Effects of Dietary Micronutrient Supplementation on the Development of Emotionality and Anxiety in a Normal Rat Population

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Abbreviations

ADHD	Attention-Deficit/Hyperactivity Disorder
ANOVA	Analysis of Variance
˚C	Degrees Celsius
EMP+	EMPowerplus
F	Female
GAD	Generalised Anxiety Disorder
g	Grams
IU	International Unit
M	Male
mcg	Micrograms
mg	Milligrams
ml	Millilitres
mm	Millimetres
ms	Milliseconds
N	Number
OCD	Obsessive-Compulsive Disorder
PND	Post Natal Day
S.E.M.	Standard Error of the Mean
TX	Treatment

Abstract

There is a growing body of research into the effects of micronutrients on human mental health. There is evidence that multi-ingredient formulas are beneficial especially in relation to serious mental health disorders such as mood and anxiety disorders, attention-deficit hyperactivity disorder and obsessive-compulsive disorders. However there is almost no scientific research which looks at the effects of these formulas in an animal population. Therefore the aim of this study was to investigate the effects of a micronutrient formula, EMPowerplus, on anxiety behaviour in rats, and whether there is a relationship between dose and anxiolytic effect. In order to investigate this 40 male and 40 female rats received a diet consisting of either 0%, 1.25%, 2.5% or 5% EMP+ from when they were weaned (post natal day 30) until the end of testing 141 days later. Animals were tested in a Y maze, a light-dark emergence box and an open field at mid-adulthood (PND 136-138) and late adulthood (PND 186-188). Results found that animals receiving the 5% supplemented diet occupied the centre squares the most, occupied the corner squares the least and ambulated the most in the open field compared to the other experimental groups and control groups. No significant differences were found in the Y maze or Light-dark box. Animals were found to display more anxiety-like behaviour at time 2 than at time 1 regardless of receiving a supplemented diet or not. Overall a higher dose of EMP+ was associated with the greatest reduction in anxiety related behaviour. Due to the impact of the September 4th, 2010 Canterbury Earthquake caution should be taken when interpreting these results.

1.0 Introduction

1.1 General Overview:

Anxiety symptoms and disorders are common in the general population and in primary and secondary medical care worldwide (Baldwin et al., 2005). While symptoms can be mild, transient and not cause functional impairment in social and occupational areas, for many individuals the symptoms they experience are severe and enduring and impact functioning across many areas of their lives (Baldwin et al., 2005; Cryan & Sweeney, 2011). Anxiety disorders are currently the most prevalent psychiatric disorder across Europe and in the United States of America. Large epidemiological studies in these regions show that they have high lifetime prevalence with estimates ranging from 13.6-28.8% (Cryan & Sweeney, 2011). There is evidence that indicates that anxiety disorders have the earliest age of onset (11 years old) when compared to other psychiatric disorders (Kessler, 2007; Kessler et al., 2005a; Kessler et al., 2005b). It has also been shown that patients with a diagnosis of an anxiety disorder are likely to present with other comorbid disorders often including, although not limited to, psychiatric disorders such as depression (Baldwin et al., 2005; Kessler et al., 2005b) along with physical health conditions including cardiovascular disease, gastrointestinal disease, respiratory difficulties such as asthma, hypertension, migraine, cancer and chronic pain (Härter et al., 2003). It is clear the wide ranging impact anxiety disorders have is not only on an individual level but also on a social and economic level worldwide. The need for effective treatment opinions to help reduce these multiple impacts is obvious.

To date, the main psychopharmacological choice of treatment for anxiety disorders are two types of pharmacological agents known as benzodiazepines and selective serotonin re-uptake inhibitors (SSRIs). Benzodiazepines have been available since the 1960s and produce their anxiolytic effects through interactions with the $GABA_A$ receptor. Although benzodiazepines reduce anxiety symptoms, this effect is accompanied with a number of less desirable side-effects following long

term use - namely the development of drug dependence and tolerance along with cognitive and behavioural effects. These side effects highlighted the need for investigation of non-GABAergic based pharmacological therapies and the discovery of the role the serotonegeric system plays in anxiety (Cryan & Sweeney, 2011; Dunlop & Davis, 2008). SSRIs were originally developed for the treatment of depression, however the recognition that anxiety and depression are often comorbid led to the clinical observation that SSRIs are also effective in treating anxiety symptoms (Cryan & Sweeney, 2011; Dunlop & Davis, 2008). In fact SSRIs are now the first-line treatment for a number of anxiety disorders due to their established efficacy, their general good tolerability profile, their efficacy for comorbid depression and their suitability for long term use (Dunlop & Davis, 2008).

Despite being effective anxiolytics both classes of drugs have their limitations. As mentioned above, benzodiazepines are not recommended for long term use due to their potential for dependence and tolerance. As mentioned above, benzodiazepines have little impact of on comorbid depressive symptoms and in fact may exacerbate these symptoms. There is also the possibility of rebound anxiety following discontinuation of medication. They produce psychomotor and cognitive impairment along with increasing rates of ataxia and falls. They have abuse potential along with the physical effect of respiratory depression increasing risk of over-dose (Dunlop & Davis, 2008). SSRIs on the other hand have slow onset of drug action, can induce agitation or anxiety, produce sleep disturbance, sexual dysfunction and weight gain. More recently, concerns have been raised about a potential link between SSRI use in some populations and an increase in suicidality.

Both classes of drugs are associated with discontinuation symptoms including increased anxiety, irritability and insomnia along with dizziness, headache, heighten sensations, tremor, nausea, seizures and excessive sweating (Dunlop & Davis, 2008).

It is important to note that although effectiveness has been established in regards to both classes of drugs not all individuals respond to this type of intervention (Koen & Stein, 2011). While

the majority of randomised controlled trials (RCTs) show that an active psychopharmacological agent (either a SSRI or benzodiazepine) is more effective in reducing anxiety symptoms than the placebo condition across a range of anxiety disorders the response rates are not one hundred percent for the treatment groups (Koen & Stein, 2011). In fact the studies reviewed by Koen and Stein (2011) reported treatment response rates between 50 to 68% with placebo rates ranging between 20 to 41%. Although initially these response rates look to be impressive with a significant difference between the treatment conditions and placebo condition, it actually means that 32 to 50% of individuals in these studies did not respond to the psychopharmacological intervention (Koen & Stein, 2011).

One can conclude that although the pharmacological agents of choice for treating anxiety are effective anxiolytics, they are also associated with a number of unpleasant side and discontinuation effects. In addition to this, not all individuals respond to a pharmacological intervention of this nature. This has lead to the search for alternative treatments that not only reduce individuals' psychiatric symptoms but also have less side effects and risks with regards to long term treatment, as well as a treatment that can produce response rates as good as, if not greater than, the current first-line agents.

One avenue of research into alternative treatments has focused on the effects of vitamins and minerals - alone or in combination - on mental health. Articles in medical journals from the 1920s onwards made reference to symptom improvement as a result of a nutrient based intervention (Kaplan & Leung, 2011). More recently, studies carried out towards the end of the 20th century and beginning of the 21st showed that some B vitamins may reduce psychiatric symptoms while other dietary minerals such as magnesium may improve mood stability. Since the year 2000 scientific studies have begun to investigate the effects of complex micronutrient interventions on mental health outcomes (Kaplan & Leung, 2011). A variety of formulas have been investigated across a range of mental health areas including obsessive-compulsive disorder, mood and anxiety disorders,

attention-deficit hyperactivity disorder, symptoms associated with autism as well as aggressive or antisocial behaviours (Kaplan & Leung, 2011). Following the Canterbury Earthquakes in New Zealand, several studies have looked at the effects of micronutrients on trauma responses (Rucklidge et al., 2012; Rucklidge et al., 2011a).

The largest body of literature in relation to complex micronutrient interventions has focused on a micronutrient formula made up of 36 vitamins and minerals known as EMPowerplus (EMP+) and this is the formula utilised in this study. At this stage in time, primarily the research in to the effects of micronutrients on mental health has focused on the human population with clinical disorders and there is no published research focusing on the effects of micronutrient supplementation in the rat population. Rat studies provide us with an opportunity to establish a framework of understanding and are useful due to the level of control one can have over the subjects and manipulations of the experiment. In this way, we can rule out confounding variables to ensure we can examine the relationships between the manipulations and outcomes with a greater degree of certainty. With animal research you are not likely to experience some of the difficulties associated with human research such as using drugs, not taking the supplement or missing appointments. In order to help increase our knowledge along with addressing some of the gaps in the current body of research, the primary goal of this study was to investigate the effects of micronutrient supplementation on anxiety behaviours in a rat population that were not animal models of any serious psychiatric disorders, in other words a normal rat population. Anxiety was chosen as the behaviour to examine due to the high prevalence of anxiety symptoms and disorders within the general and clinical population. It is thought that the beneficial effects of micronutrient supplementation reaches beyond those with serious mental health disorders and would be useful for individuals with more general health issues. Although the subjects of this study are rats, it is thought that the finding may have some implications for the use in humans and lead to further investigations in both the human and rat populations.

1.2 EMPowerplus:

The mixed results that have been reported in relation to single vitamins and minerals and their effects on mental health have lead researchers to start investigating the effects of complex nutrient formulas (multi-ingredient) may have on mental health. This recent shift in research focus has been controversial given the scientific method requires that only one independent variable, such as a single micronutrient, should be manipulated during an investigation of its effects on dependant variables to reduce the number of confounding factors (Kaplan & Leung, 2011). Research involving a number of independent variables is thrown out as having too many confounds and therefore interfering with the interpretation of results. Following this, designing studies with a multi-ingredient formula as a single intervention has required a shift in thinking in scientific and academic fields (Kaplan & Leung, 2011).

The growing body of research in this area has investigated the effects of micronutrient formulas in relation to a number of mental health areas including mood and anxiety disorders (Kaplan et al., 2002; Kaplan et al., 2004), attention-deficit/hyperactivity disorder (ADHD) (Rucklidge et al., 2011b) and obsessive-compulsive disorder (OCD) (Rucklidge, 2009). More recent research has also looked at the effects of a micronutrient formula on mental health factors such as stress and trauma following a natural disaster in clinical and community populations (Rucklidge et al., 2012; Rucklidge et al., 2011a). Although several multi-vitamin-mineral combinations have been examined the majority of the research focuses on the multi-micronutrient formula, EMP+. This is made up of 14 vitamins, 16 minerals, three amino acids and three antioxidants (a full list of ingredients can be found in Table 1 below).

For general mental health well-being eight capsules per day is the recommended dosage in humans, this is equivalent to a diet that contains 1.25% EMPowerplus. This dosage applies to those individuals who do not have a serious mental health disorder, rather it is intended for optimizing

health. Given the animals used in this study were from a normal population, this dosage was included in the study. A therapeutic dose used to target more serious mental health problems such as ADHD, Bipolar Disorder and OCD is 15 capsules per day. This dosage is thought to be equivalent to a diet made up of 2.5% EMP+. This level of supplementation was included in the study to see what effects this higher dosage would have on a normal population. Finally a dosage 5% EMP+ was also included in the study to investigate the effects of taking twice the recommended therapeutic dose and whether this would produce a toxic effect in a normal population. A control group was also included to provide a comparison group of animals not receiving a supplemented diet as well as comparisons between treatment groups.

Table 1. *Composition of EMP+ with amount of each ingredient per serving, for a general health dose, for a therapeutic dose and for double the therapeutic dose*

	Amount Per Serving (4 capsules)	General Health Dose (8 capsules) 1.25% diet	Therapeutic Dose (15 capsules) 2.5% Diet	Double Therapeutic Dose (30 capsules) 5% diet
Vitamin A	1,536 IU	3,072 IU	5,760 IU	11, 520 IU
Vitamin C	160 mg	320 mg	600 mg	1,200 mg
Vitamin D	384 IU	768 IU	1,440 IU	2,880 IU
Vitamin E	96 IU	192 IU	360 IU	720 IU
Vitamin B1	4.8 mg	9.6 mg	18 mg	36 mg
Vitamin B2	3.6 mg	7.2 mg	13.5 mg	27 mg
Vitamin B3	24 mg	48 mg	90 mg	180 mg
Vitamin B5	5.8 mg	11.6 mg	21.8 mg	43.6 mg
Vitamin B6	9.6 mg	19.2 mg	36 mg	72 mg
Vitamin B9	384 mcg	768 mcg	1,440 mcg	2,880 mcg
Vitamin B12	240 mcg	480 mcg	900 mcg	1,800 mcg
Vitamin H	288 mcg	576 mcg	1,080 mcg	2,160 mcg
Calcium	352 mg	704 mg	1,320 mg	2,640 mg
Phosphorous	224 mg	448 mg	840 mg	1680 mg
Magnesium	160 mg	320 mg	600 mg	1,200 mg
Potassium	64 mg	128 mg	240 mg	480 mg
Iodine	54.4 mg	108.8 mg	204 mg	408 mg
Zinc	12.8 mg	25.6 mg	48 mg	96 mg
Selenium	54.4 mg	108.8 mg	204 mg	408 mg
Copper	1.9 mg	3.8 mg	7.1 mg	14.2 mg
Manganese	2.6 mg	5.2 mg	9.8 mg	19.6 mg
Chromium	166.4 mcg	332.8 mg	624 mcg	1,248 mcg
Molybdenum	38.4 mcg	76.8 mcg	144 mcg	288 mcg
Iron	3.7 mg	7.4 mg	13.9 mg	27.8 mg
dl-Phenylalanine	96 mg	192 mg	360 mg	720 mg
Glutamine	48 mg	96 mg	180 mg	360 mg
Citrus bioflavanoids	64 mg	128 mg	240 mg	480 mg
Grape Seed	12 mg	24 mg	45 mg	90 mg
Choline bitartrate	144 mg	288 mg	540 mg	1,080 mg
Inositol	48 mg	96 mg	180 mg	360 mg
Ginkgo biloba	9.6 mg	19.2 mg	36 mg	72 mg
Methionine	16 mg	32 mg	60 mg	120 mg
Germanium sesquioxide	5.5 mg	11 mg	20.6 mg	41.3 mg
Boron	640 mcg	1,280 mcg	2,400 mcg	4,800 mcg
Nickel	7.8 mcg	15.7 mcg	29.3 mcg	58.5 mcg
Vanadium	318.4 mcg	636.8 mcg	1,194 mcg	2,388 mcg

1.3 Anxiety and Assessing Anxiety like Behaviour in Rats:

Anxiety is a vital emotion that has been highly preserved during evolution (Ohl, 2005). The anxiety response is an adaptive mechanism which enables humans and other animals to react to real dangers in our environment and dysfunction of this response can result in 'marked, persistent and excessive or unreasonable fear' (American Psychiatric Association, 2000). In this case it is important to define the difference between fear and anxiety. Barlow (2002) defines anxiety as being a future-oriented mood state which is related to the preparation for possible upcoming negative events, while fear is an alarm response to present or impending danger whether real or imagined. This conceptualisation of human anxiety and fear is equivalent to the animal predatory imminence continuum (Fanselow & Lester, 1988) and in animals anxiety can be thought of as the animal's state during a potential predatory attack while fear relates to the animal's state during predator contact or impending contact (Craske et al., 2009). In essence being anxious is an evolutionary reaction when confronted with danger or a threat. The behavioural and physical responses associated with anxiety enable an individual to prepare and act appropriately in such situations, for example a flight or fight response. The anxiety response allows an individual to escape from a dangerous situation and/or avoid the situation to begin with (Ohl, 2005).

