, U = 56.0, p = .821.

3.1.4.3 iNP and NP.P

No differences were found for Exploratory activity (U = 38.0, p = .277), General activity (U = 50.0, p = .808), Risk assessment (U = 49.0, p = .754), Risk taking (U = 31.5, p = .111) nor Shelter seeking behavior (U = 31.0, p = .111). Trends toward significance

for risk taking and shelter seeking observed where NP.P's scored higher and lower than iNP's, respectively, which is in line with what would be expected according to iP and iNP outomes.

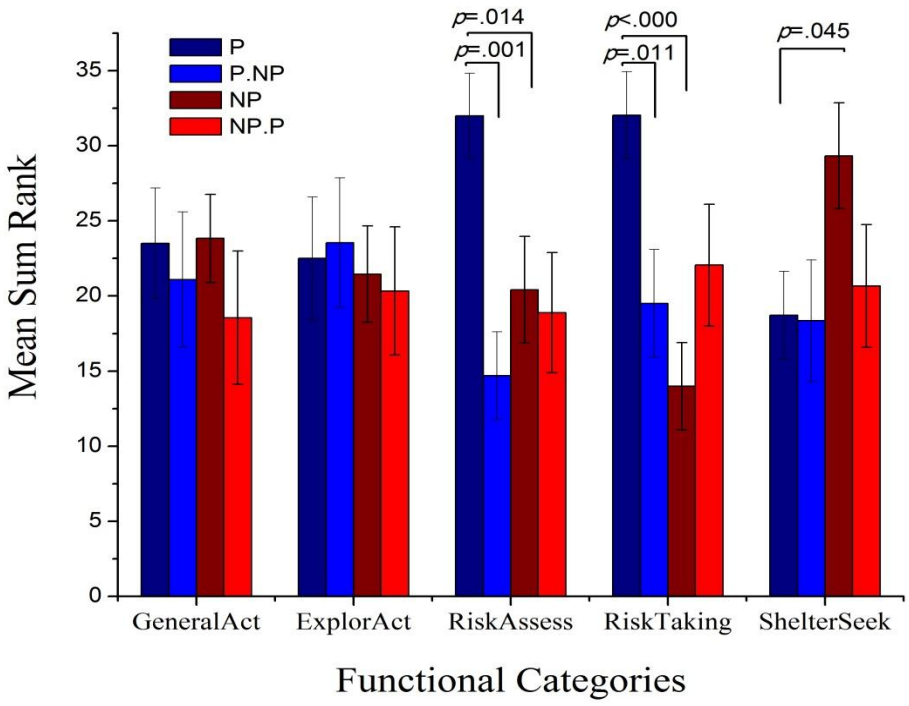

Figure 2 Trend analysis results.

Trend analysis of MCSF functional categories. Statistical significance for overall groups was found at $p = .05$. Statistical significance for pairwise genotypes, when controlling for multiple comparisons, is $p = .0167$.

3.1.5 Other behaviors

No significant differences were found overall for fecal boli output, H = 1.542, (3, N=43), $p = .673$. The lack of robust strain effect might be explained by the pair housing

approach which some have shown ameliorates behavior related to anxiety (Andrade & Guimaraes, 2003).

No significant differences were found overall for urine output, H = 2.350, (3, N=43), p = .503.

No significant differences were found overall for nosepokes, H = .747, (3, N=43), p = .862.

Significant differences were found overall for grooming, H = 18.416, (3, N=43), p < .000. Significant differences were found for iP and P.NP, U = 16.0, p = .003 with sum of ranks equal to 182 for iP and 71 for P.NP. Significant differences found for iNP and NP.P, U = 15.000, p = .004 with sum of ranks equal to 171 for iNP and 60 for NP.P.

No significant differences found overall for rearing behavior, H = .836, p = .841.

ANOVA found significant differences overall for bodyweight, F(3, 39) = .013, p = .013. Post hoc tests using Tukey HSD found that iNPs weighed significantly more than the iP group (p = .044) and the iNP group weighed more than the NP.P group (p = .010). However the iP group did not have a significant weight difference compared to P.NP group (p = .889). It should be noted that the groups compared did not have equal sample sizes, which violates one assumption of post hoc Tukey's HSD however after some consideration the test was determined to be reliable since the analysis is robust in nature and the assumption of homogeneity of variance was satisfied. These results demonstrate that the chromosome 4 QTL essentially carries the entire phenotypic difference for body weight as well as the other important behavioral differences described above between iP and iNP rats.

Table 2

MCSF parameter results of descriptive data with SEM

Functional categories	**Parameters**	**Genotype**	**P Background**	**NP Background**
General activity	TOTACT	iP/iNP	92.1±7.7	73.7±4.3
		P.NP/NP.P	69.6±8.6	59.9±9.2
	FRQ TOTCORR	iP/iNP	29.8±2.4	26±1.8
		P.NP/NP.P	21.9±2.8	18.5±3.3
	FRQ CENTER	iP/iNP	23.67±2.3	23.17±1.1
		P.NP/NP.P	23.45±3.3	23.63±2.9
	DUR CENTER	iP/iNP	284.33±22.4	403±26.3
		P.NP/NP.P	375.66±65.5	377.55±59.3
	DUR/FRQ CENTER	iP/iNP	13.75±2.1	18±1.6
		P.NP/NP.P	16.10±2.8	15.71±3.1
	OCC LEAVE CENTER	iP/iNP	12/12	12/12
		P.NP/NP.P	10/10	9/9
Exploratory activity	LAT LEAVE	iP/iNP	61.13±25.7	120.5±15.6
		P.NP/NP.P	131.12±45.7	96.13±77.1
	DUR TOTCORR	iP/iNP	546.08±27.9	522.17±23.9
		P.NP/NP.P	518.77±58.5	487.49±70.5
	DUR/FRQ TOTCORR	iP/iNP	19.88±2.3	21.42±2.3
		P.NP/NP.P	23.04±3.1	23.35±3.6
	LAT HURDLE	iP/iNP	166.5±33.9	348.67±81.1
		P.NP/NP.P	434.57±101.6	424.75±100.3
	FRQ HURDLE	iP/iNP	6.58±0.5	3.33±0.8
		P.NP/NP.P	4.05±0.8	3.26±0.5
	DUR HURDLE	iP/iNP	103.17±12.4	49.58±10.2
		P.NP/NP.P	62.58±15.5	55.74±8.4

