Effects of a Multinutrient Supplement on Anxiety, Aggression, and Impulsivity in a Normal Rat Population

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By

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Abbreviations

ADHD	Attention-Deficit/Hyperactivity Disorder
ANOVA	Analysis of Variance
ASR	Acoustic Startle Reflex
˚C	Degrees Celsius
dB	Decibels
EMP+	EMPowerplus
F	Female
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
R-I	Resident-Intruder
S.E.M	Standard Error of the Mean
SHR	Spontaneously Hyperintensive Rat
UL	Upper Intake Level

Abstract

Using natural supplementation as a treatment tool for mental health problems is becoming increasingly popular. Several studies using a multinutrient supplement called EMPowerplus (EMP+) have been conducted in humans with disorders such as Attention-Deficit/Hyperactivity Disorder (ADHD), but no studies have been conducted in animals or humans without disorders. Therefore, to address the gaps in the literature, the aim of this study was to assess the effects of EMP+ in a rat population on anxiety, aggression, and impulsivity which may be present in people who do not have a disorder. To investigate this, 40 male and 40 female rats were fed a diet consisting of 0%, 1.25%, 2.5% or 5% EMP+. They were tested during adolescence (PND 52-53) and again during mid-adulthood (PND 116-117) for anxiety and aggression, and during early adulthood (PND 92-96) and again during late adulthood (PND 127-130) for impulsivity. Due to the impact of the September 4th 2010 7.1 magnitude earthquake, data for some rats had to be excluded from anxiety and aggression analyses, leaving a sample size of 29 males and 34 females. Although there were no treatment main effects for any of the three behaviours, there were significant interaction effects between treatment and sex for measures of anxiety and aggression, showing that each sex reacted differently to the supplement. Male rats became less anxious, while female rats became more anxious. Of the five aggressive behaviours observed, significant interactions were found between treatment and sex for rough paw and allogrooming frequency. There were no significant effects involving treatment for impulsivity, but male rats were more impulsive when they were older (PND 127-130). Overall, the effects of EMP+ on the rats' anxious, aggressive, and impulsive behaviours were mixed, and it is likely that the 7.1 magnitude earthquake and its subsequent aftershocks played a role in these results.

1.0 Introduction

1.1 General Overview

The use of multinutrient supplementation as a treatment tool for disorders such as ADHD, Obsessive-Compulsive Disorder (OCD), and bipolar disorder is becoming increasingly popular. One particular 36-ingredient micronutrient formula known as EMP+ is showing great potential for treating the symptoms of these disorders. While the findings of studies using EMP+ are promising, these particular studies focus solely on people with serious disorders. It is just as important to look at the effects of this supplement in people who do not have a serious disorder. Those without disorders such as ADHD can still present with general mental health issues of lesser severity such as anxiety, aggression, and impulsivity, so this population may also benefit from the effects of the supplement. Additionally, it is necessary to establish what effect EMP+ would have on a person without a disorder because they are quite different to a person with a disorder. A supplement that is beneficial for one population, like those with a serious disorder, may not be beneficial for another, so it is necessary to establish whether any harmful effects may result if someone without a disorder were to take the supplement. It is also possible that no effects may result from taking the supplement in a person without a disorder, so it would be wasteful for them to use the supplement. Furthermore, the supplier of EMP+ makes a dose suggestion for targeting "general health" issues, but it is unclear what the benefits would be for a person without a serious disorder.

As well as the literature being limited to people with a serious disorder, there is no published research concerning effects of EMP+ in a rat population. Rat studies are important for establishing an initial framework of understanding and are useful due to the amount of control that can be exerted over the subjects and conditions of the experiments. Specifically, rats are not likely to cause the problems that humans do such as abusing drugs, missing

appointments, and not taking the supplement. Therefore, to help address the gaps in the literature outlined above, the primary aim of this study was to investigate the effects of EMP+ on anxiety, aggression, and impulsivity in a population of rats that were not animal models of any serious human disorders (from now on referred to as a normal rat population). These three behaviours were chosen because they are commonly experienced in people who do not have a serious disorder. It is hoped that the beneficial effects of EMP+ are not limited to people who have a serious disorder and that this method of treatment can be beneficial for more general health issues. While the current study involved rats, it was felt that the results might have some implications for humans and might thus warrant further research with a human population.

1.2 EMP+

Initial studies in the late 1970s to early 1980s looked at the effects of megavitamins in children. While some found reductions in problem behaviours such as hyperactivity, most were inconclusive and showed that mega-doses of vitamins might be toxic (Arnold et al., 1978; Haslam et al., 1984; Kershner and Hawke, 1979). Since then, a better knowledge and understanding of the processes involved with natural supplementation has resulted in a promising multi-ingredient formula called EMP+. This supplement contains 14 vitamins, 16 minerals, three amino acids, and three antioxidants (serving sizes of the main ingredients can be found in Table 1).

Table 1.
Composition of EMP+ showing amount of each ingredient per serving (4 capsules), for a general health dose (8 capsules), for a therapeutic dose (15 capsules), for double the highest recommended dose (30 capsules), and the upper intake levels (UL) for each ingredient.