1.3.1 The Ideal Animal Model:

The ultimate animal model for any human clinical condition must fulfil three criteria. It must provide predictive validity - in this case established effective pharmacological treatments in humans should induce equivalent effects in the animal model. Secondly it must provide face validity - the responses or behaviours seen in the animal model should be comparable to those seen in patients. Finally the model most have construct validity, the underlying theory should be the same in both human and animal models (Mckinney & Bunney, 1969). In the case of animal models of anxiety the ideal model must respond to treatment with known anxiolytics with reduced anxiety, defence

behaviour should be displayed when confronted with a threatening stimulus, and the underlying mechanisms of anxiety as well as the psychological cause must be the same (Ohl, 2005). As can be imagined, it is difficult for an animal model of anxiety to meet the three criteria given the variation in the presentation of anxiety disorders. According to the American Psychiatric Association (2000) pathological anxiety can be categorised by the following subtypes: generalised anxiety disorder, specific phobia, social phobia, panic disorder (diagnosed with or without agoraphobia), obsessive-compulsive disorder and post-traumatic stress disorder. Each subtype is characterised by differing aetiologies, presentations and symptoms of anxiety. Anxiety is a multidimensional construct and symptoms are usually divided into three groups: subjective distress (cognitive symptoms), avoidance or escape behaviour (overt behaviours), and physiological responses (Lang, 1977). Some types of anxiety disorders are very hard to model in animals given the cognitive nature of some of the symptoms that are essentially part of the human experience of the disorder. However the physiological and behavioural responses to aversive events are similar in animals and humans meaning we can effectively model anxiety in animals focusing on physiological and behavioural responses evoked by aversive situations (Ohl, 2005).

1.3.2 Behavioural Expressions of Anxiety:

It is well known that rodents tend to avoid the unprotected area of a novel environment upon entering it. Rodents typically explore the areas of new environments adjacent to the walls while avoiding an open area (Prut & Belzung, 2003). The aversive nature of the open area can be modified using differing light levels. A brightly lit area is more aversive for a rodent than a dim area and therefore produces more noticeable avoidance behaviour. Elevating of the open area can also increase the rodent's aversion towards an unprotected area (Ohl, 2005).

When confronted with a novel situation, the behaviour expressed by rodents is moderated by the conflict between the drive to explore the novel area and the desire to avoid any potential

threat. Explorative behaviours encompass a wide range of behavioural patterns such as walking, rearing, climbing, sniffing and manipulating objects (Cryan & Sweeney, 2011; Ohl, 2005). It is hypothesised that explorative behaviours are inhibited by anxiety and therefore a reduction in the above behaviours would indicate an increase in anxiety.

This study has modelled anxiety in animals using the Open-field paradigm, the Light/Dark Box and the Y-Maze. All three models draw on the unconditioned behaviour of avoidance as an analogy of anxiety in rats. Research to date has shown that known anxiolytic agents such as benzodiazepines, busipirone, paroxetine, propanolol and acute fluoxetine have produced a reduction in anxiety-like behaviour in rats using the above animal models (Cryan & Sweeney, 2011).

1.4 Rat Studies:

To date there has been minimal research looking into the effects of EMPowerplus or other micronutrient formulas on anxiety behaviours in rats. Therefore it is difficult to predict the types of changes in behaviours that we might see following micronutrient supplementation. There is slightly more research looking into the effects of individual nutrients on anxiety in rats which at least may provide a guideline of the types of effects and the size of these effects we may expect to see. This research has been reviewed below.

1.4.1 Individual Nutrients:

Studies have looked at the effects of several vitamins (A, C, and E) along with minerals - namely zinc - on anxiety behaviours in rats. The most commonly used behavioural tests to look at anxiety-like behaviours in rats are the open-field test and the elevated plus maze. Other tests that have been used in the review literature include the four plates test and the light-dark box.

Deficiencies in dietary Vitamin E have found to be associated with increases in anxiety-like behaviour in rats (Terada et al., 2011). Research using both juvenile and adult rats has shown that animals receiving a Vitamin E deficient diet over a four week period when tested in an elevated plus

maze spent less time in, and made fewer entries into, the open arms. They also displayed more stretch-out posture and reduced head dipping when compared to control animals. These behaviours are thought to indicate increased anxiety in rats. The animals receiving the deficient diet also had lower concentrations of α-tocopheral and increased levels of oxidative stress. These outcomes provide evidence that Vitamin E deficiency can produce anxiety-like behaviour in rats (Terada et al., 2011).

Consistent with this, research into the effects of Vitamin C and E alone and in combination has found that both separately and combined these vitamins reduce anxiety-like behaviour in rats in the open field (Hughes et al., 2011). Animals which received either Vitamin C, Vitamin E or both spent more time in the centre squares, spent less time in the corner squares and had greater levels of ambulation when compared to control animals. However there were no significant differences between vitamin treatment groups on any of these measures. Animals in the control group displayed significantly greater acoustic startle amplitudes than any of the three vitamin groups. Together these behavioural outcomes following supplementation of Vitamins C and E alone and in combination provide support that supplementation reduces anxiety-like behaviour in rats although there was no detectable difference between the individual vitamins or the combination treatment (Hughes et al., 2011).

Chronic dosages of vitamin A have been found to produce a higher number of refusals for entering the light side of a light-dark box which is thought to indicate higher levels of anxiety. It is important to note that the dosages of vitamin A in this study were high and the increase of anxiety is likely to be related to a toxic effect (De Oliveria et al., 2007).

Recent animal research indicates that zinc may have an effect on anxiety-like behaviour in rodents. Two recent studies indicate that zinc deficiencies may lead to an increase in anxiety-like behaviours, with animals receiving a zinc supplemented diet showing a decrease in these behaviours

(Partyka et al., 2011; Takeda et al., 2007). A zinc deficient diet in rats has been found to result in reduced activity in the open field and on the elevated plus maze with animals ambulating less and spending less time in the open arms respectively (Takeda et al., 2007). These results indicate that zinc deficiency may lead to an increase in anxiety-like behaviours in animals; however it is important to note that food restriction may have also led to an increase in anxiety-like behaviour. Researchers have found that rats with a zinc deficient diet made significantly fewer transitions into the light side and spent significantly less time on the light side of the box when tested in a Light-Dark Box. Tassabehji et al. (2008) suggest that these increases in anxiety-like behaviour were not related to decreases in food intake as the pair-fed animals consumed the same amount of food per day as the zinc deficient animals but did not display this increase in anxiety-like behaviour.

Acute Zn administration in rats and mice has been associated with some reductions in anxiety-like behaviours (Partyka et al., 2011). Rats receiving acute Zn supplementation have been found to make more entries into the open arms of the elevated-plus maze. On the other hand, in mice no significant differences were found between mice receiving Zn supplementation and control mice on the elevated-plus maze. On the four plates test mice who received Zn supplementation made more punished crossings. In the stress-induced hyperthermia test Zn supplementation was found to be associated with a reduction in stress-induced hyperthermia in mice. Zn administration was also associated with a decrease in locomotive activity in mice on a test of locomotive activity when compared to controls (Partyka et al., 2011). The researchers argued that the results supported the hypothesis that acute zinc administration would reduce anxiety-like behaviour in rodents.

1.4.2 EMPowerplus:

To date, only one animal study investigating the effects of the micronutrient formula EMP+ has been published. The study investigated the effects of chronic stress during pregnancy and/or a prefrontal injury in rats in regards to behavioural and anatomical outcomes (Halliwell et al., n.d.). It

also investigated the effects of a micronutrient supplement following chronic stress and/or a prefrontal injury on behavioural and anatomical measures. In regards to micronutrient treatment it found that the supplement improved performance on a number of behavioural measures including skilled reaching, latencies in the Morris Water Maze and increased exploratory behaviour in the elevated plus maze (Halliwell et al., n.d.). These findings indicate beneficial effects following micronutrient supplementation for cognition and emotionality in both control and frontal lesion animals.

It appears that some of the vitamin and minerals that are present in EMP+ do have an effect on anxiety-like behaviour in rats. In some cases it appears that the nutrient may decrease these behaviours while in other cases nutrients may actually increase anxiety-like behaviours. There is also evidence to suggest that a diet deficient in certain nutrients may also lead to an increase in anxiety like behaviour in rodents. Due to these discrepancies we need to keep in mind that when looking at the effects of EMP+ on anxiety-like behaviours in rats it is hard to tease apart which compounds are actually producing these changes. If a decrease in anxiety is found, we can conclude that EMP+ is effective in reducing anxiety in rodents, however we cannot say with certainty which nutrients brought about this change.

1.5 Human Studies using a Micronutrient Formula:

Although there is a growing body of research looking at the effects of single nutrients on mental health in the human population, the purpose of this literature review is to investigate the current knowledge in regards to the effects of micronutrient supplementation on psychiatric disorders and other mental health issues found in the human population.

The current study aims to investigate the effects of a micronutrient formula on stress and anxiety-like behaviours in rats using EMP+, therefore the literature reviewed here will focus mainly on studies using this formula.

1.5.1 Mood Disorders:

The majority of research in this area focuses on the effect of micronutrients on Bipolar Disorder and mood issues comorbid with other disorders such as ADHD. Research has looked at both children and adults and to date eight studies have been published showing promising results. Rucklidge et al. (2010) used a database analysis to examine the effects of micronutrient supplementation on symptoms of bipolar disorder in children and adolescents. One hundred and twenty children and adolescents with a diagnosis of pediatric bipolar (PBD) were included in the study, approximately a quarter of these individuals also had a diagnosis of ADHD (n=29) and an additional 41 individuals diagnosed with ADHD were examined to compare changes in ADHD symptoms in the Bipolar and ADHD group (Rucklidge et al., 2010). Baseline data was compared to data recorded after 3 to 6 months micronutrient supplementation using Last Observation Carried Forward (LOCF). What they found was that the mean symptom severity of bipolar symptoms for the primary sample was 46% lower than at baseline and this reduction was statistically significant ($p<0.001$). They also found that 46% of individuals reported <50% improvement at LOCF and 38% of the sample were still taking medication (compared to 52% at baseline). In individuals with PBD but not ADHD, a 44% mean decrease in symptom severity was found and this was also statistically significant ($p<0.001$) (Rucklidge et al., 2010). Similar reductions in symptom severity were found in both males and females and in both younger and older children, and these reductions were statistically significant. Overall the results indicate that micronutrient supplementation may be an alternative to drug interventions for the treatment of bipolar disorder in children and adolescents. It is important to note that this study was unblinded and that there was no control group to rule out any placebo effects.

Frazier et al. (2009) carried out a case study to investigate the effects of a micronutrient formula on bipolar symptoms using a single case design. EMP+ was used to treat a 12 year old boy who was diagnosed with Bipolar I, mixed, with psychotic features (Frazier et al., 2009). He was

initially diagnosed at age 6 with Bipolar Disorder – Not otherwise specified. He also met the criteria for Generalised Anxiety Disorder (GAD) and Obsessive Compulsive Disorder (OCD). Previously he had been treated with a number of medications however none provided a desirable mood balance or improvement in functioning over time. During the study he received 14 months of EMP+. After 19 days of supplementation it was reported that all symptoms - irritability, headache, dizziness, fatigue, hallucinations and compulsions - had remitted and improvements were noted in attention and sleep (Frazier et al., 2009). Although this case study provides further support for the use of micronutrient supplementation as a treatment for pediatric bipolar disorder, it is difficult to make further conclusions as the researchers did not use standard measures to provide quantitative data in regards to symptom reduction.

Another study looked at the effects of micronutrient on mood and behaviour in children with ODD and behavioural problems (Kaplan et al., 2004). Eleven children aged 8- to 15-years old participated the open-label trial over a minimum period of 8 weeks. The children had a range of diagnoses including Bipolar Disorder, ADHD, Oppositional Defiant Disorder (ODD), GAD, asperger syndrome, depression and anxiety. The parents completed the Child Behaviour Checklist (CBCL), Youth Outcome Questionnaire (YOQ) and the Young Mania Rating Scale (YMRS) at the beginning of the trial and again after the final visit which ranged from 8 weeks through to 17 weeks of supplementation (Kaplan et al., 2004). For the 9 children who completed a minimum of 8 weeks significant decreases were found on the YOQ ($p<0.001$) and the YMRS ($p<0.01$) from baseline to the final visit. They also found improvement on seven of the eight CBCL scales which was statistically significant, only the somatic scale did not show an improvement. The effect sizes for all significant outcome measures were relatively large (>0.80) (Kaplan et al., 2004). Based on these results, there is evidence to suggest that micronutrient supplementation may have a stabilizing effect on temper, mood and anxiety across a range of diagnostic categories.

Studies examining the effects of micronutrients on bipolar and other mood symptoms have found similar results to the above investigations. A study using a similar method to Rucklidge et al. (2010) looked at the effects of EMP+ on adults with bipolar disorder (Gately & Kaplan, 2009). Data of 358 individuals who had a diagnosis of Bipolar Disorder was selected and symptom reports for at least 60 days of the first 180 days of micronutrient supplementation were analysed. They found that micronutrient supplementation was associated with a 41% decrease in symptom severity scores after 3 months and a 45% decrease at 6 months. Both of these drops were found to be statistically significant ($p<0.001$) when compared to baseline scores (Gately & Kaplan, 2009). There was evidence to suggest that symptom decreases were associated with a reduction in medication and an increase in dosage of the micronutrient formula.