Table 2 Continued

	DUR/FRQ HURDLE	iP/iNP	16.16±1.9	15.16±3.8
		P.NP/NP.P	12.48±2.1	16.99±3.3
	OCC HURDLE	iP/iNP	12/12	10/12
		P.NP/NP.P	9/10	9/9
	Nosepokes	iP/iNP	2.58±0.9	1.67±0.6
		P.NP/NP.P	3.1±1.0	2.22±1.2
	OCC VIST ALL ZONES	iP/iNP	11/12	8/12
		P.NP/NP.P	6/10	4/9
	REARING	iP/iNP	61.75±3.2	61.17±2.8
Risk assessment	LAT SLOPE	iP/iNP	206±51.1	364±47.9
		P.NP/NP.P	547±114.1	466.83±92.7
	FRQ SLOPE	iP/iNP	7.25±0.83	3.42±0.7
		P.NP/NP.P	3.17±0.9	2.78±0.9
	DUR SLOPE	iP/iNP	35.75±4.6	17.67±4.2
		P.NP/NP.P	11.33±3.6	16.66±4.6
	DUR/FRQ SLOPE	iP/iNP	5.0±0.5	4.0±0.9
		P.NP/NP.P	2.52±0.6	5.25±1.8
	OCC SLOPE	iP/iNP	12/12	9/12
		P.NP/NP.P	8/10	7/9
	LAT BRIDGE ENT	iP/iNP	211.0±51.4	438.20±44.3
		P.NP/NP.P	551.33±111.4	500.20±143.4
	FRQ BRIDGE ENTRANCE	iP/iNP	7.67±0.9	3.58±0.8
		P.NP/NP.P	3.75±1.0	3.09±1.2

Table 2 Continued

	DUR BRIDGE ENT	iP/iNP	45.92±7.2	22.67±5.9
		P.NP/NP.P	22.43±5.3	21.02±10.8
	DUR/FRQ BRIDGE ENT	iP/iNP	5.92±0.5	4.83±1.2
		P.NP/NP.P	4.55±0.9	3.15±1.1
	OCC BRIDGE ENTRANCE	iP/iNP	12/12	9/12
		P.NP/NP.P	8/10	6/9
	SAP TO CENTER	iP/iNP	3.67±0.9	2.33±0.4
		P.NP/NP.P	2.50±0.6	3.33±0.5
	OCC SAP to CENT	iP/iNP	11/12	11/12
		P.NP/NP.P	9/10	9/9
	SAP TO SLOPE	iP/iNP	4.25±0.7	6.0±0.6
		P.NP/NP.P	3.90±0.7	5.0±0.5
	OCC SAP TO SLOPE	iP/iNP	12/12	12/12
		P.NP/NP.P	10/10	9/9
Risk taking	LAT BRIDGE	iP/iNP	213.88±51.3	441.60±44.8
		P.NP/NP.P	555.5±111.5	502.80±143.6
	FRQ BRIDGE	iP/iNP	4.0±0.4	1.92±0.5
		P.NP/NP.P	2.07±0.5	1.84±0.8
	DUR BRIDGE	iP/iNP	97.33±11.8	35.17±8.9
		P.NP/NP.P	43.64±10.6	45.08±21.1
	DUR/FRQ BRIDGE	iP/iNP	24.50±1.5	13.75±2.6
		P.NP/NP.P	16.97±3.6	15.55±4.7
	OCC BRIDGE	iP/iNP	12/12	9/12
		P.NP/NP.P	8/10	6/9

Table 2 Continued

	LAT CTRCI	iP/iNP	63.88±68.0	135.38±67.4
		P.NP/NP.P	130.86±113.4	45.0±17.9
	FRQ CTRCI	iP/iNP	8.42±1.3	7.08±0.5
		P.NP/NP.P	9.38±2.3	9.03±1.2
	DUR CTRCI	iP/iNP	15.25±2.3	12.67±1.8
		P.NP/NP.P	13.22±3.4	15.93±3.5
	DUR/FRQ CTRCI	iP/iNP	2.17±0.5	1.58±0.2
		P.NP/NP.P	1.20±0.2	1.55±0.3
	OCC CTRCI	iP/iNP	12/12	12/12
		P.NP/NP.P	10/10	9/9
Shelter seeking	LAT DCR	iP/iNP	234.63±53.3	430.71±69.0
		P.NP/NP.P	430.50±99.8	525.67±130.6
	FRQ DCR	iP/iNP	4.67±0.8	5.17±0.8
		P.NP/NP.P	3.16±0.9	2.58±0.7
	DUR DCR	iP/iNP	72.17±16.2	136.83±25.7
		P.NP/NP.P	61.28±22.5	70.82±21.1
	DUR/FRQ DCR	iP/iNP	13.42±1.8	24.25±3.6
		P.NP/NP.P	12.21±3.1	19.28±6.3
	OCC DCR	iP/iNP	11/12	11/12
		P.NP/NP.P	8/10	7/9
Anxiety-like behavior		iP/iNP	-0.236±0.1	0.574±0.1
		P.NP/NP.P	.028±0.2	0.256±0.3
Impulsive-like behavior		iP/iNP	0.057±0.04	0.527±1.0
		P.NP/NP.P	0.012±0.02	0.305±1.1

The data table is organized with respect to the genotype of the four animal groups. The two columns denote the recipient's background genome. There are two rows, where data for inbreds are on the top row and congenics is on the second row. The animal genotype column identifies the first and second column. Occurrence (OCC) indicates the number of animals out of the total number of animals in a group that visited a zone or participated in a behavior (#visited or #performed a behavior/n). *Abbreviations:* CTRCI, Center Circle; DCR, dark corner room; DUR, duration (s); DUR/FRQ, duration per visit (s); FRQ, frequency; LAT, latency (s); OCC, occurrence; SAP, stretched attend posture; TOTACT, total activity, i.e. the sum of all frequencies; TOTCORR; total corridor, i.e. the sum of all corridors.

Table 3

Summary of between group differences for MCSF functional categories.

Functional categories	**Parameters**	**iP vs iNP**	**iP vs P.NP**	**iNP vs NP.P**
General activity	TOTACT	#	#	#
	FRQ TOTCORR	#	#	#
	FRQ CENTER	#	#	#
	DUR CENTER	iP < iNP**	iP ≤ P.NP p=.03	#
	DUR/FRQ CENTER	#	#	#
Exploratory activity	LAT LEAVE	iP < iNP*	iP < P.NP*	#
	DUR TOTCORR	#	#	#
	DUR/FRQ TOTCORR	#	#	#
	LAT HURDLE	iP < iNP*	iP < P.NP*	#
	FRQ HURDLE	#	#	#
	DUR HURDLE	iP > iNP**	#	#
	DUR/FRQ HURDLE	#	#	#
	FRQ NOSEPOKE	#	#	#
	OCC VISIT ALL ZONES	#	#	#
	REARING	#	#	#
Risk assessment	LAT SLOPE	#	iP < P.NP*	#
	FRQ SLOPE	iP > iNP**	iP > P.NP**	#
	DUR SLOPE	iP > iNP*	iP > P.NP**	#
	DUR/FRQ SLOPE	#	iP > P.NP**	#
	OCC SLOPE	#	#	#
	LAT BRIDGE ENTRANCE	iP < iNP*	iP < P.NP*	#