	Amount Per Serving* (4 capsules)	General Health Dose (8 capsules) 1.25% EMP+	Therapeutic Dose (15 capsules) 2.5% EMP+	Double Highest Dose (30 capsules) 5% EMP+	UL**
Vitamin A	1,536 IU	3,072 IU	5,760 IU	11,520 IU†	10,000 IU
Vitamin C	160 mg	320 mg	600 mg	1,200 mg	2,000 mg
Vitamin D	384 IU	768 IU	1,440 IU	2,880 IU†	2,000 IU
Vitamin E	96 IU	192 IU	360 IU	720 IU	1,500 IU
Vitamin B1	4.8 mg	9.6 mg	18 mg	36 mg	none set
Vitamin B2	3.6 mg	7.2 mg	13.5 mg	27 mg	none set
Vitamin B3	24 mg	48 mg†	90 mg†	180 mg†	35 mg
Vitamin B5	5.8 mg	11.6 mg	21.8 mg	43.6 mg	none set
Vitamin B6	9.6 mg	19.2 mg	36 mg	72 mg	100 mg
Vitamin B9	384 mcg	768 mcg	1,440 mcg†	2,880 mcg†	1,000 mcg
Vitamin B12	240 mcg	480 mcg	900 mcg	1,800 mcg	none set
Vitamin H	288 mcg	576 mcg	1,080 mcg	2,160 mcg	none set
Calcium	352 mg	704 mg	1,320 mg	2,640 mg†	2,500 mg
Phosphorous	224 mg	448 mg	840 mg	1680 mg	4,000 mg
Magnesium	160 mg	320 mg	600 mg†	1,200 mg†	350 mg
Potassium	64 mg	128 mg	240 mg	480 mg	none set
Iodine	54.4 mcg	108.8 mcg	204 mcg	408 mcg	1,100 mcg
Zinc	12.8 mg	25.6 mg	48 mg†	96 mg†	40 mg
Selenium	54.4 mcg	108.8 mcg	204 mcg	408 mcg†	400 mcg
Copper	1.9 mg	3.8 mg	7.1 mg	14.2 mg†	10 mg
Manganese	2.6 mg	5.2 mg	9.8 mg	19.6 mg†	11 mg
Chromium	166.4 mcg	332.8 mcg	624 mcg	1,248 mcg	none set
Molybdenum	38.4 mcg	76.8 mcg	144 mcg	288 mcg	2,000 mcg
Iron	3.7 mg	7.4 mg	13.9 mg	27.8 mg	45 mg
Proprietary Blend	444.1 mg	882.2 mg	1,654.1 mg	3,308.2 mg	none set

Proprietary blend consists of dl-phenylalanine, glutamine, citrus bioflavanoids, grape seed extract, chlorine bitartrate, inositol, ginkgo biloba, methionine, germanium sesquioxide, boron, vanadium, nickel.
* Supplement composition information is available from the developer's website (http://www.Truehope.com)
** ULs found in Simpson et al., 2011.
† UL is exceeded for this dose.

For general mental health issues such as anxiety, two servings of four capsules each per day are recommended, which is equivalent to a diet that contains 1.25% EMP+. This dosage applies to people without a more serious disorder who have general problems such as anxiety, aggression, and impulsivity. This was the target population of this study so was

included as a treatment condition. This dosage is increased to approximately 15 capsules per day for a therapeutic dose aimed at targeting mental health disorders such as ADHD and bipolar disorder. This is equivalent to a diet consisting of 2.5% EMP+ and was also included in this study to see if it is safe for a normal healthy population to take a dose aimed at those with a mental disorder. Additionally, a dose of 5% EMP+ was included to evaluate the effects of taking double the highest recommended dose, and a control group was also included to allow comparison between all three treatment groups and rats not on the supplement.

For four of the ingredients, vitamin B3, vitamin B9, magnesium, and zinc, the full daily dose exceeds the upper intake level (UL) when a therapeutic dose is taken. However, there have been no problems reported so far resulting from taking this supplement, and there are accordingly no safety concerns (Simpson et al., 2011). Skin flushing is only a concern when vitamin B3 is taken in excess, and the UL was set at 35mg to try and prevent this. Taking vitamin B9 in excess of 1000mcg could mask a vitamin B12 deficiency, but this is of no concern because of the B12 content in the supplement. Diarrhoea is of concern if the UL of magnesium is exceeded, but there have been no reports of this so far. Finally, the zinc UL was set to avoid an imbalance of copper, but this is unlikely because of the copper content of the supplement (Simpson et al., 2011). More importantly, only one of the UL's is exceeded when a dose for general health purposes is taken, which is vitamin B3, and not by much. Therefore, if the results of the current study on rats prove promising, there should be minimal concerns over using EMP+ to treat general health problems such as anxiety, aggression, and impulsivity in people without a disorder.

It is not surprising that several more UL's are exceeded when double the recommended therapeutic dose is taken. Table 2 describes some of the symptoms associated with toxicity of these, including symptoms associated with the four ingredients already covered when a therapeutic dose is taken.

Table 2.
Toxicity symptoms of EMP+ ingredients that are exceeded when a general health dose is taken (8 capsules), when a therapeutic dose is taken (15 capsules), and when double the highest recommended dose is taken (30 capsules).

Nutrient	Associated Toxicity Symptoms
Vitamin A*	Headache, diplopia, dermatitis, anemia, insomnia, bone abnormalities, liver damage, hypercalcemia, and menstrual irregularities.
Vitamin D*	Nausea, vomiting, excessive thirst and urination, muscular weakness, joint pain, and calcification of the heart, lungs, and kidneys.
Vitamin B3#†*	Nausea, heartburn, fatigue, dry hair, sore throat, inability to focus eyes, vascular dilation, and gastrointestinal irritation.
Vitamin B9†*	Can obscure the diagnosis of pernicious anemia, may reduce zinc absorption, and precipitates in kidneys of laboratory rats.
Calcium*	Nausea, constipation, hypertension, hypercalcemia, kidney stones, myopathy, and may inhibit absorption of iron and zinc.
Magnesium†*	Nausea, diarrhoea, hypotension, bradycardia, vasodilation, EKG changes, coma, and cardiac arrest.
Zinc†*	Gastrointestinal irritation, vomiting, impairment of copper status, microcytic anemia, and impairment of immune responses.
Selenium*	Fingernail changes, hair loss, nausea, abdominal pain, diarrhoea, fatigue, irritability, and peripheral neuropathy.
Copper*	Nausea, gastric pain, diarrhoea, vascular collapse, and interacts with zinc, cadmium, and molybdenum in the body.
Manganese*	Can cause severe psychiatric disorder, reproductive and immune system dysfunction, and kidney and liver disorders.