1.5.2 Obsessive Compulsive Disorder:

To date, two studies have looked at the effects of micronutrient consumption on the symptoms of Obsessive Compulsive Disorder (OCD). The first of these studies is a case study of an 18 year old male who was diagnosed with OCD and Asperger's Disorder at age 16 (Rucklidge, 2009). Treatment followed a standard cognitive behavioural approach over a 1 year period and he showed a moderate response with symptom severity reducing to moderate from severe range on the Yale-Brown Obsessive Compulsive Scale (Y-BOCS) at time of discharge (Rucklidge, 2009). After almost a year he was re-assessed as family reported that his symptoms had worsened and there were concerns around risk of harm to self. At this time, he still met the DSM-IV-TR criteria for OCD as well as Major Depressive Disorder (MDD). The following measures were used to monitor treatment change over time: the Beck Depression Inventory (BDI-II), the Beck Anxiety Inventory (BAI), the Global Assessment of Function (GAF), the CBCL and the Outcome Questionnaire (OQ) and these were completed at baseline, after 8 weeks of micronutrient supplementation, after 8 weeks off supplementation and after 8 weeks back on the supplement, in essence an ABAB design (Rucklidge, 2009).

After the first 8 weeks of supplementation BDI scores remained relatively high but the CBCL, the BAI and the Y-BOCS scores were low and the OQ was in the range which is found within community samples. When supplementation was discontinued, mild-symptoms reappeared after 10 days and after 8 weeks there was an increase in the severity of his anxiety, obsessions and depressed mood. 12 days following the reintroduction of the micronutrient his scores on the BDI, BAI and Y-BOCS had improved and after 4 weeks his score on the Y-BOCS indicated that OCD symptoms were in remission (Rucklidge, 2009).

An earlier study, also using an ABAB design, investigated the effects of a micronutrient supplement on mood lability and explosive rage in 2 boys, one diagnosed with atypical OCD along with ADHD, mood lability and explosive rage (Kaplan et al., 2002). At baseline, this child's score on the Conner's Parent Rating Scale (CPRS) was 1.6 on the mood lability and on the tantrums item – these scales range from 0-3. The CBCL indicated significant behavioural difficulties with 7 of the 8 scales with *T* scores above the clinical cutoff of 70, in other words, 2 standard deviations above the normative mean. On the Y-BOCS compulsive scale, his baseline score was 13 (Kaplan et al., 2002). Following 16 weeks of micronutrient supplementation his parents reported no significant symptoms and he reported being able to go all day without thinking of his obsessions. All scales on the CBCL were below clinical cutoff levels. His score on both the mood lability and tantrums item had reduced to 1 on the CPRS indicating the child having the behaviour 'just a little' and his score on the Y-BOCS compulsion scale was 2 (Kaplan et al., 2002).

Following this, the child discontinued micronutrient supplementation and after three weeks without the supplement pre-treatment symptoms reappeared, namely his obsessive thoughts. After 6 weeks without supplementation, the parents reported a clinically significant regression and his responses on the CBCL showed that three of the scales again exceeded the clinical cutoff with *T* scores about 70. Scores on the CPRS had also increased for both temper outbursts and mood lability. His responses on the Y-BOCS compulsion scale indicated a score of 7. However while during the

interview he admitted to having significant intrusive thoughts he also emphasized his ability to fight them, something he had not recognised during the baseline assessment (Kaplan et al., 2002). The micronutrient was then re-introduced and after being back on the micronutrient supplement for 11 weeks his Y-BOCS compulsion scale score had reduced to 2 and by week 18 the school psychologist felt the his behaviour and attention had improved enough for him to be reintegrated into a regular school. Scores on the CPRS mood lability and temper scales had also reduced to the same levels as the first phase when micronutrients were introduced. One year later, the final follow-up assessment noted that he was succeeding with his education in a regular classroom, that no behavioural or attentional difficulties were detected and that his Y-BOCS compulsion score had further reduced to 1. The other boy, diagnosed with pervasive developmental delay also showed a reduction in anger outbursts and obsessional symptoms along with an improvement in mood while receiving EMP+ and these symptoms returned after cessation of the formula (Kaplan et al., 2002)

Overall, for these individuals, taking the micronutrient supplement appeared to be linked with a clinically significant reduction in obsessional thoughts along with improvements in mood lability and temper control (Kaplan et al., 2002).

1.5.3 Attention-Deficit/Hyperactivity Disorder:

Rucklidge et al. (2011) investigated the effects of a micronutrient formula on behaviour and mood in 14 adults diagnosed with ADHD and severe mood dysregulation (SMD). All participants were medication free and completed an 8 week open-label trial. Outcome measures in this study were the clinical global impressions severity (CGI-S) and clinical global impressions involvement (CGI-I) scales which were used to measure the severity and improvement of depression, mania and ADHD symptoms separately; the Young Mania Rating Scale (YMRS); the global assessment of functioning (GAF) and the Montgomery-Asberg Depression Rating Scale (MADRS). Along with these clinician administered measures, self report measures such as the Conners Adult ADHD Rating Scale (CAARS)

and the Outcome Questionnaire (OQ) were also used. Each participant completed the measures at baseline, after 8 weeks on the supplement and then at follow up (Rucklidge et al., 2011b). The results after 8 weeks supplementation showed statistically significant improvements on measures of inattention and hyperactivity/impulsivity, mood, quality of life, anxiety and stress with P values <0.01. The inattention mean score remained in the clinical range. Follow up data that was collected approximately 2 months after the trial ceased indicated those who had opted to stay on the micronutrient supplement maintained changes or showed further improvement on outcome measures while those who came off the supplement tended to show some maintenance or regression of symptoms; however, these did not return to baseline levels (Rucklidge et al., 2011b). This study had a number of strengths including the confirming of diagnoses through structured interviewing, the factor that the sample was made up of 'difficult to treat combination of symptoms' and that multiple sources were used to monitor symptom change (self, observer and clinician). Along with these, the natural extension of the trial allowed comparison of those who opted to continue with supplementation and those who did not (Kaplan & Leung, 2011).

1.5.4 Autism and the Autistic Spectrum:

One study has investigated the effects of multi-micronutrient supplementation on symptoms associated with the autism spectrum disorders compared to standard medication management (Mehl-Madrona et al., 2010). The 44 participants in the experimental group had opted for treatment without pharmaceuticals and their records were matched with similar children who were undergoing conventional treatment for autism spectrum disorders. The Childhood Autism Rating Scale, the Childhood Psychiatric Rating Scale and the Aberrant Behaviour Checklist were used as outcome measures. What they found was that both groups of children improved significantly on Childhood Autism Rating Scale, Clinical Global Impressions Scale and Childhood Psychiatric Rating Scale. (all p values <0.0001) and both groups showed significant reductions on the Aberrant Behaviour Checklist. However those receiving the micronutrient formula showed a statistically significant improvement

compared to those receiving standard medication ($p<0.0001$). Self injurious behaviour intensity was also found to be significantly lower in the micronutrient group ($p=0.005$). The micronutrient group showed a significantly greater improvement on the CGI-S compared to the standard medication group. Although the study shows reductions in activity level and social withdrawal along with less anger and irritability, increased spontaneity, less intense SIB, fewer adverse events and less weight gain than standard medications, it is important to note that it is difficult to say whether the observed improvements were associated with changes in mood disorder or a specific effect on autistic disorder (Mehl-Madrona et al., 2010). Although the study provided an age, gender and symptom severity matched comparison group it was affected by self-selection bias, with those participants' families choosing their treatment and the fact that it was unblended leading to possible observer and participant biases (Kaplan & Leung, 2011).

1.5.5 Stress and Trauma Reactions:

Two recent studies from Christchurch, New Zealand have looked at the effects of a multi-micronutrient supplement on stress and trauma reactions following natural disasters, in this case earthquakes (Rucklidge et al., 2012; Rucklidge et al., 2011a). The first of these studies looked at the effects of micronutrient supplementation during and following a 7.1 earthquake (September 2010) on stress and anxiety levels in adults with ADHD (Rucklidge et al., 2011a). The participants were recruited from ongoing and completed studies examining the effects of micronutrients on symptoms of ADHD in adults which resulted in 33 adults with ADHD taking part in the study. Seventeen participants were not taking micronutrients at the time of the September (7.1) earthquake and they made up the control group. The experimental group comprised of 16 participants who were taking the micronutrient supplement at this time. All participants were contacted within 7 to 10 days post earthquake and were asked to complete the Depression, Anxiety and Stress Scale (DASS) with answers reflecting on the first week post earthquake (Time 1). Within 7 to 10 days of this initial contact participants were contacted again to re-administer the DASS and were asked to base their

answers on the week following the first contact (Time 2). All participants had completed the DASS as part of the baseline assessment for the original studies they had been involved in and these results were used as a baseline for this study.

The results indicated that there were no significant differences between the control and experimental group 1-week post earthquake but at time 2 those taking the micronutrient supplement reported significantly less stress and anxiety than those in the control group. Interviews with the participants indicated that individuals in the both the control and experimental groups experienced similar levels of personal injury and loss. The researchers state that the differences between the micronutrient group and controls could not be explained by other factors such as demographics, psychiatric status or pre-earthquake measures of emotions and argue that the results suggest that micronutrient supplementation positively affects resilience in regards to ongoing stress and anxiety in individuals with ADHD following a highly stressful event.

The other study compared two micronutrient formulas and examined their impact on stress related to the 6.3 earthquake on February 22nd, 2011 in Christchurch, New Zealand (Rucklidge et al., 2012). The study was made up of 91 adults who reported increased anxiety or stress over a 2- to 3-month period following the earthquake. The micronutrient formulas used in this study were Berocca and CNE. Berocca is made up of 9 vitamins (B_1, B_2, B_6, B_{12}, C, biotin, folic acid, nicotinamide and panthothenic acid) and 3 minerals (calcium, magnesium and zinc). The other micronutrient formula was CNE which is the same formula as EMP+. The participants were randomised to receive Berocca, CNE low dose (CNE4) or CNE high dose (CNE8) over a 28 day period and completed weekly online questionnaires during this period. They were followed up one month post trial. A control group made up on 25 non-randomised participants were also monitored, completing questionnaires at baseline and 4 weeks. The measures used to monitor symptoms were the Depression, Anxiety and Stress Scale (DASS), the Impact of Event Scale-Revised (IES-R) and the Perceived Stress Scale (PSS) (Rucklidge et al., 2012).

The results showed that all three treatment groups experienced a significant decline in symptoms across all measures (all *p* values <0.001) over the 4 week period. There were no statistically significant differences between the treatment groups in regards to change over time although a trend was detected on the IES-R intrusion scale suggesting that CNE may have a greater effect on reducing intrusive thoughts related to the earthquake trauma compared to Berocca. Significant differences were also found between treatment groups and controls on most measures with those in the treatment groups reporting greater decreases. In regards to specific symptom groups, there were significant treatment differences in mood, anxiety and energy with the CNE8 group having the most improved mood ($p<0.05$), anxiety ($p<0.05$) and energy ($p<0.01$) compared to Berocca. No treatment difference was found for change in stress. When comparing treatment groups to controls, the treatment groups reported statistically significant improvements in stress ($p<0.0001$), mood ($p<0.001$) and anxiety ($p<0.001$) (Rucklidge et al., 2012).

Eighty-four participants completed the 1 month follow up and given the number of changes in dose and micronutrient type the results were grouped into those who continued to take a micronutrient supplement, those who switched micronutrients and those who did not continue to take a supplement. Those who initially received CNE8 were more likely to continue to take the supplement than those who received Berocca. Those who continued with the supplementation showed greater ongoing improvement of depressive symptoms than who discontinued and there were significant differences in the amount of changed reported in anxiety, stress and energy, with greater improvements in these areas being reported by those who continued with micronutrient supplementation (Rucklidge et al., 2012). Overall, this study is consistent with previous research in this area with ongoing micronutrient supplementation associated with a decrease in symptoms and greater improvement in symptomatology over time. There is evidence to suggest that greater amounts of micronutrient consumed may have more beneficial effects on mood, anxiety and energy.

The authors also suggest that the results support micronutrients as an inexpensive and practical way to manage symptoms of acute stress and trauma following a natural disaster.

Overall, the current body of literature appears to support the use of micronutrient formulas as an alternative treatment for a number of mental health disorders. Alongside this, there is evidence to suggest that a micronutrient supplemented diet may help reduce stress and trauma responses in the healthy adult population. Despite the positive results there are some holes in the current body of research. More randomised controlled trials need to be carried out to assist in drawing conclusions with regards to the effects of micronutrients on human behaviour. Studies looking into short-term compared to long term supplementation are also needed in order to gain an understanding in regards to ongoing benefits of a supplemented diet, and given the concerns around toxicity of some of the ingredients at high doses, investigations need to look at the effect of dosage of micronutrient formulas to provide further information with regards to the most effective therapeutic dose for both clinical and community populations. This study aims to address some of these issues through the use of an animal model of anxiety.

1.6 Mechanism of Action:

There are several hypotheses concerning how micronutrients could affect mental functioning. It is important to note that these theories are not mutually exclusive and that they may in fact overlap or interact with each other and that there are other possibilities out there (Kaplan & Leung, 2011).

The first explanatory model put forward relates to inborn errors of metabolism. It has been shown that at least a third of the currently known genetic mutations cause a reduced binding affinity for a coenzyme by a known enzyme (Ames, 2004). Therefore lower levels of a coenzyme, a number of which are made up of micronutrients, would cause a decrease in the binding affinity along with reduced metabolic activity (Kaplan & Leung, 2011). Ames et al. (2002) looked at a number of genetic

diseases which were associated with this type of genetic defect and found that in the majority of cases the symptoms were successfully treated by giving individuals additional cofactors, for example, micronutrients. From this we can conclude that it is possible that some mental health disorders that have a well established genetic predisposition may be due to inborn errors in metabolism that decrease the speed of metabolic activity in the brain affecting the neurotransmitters. Therefore micronutrient supplementation may increase the level of metabolic activity (Kaplan & Leung, 2011).

The second theory is based on the effects of deficient methylation. Methylation is where a methyl group is added to a molecule and is essential to proper biological functioning. This process turns on genes, starts enzymatic processes and regulates the amount of protein created by genes. It is vital in DNA transcription and the synthesis of neurotransmitters (Kaplan & Leung, 2011). A micronutrient, S-adenosyl-l-methionine (SAMe) is one of the methyl donors. Research suggests that SAMe may be a form of treatment for depression. What this indicates is that micronutrients play an important role in methylation activity and that it may be that micronutrients augment the methylation of enzymes in the brain, which in turn leads to increased neurotransmitter synthesis (Kaplan & Leung, 2011). Related to this hypothesis is the theory that altered gene expression may be the mechanism of action through which micronutrients affect mental health. Low levels of nutrients can lead to deficiencies in the methylation process. It is well known that there appears to be a strong genetic component to a number of mental health disorders; however, it may be that the expression of these genetic predispositions may depend on the nutrient status of an individual. There is a growing body of research that indicates that an individuals' nutrient level is a strong modifier of genetic expression (Kaplan & Leung, 2011).