Table 3 Continued

	FRQ BRIDGE ENTRANCE	iP > iNP**	iP > NP.P*	#
	DUR BRIDGE ENTRANCE	iP > iNP*	iP > NP.P*	#
	DUR/FRQ BRIDGE ENTRANCE	#	#	#
	OCC BRIDGE ENTRANCE	#	#	#
	SAP TO CENTER	#	#	#
	OCC SAP TO CENTER	#	#	#
	SAP TO SLOPE	#	#	#
Risk taking	LAT BRIDGE	iP < iNP*	iP < P.NP*	#
	FRQ BRIDGE	iP > iNP**	iP > P.NP*	#
	DUR BRIDGE	iP > iNP***	iP > P.NP**	#
	DUR/FRQ BRIDGE	iP > iNP**	#	#
	OCC BRIDGE	#	#	#
	FRQ CTRCI	#	#	iNP < NP.P**
	DUR CTRCI	#	#	#
	DUR/FRQ CTRCI	#	#	#
	OCC CTRCI	#	#	#
Shelter seeking	LAT DCR	#	#	#
	FRQ DCR	#	#	#
	DUR DCR	iP < iNP*	#	#
	DUR/FRQ DCR	iP < iNP**	#	#

Table 3 Continued

Anxiety-like behavior	DUR SHELTER/RISK INDEX	iP < iNP**	#	#
Impulsive-like behavior	SLOPE/BRIDGE INTERVAL	#	#	#

Behavioral parameters for which there were significant differences between the respective inbred preferring and nonpreferring rats and inbred and congenic rats. Occurrence (OCC) indicates the number of animals out of the total number of animals in a group that visited a zone or participated in a behavior (#visited or #performed a behavior/n). *$p \leq 0.0167$, **$p < 0.01$, ***$p < 0.001$, comparing iPviNP, iPvP.NP and iNPvNP.P groups (Mann-Whitney U-test); ≤ or ≥ denotes trend toward significance; # denotes no significant differences found. *Abbreviations:* CTRCI, Center Circle; DCR, dark corner room; DUR, duration; DUR/FRQ, duration per visit; FRQ, frequency; LAT, latency; OCC, occurrence; SAP, stretched attend posture; TOTACT, total activity, i.e. the sum of all frequencies; TOTCORR; total corridor, i.e. the sum of all corridors.

Table 4

Descriptive data and SEM of other behaviors in the MCSF.

	Parameters	Genotype	P Background	NP Background
Other	GROOMING	iP/iNP	2.25±0.4	1.58±0.3
		P.NP/NP.P	1.9±0.3	1.22±0.3
	OCC GROOMING	iP/iNP	10/12	10/12
		P.NP/NP.P	9/10	7/9
	BOLI	iP/iNP	2.17±0.6	3.00±0.6
		P.NP/NP.P	2.50±0.7	2.78±0.6
	OCC BOLI	iP/iNP	9/12	10/12
		P.NP/NP.P	6/10	7/9
	URINE	iP/iNP	2.42±0.6	2.67±0.4
		P.NP/NP.P	1.7±0.4	2.33±0.6
	OCC URINE	iP/iNP	9/12	11/12
		P.NP/NP.P	7/10	7/9
	BODY WEIGHT	iP/iNP	536.81±9. 3	566.42±6.3
		P.NP/NP.P	544.88±11.8	528.19±5.2

The data table is organized with respect to the genotype of the four animal groups. The two columns denote the recipient's background genome. There are two rows, where data for inbreds are on the top row and congenics is on the second row. The animal genotype column identifies the first and second column. Occurrence (OCC) indicates the number of animals out of the total number of animals in a group that participated in a behavior (#performed a behavior/n).

3.2 Experiment 2 – Open Field

A summary of means and standard error means of all data can be found in Table 5 and a summary of significant differences can be found in Table 6.

3.2.1 Inbred & congenic pairwise comparisons

3.2.1.1 iP and iNP

Inbred Ps tended to make more line crossings than iNPs (p = .041). Inbred Ps appeared to spend more time and took more visits to the Center area (p = .127, .031, respectively), while iNPs tended to spend more time performing thigmotaxic behavior by spending more time per visit in Periphery (p = .031) than iPs. These results provide support for MCSF data which demonstrated an increase in risk-taking in the iP genetic strain.

3.2.1.2 iP and P.NP

Significant differences were found between iP and P.NP on time spent per visit to Center (CenterDUR/FREQ) where iPs scored higher than P.NPs, p = .008. Time spent in Periphery trended towards significance where P.NP scored higher than iP on this variable. Perhaps these differences are not conclusive due to lack of statistical power to elucidate those differences, however it is noteworthy that the trending results show values in the direction predicted by the introgressed QTL region.

3.2.1.3 iNP and NP.P

No significant differences were found for behavior in OF.

Table 5

Descriptive data and SEM in the Open field.

Open Field	Genotype	P Background	NP Background
PeripheryFREQ	iP/iNP	15.17±3.9	11.0±4.0
	P.NP/NP.P	13.33±6.5	12.1±3.7
PeripheryDUR	iP/iNP	505.67±44.8	534.33±61.7
	P.NP/NP.P	548.33±32.4	537.70±27.0
PeripheryDUR/FREQ	iP/iNP	36.75±14.0	56.67±25.2
	P.NP/NP.P	51.2±25.4	50.4±21.7
CenterFREQ	iP/iNP	14.25±4.0	10.08±4.1
	P.NP/NP.P	12.33±6.5	11.20±3.8
CenterDUR	iP/iNP	94.33±44.8	65.67±61.7
	P.NP/NP.P	51.67±32.4	62.30±27.1
CenterDUR/FREQ	iP/iNP	6.50±2.0	5.58±3.2
	P.NP/NP.P	3.89±0.7	5.6±1.0
CrossingFREQ	iP/iNP	190.33±30.9	161.8±32.9
	P.NP/NP.P	209.67±43.0	171.10±24.7
Fecal Boli	iP/iNP	2.67±1.6	3.58±1.7
	P.NP/NP.P	3.89±2.8	2.90±2.1
Urine	iP/iNP	0.25±0.4	0.17±0.3
	P.NP/NP.P	0.44±0.7	0.30±0.4

The data table is organized with respect to the genotype of the four animal groups. The two columns denote the recipient's background genome. There are two rows, where data for inbreds were on the top row and congenics is on the second row. The animal genotype column identifies the first and second column. *Abbreviations:* FRQ, frequency (s); DUR, duration (s); DUR/FRQ, duration per visit (s)

Table 6

Summary of between group differences in Open field

Open Field	**iP v iNP**	**iP v P.NP**	**iNP v NP.P**
PeripheryFREQ	#	#	#
PeripheryDUR	#	#	#
PeripheryDUR/FREQ	iP≤iNP	#	#
	p =.031		
CenterFREQ	iP≥iNP	#	#
	p = .031		
CenterDUR	iP≥iNP	#	#
	p = .127		
CenterDUR/FREQ	#	iP > P.NP**	#
CrossingFREQ	iP ≥ iNP	#	#
	p = .041		
Fecal Boli	#	#	#
Urine	#	#	#

Results for significant differences between the inbred preferring and nonpreferring rats and inbred and congenic rats. *$p < 0.0167$, **$p < 0.01$, ***$p < 0.001$, comparing iPviNP, iPvP.NP and iNPvNP.P groups (bonferonni adjusted, ANOVA and post hoc LSD); ≤ or ≥ denotes trend toward significance; # denotes no significant differences found.