Note: Adapted from Driskell, 1992.
exceeded when a general health dose is taken
† exceeded when a therapeutic dose is taken
* exceeded when double the highest recommended dose is taken

Table 2 illustrates the undesirability of taking too much of any of these nutrients, which has implications for EMP+. Health warnings should highlight these possible risks to ensure that no-one takes too much of the supplement.

1.3 Anxiety, Aggression, and Impulsivity

Anxiety is one of the most prevalent health mental disorders, with children and adolescents having rates as high as 7-25% (Jimerson, 2003). It is likely that the rate of anxiety symptoms in a population of children and adolescents that are not diagnosed with an anxiety disorder is much higher. In well functioning older adults, anxiety symptoms occurred in 15% of those without depression, with women more likely to have symptoms than men (Mehta et al., 2003). Anxiety in children is characterised by excessive worry and concern,

exaggerated fear, stranger and separation anxiety, and inhibition, which are often associated with physical complaints such as headaches and nausea (Minnesota Association for Children's Mental Health, 2011). Anxiety symptoms in adults are similar to those of children, with the excessive worry and concern focused more on their daily activities as opposed to fear of separation. Normal healthy individuals will experience these symptoms of anxiety from time to time, but those who worry so excessively that it interferes with their lifestyle may qualify for a diagnosis (Ciechanowski and Katon, 2011). In rats, the anxiety response is characterised by freezing, particularly when they are in an open space, with faecal boli more common when a rat is more anxious (Ennaceur et al., 2006). A more anxious rat will also startle more, as evidenced by higher startle amplitudes in response to a sudden noise (Koch, 1998).

Aggression is a common and disruptive behavioural problem that can be described in a number of ways depending on the target, type, and cause of aggression, and is commonly classified as being either premeditated or impulsive (Siever, 2008). In psychiatric inpatients, aggression was recorded in 13.7% of patients, with the most common forms of aggression being verbal threats, loud and demanding manner, and battery of a person with physical force (Barlow et al., 2000). In children, aggressive behaviour is a common reason for clinical referral, with aggression being more maladaptive in children with a psychiatric disorder than those who are not referred (Bambauer, 2005). Aggressive behaviours in rats are usually referred to as defensive responses, and include behaviours such as bite attacks, rough grooming, chasing, and mutual upright boxing (Johns and Noonan, 1995; Miczek and de Boer, 2004).

Impulsivity is a behaviour commonly associated with ADHD, and is characterised by a tendency to act without thinking or engage in risk taking behaviours without considering consequences (Virtual Medical Centre, 2011). The worldwide prevalence of ADHD is

estimated to be 5.29% (Polanczyk et al., 2007), with the adult prevalence rate estimated to be 4.4% (Kessler et al., 2006). Impulsive behaviour is not limited to ADHD, and is instead referred to as trait impulsivity which can be measured using the Barratt Impulsiveness Scale (Hollander et al., 2005; Steinberg et al., 2008). In rats, impulsivity is commonly assessed by measuring their tolerance to the delay of a reward, or in other words their decisions regarding the costs and benefits of delay and effort to gain a reward (Thiébot et al., 1985; Evenden and Ryan, 1996; Denk et al., 2005). Impulsivity is displayed when the rat chooses to gain a small immediate reward instead of a larger delayed reward.

1.4 Rat Studies

To date there has been no research into the effects of EMP+ on any types of behaviour in rats. Therefore, it is difficult to make predictions about how the current rat population might behave when on the supplement. However, it is possible to look at how particular ingredients in EMP+ could affect the rats' anxiety, aggression, and impulsivity levels, as outlined by previous research. This is done in the following three sections.

1.4.1 Anxiety

Studies have looked at the effects of vitamins A, C, D, and E on anxiety in rats. Rats tested in an open field and elevated plus maze, both of which are commonly used to examine anxiety levels in rats, showed no alterations in anxiety levels after chronic administration of 13-*cis*-retinoic acid (a derivative of vitamin A) (Dopheide and Morgan, 2008). It has been shown that pharmacological doses of vitamin A can induce anxiety-like behaviour in rats (de Oliveira et al., 2010). Rats exposed to a chronic dose of vitamin A exhibited increased anxiety as evidenced by a higher number of refusals for entering the light compartment in the

light-dark task (de Oliveira et al., 2007). However, the doses of vitamin A in these studies were large, and the increases in anxiety were likely a result of toxic effects.

Rats have shown decreased anxiety levels as measured by the open-field task and acoustic startle when consuming vitamins C and E either separately or combined together in their drinking water (Hughes et al., 2011). Conversely, vitamin E has been shown to increase anxiety in Wistar rats while not affecting anxiety levels in OXYS rats (Kolosova et al., 2006). A further study has shown that vitamin D deficiency doesn't affect anxiety in mice (Harms et al., 2008).