A well known theory in the area of micronutrients and mental health is the hypothesis put forward by Ames (2006) known as the triage hypothesis, which argues that our survival in nature is ensured by the rebalancing of our metabolism in situations where the availability of approximately 40 micronutrients is limited. In this case, nature directs any available micronutrients to the processes

that are vital for the individual's immediate survival even if this comes at the expensive of their long term health. Although this theory is accepted in certain areas relating to physical health, it may also apply to mental health. In this case it may be that the underlying dysfunction develops with age and is evidenced by the fact a number of people do not experience their first episode of mental illness until adulthood. Therefore it may be that mental health disorders are in fact the cumulative effect of suboptimal levels of micronutrients being triaged to those areas essential to our survival at the expense of other brain-related functions critical in maintaining good mental health (Kaplan & Leung, 2011).

Other theories include the down regulation of micronutrient receptors which has come out of research looking at niacin-receptors in individuals with schizophrenia, where individuals showed significantly decreased levels of a critical protein for niacin receptors compared to those with a diagnosis of bipolar disorder and even more so when compared with healthy controls (Kaplan & Leung, 2011). Although this research is restricted to niacin it is possible that future research will reveal similar processes in relation to other micronutrients. It is possible that the dysfunctional brain activity in those with psychiatric disorders may, in some occasions, be associated with inherited deficiencies in protein regulation of micronutrient receptors which can be ameliorated by micronutrient therapy (Kaplan & Leung, 2011). The impaired growth and development of neurons may also be another mechanism in which micronutrient levels affect mental health. There is a large and growing body of evidence that suggests decreased brain tissue may be associated with poorer mental health across a variety of disorders such as depression, bipolar disorder and schizophrenia (Kaplan & Leung, 2011). In regards to nutrient deficiencies it is important to note that studies on cortical thinning demonstrate progressive changes where the length of illness appears to be related to a reduced ability for the brain to produce certain amino acids. It could be that the long term experience of reduced nutrition leads to a decreased ability to maintain and initiate neuronal growth and ongoing development, increasing the vulnerability to mental health difficulties. This may be

especially so in individuals who have a genetic predisposition to mental illness (Kaplan & Leung, 2011).

What can be taken away from this review is that there are a number of theories that attempt to explain the mechanism in which micronutrients affect mental health. Each of these theories have growing bodies of evidence to support them although nothing certain can be concluded at this stage. It is important to emphasise that these processes may interact and perhaps affect one another in numerous combinations. What it highlights is the need for further research into this area so we can increase our understanding of these mechanisms. Animal research provides us the opportunity to carry out randomised controlled trials in which we are able to keep constant a number of variables with more control than in human research. This in turn, means we are able to have a greater confidence that results are due to experimental manipulations rather than other confounding factors including prior medication history, other drug use and participant's expectations.

1.7 The Current Study:

The current study investigated the long term behavioural effects of micronutrient supplementation. Rats were randomly assigned to one of three treatment groups or to a control group. Animals received a supplemented diet and were tested on a range of behavioural measures at PND 136-138 and again at PND 186-188. The importance of such a study on micronutrient supplementation is evidenced by the lack of current animal research into this treatment and the growing body of literature and interest in the effects of micronutrients on human populations. Valid and reliable data is needed to gain valuable insight into the behavioural effects of this alternative treatment in both normal and clinical populations. It is imperative that further research is undertaken in this area to evaluate the effectiveness of micronutrient supplementation as an alternative treatment for certain psychological disorders.

2.0 Aims and Hypotheses

Given the growing body of research investigating the effects of micronutrients in human populations on mental health, there is an increasing need for randomised controlled trials using both human and animal populations to provide further information with regards to the effectiveness of micronutrient based treatments. To date the main focus of research has been on human clinical populations, therefore research investigating the effects of micronutrient supplementation on emotionality in a normal rat population is needed to help guide further research in this area.

1) Firstly it is hypothesised that animals receiving a micronutrient supplemented diet will display lower levels of anxiety and emotionality than control animals in the open field, the Y-maze and the Light-dark box. It is thought that this effect will be dose dependant with those animals receiving higher levels of micronutrients in their diet showing a greater reduction in the behaviours than animals receiving a lower dose. It is important to note that there may be an exception to this with the possibility of those animals receiving the highest dose (5%) displaying increased levels of anxiety and emotionality due to toxic levels of micronutrients in their diet as this dosage is twice the recommended therapeutic dose.
2) Secondly, it is expected that any effects of the micronutrient would increase over time - that is animals will show lower levels of anxiety and emotionality during the second phase of testing when compared to their performances during the first phase of testing on the three tests. Currently little is known about the possible differences micronutrient supplementation may have between sexes, especially within a rat population. Therefore it is difficult to predict a direction of results although some sex differences are expected.

3.0 Method:

3.1 Subjects:

The subjects of the current study were 80 PVG Hooded rats from the breeding colony in the Psychology Department, University of Canterbury, Christchurch, New Zealand.

Of these 80 animals, 40 were females and 40 were males. All animals were kept in the Animal Facility within the Psychology Department in a temperature regulated (22 ˚C ± 2˚C) and humidity controlled (48% ± 10%) environment. The rats were maintained in a constant light-dark cycle of 12 hours light, 12 hours dark and had free access to water. Fresh food mix, with the micronutrient formula added dependant on experimental group, was provided daily.

The rats were weaned on post natal day (PND) 30 and housed in plastic cages (475-mm x 280-mm x320-mm) in small groups of 3-4 of the same sex for the duration of the experiment. All procedures were approved by the University of Canterbury Animal Ethics Committee.

On PND 30 the rats were randomly separated in to four experiment groups, each made up of 10 females and 10 males, with a total of 20 animals in each group. The first group (n=20) were to become the control group and were immediately started on the food mix without the micronutrient formula added. The second group (n=20) was assigned to the highest percentage of diet group where 5% of their diet was made up of the micronutrient formula (5g of EMP+ to 95g of standard rat food combined with water). The third group (n=20) was assigned to the 2.5% of diet group (2.5g of EMP+ to 97.5g of standard rat food combined with water). The final group was assigned to the lowest percentage of diet group where 1.25% of the diet was made up of the micronutrient formula (1.25g of EMP+ to 98.75g of standard rat feed combined with water).

Initially it was planned that the animals would receive the supplemented diet for 30 days and then undergo a series of tests made up of the open-field, Light-dark box, the Y-maze and the acoustic

startle response. Following this period of testing the animals would remain on the supplemented diet for another 30 days and then a second testing block was to take place using the same battery of tests. This planned procedure was interrupted by the September 4th, 2010 Canterbury Earthquake.

3.1.1 The Impact of the Canterbury Earthquake on Experimental Procedure:

It is important to note that the animals were made up of three separate birth cohorts. This led to different ages of the animals when the September 4th, 2010 Canterbury Earthquake occurred. The first 40 animals (control group and 5% group) were PND 62 and had been receiving the micronutrient formula for 32 days; the second cohort (2.5% group and 6 1.25% female animals) were PND 49 and had been receiving the micronutrient formula for 18 days and the third cohort (4 1.25% female and the 1.25% males) were PND 34 and had been receiving the micronutrient formula for 4 days. The September 2010 earthquake led to the animals being placed on regular rat feed for 16 days while access to the University was restricted. Therefore testing was delayed and each group was tested at PND 136-138 (time 1) after receiving the diet for 91 days and PND 186-188 (time 2) after receiving the diet for 141 days on a number of measures of anxiety, curiosity, memory, habituation and activity. For cohort 1 this meant that they were tested at 59 days post earthquake (T1) and 99 days post earthquake (T2). Cohort 2 were tested 73 days post earthquake (T1) and 123 days post earthquake (T2). Cohort 3 were tested 87 days post earthquake (T1) and 147 days post earthquake (T2). Please refer to Figure 1 below.

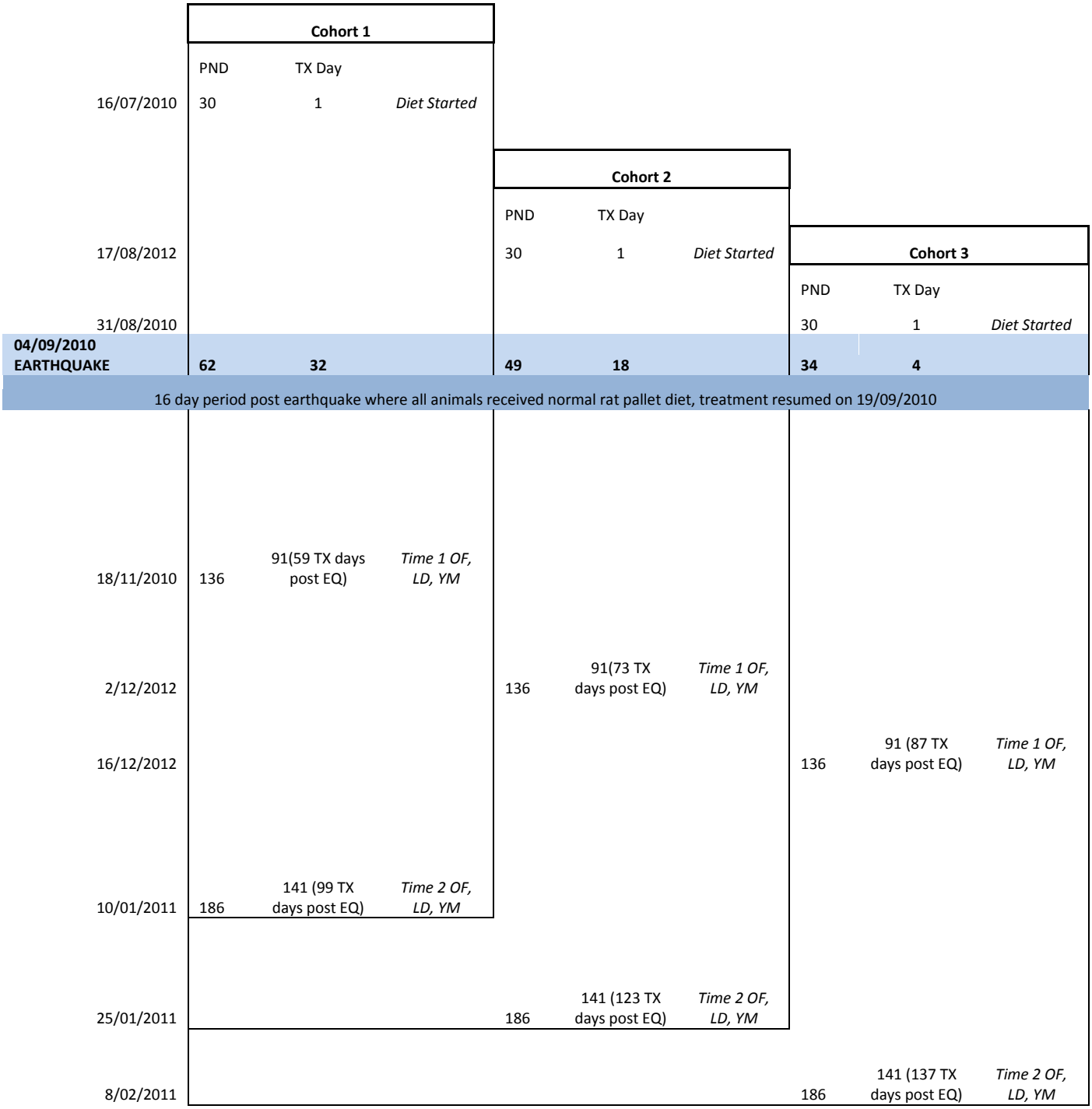

Figure 1. *Timeline showing when each birth cohort began the diet, underwent each experimental procedure, PND at time of procedure and the occurrence of the earthquake. Cohort 1 – made up of control (N=20) and 5% (N=20), Cohort 2 made up of 2.5% (N=20) and 1.25% (N=6) and Cohort 3 made up of 1.25% (N=14). PND = post natal day, TX = treatment, OF = Open Field, YM = Y-Maze and LD = Light-dark box.*

3.2 Micronutrient Use and Rationale for Dosage:

The micronutrient formula used in this study was EMP+ which is a 36-ingredient formula (see Table 1). EMP+ was donated by TrueHope Canada in powder form.

The micronutrient formula was combined with a powdered version of the animals' normal rat chow and water. For those animals receiving a diet with 1.25% micronutrient 1.25g was weighed out and mixed with 98.75g of the ground up rat chow to equal a total 100g. This was then mixed with water to create a moist mash that the animals found palatable. For those receiving a diet including 2.5% and 5% micronutrient formula the same procedure was followed as above however 2.5g and 5g of micronutrient formula were combined with 97.5g and 95g of ground rat chow respectively which was then mixed with water as described above. Each cage of animals received between 100-200g of food daily and this was made fresh for the animals each day to prevent the mash from drying out. The control animals received a mash made up of the ground rat chow and water to ensure that all treatment groups received the chow in the same form.

The animals received the diet after they had been weaned (PND30) until testing was complete (PND188). During testing periods food and water was readily available to the animals. As mentioned previously the animals did not received the mash over a two week period following the September 4th, 2010 earthquake. During this period the animals received standard rat chow.

The rationale for dosage relates to the recommended dose for human consumption. EMP+ makers, Truehope, recommend that 17g of the powdered supplement to be taken twice daily or eight capsules of the formula to be taken daily as a general health dose, the 1.25% dose for animals is thought to be equivalent to this. The 2.5% diet is proposed to be equivalent to the therapeutic dose, 15 capsules daily, while the 5% diet is double the therapeutic dose, equivalent to 30 capsules daily in humans (Please refer to Table 1.).

Recent research indicates that micronutrients may be effective in the treatment of individuals with psychiatric disorders such as anxiety disorder, affective disorders such as depression and bipolar disorder and disorders such as attention-deficit/hyperactivity disorder (Kaplan & Leung, 2011). Studies which have shown that micronutrients may help reduce psychopathology indicate that a higher than normal dose may be required, in some studies this has been twice the recommended dose (Kaplan & Leung, 2011). The 5% treatment group was included following this rationale.

3.3 Apparatus and Behavioural Measures:

In this experiment four empirically supported tests of anxiety-like behaviour were used: responsiveness to change in a Y maze; an emergence test (Light-dark test); and behaviour in an open field. Each of these tests are simple and do not require any training prior to testing of the animals, nor do they involve any form of deprivation or physical harm. The tests elicit behaviours which are caused by being placed in novel situations and which are measured as an index of anxiety. The different tests provoke different behaviours in the animals which are thought to be comparable to anxiety behaviours in humans.