Abbreviations: FRQ, frequency; DUR, duration; DUR/FRQ, duration per visit.

3.3 Experiment 3 – Elevated plus maze

A table of all means and SEMs can be found in Table 6 and a summary of significant differences is shown in Table 7.

Significant differences were found for visits to Center, F (3,41) = 9.989, $p < 0.00$. Inbred Ps took more visits to the Center than iNPs, which provides some support for higher risk assessment in iP rats. For Total Arm Entries, F (3, 41) = 9.56, $p < .049$, iPs had significantly more arm entries than iNP rats. This supports previous research that showed iP rat's greater general locomotor activity (Badishtov, et al., 1995; Roman, et al., 2011).

Inbred NP rats tended to have greater fecal boli output than iPs. Inbred NP rats tended to have greater urine output than iPs.

Table 7

Descriptive data and SEM in Elevated plus maze.

Elevated Plus Maze	**Genotype**	**P Background**	**NP Background**
Open	iP/iNP	11.33±3.5	9.75±1.9
	P.NP/NP.P	12.20±2.1	2.25±.79
DUR Open	iP/iNP	122.91±36.0	141.58±33.4
	P.NP/NP.P	114.00±26.4	131.12±38.4
DUR/FREQ Open	iP/iNP	10.08±2.5	14.83±4.3
	P.NP/NP.P	9.3±3.7	20.6±12.7
Closed	iP/iNP	6.25±1.9	4.83±2.2
	P.NP/NP.P	7.00±3.1	5.00±3.2

Table 7 Continued

DUR Closed	iP/iNP	55.08±27.2	58.17±33.8
	P.NP/NP.P	62.50±39.4	66.37±36.8
DUR/FREQ Closed	iP/iNP	12.75±5.1	16.67±4.9
	P.NP/NP.P	10.40±2.4	20.11±9.3
Center	iP/iNP	16.00±3.2	12.67±1.8
	P.NP/NP.P	17.70±3.2	11.00±3.4
DUR Center	iP/iNP	124.00±26.3	102.33±19.4
	P.NP/NP.P	125.40±28.4	113.86±27.9
DUR/FREQ Center	iP/iNP	8.00±2.2	8.00±1.7
	P.NP/NP.P	7.20±2.1	10.88±4.0
TotalArmEntries	iP/iNP	15.58±3.1	12.58±1.7
	P.NP/NP.P	17.20±2.9	11.25±3.5
Fecal Boli	iP/iNP	0.67±1.2	1.58±1.4
	P.NP/NP.P	0.70±1.2	2.22±1.2
Urine	iP/iNP	0.25±0.4	0.75±0.7
	P.NP/NP.P	0.20±0.4	1.11±0.6

The data table is organized with respect to the genotype of the four animal groups. The two columns denote the recipient's background genome. There are two rows, where data for inbreds are on the top row and congenics is on the second row. The animal genotype

column identifies the first and second column. *Abbreviations:* FRQ, frequency (s); DUR/FRQ, duration per visit(s).

Table 8

Summary of between group differences in Elevated Plus Maze.

ELEVATED PLUS MAZE			
Zones	**P vs NP**	**iP vs P.NP**	**iNP vs NP.P**
Open	#	#	#
DUROpen	#	#	#
DUR/FREQ Open	#	#	#
Closed	#	#	#
DURClosed	#	#	#
DUR/FREQ Closed	#	#	#
Center	iP > iNP*	#	#
DURCenter	#	#	#
DUR/FREQ Center	#	#	#
TotalArmEntries	iP > iNP*	#	#
Fecal Boli	#	#	#
Urine	#	#	#

Behavioral data for which there were significant differences between the respective inbred preferring and nonpreferring rats and inbred and congenic rats. *$p \leq 0.0167$, **$p < 0.01$, ***$p < 0.001$, # = no significant differences found. Comparisons: iPviNP, iPvP.NP and iNPvNP.P groups (ANOVA, LSD posthoc tests with bonferonni adjustment); *Abbreviations:* FRQ, frequency; DUR, duration; DUR/FREQ, duration per visit.

3.4 PCA analysis

PCA was mainly used to examine the overall relationships between the animal groups performance in the MCSF and possibly reveal trends in behavior (Appendix B for graphic results). For each pairwise comparison all MCSF behavioral observation data were input into the analysis. The statistical technique was blind to which observations belonged to which genotype. The outcomes were graphed in a plot that shows the relationship among observations. Randomly distributed data points indicate no apparent pattern or relationship among the input data. Grouped data points or separation among data points, in terms of genotype, points to differences behavioral strategy in the MCSF.

The analysis for iP and iNP showed good separation between the groups (Appendix B1). Relevant loading parameters for iP's were performance on the Bridge and Center circle (risk taking), SAP to Center and frequency and duration on Slope and Bridge Entrance zones (risk assessment). Inbred NP's loaded highly on latency to risk areas, latency to leave initial Center start point, and shelter seeking (performance on DCR). Overall iNPs took less risky options in the MCSF, while iP's clearly tended to take more risky behavior.

The analysis for iP and P.NP showed moderate amount of separation (Appendix B2).

For iPs the relevant loading parameters are performance on DCR, risk/shelter index, total activity and Hurdle zone. Latency to Slope, Bridge Entrance, and Bridge, SAP to Slope (risk assessment), slope/bridge index (impulsive-like behavior) parameters loaded in the same quadrant as P.NP's. Overall, these two groups showed more overlap

in their behavioral strategies than iP and iNPs, nevertheless separation between groups is demonstrated.

The analysis for iNP and NP.Ps showed no separation (Appendix B3). This result is in agreement with the lack of significant differences elucidated for most behavioral paramenters analyzed for iNP and NP.Ps. This study found, for the most part, their behavioral strategies were indistiguishable.

3.5 Shelter/Risk Index (Anxiety-like Behavior)

The shelter/risk index is calculated from the difference in time spent in the dark corner room (DCR) and on the BRIDGE, relative to the total time spent in the two zones and is used as a measure of anxiety-like behavior. A positive value indicates that the animals spent more time in the DCR than on the Bridge and is interpreted as higher anxiety-like behavior. Bonferonni corrected, Mann Whitney U test found statistically significant differences for the Shelter Risk Index Duration variable (p = .002) finding that iNPs spent more time seeking shelter in DCR than on the Bridge when compared to iPs. No differences were detected for iP and P.NP groups and no differences were detected for iNP and NP.P groups. The outcome for this index was supported by behavior parameter results that showed for time spent on bridge and number of visits to the bridge, iPs spent more time doing both than iNP and P.NP. Inbred iNP rats spent significantly more time per visit to DCR than iPs. Also, iNPs tended to spend more time in Periphery of OF.

3.6 Slope/Bridge Interval (Impulsive-like Behavior)

The impulsive-like behavior is measured in terms of latency to visit risk zone (Bridge) once the risk assessment zone has been entered (Slope). A value close to zero indicates less risk assessment and a short latency before making the risk-taking response. No differences were found between any of the groups.