The effects of a few minerals on rats' anxiety have also been assessed. Anxiety was unchanged in a study that maintained rats on water containing 1% calcium gluconate for three days (Godinho et al., 2002). Increased anxiety was observed in the elevated plus maze task in rats after exposure to manganese, as evidenced by a reduction in the percentage of time spent in the open arms of the maze (Bouilleret et al., 2011). Lastly, it has been shown that zinc deficiency significantly increases anxiety-like behaviours, which was also shown by a reduction in time spent in the open arms of the elevated plus maze (Cope et al., 2011).

It is clear that the vitamins and minerals in EMP+ have different effects on anxiety, with some increasing and some decreasing anxiety levels. This highlights an important issue. When testing the rats for anxiety using EMP+, it will be difficult to know which ingredients are responsible for any behavioural changes. If decreases in anxiety are found we can say that EMP+ was beneficial, but only a few of the ingredients may have been responsible for the changes.

1.4.2 Aggression

Other studies have looked at the effects of vitamin A, potassium, manganese, copper and zinc on aggression in rats. Chronic administration of 13-*cis*-retinoic acid reduced aggression related behaviours such as bite attacks in adults when tested in the resident-

intruder paradigm (Trent et al., 2009). Aggression levels of those in the treatment group significantly decreased at seven and 14 days after treatment, and these aggression levels were significantly lower than the controls. These were reversed to pre-treatment levels following one week cessation of drug treatment. Studies suggest that prenatal zinc deficiency can be a cause of aggression in the adult rat (Halas et al., 1975; Peters, 1978). It is possible that a diet supplemented with zinc could decrease levels of aggression, although there is no direct evidence for this. Following long-term ingestion of manganese or copper salt, male rats exhibited lowered levels of aggression as evidenced by significant decreases in aggressive behaviours such as boxing and fighting (Bataineh et al., 1998). These studies all demonstrate the beneficial effects of vitamins and minerals for aggression.

Interestingly, potassium given to rats on a chronic large dose schedule has been shown to facilitate shock-induced aggressive behaviours, increasing them by 11.4% ($p<0.05$) (Eichelman et al., 1973). This may have been due to toxicity as a result of the chronic dose, so it would be necessary to review the effects of lower potassium doses on rat behaviour to see if they reduce aggression. While this result is not in support of using EMP+ to treat aggression, it is necessary to consider the larger number of benefits of its other ingredients in treating aggression. As with testing for anxiety, any changes associated with EMP+ can only be attributed to the supplement as a whole, and not to any of the individual ingredients.

1.4.3 Impulsivity

There appears to be no published available research on the effects of any of the ingredients in EMP+ on impulsivity in rats. Research is limited to the effects of drugs such as amphetamines, heroin, cocaine, and methylphenidate (a psychostimulant drug approved for the treatment of ADHD).

1.5 Human Studies

Even though the primary focus of this study is rat behaviour, this section will outline the research conducted on humans using EMP+ to show how effective the supplement has been so far. The literature describing the effects of EMP+ in humans is limited to populations with disorders such as ADHD, so there is no literature on the effects of EMP+ in people without a serious disorder. However, these studies still look at symptoms such as anxiety, aggression, and impulsivity in conjunction with the symptoms associated with the disorders. Therefore, since the focus of this study was on anxiety, aggression, and impulsivity, and not on disorders themselves, the following sections will draw from the parts of the literature that evaluate these particular symptoms. Even though the anxiety, aggression, and impulsivity experienced by those with a disorder may be different from that experienced by those without a disorder, effects found on these behaviours in the population with a disorder are indications of how the supplement will affect these behaviours in a population who do not have serious disorders.

In the early 2000s, the use of EMP+ for treating mental health problems focussed mainly on bipolar disorder and mood. More recently, this has been expanded to look at other disorders such as OCD, autism (Mehl-Madrona et al., 2010), and ADHD. An open-label case series on nine children with a range of mood and behaviour problems, including ADHD, produced significant improvements with all effect sizes being larger than 0.8 (Kaplan et al., 2004). At study entry, at least six of the children scored in the clinical range for anxiety and aggression. After approximately 12 weeks on EMP+, there were statistically significant decreases in anxiety ($p<0.05$) and aggression ($p<0.01$). Participant compliance was high, and side effects were minimal. Most importantly, the three children who were on supplement during the trial were able to decrease their intake and still benefit from the effects of the supplement, thereby reducing any negative side effects as well as any interaction with the

supplement. Similar effects have been found in adults (Rucklidge et al., 2011). Of 14 participants, all with a diagnosis of ADHD and three with generalised anxiety disorder, a therapeutic dose of EMP+ significantly decreased the measure of impulsivity in 10 of them (p<0.001), lowering their scores to a normal nonclinical range. An effect size of >0.8 was found for the drop in self-reported anger and an improvement in the ability to regulate anger. Additionally, anxiety as measured by the Depression and Anxiety Stress Scales decreased significantly (p<0.01). Like the study with children, compliance was high (97.2%) and side effects were minimal. There were some reports of nausea and headaches, but these did not last for longer than the first few weeks. A case study of an 18-year old male with OCD showed significant reduction in his clinical levels of anxiety after using EMP+ (Rucklidge, 2009a). After discontinuation of the supplement, his anxiety worsened, with the reintroduction of the supplement improving his symptoms again. Another case study of a 21 year old female with a range of problems including ADHD showed significant improvements in anxiety and impulsivity after eight weeks of being on EMP+ (Rucklidge and Harrison, 2010). As well as exhibiting on and off control of her symptoms with the introduction and removal of the supplement, she showed remission from all mental illness after a year of being on the supplement. No side effects were reported as a result of the treatment.