When placed in the Y maze it is thought that a more anxious rat will enter the changed arm less often, and that the time spent in the changed arm will be less than an animal that is less anxious (Atchison & Hughes, 2006). In the Light-Dark emergence test it is thought that the higher the level of anxiety the animal is experiencing, the longer will be the latency to enter the light side of the box and the shorter the duration of time spent in the light side compared to an animal experiencing lower levels of anxiety (Hascoët et al, 2000). With the Open Field test the more anxious animal will spend a greater proportion of time in the corners of the apparatus and less time in the exposed centre squares (Hall, 1934a). It is also thought that the more anxious animal will display less rearing and grooming behaviours (Prut & Belzung, 2002). It is thought that the higher the level of anxiety the

animal is experiencing the more it will defecate (Hall, 1934b; (Wills et al., 1983)), therefore the number of faecal boluses was also recorded.

The tests were carried out in the same experimental room which maintained a stable temperature of 22˚C ± 2˚C with a humidity control 48% ± 10%. All tests were carried out between 10.00 and 16.00 hours in the light phase of the rats' normal light/dark cycle. The tests were completed over three consecutive days at two times, PND 136-138 and PND 186-188. Over each three day testing period, each animal completed the open field test once, the light-dark preference box test once and the Y-maze test once. Each animal completed one test per day except on the third day of testing in the second testing period where the animals completed two tests - the open field and the acoustic startle test. To ensure that the animals received ample rest between the tests on day three there was a minimum of one hour between the two tests.

3.3.1 Responsiveness to Brightness Change in the Y Maze:

The Y maze exploits the rats' natural tendency to explore novel areas. It is thought that less anxious animals will enter the novel arm more often than the familiar arm, whereas a more anxious animal will show a preference for familiarity rather than novelty (Hughes, 2001; Hughes & Neeson, 2003; Samyai et al., 2000).

In this study, a wooden apparatus was placed on a table 700mm high which was illuminated overhead by low (45 lux) fluorescent lighting 1390mm above the maze. An infrared video camera was mounted 1390mm directly above the apparatus and this was used by the experimenter to monitor the rats' location within the maze. The arms of the maze were 450mm long with a 120˚ angle between them. The stem of the maze was 300mm long with a black compartment at the base which was separated from the rest of the stem by a guillotine slide. Both the stem and arms were 100mm wide and 140mm high. One arm contained a black painted metal insert while the other contained a

white painted metal insert which occupied the width, height and 400mm length of each arm. A clear Perspex lid covered the entire apparatus.

At both Time 1 and Time 2, each animal experienced a pair of acquisition and retention trials. Each animal was placed in the black compartment at the base of the stem and then released. It was allowed to roam freely within the maze for 6 minutes. This is known as the acquisition trial. During this trial one arm contained a black insert while the other arm contained a white insert. After 6 minutes the animal was returned immediately to its home cage where it had free access to food and water. The inserts were replaced with two clean black inserts and the entire apparatus was wiped down and disinfected with 20% Paraquat Blue solution. The rat was returned to the black compartment at the base of the stem 25 minutes after completing the acquisition trial, the guillotine slide was opened and then closed once the animal had fully emerged. The animal was allowed to roam freely for exactly 3 minutes (retention trial). Timing for the retention trial began once the rat had fully emerged (all four paws) from the black compartment. Entries into either arm were recorded when the animal had fully entered the arm, all four paws within the insert. During the three minute period, the experimenter recorded the animals' movement via keyboard using a specialised computer program. The records enabled the calculation of (1) the percentage of entries into the novel arm; (2) the percentage of time spent in the novel arm; (3) the total entries of both arms and (4) the total time spent in both arms.

During the acquisition trial, one half of the animals (n = 40) experienced the maze with the black insert in the left arm while the remaining half experienced it with the black insert in the right arm. It was randomly determined which side the black insert would be on for each animal. Within each testing group five rats completed the maze with the black insert on the left and five rats completed it with the black insert on the right. At Time 2 the rats were tested with the black insert being placed in the opposite arm to what they first experienced at Time 1.

3.3.2 Light-Dark Preference Box:

The Light-Dark Preference Box as a test of emotionality and anxiety behaviour in rats is based on the rats' innate avoidance of brightly illuminated areas. In response to mild stressors - in this case a novel environment and bright light - the rat faces a natural conflict between the desire to explore the new environment (curiosity) verses the tendency to avoid the unfamiliar and a fear of brightness (Hascoët & Bourin, 2009; Sanchez, 1996). Higher emotionality, thus anxiety, is indicated by the tendency to avoid the light side (Hughes et al., 2004; Hascoët & Bourin, 2009).

Each animal was placed in the dark component of the apparatus for 60 seconds with the guillotine slide closed, separating the light and dark compartments. The slide was then raised allowing the animal access to the light side and the latency of the animal fully entering (all four paws) the light side was measured using a hand held stop watch. Once the animal entered the light side, it was allowed free access to both light and dark sides of the box for five minutes with the total period of time spent in the light side and total period of time spent in the dark side being recorded along with the number of entries using a computer program. If the rat did not emerge from the dark side after five minutes, the trial was terminated and a latency of 300 seconds was recorded. After each trial the box was cleaned down and disinfected with 20% Paraquat Blue.

3.3.3 Open Field:

The open field test is designed to elicit anxiety by separating the animal from its home cage and social group and placing it in an inescapable novel environment (Prut & Belzung, 2002; Walsh & Cummings, 1976). The test was originally developed to measure emotionality in rats by Hall (1934) and now is one of the most widely used measures of anxiety-like behaviours in animal studies (Prut & Belzung, 2002). As well as being used to measure anxiety behaviours, it has also been used to demonstrate the behaviourally stimulant and sedative properties of drugs in animals (Gould et al, 2009).

In this study, the apparatus consisted of a wooden open field measuring 600 x 600 x 250mm which was placed on a table 700mm high. The floor was painted flat black and divided in a 4 x 4 grid of sixteen identical squares each measuring 150 x 150 mm. There were twelve peripheral squares and four central squares. The wooden walls were also painted flat black and were 250mm high. Garau et al. (2000) suggest that uncertainty (therefore anxiety) is increased by placing an animal in a novel environment and also by altering light levels. In this study, the light level was low (45 lux) and the testing arena was unfamiliar to the animals producing low to moderate anxiety (File & Seth, 2003). An infrared camera was mounted on a single wooden arm 850mm above the open field and this was connected to a television monitor in the same experimental room which was used by the observer to monitor the animal's position and behaviour within the open field. The arena was washed down and disinfected with 20% Paraquat Blue after each animal.

Each rat was placed in the centre of the wooden open field and its location and behaviours were recorded every three seconds for a total time period of five minutes following a six second delay before recording commenced. This test and the behaviours that are observed are designed to provide a measure of general activity and emotional reactivity in animals (Hall, 1934a; Royce, 1977; Walsh & Cummings, 1976).

The recorded behaviours included location, rearing up or leaning on hind legs, and grooming. As previously mentioned, the total number of faecal boluses was also recorded as defecation increases when the animals are under stress. In this experiment, grooming was defined as the touching of hands to mouth, hands to ears, mouth to sides, mouth to tail, mouth to feet, mouth to genitals, mouth to abdomen, feet to head/ears and feet to sides (Mayaho et al., 1995; Pleskacheva, 1995). The number of times the animal was located in a different square to where it had been 3 seconds prior was also calculated to indicate the number of transitions the animal made and to provide a measure of locomotive activity (ambulation). As it has been argued that animals prefer the outer squares to the exposed central squares (Prut et al., 2002) the total frequency of

centre and corner square occupation was calculated. Each animal completed the open field test twice, first on PND 136 and again on PND 186. This was to measure whether prolonged exposure to a micronutrient formula as part of a typical diet led to a reduction in emotionality and anxiety-like behaviour in the animals.

4.0 Statistical Analyses

The main focus of this study was to examine the effects of receiving micronutrients on animal emotionality and whether this effect was maintained over time while receiving micronutrients as part of a normal diet.

All raw data sets were analysed using the statistical program *Statistical Package for the Social Sciences (SPSS)* 19.0. Each measure was treated to a separate 4 (treatment group) x 2 (sex) x (age) factorial repeated measure ANOVA. Post hoc Tukey tests were carried out when a main effect was significant ($p<.05$). Sex differences were included in the analyses due to sex differences in brain maturation and in brain maturation rates (Anderson, 2003). Given male and female brains develop differently, receiving micronutrients as part of their diet would likely affect the two sexes differently over time. Differences in behaviour at each testing age were investigated to detect any additional changes after receiving micronutrients over an extended period of time.

5.0 Results

5.1 Responsiveness to Brightness Change in a Y-Maze Results:

Each animal was tested twice in the Y Maze, once at PND 136 and again at PND 186. ANOVAs were used to assess the effects of the micronutrient diet, gender and any interactions between them, along with a repeated measures analysis which was used to look at the differences between testing at time 1 and testing at time 2. The measures included in the analysis were time in both arms, entries into both arms, percentage of entries into the novel arm and percentage of time spent in the novel arm.

Table 2: *Mean (Standard Deviation) for responses in the Y Maze for controls (n=20), 1.25% (n=20), 2.5% (n=20) and 5% (n=20) of micronutrient in diet treatment groups for male (n=40) and female (n=40) rats at PND 136-138 (n=80) and at PND186-188 (n=80) and results of ANOVAs.*

Percentage of Micronutrient in Diet	**0%**	**1.25%**	**2.50%**	**5%**	***F(1,15)***	**P**
Entries of both arms	4.70 (2.44)	5.23 (2.61)	4.83 (2.48)	5.10 (1.85)	0.508	0.678
Time in both arms	73.24 (34.74)	67.95 (34.05)	59.30 (29.62)	66.67 (24.08)	1.399	0.245
% entries of novel arm	51.59 (20.49)	51.60 (17.24)	52.29 (22.85)	53.42 (13.21)	0.081	0.970
% time in novel arm	45.31 (21.80)	48.93 (20.02)	51.58 (23.38)	52.61 (19.84)	0.948	0.419
Sex	**Female**	**Male**			***F(1,15)***	**P**
Entries of both arms	5.36 (2.04)	4.56 (2.57)			5.548	**0.020**
Time in both arms	67.23 (24.68)	66.38 (36.44)			0.031	0.860
% entries of novel arm	53.70 (16.27)	50.75 (20.73)			0.956	0.330
% time in novel arm	51.90 (18.56)	47.31 (23.63)			1.875	0.173
Testing Block	**PND 136-138**	**PND186-188**			***F(1,15)***	**P**
Entries of both arms	5.88 (2.17)	4.05 (1.87)			37.754	**0.000**
Time in both arms	74.89 (25.36)	58.73 (34.09)			19.22	**0.000**
% entries of novel arm	51.89 (14.68)	52.56 (21.98)			0.043	0.836
% time in novel arm	47.29 (17.83)	51.93 (24.17)			1.678	0.199

5.1.1 Entries into Both Arms:

There was a significant main effect in relation to sex for the number of entries into both arms with female animals making significantly more entries to both arms than male animals. There was also a significant effect in relation to testing blocks with animals making more entries at time 1 (5.88 entries) than at time 2 (4.05 entries). No significant differences were found in regards to the percentage of micronutrient in diet received by the rats. No interaction effects were found to be statistically significant.

5.1.2 Time Spent in Both Arms:

There was a statistically significant difference between the amount of time spent in both arms at time 1 and time 2 with animals spending more time in both arms at time 1 (74.89 seconds) when compared to time 2 (58.73 seconds). No significant main effects were found for sex or the percentage of micronutrient received. No interaction effects were found to be significant for this measure.

5.1.3 Percentage entries of Novel Arm:

There were no significant main effects of percentage of micronutrient in diet, sex or time at which testing took place for percentage of entries of novel arm. No interaction effects were found to be statistically significant.

5.1.4 Percentage time in Novel Arm:

No significant main effects were found in relation to micronutrient in diet, sex or time at which testing took place for percentage of time spent in the novel arms. No statistically significant interaction effects were detected either.

5.2 Light/Dark Box Results:

In the light-dark box emergence test, each animal was tested twice, first at PND 136-138 and then again at PND 186-188. ANOVAs were used to detect whether there were effects due to the percentage of micronutrient in the animal's diet, and sex differences. A repeated measures analysis was also used to assess whether there was any difference in the animals performance on the measures over time.

Table 3: *Mean (Standard Deviation) responses in the Light/Dark Box for controls (n=20), 1.25% (n=20), 2.5% (n=20) and 5% (n=20) of micronutrient in diet treatment groups for male (n=40) and female (n=40) rats at PND 136-138 (n=80) and at PND186-188 (n=80) and results of ANOVAs.*

Percentage of Micronutrient in Diet	**0%**	**1.25%**	**2.50%**	**5%**	***F(1,15)***	**P**
Transitions	4.68 (3.52)	4.20 (3.80)	4.90 (3.75)	5.50 (3.84)	1.235	0.299
Time spent in light side	46.98 (35.53)	35.77 (40.24)	40.32 (30.77)	42.39 (36.89)	0.825	0.482
Sex	**Female**	**Male**			***F(1,15)***	**P**
Transitions	6.64 (3.66)	3.00 (2.79)			56.093	**0.000**
Time spent in light side	55.74 (38.30)	26.99 (26.63)			31.454	**0.000**
Testing Block	**PND 136-138**	**PND186-188**			***F(1,15)***	**P**
Transitions	5.93 (3.40)	3.71 (3.72)			29.88	**0.000**
Time spent in light side	46.79 (28.98)	35.94 (41.17)			5.358	**0.023**

5.2.1 Transitions:

There was a significant main effect of sex for the number of transitions animals made. Females (6.64 transitions) made significantly more transitions than males (3.00 transitions). There was also a statistically significant difference in the number of transitions made by the rats at time 1 compared to time 2. Animals made more transitions at time 1 (5.93 transitions) than at time 2 (3.71

transitions). The percentage of micronutrient in the animal's diet was not found to have a significant effect on the number of transitions made. No significant interaction effects were detected.

5.2.2 Time in Light Side:

There was a significant difference in the amount of time spent on the light side of the apparatus between testing at time 1 and testing at time 2. Animals spent more time in the light side of the box at time 1 (46.79s) than they did at time 2 (35.94s) Sex was also found to have a statistically significant effect on the amount of time spent in the light side of the box. Female rats were found to spend more time in the light side than male rats, 55.74s for females compared to 26.99s for males. No significant main effect was found in regards to the percentage of micronutrients the rats received in their diet in relation to the amount of time spent on the light side of the apparatus.