CHAPTER 4 DISCUSSION

This study accomplished the task of behaviorally profiling the P.NP and NP.P congenics for the first time outside of alcohol consumption behavior and also profiled the inbred P and NP rats for the first time using the MCSF apparatus. This study's findings were in line with previous work that showed the chromosome 4 QTL influence on behavioral traits in reciprocal congenics (Carr et al., 2006) and demonstrated that the QTL determines some significant portion of phenotypic differences between iP and iNP rats. The iP and iNP rats showed different behavioral strategies in the MCSF and there was some support provided by the OF and EPM for this result. The P.NP congenics primarily demonstrated the expected shift in behavior on many behavioral parameters compared to its background strain while the NP.P congenics failed to convincingly demonstrate the expected shift in behavior. Inbred P genes that control risk assessment and risk taking behavior appear to lie within the chromosome 4 QTL region and further this QTL appears to be essential but not sufficient to generate "P" phenotypic behavior. In general, this study confirms that the QTL contributes significantly to the divergent behavior between P and NP rats; however more research is necessary since some results in this study did not replicate previous research using outbred P and NP rats.

4.1 Behavioral profiles

4.1.1 iP and iNP

In this study iPs were the same as iNPs concerning general activity in the MCSF which is in opposition to the hypothesis and previous research showing iPs being less active (Roman, et al., 2011). This research confirmed the hypothesis that iPs show more activity than iNPs in the OF, in terms of frequency of line crosses, and further support came in the form of iPs having more total arm entries in the EPM than iNPs. More line crossings in the OF by iPs replicated previous research in P rats (Badishtov, et al., 1995; Roman, et al., 2011). Contextual differences between MCSF and OF environments might explain the differences in activity outcomes. The MCSF is constructed with corridors and many zones so overall has a more complex construction which may have mitigated activity in the MCSF.

Inbred Ps showed more risk assessment than iNPs, this outcome contradicted our hypothesis and previous research (Roman, et al., 2011), but speculatively this factor may have contributed to the decreased activity displayed in the MCSF. As long as it is necessary to perform more risk assessment, due to the more complex MCSF environment, again it is possible that activity was mitigated in the MCSF. Alternatively, cognitive processing of environmental cues might differ between Ps and NPs hence moderate behavioral outcomes in P and NP rats. This study per se cannot provide convincing support for anxiety-related behavioral differences directly affecting risk assessment differences.

From previous research in the MCSF with outbred P rats (Roman, et al., 2011) we expected iPs to show less risk taking behavior and take less visits to the Center zone, however this study found iPs displayed more risk taking behavior than iNPs and no difference was observed in visits to the Center (Roman, et al., 2011). As shown by the same previous research, it was expected that iPs would take more visits to the Slope, Bridge entrance, and Bridge than iNPs. This study was able to replicate these outcomes, which is congruent with the higher risk taking finding for iPs in this study. The OF also supported an element of risk taking and showed that iPs tended to spend more time in the Center while iNP's tended to spend more time in the Periphery.

This study showed no differences in exploratory behavior between iPs and iNPs and this outcome replicated previous research (Roman, et al., 2011). This study showed that iNPs sought safety more often than iPs, ie more shelter seeking, where previous research on outbred Ps and NPs did not find these differences (Roman, et al., 2011).

On the Shelter/Risk index that measured anxiety-like behavior iP rats scored lower than iNPs thus, iPs spent more time on the Bridge than in the DCR. Previous research did not find this difference for outbred Ps. Previous research has demonstrated significant reduction in the number of total arm entries in the EPM following intraperitoneal administration of anxiogenics (Pellow & File, 1986) hence total arm entries has been used as a measure for anxiety-like behavior even though it is an uncommon measure of anxiety in the EPM. In this study, iPs scored higher than iNPs for total arm entries in the EPM, which signals that iNP rats may be showing anxiety-related behavior, this result is in line with the shelter/risk index outcome. EPM findings could not provide any additional support for differences in anxiety-like behavior between

iNPs and iPs in relation to their performance in the open or closed arms, so this outcome could not replicate previous research showing that outbred Ps displayed more anxiety-like behavior than NPs in the EPM (Stewart, Gatto, Lumeng, Li, & Murphy, 1993). In the same Pellow & File (1986) study, no effects of other drugs with anxiogenic activity were found in the EPM when compared with controls, however these drugs had demonstrated anxiogenic activity in other tests of anxiety-like behavior, such as the Vogel and social interaction tests. It has been suggested that behavioral actions of benzodiazepine receptor antagonists are dependent on the test situation and differences may reflect the level of the endogenous nature within the system (File & Pellow, 1986). Fecal boli output has been used as an indicator of anxious emotionality in rats. Outbred NP rats in novel environments have been reported to have higher fecal boli output (Badishtov et al., 1995; (Roman, et al., 2011). Inbred NP rats tended to have greater boli output than iPs in the EPM.

Impulsive-like behavior was characterized by latency to enter the Bridge, risk area once entering the risk assessment zone. Inbred Ps and NPs scored the same on the Bridge/Slope interval that measures impulsive-like behavior and hence did not support previous research that showed iPs tendency toward more impulsive-like behavior in the MCSF (Roman, et al., 2011).

As seen by the above stated conclusions, many differences could be elucidated between iPs and iNPs and the PCA analysis was able to provide support that showed good separation overall between the two groups. It was most evident in the separation between the two groups in their performances on parameters related to risk assessment and risk taking such as bridge, slope, SAP to slope as well as latencies to these areas.

4.1.2 P.NP and NP.P

This study found that the congenics were very much like their inbred counterparts, but some important differences were found especially between iPs and P.NPs. Environmental differences are controlled as much as possible so the only difference between the iP and P.NP and likewise the iNP and NP.P is the introgressed QTL region. Consequently, it is presumed that the QTL is accountable for any observed differences in behavioral strategies in the MCSF, OF and EPM. Specifically, in regards to the QTL the study hypothesized that variables where iPs scored higher or lower than iNPs, congenic NP.Ps should have scored higher or lower than NPs, respectively. The reciprocal case was expected for how iNP scores related to P.NP scores.

Each inbred strain and corresponding congenic strain ranked the same on general activity and exploratory behavior. Congenic P.NPs scored lower on risk assessment than iPs, demonstrating that the "NP" QTL mitigated risk assessment in P.NPs. Congenic NP.Ps tended to score higher than iNPs. Congenic P.NPs scored lower on risk taking than iPs, while NP.P's tended to score higher than iNPs. Concerning shelter seeking, inbred and congenic strains were not different. Congenics were the same as their inbred background strains on indices of anxiety-like and impulsive-like behavior. Additionally the PCA analysis confirmed more similarities between the congenics and their respective inbred strain. A moderate amount of separation was shown in the PCA plot between iP and P.NP, however no separation was apparent between iNP and NP.P.