While the above studies tested the effects of EMP+ on people with mental disorders, they still looked at the effects of the supplement on participants' anxiety, aggression, and impulsivity ratings. These studies demonstrate the effectiveness of using EMP+ to treat the symptoms of anxiety, aggression, and impulsivity, showing that it is a promising method of treatment for a population without a disorder who are experiencing symptoms of anxiety, aggression, and impulsivity. It was expected that the rat population in the present study might be similarly affected.

1.5.1 Related Literature

Several studies illustrating the benefits of supplementation on the aggressive behaviours of delinquent juveniles and criminals demonstrate the advantages of micronutrient formulas for ameliorating problem behaviour. A well designed double-blind, placebo-controlled, randomised experiment of 231 young adult prisoners found that those who were administered nutritional supplements committed an offence on average 26.3% times less (p<0.03) than controls (Gesch et al., 2002). This was equivalent to a 35.1% decrease (p<0.001) in offences from baseline in those who received supplementation. Consumption of a vitamin-mineral supplement by 32 adolescent delinquents resulted in 28% fewer rule infractions of a violent nature than for a placebo group (Schoenthaler et al., 1997). The effects of vitamin-mineral supplementation on delinquency have also been investigated in children. Schoenthaler and Bier (2000) found a 47% reduction in the need for discipline in the 40 children aged six to 12 years who took a daily vitamin-mineral supplement for four months compared to the 40 children who took placebos. Behaviours that decreased the most were vandalism, refusal to do work, uttering obscenities, and disorderly conduct. In all cases, the supplements used contained a wide range of vitamins and minerals similar to those in EMP+. The results of these studies outline the effectiveness of combinations of vitamins and minerals for reducing aggression-related behaviours.

Of particular interest are studies involving over the counter supplements. Berocca is a dissolvable tablet containing B vitamins and vitamin C, and has been shown to reduce anxiety. A double-blind randomised-control trial of 80 healthy male volunteers found statistically significant reductions in anxiety levels of those taking Berocca once daily for 28 days compared to controls (Carroll et al., 2000). A similar study which included a sample of 204 women and 96 men found significant reductions in stress, which is closely related to anxiety, after taking Berocca for 30 days (Schelbush et al., 2000). The males and females

were equally distributed across the control group and treatment group, suggesting that both males and females benefited from taking Berocca. Conversely, a review of herbal and dietary supplements claims that there is no clinical trial evidence on the effectiveness of Berocca for anxiety disorders (Saeed et al., 2007). Additionally, administration of a high dose vitamin B-complex formula found no significant reductions in anxiety in a three month, double-blind, randomised, placebo-controlled trial of 60 participants (Stough et al., 2011). It is likely that the effect of a single vitamin is not enough to reduce anxiety by itself, and that a combination of vitamins and minerals is necessary for targeting underlying deficiencies in order to reduce anxiety.

1.6 Mechanisms of Action

The term mechanism of action refers to the specific action through which a substance produces its effect on an organism. It is beyond the scope of this article to discuss the mechanisms of action of all ingredients in EMP+ in depth. Instead, a brief summary of some of the vitamins and minerals in EMP+ that have been more extensively researched will be covered to illustrate how they might help reduce anxiety, aggression, and impulsivity.

Table 3.
Brain functions of selected micronutrients in EMP+.

	Important functions of selected EMP+ ingredients
Vitamins A, C, and E	Inhibit the destructive effects of oxidation[a]
Vitamin B1	Involved in the presynaptic release of acetylcholine[b]
Vitamin B6	Neurotransmitter synthesis and normal brain development[c]
Vitamins B9 and B12	Involved in processes important for central nervous system function[d]
Magnesium	Neurotransmitter synthesis,[c] post-synaptic receptor function,[e] active transport of ions across cell membranes and for cell signalling[f]
Zinc	Normal growth, immune function, neurological development,[c] synaptic transmission and DNA synthesis[e]
Selenium	Protects cells from the effects of free radicals[f]
Chromium	Affects norepinephrine release and tryptophan avaliability[g]
Iron	Normal brain growth, neurotransmitter synthesis and catabolism and many cellular metabolic processes[c]

[a] Brigelius-Flohé and Traber, 1999
[b] Meador et al., 1993
[c] Rucklidge et al., 2009
[d] Bjelland et al., 2003
[e] Kaplan and Shannon, 2007
[f] Kaplan et al., 2007
[g] McLeod and Golden, 2000

It is clear from Table 3 that the vitamins and minerals present in EMP+ are necessary for some very important brain processes, and are therefore vital for normal and healthy development. Deficits in these essential micronutrients would result in sub-optimal functioning, therefore increasing the likelihood of a disorder. In fact, the approach of using natural supplementation for the treatment of disorders such as ADHD is often based on the assumption that the disorder is the result of an underlying nutritional deficiency (Rucklidge, 2009).

Particular attention has been paid to the B vitamins and their role in altering behaviours associated with ADHD, which therefore has implications for impulsivity. It is proposed that the molecular structures of the B vitamins, particularly vitamin B1, are similar to those of dopamine (Shaw et al., 2011). They may occupy the dopamine transporter (DAT) binding site, resulting in an increase in synaptic dopamine level, consequently activating the postsynaptic D_2 receptor. This receptor has been implicated in the amelioration of symptoms

associated with ADHD, such as impulsivity. Methylphenidate (Ritalin) is commonly prescribed for ADHD and works by this same mechanism, therefore possibly sharing a common neurochemical mechanism of action with the B vitamins.