5.3 Open Field Results:

In the Open Field apparatus, each rat was tested once at each testing age. ANOVAs were used to detect whether there was an effect due to the percentage of micronutrient in the animal's diet, and sex differences. A repeated measures analysis was also used to assess whether there was any difference in the animals performance on the measures over time.

Table 4: *Mean (Standard Deviation) for responses in the Open Field for controls (n=20), 1.25% (n=20), 2.5% (n=20) and 5% (n=20) of micronutrient in diet treatment groups for male (n=40) and female (n=40) rats at PND 136-138 (n=80) and at PND186-188 (n=80) and results of ANOVAs.*

Percentage of Micronutrient in Diet	**0%**	**1.25%**	**2.50%**	**5%**	***F(1,15)***	**P**
Transitions	37.42 (13.10)	32.85 (12.13)	39.38 (12.78)	40.35 (14.54)	4.203	**0.007**
Centre Square Occupancy	4.47 (2.47)	3.93 (2.44)	4.72 (2.66)	7.90 (4.74)	14.33	**0.000**
Corner Square Occupancy	66.20 (12.79)	71.60 (11.35)	68.52 (9.87)	61.33 (12.80)	5.961	**0.001**
Rearing	32.70 (11.93)	33.18 (12.35)	33.23 (12.59)	34.98 (12.08)	0.288	0.834
Grooming	2.05 (2.74)	2.18 (2.44)	2.62 (2.32)	2.13 (2.45)	0.447	0.720
Faecal Boluses	0.30 (1.09)	0.43 (1.13)	0.10 (0.63)	0.47 (0.95)	0.983	0.403
Sex	**Female**	**Male**			***F(1,15)***	**P**
Transitions	42.23 (12.39)	32.78 (12.67)			33.849	**0.000**
Centre Square Occupancy	4.94 (3.26)	5.57 (3.83)			1.81	0.181
Corner Square Occupancy	68.33 (10.87)	65.50 (13.40)			2.535	0.114
Rearing	33.64 (10.72)	33.40 (13.50)			0.016	0.899
Grooming	2.37 (2.48)	2.11 (2.49)			0.458	0.500
Faecal Boluses	0.01 (0.11)	0.47 (1.33)			9.633	**0.002**
Testing Block	**PND 136-138**	**PND186-188**			***F(1,15)***	**P**
Transitions	44.31 (10.31)	30.69 (12.60)			101.258	**0.000**
Centre Square Occupancy	6.18 (3.96)	4.34 (2.84)			18.903	**0.000**
Corner Square Occupancy	63.47 (10.90)	70.35 (12.62)			21.918	**0.000**
Rearing	37.43 (10.92)	30.10 (12.39)			14.397	**0.000**
Grooming	2.76 (2.72)	1.73 (2.11)			10.17	**0.002**
Faecal Boluses	0.30 (1.08)	0.19 (0.84)			0.69	0.409

5.3.1 Transitions:

A significant treatment effect was found for the number of transitions. There was a statistical difference between the number of transitions made with animals receiving micronutrients at a 5% level making the greatest number of transitions and those receiving 1.25% in their diet made the least number of transitions. Post hoc analysis showed that there was a statistically significant difference between the number of transitions made by the animals receiving a 5% diet and those receiving the 1.25% diet (p=0.007). A statistically significant difference was also found between animals receiving the 2.5% and animals on the 1.25% diet (p=0.026). In both cases, the 1.25% diet group made significantly less transitions. No significant differences were found between the control animals and any treatment group or between the animals receiving 5% diet and those receiving the 2.5% diet (see Figure 2).

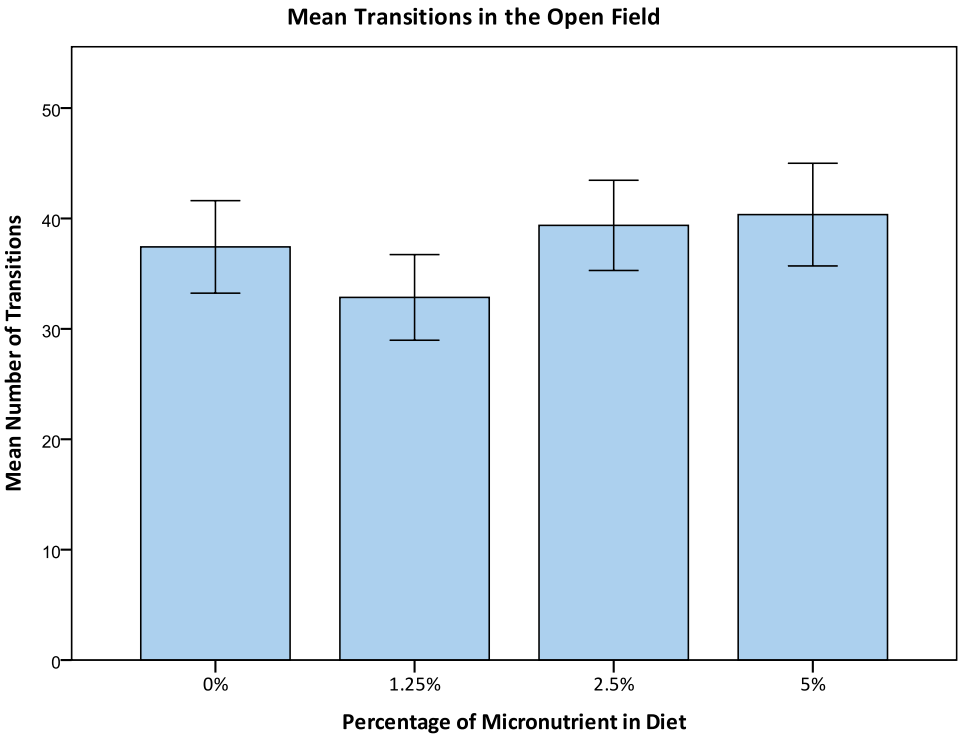

Figure 2. *Mean transitions for rats as a function of percentage of micronutrient in diet. Vertical bars denote the 95% confidence interval.*

Sex was found to have a significant effect on transitions with females making more transitions than males. It was also found that animals made significantly more transitions at when tested at time 1 than they did at time 2. No interaction effects were found.

5.3.2 Centre Square Occupancy:

The percentage of micronutrient in the animals' diet had a significant effect on the amount of centre square occupancy with those animals receiving a 5% micronutrient diet occupying the centre squares the most. Animals receiving 1.25% diet occupied the centre squares the least out of the treatment groups. Post hoc analysis showed that there was a significant difference in centre square occupancy with the animals receiving the 5% diet occupying the centre squares significantly more than the control animals ($p=0.000$); the animals receiving 1.25% diet ($p=0.000$) and the animals on the 2.5% diet ($p=0.000$). No statistically significant differences were found between the control animals, the 1.25% diet group or the 2.5% diet group (see Figure 3).

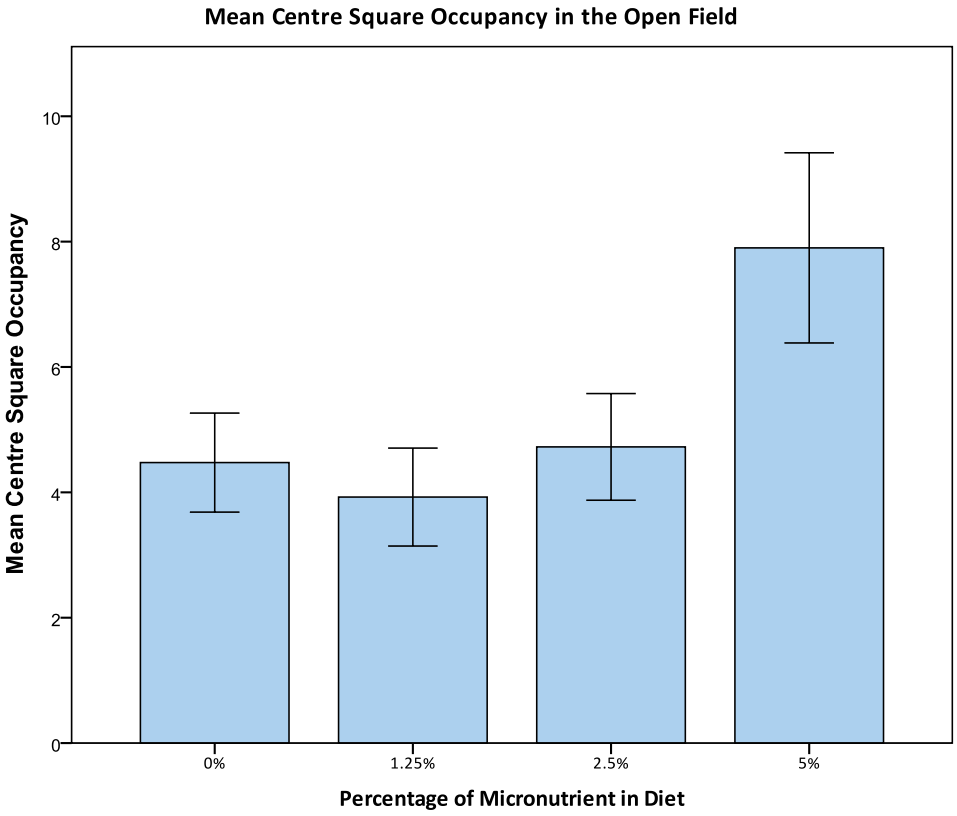

Figure 3. *Centre square occupancy as a function of percentage of micronutrient in diet. Vertical bars denotes the 95% confidence intervals.*

Time of testing also had a significant effect on centre occupancy with the animals occupying the centre more at time 1 than at time 2.

5.3.3 Corner Square Occupancy:

Corner square occupancy was significantly affected by the percentage of micronutrients the rats received in their diet. In this case, those animals receiving 1.25% occupied the corner squares the most, followed by those receiving the 2.5% diet and then animals receiving the control diet. Those that receiving the 5% diet occupied the corner squares the least. Post hoc analysis revealed a significant difference between corner square occupancy with the animals receiving the 5% diet occupying the corner squares significantly less than the animals receiving the 1.25% diet (p=0.000)

and those on the 2.5% diet (p=0.024). A significant difference was also found between the control animals and those on the 1.25% with control animals occupying the corner squares significantly less than the animals receiving 1.25% diet (p=0.033) (see Figure 4).

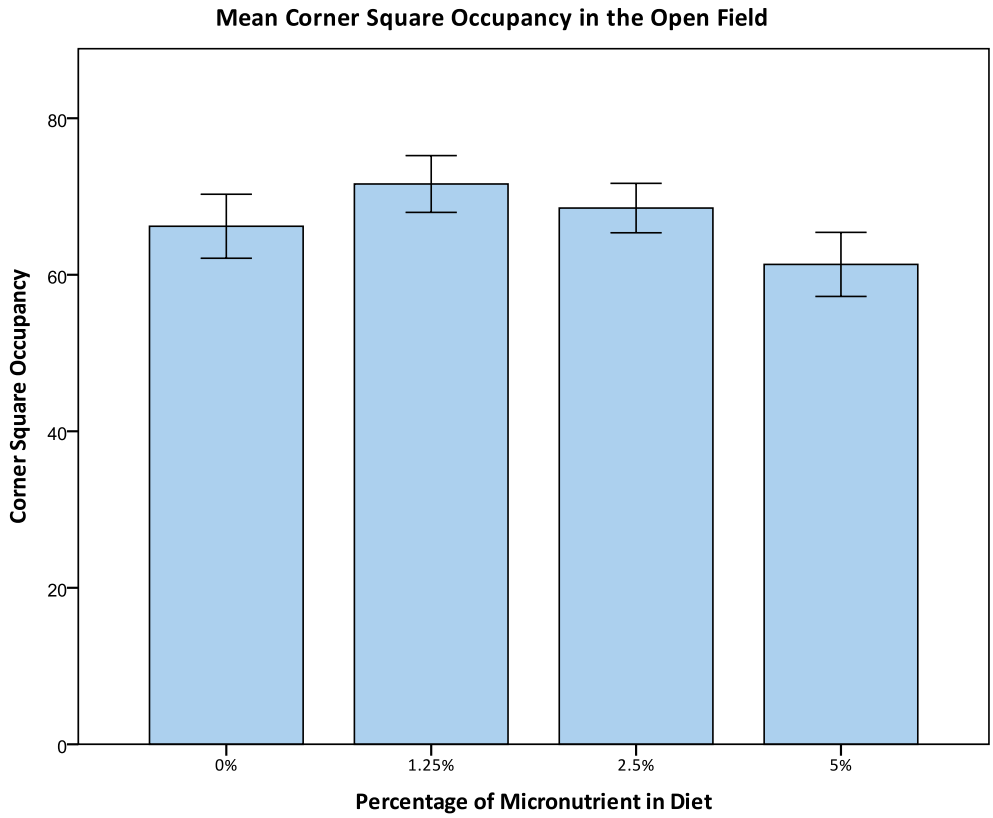

Figure 4. *Corner square occupancy as a function of percentage of micronutrient in diet. Vertical bars denote the 95% confidence intervals.*

There was also a significant difference in corner occupancy depending on the testing block. Animals occupied the corner squares more at time 2 than at time 1

5.3.4 Rearing:

A significant effect was found of the testing block for rearing behaviours. Animals reared more at time 1 than at time 2.

5.3.5 Grooming:

The testing block was found to have a statistically significant effect on grooming behaviours with animals exhibiting more grooming behaviour at PND136-138 than at PND186-188.

5.3.6 Faecal Boluses:

Sex was found to have a significant main effect in relation to the number of faecal boluses recorded. Females produced significantly less faecal boluses than males.

6.0 Discussion of Results

In the current study, 80 rats were divided into treatment groups consisting of a control group which received regular rat chow, animals whose diet contained 1.25% of the micronutrient formula EMP+, those whose diet was made up of 2.5% of EMP+ and animals whose diet consisted of 5% EMP+. Animals were fed the diet after been weaned on PND30 and remained on the diet until the final day of testing (PND188). As mentioned earlier, due to the September 2010 Canterbury earthquake all animals had a two week period where they received a regular rat chow diet. Animals were tested at two times using common behavioural tests at PND136-138 and PND 186-188. Different dosages of the micronutrient formula were used in this study to allow for comparative data to be gathered and for any possible effects of dose to be investigated. Interpretations could then be made from any significant results about the behavioural outcomes of a micronutrient supplemented diet over a prolonged period and whether the level of supplementation produced differing outcomes.