4.2 Quantitative trait locus

In the process of creating the congenics, it was not clear whether the iP donor QTL was actually contributing to the high alcohol consumption phenotype, based on the more than expected variation observed in drinking scores for the NP.P background strains (Carr et al., 2006). The present study found a lower rate of significant differences between iNP and NP.P animals than for iP and P.NPs, in other words, the "P" QTL failed more often to augment "P" phenotypic traits in iNPs. In a previous study a chromosome 4 QTL was found to segregate in high alcohol drinking (HAD)/low alcohol drinking (LAD) rats, however wasn't found to be linked to the alcohol consumption phenotype in these lines (Foroud et al., 2000) which suggests that this QTL is not a trait that is necessary and sufficient for the alcohol preference phenotype. The P.NP strains displayed the expected difference in consumption compared to the iP parent strain and so it appears as though the iNP QTL does contribute to the alcohol avoidant phenotype of the iNP rats (Carr et al., 2006). This previous research and outcomes found in this study provide evidence that the chromosome 4 QTL is not singly responsible for traits underlying the alcohol preferring phenotype and support for epistasis as an important factor in the development of the alcohol preferring phenotype. These results also underline the fact that alcohol dependence is a complex, pleiotropic disease.

The NPY precursor is encoded within the QTL chromosome 4 region and NPY is divergently expressed in the brains of P and NP rats (Kimpel, et al., 2007). NPY is involved in the stress-anxiety circuit of the nervous system and is presumed to be responsible, in part, to the differences in scores on anxiety measures in Ps and NPs (Badia-Elder, et al., 2007). Although, this study cannot determine whether any observed

effect was a direct result of NPY expression differences the NPY phenotype did not appear to affect any anxiety construct as it relates to the EPM. It is not immediately clear as to how NPY might affect measures of anxiety-related behavior as it is gauged in the MCSF and OF.

Body weight differences were found where iPs weighed less than iNPs and NP.P weighed less than iNPs. Significant differences were not found for P.NPs and iPs. This weight difference result is in line with previous work (Alam, et al., 2005) and demonstrates that the QTL controls this phenotype in P and NP rats. This trait cosegregates with the selection trait in P and NP rats, however the above mentioned previous work has found that this difference is caused by bone mass differences. This pattern of weight difference is also observed in High Alcohol Drinking (HAD) rats, however the opposite is observed in its HAD2 replicate line, which is further evidence that this trait is irrelevant to the alcohol consumption trait.

4.3 General discussion

Alcohol dependence and many of its identifying features are highly heritable as evidenced by twin studies that puts heritability between 50-60% (Hiroi & Agatsuma, 2005) and also supported by selective breeding pressures that maintain divergent alcohol consumption phenotypes in rats that put heritability estimates in the neighborhood of 30-40% in rodent animal models (Li, et al., 1987). Selectively bred and inbred animal models are an important experimental tool to examine genetic factors that underlie phenotypic traits related to high alcohol consumption (Grahame, 2000).

The MCSF environment allowed observation of a multitude of behaviors related to anxiety, exploration, impulsivity, safety and risk. The MCSF exposes the heterogeneity that has been found to exist in the large collection of selected lines bred for alcoholism research as it relates to the human condition (Roman, et al., 2011) and this present study adds to that body of work. This study could not duplicate some of this previous work, but inconsistencies might be explained by the differences in the model of animal used. The differences between genetically selected rats and inbred rats, are exacerbated by forces of genetic drift and spontaneous genetic mutation, respectively (Grahame, 2000). There is no inter-observer reliability to ensure standardized scoring, so outcomes evaluated by the MCSF could differ between different laboratories. Also, the observer of behavior for the MCSF, OF and EPM were not blind to the genotype of the rats. Finally, since the animals were no longer experimentally naïve for the OF and EPM tests carryover effects cannot be ruled out, even though previous work has provided evidence of minimal effect (Augustsson, 2004).

Since the animals used in this study were inbred, their genetic traits leading to the high alcohol consuming phenotype are fixed but other correlated traits unrelated to the high consuming phenotype are also fixed, so it must be acknowledged that differences between groups, namely iP and iNP, may not bear upon alcohol dependence. These differences may be important to that cohort as an animal model but may not have any translational value to the human condition.

In the future it may be useful to retest the same cohorts of animals while applying some additional methods. A continuous access, voluntary alcohol consumption component could be added and run concurrently with these behavioral profiles in order to

replicate the QTL effect. Since calculations were made that suggest a study needs 20-25 animals to gain the 80% power necessary to detect differences on alcohol consumption (Carr et al., 2006), it would be useful to amplify the sample size numbers in order to obtain the statistical power needed to further sharpen differences between the inbred and congenic groups in their behavioral strategies in the MCSF and other tests. The MCSF provides the necessary context that permits differences in behavioral strategies to be clarified and allows the assessment to be executed simultaneously in one test session and it will be beneficial in the future to avoid serial testing which might introduce carryover effects into behavioral outcomes on subsequent testing, otherwise a different naïve group for each genotypes used can be tested on each apparatus.

In summary, while behaviorally profiling the selectively bred animals using the MCSF, this study was able to meet its objectives by (i) behaviorally profiling iP, iNP, P.NP and NP.P groups while contributing new research to the current body of literature for these genetically selected breeds, (ii) providing support for the chromosome 4 QTL as a major element in determining differences between iP and iNP rats and finally this study (iii) adds to a growing body of literature using the MCSF behavioral assessment test.