Apart from the B vitamins, proposed mechanisms of action for the ingredients in EMP+ and their specific link to effects on anxiety, aggression, or impulsivity are hard to come by. We know what the effects of the vitamins and minerals are on the brain, and we know how some of them can affect behaviour, but we cannot assume a causal link. For example, it is known that magnesium deficiency is associated with anxiety (Watts, 1988). It is also known that magnesium is important for processes such as neurotransmitter synthesis and cell signalling (see Table 3). However, it does not necessarily follow that impairment of neurotransmitter synthesis and cell signalling processes cause anxiety. This idea is represented generally in Figure 1. To further complicate the issue is the fact that EMP+ is a multi-ingredient formula so the mechanism of action of the supplement as a whole may not equal the sum of the mechanisms of action of each of its individual ingredients.

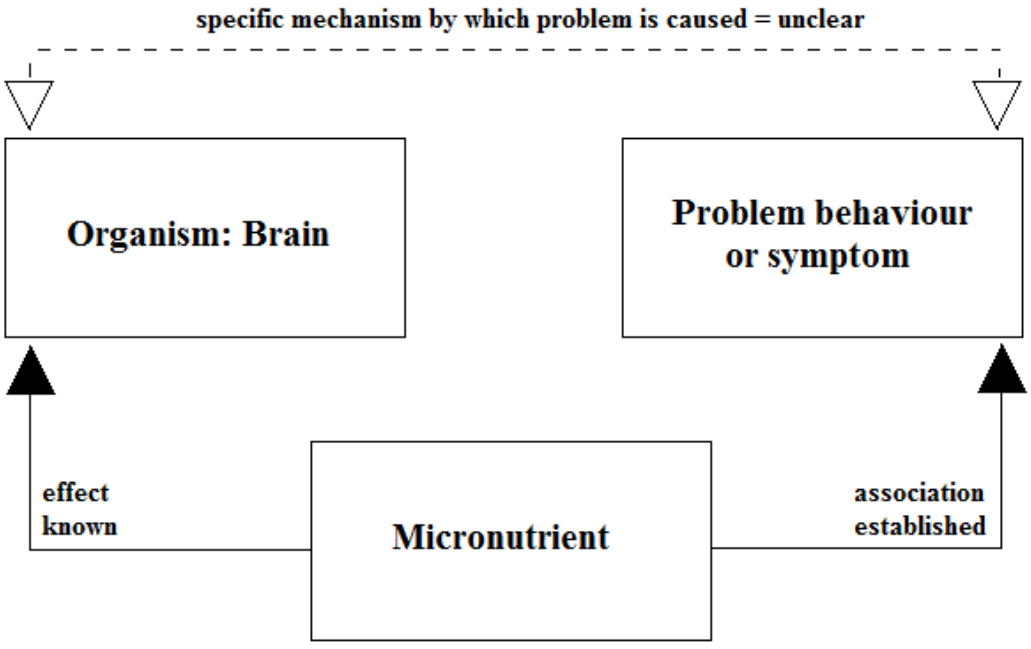

Figure 1. Diagram summarising the current state of knowledge of the mechanisms of action of EMP+.

Kaplan and colleagues (2007) have proposed four explanatory models in an attempt to understand the mechanisms by which the micronutrients of EMP+ influence mood and these may be helpful for explaining the same processes related to anxiety, aggression, and impulsivity. In brief, these hypotheses are that mental problems are a result of inborn errors of metabolism, manifestations of deficient methylation processes, the result of the alteration of gene expression by nutrient deficiency, or that disorders are long-latency deficiency diseases. While these are not specific mechanisms of action like that proposed by Shaw and colleagues (2011) for the B vitamins, they allow for an understanding of how EMP+ may benefit anxiety, aggression, and impulsivity symptoms when knowledge on the specific mechanisms of action is lacking.

1.7 The Present Study

The present study investigated the effects of EMP+ on levels of anxiety, aggression, and impulsivity in a normal rat population. Male and female rats were fed a diet containing 0%, 1.25%, 2.5%, or 5% EMP+ which was first administered on post natal day (PND) 30 (childhood) and they had continuous access to this diet during testing. They were tested for anxiety and aggression during adolescence (PND 52-53) and again during mid adulthood (PND 116-117), and were tested for impulsivity during early adulthood (PND 92-96) and again during late adulthood (PND 127-130) to see if being on the supplement further affected behaviour over time, and to assess the effects of the supplement at different developmental ages. A summary of these ages and their relevant developmental stages can be found in Table 4 for easy future reference. Male and female rats were used to investigate possible sex differences. While findings from animal studies should be applied to humans with caution, the results can provide a valid reason for conducting research with a population of humans who do not have a disorder.

Table 4.
Summary of developmental stage of rats during each test for both testing phases, and the corresponding age in days.

	Testing Phase One		Testing Phase Two	
	Developmental Stage	PND	Developmental Stage	PND
Anxiety and Aggression	Adolescence	52-53	Mid Adulthood	116-117
Impulsivity	Early Adulthood	92-96	Late Adulthood	127-130

2.0 Aims and Hypotheses

To date the literature on the effects of EMP+ on behaviours such as anxiety, aggression, and impulsivity in a normal rat population is severely lacking. It is important that such research is carried out as testing of this supplement on humans is, while relatively new, well underway. The current research aimed to investigate the effects of EMP+ on anxiety, aggression, and impulsivity in a normal rat population, and to throw some light on where future investigations should be directed.