6.1 Summary of Results:

Results indicated several differences in behaviours displayed between the control and treatment groups mainly on one test of emotionality, the open field test. The rats that had received the highest proportion of micronutrient in their diet (5%) showed significantly less emotionality than those receiving a smaller percentage of micronutrient in their diet.

In the open field test animals receiving the 5% diet made more transitions, occupied the centre squares more often and occupied the corner squares the least when compared to controls and the other 2 treatment groups. Results from the two other behavioural measures, the Y-maze and Light-Dark box did not reveal any significant differences between the treatment groups in regards to performance. The results of these three behavioural measures provide partial support for the main hypothesis in that animals receiving the highest dose showed the least amount of anxiety-related

behaviour and there is some evidence to suggest that the reduction in anxiety-like behaviour was dose dependant with animals receiving the lowest dose displaying higher levels of anxiety.

6.1.1. Treatment Effects:

In the Open Field test, animals receiving the 5% micronutrient diet ambulated more when compared to the other micronutrient treatment groups with the 1.25% group making the least number of transitions (low ambulation). Low ambulation is thought to be indicative of higher anxiety and therefore this result provides support for the interpretation that animals receiving the highest dose of micronutrient displayed less emotionality in the open field. Increased centre square occupancy is indicative of reduced anxiety (Prut & Belzung, 2003) and the fact that animals receiving the 5% diet had the greatest amount of centre square occupancy compared to control animals and animals receiving lower doses of micronutrients provides further support for reduced anxiety and emotionality in the highest dosage group. A further treatment effect was found with animals in the 5% group occupying the corner squares less than those in the 1.25% and 2.5% groups. The 1.25% group occupied the corner squares the most compared to controls and the 5% group. High corner square occupancy in the Open Field suggests higher anxiety as the animal is avoiding open areas within the apparatus (Kontinen et al., 1999). Combined, these results indicate that in the open field test, the higher the percentage of micronutrient in the animals' diet, the less anxiety and emotionality is displayed. Interestingly, in all of these measures the most anxious group appeared to be those receiving the 1.25% diet rather than control animals.

The above findings are in contrast to the results from the Y-maze and the Light-Dark test where no significant differences between treatment groups were found.

6.1.2 Length of Supplementation:

In contrast to the hypothesis that the longer the animals received the supplemented diet the greater would be the reduction in anxiety, the results indicated that animals displayed lower levels of anxiety-like behaviour at time 1 than at time 2 on all three behavioural measures.

On the Y-maze, animals made significantly fewer entries of and spent less time in both arms after 141 days of supplementation compared to 91 days of supplementation. Although not statistically significant, this trend was also seen in the percentage of entries into the novel arm and the percentage of time spent in the novel arm with both measures being greater at time 1 than at time 2 indicating more time and entries into the novel arm at 91 days of supplementation. An animal which enters the novel arm more is thought to be less anxious while a more anxious animal will show a preference for the unchanged arm (Hughes, 2001).

In the Light-dark box animals appeared to be more anxious at time 2, after prolonged micronutrient supplementation, than at time 1. Higher anxiety is indicated by the animal's tendency to avoid the light side of the apparatus (Hughes et al., 2004). Both the number of transitions and the time spent in the light side were significantly greater at time 1 than at time 2 which suggests that at time 2 animals were avoiding the light side more and therefore more anxious.

In the Open-field test the animals spent significantly less time in the centre squares and significantly more time in the corner squares at time 2 compared to time 1 regardless of whether they were receiving the supplemented diet or not. As mentioned above, reduced centre square occupancy and increased corner square occupancy is suggestive of increased anxiety as the animal is avoiding the open areas of the apparatus (Prut & Belzung, 2003). The animals had significantly lower levels of ambulation at time 2 and lower levels are thought to be associated with higher levels of anxiety. The animals also reared less at time 2 than at time 1 which suggests that the rats were more anxious at time 2 than at time 1 as lower rearing in rats is suggestive of higher emotionality as less

exploratory behaviour is being displayed (Aitchison & Hughes, 2006; Herbert & Hughes, 2006). Conversely, the animals showed significantly lower levels of grooming behaviour at time 2 than at time 1. This is contradictory to the other findings as higher levels of grooming are associated with higher levels of anxiety in rats while lower levels are indicative of reduced anxiety (Archer, 1973).

Overall, the results strongly suggest that the animals in this study were more anxious at time 2 than at time 1. Although this effect may be attributed to the prolonged exposure to the micronutrient formula in their diet, it is important to note effect of the September 2010 earthquake and the on-going aftershocks. Research into the effects of the earthquake and aftershocks indicate that the on-going aftershocks following the September earthquake continued to stress animals with rat breeding at the University of Canterbury reduced approximately 10% in the weeks following, while fish housed at the University of Canterbury stopped eating for up to a week (Glassey & Wilson, 2011). Furthermore, there were numerous reports from pet owners and farmers in the region of animals being 'spooked' by the initial earthquake event and following aftershocks (Glassey & Wilson, 2011). Therefore the increase in anxiety-like behaviour may actually be reflective of the effects of the initial earthquake and prolonged exposure to on-going earthquakes rather than to prolonged exposure to the micronutrient formula. This is further supported by the increase in anxiety being found across all groups including controls animals. Although research indicates that micronutrient supplementation may actually reduce the stress/anxiety response in human populations in relation to the Canterbury earthquakes (Rucklidge et al., 2012; Rucklidge et al., 2011a) it is important to note that for the two weeks following the September earthquakes the animals did not receive the micronutrient formula due to restricted access which may have also affected the animals initial stress-response to the earthquake and aftershocks.

6.1.3 Sex Differences:

Several significant differences between male and female animals were found in the current study of behavioural measures. In the Y maze, females made more entries into both arms which suggests a greater level of activity and more exploratory behaviour than the male rats and therefore lower anxiety (Hughes & Neeson, 2003). This result is consistent with the light-dark box results where female rats made a greater number of transitions between the light and dark side and spent longer in the light side of the box. It is assumed that rats that spend longer in the light side of the apparatus are generally less anxious which would indicate that the females were displaying less anxiety-like behaviour than the males.(Hascoët & Bourin, 2009). The results from the open-field test also indicates that the female rats were less anxious than male rats with the females showing significantly higher levels of ambulation and significantly less defecation (faecal boluses) than males. As mentioned previously, higher ambulation levels are thought to be indicative of lower anxiety while increased defecation is thought to suggest the rat is anxious or in a stressful state (Hall, 1934a).

7.0 General Discussion

7.1 Methodological Strengths:

The methodological strengths and weaknesses of the current study need to be examined in order to evaluate the degree to which these results can be generalised. The current experiment has several strengths, the first being the use of rats. Rats develop quickly and reach adulthood approximately after 90 days (Anderson, 2003). This means that any research using rats can be completed in a shorter period of time that human research as the long term behavioural effects of exposure can be detected more quickly than in human populations. Given that the study was designed to measure the long term behavioural effects of micronutrient supplementation, the use of rats meant behavioural measures could be completed at early adulthood and late adulthood providing a more detailed picture of the long term effects of supplementation. Using rats also allows us to ensure that all subjects are exposed to the same experiences such as handling, living conditions and also that there has been no prior history of medication use or expectations.

The use of different dosages of the micronutrient formula enables us to establish the most effective dose in relation to reduction in anxiety-like behaviour in normal rat populations. Comparing low, medium and high doses of the formula alongside the control animals provides us with information in regards to the effects of what are thought to be a general health dose, a therapeutic dose and a dose twice the therapeutic dose in normal populations. Previous human research indicates that individuals with psychiatric disorders may require higher doses than the normal population to produce beneficial effects (Kaplan & Leung, 2011). What this study shows is that animals receiving a high dose of micronutrient supplementation showed the greatest reduction in anxiety-like behaviour on some measures while those receiving the general health dose and recommended therapeutic dose did not differ greatly to controls and on some measures performed worse than controls. Although there were concerns that the highest dose could produce a toxic

effect where animals would display greater anxiety, this was not found in this study with those animals actually displaying significantly less anxious behaviours in the open field. No significant differences in behaviour in relation to dose were found on the two other measures included in this study.

The presence of the observer during the testing phases may have also affected the rats' responses on the behavioural measures, namely in the open-field and light dark preference box. To ensure that this effect was kept to a minimum an external video camera was mounted above each apparatus and connected to a television monitor in the experimental room where the observer was able to record the animals' movements without sitting in close proximity to either apparatus. Alongside the use of an external video camera, the same testing room was used during both testing phases and the apparatus were orientated in the same location as well.

This study served as a pilot study for the behavioural effect of micronutrients in rats as only one other study in this area has been reported, and consequentially very little is known about the behavioural effects. The purpose of the study was not to mimic the exact human use of micronutrient formulas as research to date has followed a more individualistic treatment approach but rather to detect any differences between the levels of supplementation and the long term behavioural effects. As there is a lack of animal research in this area, any results can be seen as preliminary and providing a starting point for future research in this area.

7.2 Methodological Limitations:

During this study, several limitations became apparent, mainly as a result of the September 4th 2010 Canterbury Earthquake. The way the animals had initially been randomised into treatment groups meant that the treatment groups were at different developmental ages and stages when the earthquake struck which could have led to different emotional and behavioural responses in relation to the main earthquake and aftershock sequence across the treatment groups.

As mentioned in the introduction, the treatment groups had been receiving the micronutrient supplement for different lengths of time when the earthquake occurred. The animals in the high dose group had been receiving the formula for a month prior to the earthquake, those receiving the medium and some receiving the low dose diets had been supplemented for 3 weeks and the majority of those in the low dose group had only received the supplemented diet for four days prior to the earthquake. This difference in period of supplementation may have also led to different behavioural and emotional responses following the earthquake and during the on-going aftershocks, especially given that recent research using human populations found that micronutrient use leading up to and during the Canterbury earthquake produced increased resilience to ongoing stress and anxiety (Rucklidge et al., 2011a). Another limitation caused by the earthquake was the two week break in supplementation immediately following the quake due to restricted access to animal labs. During this time all treatment groups received regular rat chow, however as outlined above the treatment groups were at different developmental ages and had been receiving the supplement for differing lengths of time leading up to this two week break. It is hard to determine to what to what extent or effect this may have affected the animals' behaviour.

The method of administering the supplement was also a limitation in this study. As the animals lived in shared cages with 3 to 4 animals per cage it is hard to estimate the amount of supplement each animal was actually consuming on a daily basis. Although the ground rat chow and supplement were combined to create a diet made up of 1.25%, 2.5% or 5% of micronutrient formula it is likely that animals would have consumed different proportions of the food given daily and therefore it is likely that there was a variation in the level of supplement each animal received on a daily basis. It is important to note that each animal was weighed regularly to ensure that they maintained normal body weight and that growth was similar to other animals in the home cage. If lower body weight had been detected the animal would have been removed from the home cage and house separately until a normal body weight had been reached and then returned to the home

cage. It is acknowledged that if rats had been housed individually, food consumption could have been recorded and the exact level of micronutrient consumed could have been calculated.

Another limitation or strength, depending on which way you come at it, is the fact the animals were from a normal rat population. In one way this is a strength as it provides us with an understanding of the effects of differing dosages of micronutrient formula on anxiety behaviour in normal rats. On the other hand, the majority of human research in this area has focused on individuals with serious mental health disorders and perhaps the use of an animal model of anxiety using genetic manipulation to produce animals that show high levels of anxious behaviour would provide more insight in regards to the effects of EMP+ in an anxious population.

7.3 Implications:

This is a pilot study designed to assess the effects of a micronutrient supplement on rat behaviour. Given the lack of research in this area, the results of this study are important and provide additional knowledge to a body of research that is plagued with mixed results across a variety of individual vitamins and minerals and their effects on a number of animal behavioural measures. The current study highlights some important issues as well as providing a base for future research to take into consideration. Due to the increasing numbers of studies looking at the effects of micronutrients in the human population, it is important that animal research is continued as it adds to our understanding of the effects of micronutrient supplementation.

One important finding from this study is the relationship between the level of micronutrient supplementation and the reduction in anxiety-like behaviour in rats. This study found that on some behavioural measures animals receiving the highest dose of micronutrient formula which is equivalent to twice the recommended therapeutic dose showed significantly less anxious behaviour. This was compared to both the other treatment groups and controls. This has important clinical implications because the results suggest that a higher dose of EMP+ may be more effective in

reducing anxiety-related behaviours in a normal population. Similar results have been found when looking at stress and trauma responses following a natural disaster in a healthy human population (Rucklidge et al., 2012). However it is important to note that on the majority of behavioural measures no significant differences where found between supplemented groups or control animals.

In regards to negative effects of EMP+ on anxiety like behaviour in rats, it was found that the longer the animals received the supplement the more anxious they became with animals displaying more anxiety related behaviours on all measures at time 2 than at time 1. However, it should be noted that this pattern of results was also found in control animals which received no micronutrient supplementation. Therefore, perhaps this pattern of increased an anxiety can be more associated with the effects of the ongoing aftershocks rather than long term micronutrient supplementation.

Another interesting finding was the fact that animals receiving the 1.25% supplemented diet actually displayed significantly greater levels of anxiety-like behaviours than control animals on several measures. This dosage is the recommended dose for general good health and one would expect these animals to have shown less anxiety-like behaviour when compared to controls. It is hard to conclude whether this is related to the micronutrient supplement or the fact that the control animals were older when the initial earthquake struck. This meant that the control animals would have experienced less aftershocks during their experimental procedure therefore experienced lower levels of additional stress compared to those animals receiving the 1.25% diet. Once again, this result displays the number of confounding variables that were introduced following the September earthquake.

Overall, it appears that the 5% dose has the greatest effect on anxiety in a normal rat population. These animals showed the greatest reduction in anxiety related behaviours in the open field compared to the other diet strengths. In contrast, those animals receiving the 1.25% diet showed greater levels of anxiety-like behaviours such as increased corner square occupancy, reduced

centre square occupancy and reduced ambulation in the open field when compared to control animals and other diet strengths. Based on these results, it may be that a diet strength of 5% could be suitable for a health human population, however future research is necessary before any firm conclusions can be made.

8.0 Future Directions

This study was designed to be a pilot study intended to provide direction for future research. Although it may be obvious, and in fact out of our control, any future research in this area needs to take place without the inference from earthquakes. Randomised controlled trials are especially designed to eliminate confounding variables in order to provide us with a greater degree of certainty in which we can claim the results of the study are a result of our experimental manipulations rather than those of uncontrolled factors. In this case, the number of confounding variables produced by the earthquake and ongoing aftershocks makes it difficult to conclude whether or not the results were in fact due to the experimental manipulations.