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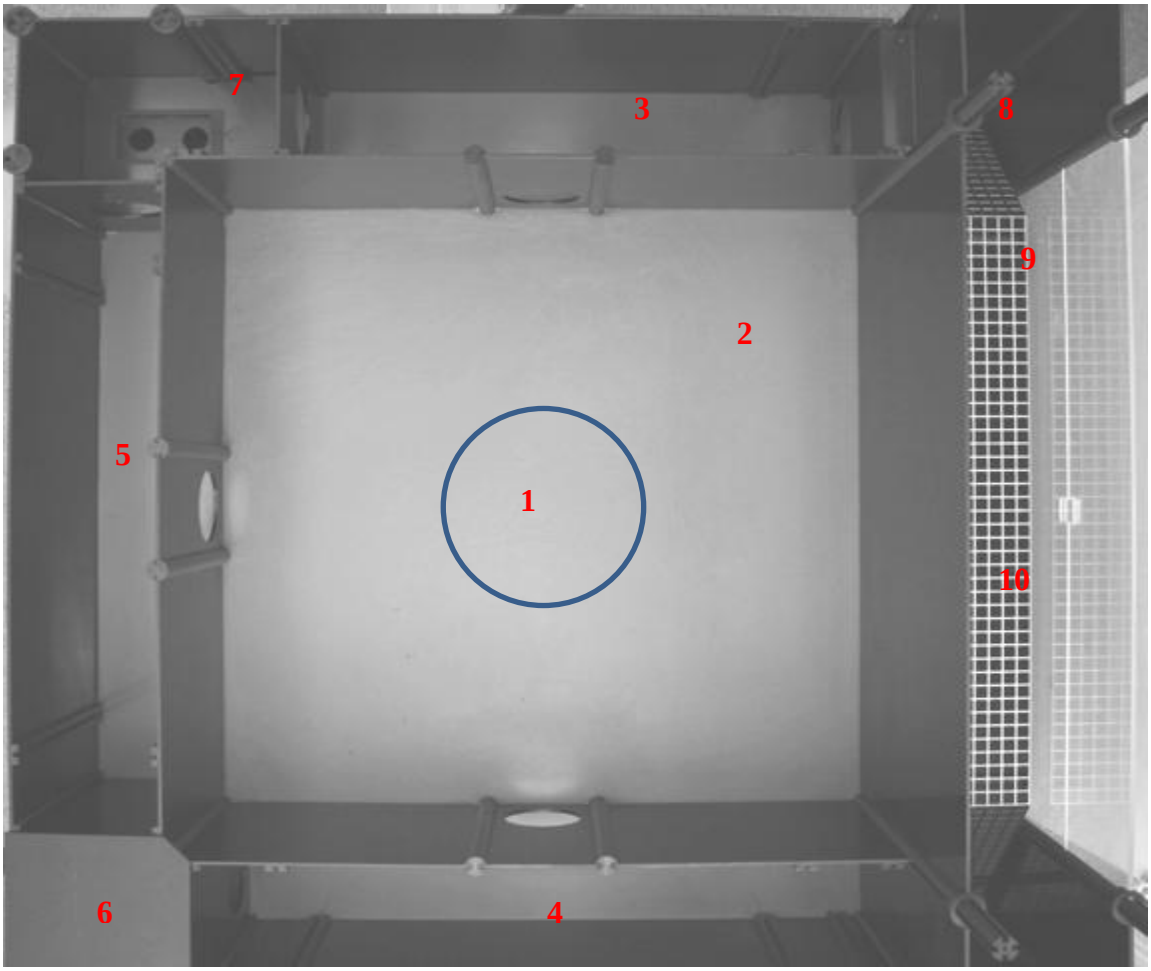
APPENDICES

Appendix A



1 = Center circle	6 = dark corner room (DCR)
2 = Center	7 = Hurdle
3 = North Corridor	8 = Slope
4 = South Corridor	9 = Bridge entrance
5 = West Corridor	10 = Bridge

Figure A.1 Multivariate Concentric Square Field (MCSF)