Firstly, it was expected that compared to controls, rats on the supplement would exhibit lower anxiety, aggression, and impulsivity levels. Presumably, this effect would be dose dependent i.e. those on a higher dose of EMP+ would exhibit lower levels of the behaviours than rats on a lower dose, with the exception of those on the 5% diet that might exhibit increased levels of anxiety, aggression, and impulsivity due to toxic levels of the supplement. Secondly, it was expected that any effects of the supplement would become more pronounced over time. Specifically, if the supplement resulted in reduced anxiety, aggression, and impulsivity levels at one phase of testing, it should continue to do so over time, producing even lower levels of anxiety, aggression, and impulsivity at a second phase of testing. Little is known about any differences in effects of EMP+ on each sex in a rat population. Therefore, while it was expected that some sex differences would be apparent, the direction and extent of these for each of the behaviours measured could not be predicted.

3.0 Method

3.1 Subjects

Eighty male and 80 female hooded rats of the PVG/C strain bred in the Animal Facility, Department of Psychology at the University of Canterbury, were used as subjects for the present study. On PND 30, the rats were weaned and housed in plastic cages with inner dimensions of 440×270×210mm. They were housed in small groups of two to four rats of the same sex for the duration of the experiments, unless specified otherwise. They were maintained on a 12-hour light/dark cycle (lights on at 0800 hours) and behavioural testing was conducted during the light portion of the cycle. All animals were kept in a holding room with a regulated temperature of 21°C (± 2°C) and humidity of 45-50%. They had free access to food and water in their holding room throughout the duration of experimentation, unless specified otherwise. Procedures, approved by the University of Canterbury Animal Ethics Committee, minimized the number of animals used in the experiments and their level of suffering (see Appendix A).

Forty of the male and 40 of the female rats were used as intruders for the resident-intruder paradigm, so were not fed an experimental diet. They always had free access to rat pellets and water. Of the rest of the rats, 10 of each sex were randomly allocated on PND 30 to a diet of 0%, 1.25%, 2.5% or 5% EMP+ resulting in 20 rats in each experimental condition. The age when they began the diet corresponds to the childhood stage of development in humans (for a relative comparison of human and rat ages and stages of development, see Figure 2). For phase one of experimentation, the rats were behaviourally tested on measures of anxiety and aggression during adolescence (PND 52-53), and for impulsivity during early adulthood (PND 92-96). For phase two of experimentation, the rats were tested on measures of anxiety and aggression during mid adulthood (PND 116-117), and for impulsivity during late adulthood (PND 127-130).

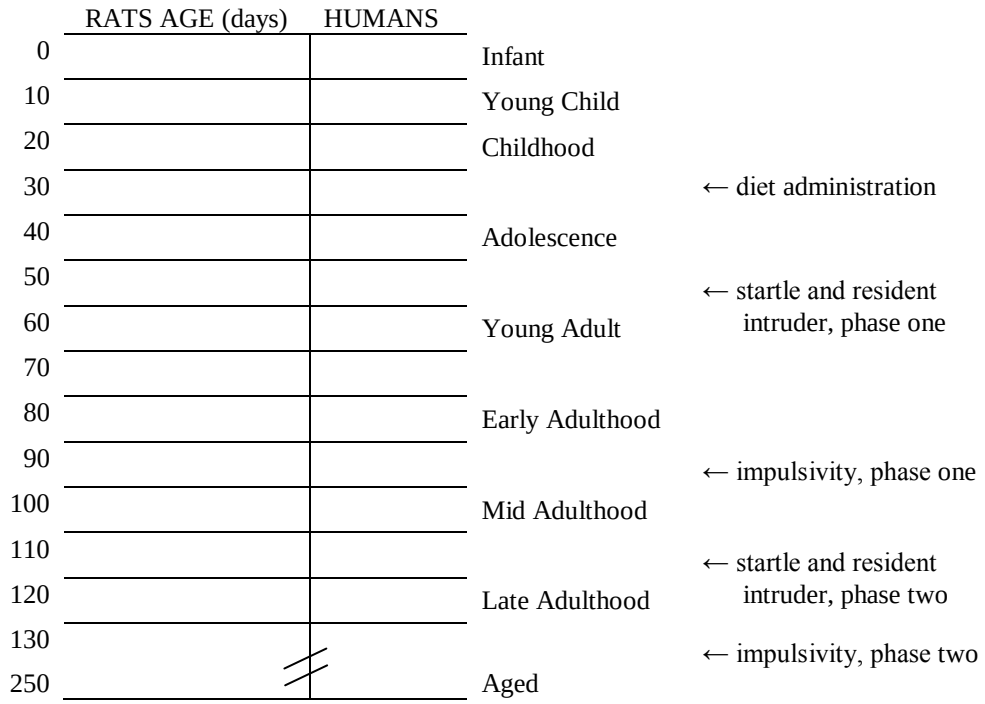

Figure 2. A relative comparison of ages and stages of human versus rat development, showing at which stage each procedure occurred (Spear, 2000).

The rats were supplied in four different cohorts every two weeks, and the number of rats in each cohort varied depending on how many rats were available. Therefore, the number of rats in each cohort was not exactly the same. Consequently, even though best efforts were made to produce an equal representation of both sexes and all four diet groups in each cohort, this was not possible. Table 5 shows how many rats were received in each cohort and what diet group they were allocated to.

Table 5.
Description of each cohort showing how many of each sex were allocated to which diet group.