The other limitations of this study also need to be addressed. The fact the supplement was administered through the animal's shared rat chow led to some uncertainty with regards to what percentage of the diet of each animal was made up of the micronutrient. To remedy this, the supplement could be added to the animal's drinking water and the rats housed in cages divided into two sections with an animal in each section. This would allow animals to interact socially but for them to have individual access to drinking water and food. This way, water consumed daily by each animal could be measured and from this, the exact amount of supplement received could be calculated. Using this method would allow for the exact amount of micronutrient consumed to be calculated however it would not ensure that animals from the same treatment groups were consuming the same amount of supplement on a daily basis. An alternative which would allow greater control over the amount of supplement received would be supplement administration via injection. The supplement could be combined with an agent which would make it possible for it to be injected on a daily basis however this procedure would be time consuming in a study using the same number of rats receiving the supplement for an extend period of time.

Given the fact that research using human populations mainly focuses on individuals with serious mental health disorders, another avenue of research could be to use animal models of these disorders to provide further knowledge in to the effects and mechanism of action of micronutrient supplementation. Genetic models of some of these disorders have been developed such as the BALB/c mouse which displays high levels of anxious behaviour in the open field and greater levels of anxiety in the light-dark box (Cryan & Sweeney, 2011). In line with this, human research in this area has begun to look at the level of nutrient deficiency pre- and post-supplementation through blood tests. This could be followed up in the animal research as well providing us with a greater understanding of the mechanism of action and the effects on behaviours. It would also allow future researchers to ensure that high doses of the micronutrient do not cause a toxic level of individual ingredients in the blood stream which in turn could produce toxic effects.

As this body of research continues to grow, further investigations should be done into the effects of micronutrients both human and animal populations to further our understanding of the role micronutrients can play in treatment of psychiatric disorders and to help increase of knowledge of the exact mechanism of action that produces changes in symptomatology. Future research should investigate the exact role each of the nutrients within the formula play and what combination of these is the most effective for individual disorders.

9.0 Conclusions

Despite earthquakes and ongoing aftershocks this study provides a starting point for a new area of research with some promising results in relation to the effects of EMP+ in an animal population. It has shown that a high dose of micronutrient supplementation leads to reduced levels of anxiety behaviours in a normal rat population and that a lower dose may increase anxiety-like behaviour, suggesting that there may be a dose dependant response. Unfortunately it is hard to reach any firm conclusions due to the Canterbury Earthquake and aftershock sequence; however this does not mean the results should be discounted.

Being a pilot study it has highlighted several limitations that can be addressed in future research including the administration of the micronutrient supplement and the animal model used. There is definitely evidence to suggest that micronutrient supplementation does affect behavioural responses in animals and further research using randomised controlled designs in both human and animal populations is needed to help us gain a greater understanding of exactly what this effect may be and how we can integrate micronutrient supplementation into clinical practice.

References

Aitchison, L. K., & Hughes, R. N. (2006). Treatment of adolescent rats with 1- benzylpiperazine: A preliminary study of subsequent behavioural effects. *Neurotoxiology and Teratology, 28*, 453-458.

Ames, B. N. (2004). A role for supplements in optimizing health: The metabolic tune up. *Archives of Biochemistry and Biophysics, 423*, 227-234.

Ames, B. N. (2006). A theory of evoulationary allocation of scarce micronutrients by enzmye triage: Adequate micronutrient nutrition to delay the degenerative diseases of aging. *Proceedings of the National Academy of Sciences*(103), 17589-17594.

Ames, B. N., Elson-Schwab, I., & Silver, E. A. (2002). High-dose vitamin therapy stimulates variant enzymes with decreased coezyme binding affinity (increased k(m)): Relevance to genetic disease and polymorphisms. *American Journal of Clinical Nutrition, 75*, 616-658.

Anderson, S. L. (2003). Trajectories of brain development: Point of vulnerability or window of opportunity? *Neuroscience and Biobehavioral Reviews, 27*, 3-18.

Archer, J. (1973). Tests for emotionality in rats and mice: A review. *Animal Behaviour, 21*, 205-235.

Baldwin, D. S., Anderson, I. M., Nutt, D. J., Bandelow, B., Bond, A., Davidson, J., . . . Wittchen, H. U. (2005). Evidence-based guidelines for the pharmacological treatment of anxiety disorders: Recommendations rom the bristish association for psychopharmacology. *Jouranl of Psychopharmacology, 19*(6), 567-596.

Craske, M. G., Rauch, S. L., Ursano, R., Prenoveau, J., Pine, D. S., & Zinbarg, R. F. (2009). What is an anxiety disorder. *Depression and Anxiety, 26*, 1066-1085.

Cryan, J. F., & Sweeney, F. F. (2011). The age of anxiety: Role of animal models of anxiolytic action in drug discovery. *British Journal of Pharmacology, 164*, 1129-1161.

de Oliveria, M. R., Silvestrin, R. B., Souza, T. M., & Moreira, J. C. F. (2007). Oxidative stress in the hippocampus, anxiety-like behaviour and decreased locomotory and exploratory activity of

adult rats: Effects of sub actue vitamin a supplementation at therapeutic doses. *Neuro Toxicology, 28*, 1191-1199.

Dunlop, B. N., & Davis, P. G. (2008). Combination treatment with benzodiazepines and ssris for comorbid anxiety and depression: A review. *The Primary Care Companion to the Journal of Clinical Psychiatry, 10*(3), 222-228.

Fanselow, M. S., & Lester, L. S. (1988). A functional behavioristic approach to aversively motivated behavior: Predatory imminence as a determinant of the topography of defensive behavior. In R. C. Bolles & M. D. Beecher (Eds.), *Evolution and learning.* (pp. 185-212). Hillsdale, NJ: Lawrence Erlbaum Associates.

Frazier, E., Fristad, M., & Arnold, L. E. (2009). Multinutrient supplement as treatment: Literature review and case report of a 12-year-old boy with bipolar disorder. *Journal of Child and Adolescent Psychopharmacology, 19*(4), 453-460.

Gately, D., & Kaplan, B. (2009). Database analysis of adults with bipolar disorder consuming a micronutrient formula. *Clinical Medicine Insights: Psychiatry, 4*, 3-16.

Glassey, S., & Wilson, T. (2011). Animal welfare impact following the 4 september 2010 canterbury (darfield) earthquake. *Australasian Journal of Disaster and Trauma Studies, 2011-2*, 49-59.

Hall, C. S. (1934a). Emotional behavior in the rat: I. Defecation and urination as measures of individual differences in emotionality. *Journal of Comparative Psychology, 18*, 382-403.

Halliwell, C. I., Kolb, B. E., & Gibb, R. L. (n.d.). *The effects of a vitamin and mineral supplemented diet on recovery from early cortical injury at postnatal day 10 in prenatally stressed rats.*

Härter, M. C., Conway, K. P., & Merikangas, K. R. (2003). Associations between anxiety disorders and physical illness. *European Archives of Psychiatry and Clinical Neurosciences, 253*, 313-320.

Hascoët, M., & Bourin, M. (2009). *The mouse light-dark box test. Mood and anxiety related phenotypes in mice: Characterization using behavioral tests*. Totowa, NJ, US: Humana Press.

Herbert, C. E., & Hughes, R. (2006). A comparison of 1-benzylpiperazine and methamphetamine in their acute effects on anxiety-related behavior of hooded rats. *Pharmacology, Biochemistry and Behavior, 92*, 243-250.

Hughes, R. (2001). Responsiveness to brightness change in hooded rats: Effects of sex and procedure. *Journal of Behavioural Processes, 55*(143-155).

Hughes, R., Desmond, C. S., & Fisher, L. C. E. (2004). Room novelty, sex, scopolamine and their interactions as determinants of general activity and rearing, and light-dark preferences in rats. *Behavioural Processes, 67*, 173-181.

Hughes, R., Lowther, C., & van Nobelen, M. (2011). Prolonged treatment with vitamins c and e separately and together decreases anxiety-related open field behaviour and acoustic startle in hooded rats. *Pharmacology, Biochemistry and Behavior, 97*, 494-499.

Hughes, R., & Neeson, L. (2003). Prevention of memory loss for a brightness change in adult and middle-aged rats by postacquisition treatment with glucose. *Pharmacology, Biochemistry and Behavior, 76*(1), 119-123.

Kaplan, B., Crawford, S. G., Gardener, B., & Farrelly, G. (2002). Treatment of mood lability and explosive rage with minerals and vitamins: Two case studies in children. *Journal of Child and Adolescent Psychopharmacology, 12*(3), 205-219.

Kaplan, B., Fisher, J., Crawford, S. G., Field, C., & Kolb, B. E. (2004). Improved mood and behaviour during treatment with a mineral-vitamin supplement: An open label case series of children. *Journal of Child and Adolescent Psychopharmacology, 14*(1), 115-122.

Kaplan, B., & Leung, B. (2011). Micronutrient treatment of mental disorders. *Integrative Medicine, 10*(3), 32-39.

Kessler, R. C. (2007). The global burden of anxiety and mood disorders: Putting the european study of the epidemiology of mental disorders (esmed) findings into perspective. *Journal of Clinical Psychiatry, 62*, 10-19.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangs, K. R., & Walters, E. E. (2005a). Lifetime prevalence and age-of-onset distrubutions of dsm-iv disorders in the national comorbidity survey replication. *Archives of General Psychiatry, 62*(6), 593-602.

Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., & Walters, E. E. (2005b). Pervalence, severity and comorbidity of 12-month dsm-iv disorders in the national comorbidity survey replication. *Archives of General Psychiatry, 62*(617-627).

Koen, N., & Stein, D. J. (2011). Pharmacotherapy of anxiety disroders: A critical review. *Dialogues of Clinical Neuroscience, 13*, 423-437.

Kontinen, V. K., Kauppila, T., Paananen, S., Pertovaara, A., & Kalso, E. (1999). Behavioural measures of depression and anxiety in rats with spinal nerve ligation-induced neuropathy. *Pain, 80*(341-346).

Lang, P. J. (1977). Physiological assessment of anxiety and fear. In J. D. Cone & R. P. Hawkins (Eds.), *Behavoural assessment: New directions in clinical psychology* (pp. 178-195): Brummer/Mazel.

McKinney, W. T. J., & Bunney, W. E. J. (1969). Animal model of depression: I. Review of evidence: Implications for research. *Arch Gen Psychiatry, 21*, 240-248.

Mehl-Madrona, L., Leung, B., Kennedy, C., Paul, S., & Kaplan, B. (2010). Micronutrients versus standard medication management in autism: A naturalistic case-control study. *Journal of Child and Adolescent Psychopharmacology, 20*(2), 95-103.

Ohl, F. (2005). Animal models of anxiety. In F. Holsboer & A. Strohle (Eds.), *Anxiety and anxiolytic drugs*: Springer Berlin Heidelberg.

Partyka, A., Jastrzebska-Wiesek, M., Szewczyk, B., Stachowicz, K., Slawinska, A., Poleszak, E., . . . Nowak, G. (2011). Anxiolytic-like activity of zinc in rodent tests. *Pharmacological Reports, 63*, 1050-1055.

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviours: A review. *European Journal of Pharmacology, 463*, 3-33.

Rucklidge, J. (2009). Successful treatment of ocd with a micronutrient formula following partial response to cognitive behavioural therapy (cbt): A case study. *Journal of Anxiety Disorders, 23*, 836-840.

Rucklidge, J., Andridge, R., Gorman, B., Blampied, N., Gordon, H., & Boggis, A. (2012). Shaken but unstirred? Effects of micronutrients on stress and trauma after an earthquake: Rct evidence comparing formulas and doses. *Human Psychopharmacology Clinical Experiement*.

Rucklidge, J., Gately, D., & Kaplan, B. (2010). Database analysis of children and adolescents with bipolar disorder consuming a micronutrient formula. *BMC Psychiatry, 10*(74).

Rucklidge, J., Johnstone, J., Harrison, R., & Boggis, A. (2011a). Micronutrients reduce stress and anxiety in adults with attention-deficit/hyperactivity disorder following an 7.1 earthquake. *Psychiatry Research, 189*, 281-287.

Rucklidge, J., Taylor, M. J., & Whitehead, K. (2011b). Effect of micronutrients on behavior and mood in adults with adhd: Evidence from an 8-week open label trial with natural extention. *Journal of Attention Disorders, 15*(1), 79-91.

Takeda, A., Tamano, H., Kan, F., Itoh, H., & Oku, N. (2007). Anxiety-like behaviour of young rats after 2-week zinc deprivation. *Behavioural Brain Research, 177*, 1-6.

Terada, Y., Okura, Y., Kikusui, T., & Takenaka, A. (2011). Dietary vitamin e deficiency increases anxiety-like behaviour in juvenile and adult rats. *Bioscience, Biotechnology, and Biochemistry, 75*(10), 1894-1899.

Wills, G., Wesley, A., Moore, F., & Sisemore, D. (1983). Social interactions among rodent conspecifics: A review of experimental paradigms. *Neuroscience and Biobehavioral Reviews, 7*(3), 315-323.

Appendix A

AEC Ref: 2010/13R

4 June 2010

Phoebe Thomass
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Phoebe

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Effects of dietary vitamin supplementation on development of emotionality"

Approval has been granted:

(a) for the use of 80 Rattus Norvegicus
(b) for your research project to be undertaken from 4 June 2010 to 30 October 2010. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MAF statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MAF Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, which ever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

Associate Professor Jim Briskie
Chair
Animal Ethics Committee

cc Animal Ethics Committee

Ref: 2010/13R

27 October 2010

Phoebe Thomass
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Phoebe

I am pleased to inform you that the Animal Ethics Committee has approved your amendment to the application entitled: "Effects of dietary vitamin supplementation on development of emotionality".

Approval has been granted:

(a) for an extension of your research project with an expected completion date of 15 December 2010. Please note that if you require a further extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

Yours sincerely

Associate Professor Jim Briskie
Chair
Animal Ethics Committee

Ref: 2010/13R

27 January 2011

Phoebe Thomass
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Phoebe

I am pleased to inform you that the Animal Ethics Committee has approved your amendment to the application entitled: "Effects of dietary vitamin supplementation on development of emotionality".

Approval has been granted for the revised manipulations outlined in your amendment application and for an extension of your research project with a new expected completion date of 31 August 2011. Please note that if you require a further extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

Yours sincerely

Associate Professor Jim Briskie
Chair
Animal Ethics Committee

c.c. AEC Committee