Appendix B

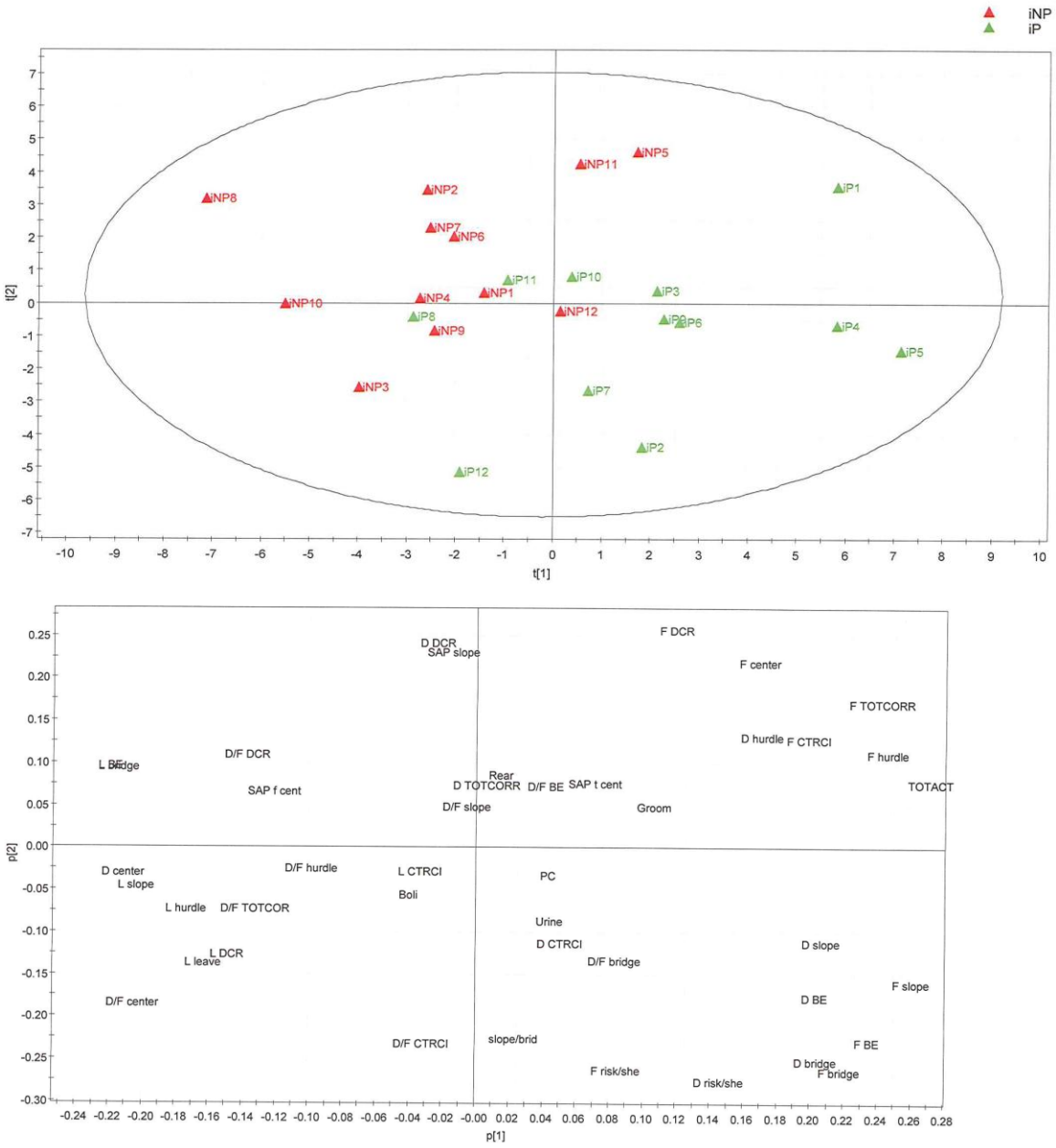


Figure B.1 PCA analysis for iP and iNP

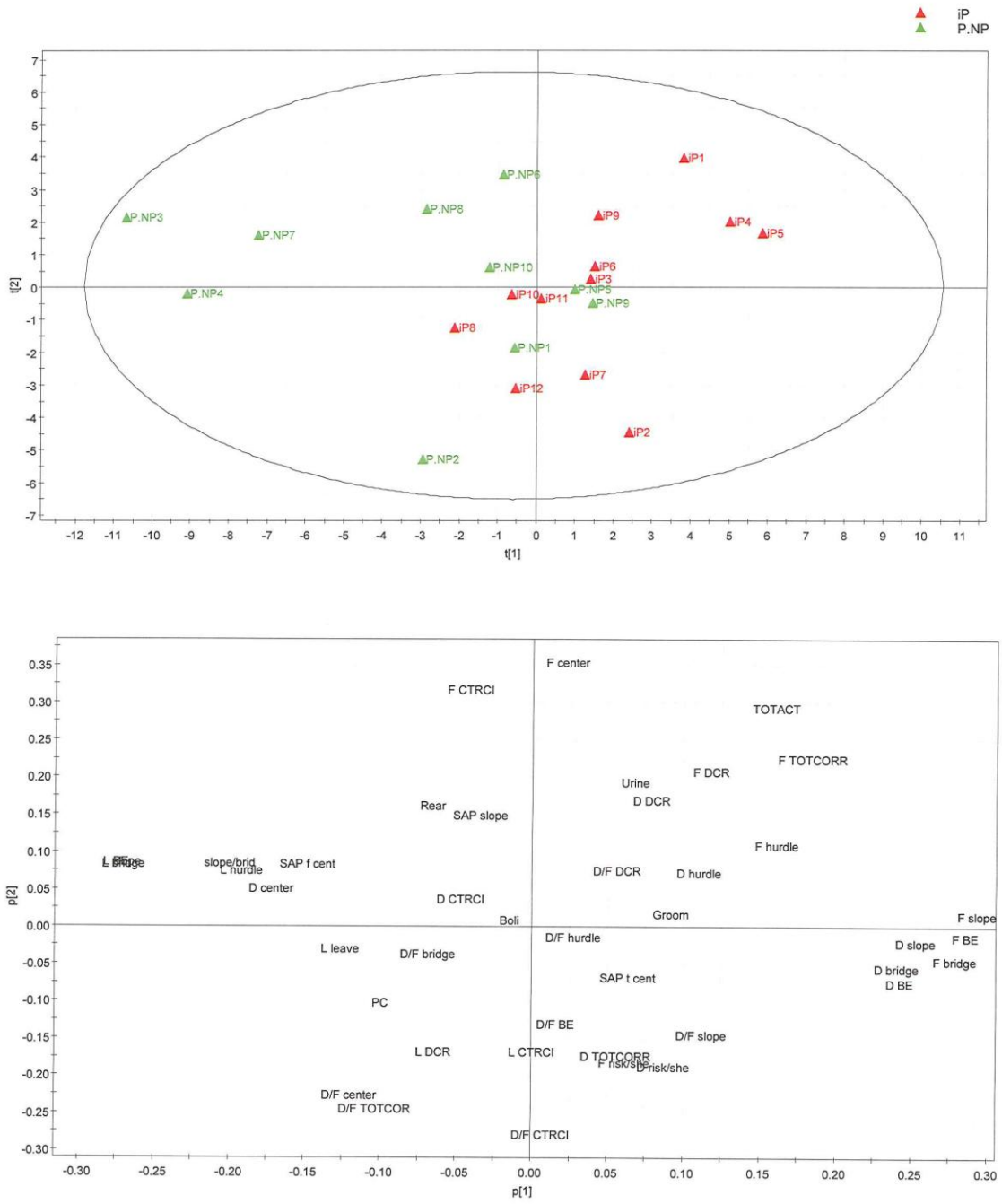


Figure B.2 PCA analysis for iP and P.NP

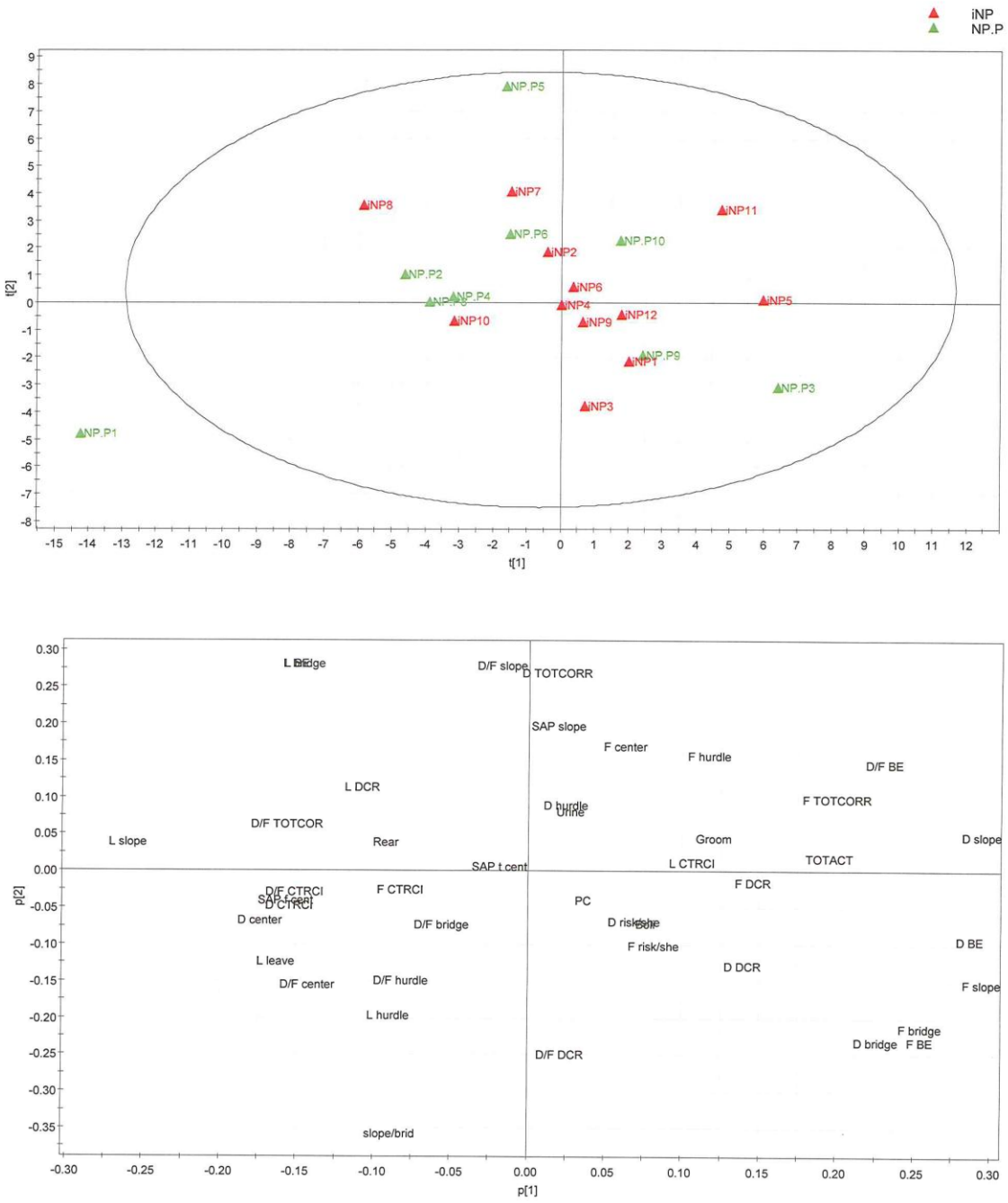


Figure B.3 PCA analysis for iNP and NP.P