	Sex	Diet	N
Cohort 1	F	0%	4
(N=24)	F	1.25%	4
	F	2.5%	4
	M	1.25%	4
	M	2.5%	4
	M	5%	4
Cohort 2	F	0%	4
(N=20)	F	2.5%	4
	F	5%	4
	M	0%	4
	M	1.25%	4
Cohort 3	F	0%	2
(N=19)	F	1.25%	3
	F	2.5%	2
	F	5%	3
	M	0%	3
	M	2.5%	3
	M	5%	3
Cohort 4	F	1.25%	3
(N=17)	F	5%	3
	M	0%	3
	M	1.25%	2
	M	2.5%	3
	M	5%	3
		Total N =	80

Note: There was an equal number of male and female rats received in each cohort. Half were used as intruders for the R-I paradigm and are not represented in this table because they were not allocated to an experimental diet group. Therefore, the total number of rats received in cohort 1 was 48, in cohort 2 was 40, in cohort 3 was 38, and in cohort 4 was 34, giving a total of 160 rats. The above resulted in 10 males and 10 females being in each of the four diet concentration groups.

3.2 Diet and Rationale for Doses

The EMP+ supplement was donated by the manufacturer, TrueHope (Canada). Rat pellets were crushed and the appropriate amount of EMP+ powder was weighed and mixed with this to attain the correct diet percentages. Approximately 60ml of distilled water per 100g of diet was added to produce the desired consistency, and placed in a small bowl. Each cage of rats was given 100-300g of food each time they were fed which was approximately three times a week. Because rats were housed in groups of two to four per cage, it was

difficult to tell exactly how much each rat ate. Additionally, rats often scattered the food around their cage, making it even more difficult to measure how much was consumed. Therefore, even though the rats were eating food that contained the appropriate percentage of EMP+, it was difficult to measure whether the rats were consuming the equivalent to the number of pills that a human would take for either a general health dose (eight capsules), a therapeutic dose (15 capsules), or double the highest recommended dose (30 capsules). Additionally, all rats were weighed frequently and in all instances every rat was putting on an appropriate amount of weight, suggesting they were eating a suitable and healthy amount of food. Table 6 shows the composition of the rat pellets that were crushed and used for mixing with the EMP+.

Table 6.
Composition of rat pellets showing the ingredients per tonne and the percentage contribution of each ingredient.

Rat Pellet Ingredients	Units Per Tonne	Contribution (%)
Barley bulk	100	10
Soybean meal 48%	300	30
Wheat bulk	450	45
Salt, fine	4	0.4
Methionine	1.25	0.125
Vitamin E	0.1	0.01
Lime	12	1.2
Bentonite	50	5
Dicalcium phosphate	10	1
Seltec	0.25	0.025
Soya oil	40	4
Molasses	30	3
PMX RAT 27	1	0.1

The PMX RAT 27 formula is a mix of vitamins and minerals and its details are commercially sensitive and not available for the purposes of this research. The rat pellets consisted mainly of wheat, soybean meal, and barley. The contribution of vitamins and minerals to the percentage composition of the rat pellets is particularly low, so we can be

confident that the rats were getting the concentration of diet that they were allocated, whether it be 0%, 1.25%, 2.5%, or 5%, and were not receiving extra nutritional supplementation from the rat pellets. Therefore, using the rat pellets as a base mix for the EMP+ powder was not likely to enhance any toxic effects of the supplement.

A diet consisting of 1.25% EMP+ is the recommended daily dose for general health. Since the focus of the study is on reducing general health problems in a normal population, this dose was included as an experimental condition. The recommended daily therapeutic dose for humans with a disorder such as ADHD is twice this, which equates to a diet consisting of 2.5% EMP+. This diet strength was also included to see if it is appropriate to give a dose of EMP+ that is aimed at a population that has a disorder, to a population that does not have a disorder. Additionally, double the highest recommended dose, i.e. 5% EMP+, was included to see if higher levels were potentially detrimental due to toxicity therefore worsening the rats' behaviour. Lastly, a diet of no EMP+ was included to allow for comparisons between treatment groups and controls.

3.3 Apparatus and Behavioural Measures

In this study, three tests were used to investigate anxiety, aggression, and impulsivity in a normal rat population. Anxiety was measured using the startle response, aggressive behaviour was measured by the resident-intruder test, and impulsivity was measured using a T-Maze. In the startle test, a more anxious animal will produce a higher startle response than a calmer or less anxious rat (Koch, 1998). During the resident-intruder test, a more aggressive rat will exhibit more aggressive behaviours towards an intruder rat than a less aggressive rat (Miczek and de Boer, 2004). Finally, during the T-Maze paradigm, a less impulsive rat chooses a larger delayed reward over a smaller immediate reward than a rat that is more

impulsive (Thiébot et al., 1985). The experimental room was controlled at 21°C (± 2°C) with a humidity of 30% (± 10%).

On September the 4th, 2010, a 7.1 magnitude earthquake hit Christchurch resulting in the closure of the University of Canterbury for nearly two weeks and consequently a disruption in the testing of the rats. Since the rats were supplied in cohorts of approximately 20 experimental and 20 intruder rats at a time, two weeks apart, some rats were tested before the earthquake and some were tested after (see Figure 3). For phase one of testing, the first three rat cohorts were tested for anxiety and aggression before the earthquake, while the fourth and final cohort was tested for anxiety and aggression after the earthquake. Analysis of the data showed that startle responses for cohort four were significantly higher than those of the other three cohorts. It was presumed that these rats' responses were affected by the earthquake, so they were excluded from all startle analyses (including phase two). Although these differences were not significant for the resident intruder data at phase one, it made sense to also exclude the resident intruder results of cohort four, as they completed this test very soon after the 7.1 earthquake and its subsequent large aftershocks. More detail on this is provided in the results section. The impulsivity data for cohort four however was collected a substantial time after this and was not affected by the earthquake, so their data remained for analyses. All testing for phase two occurred after the initial 7.1 earthquake and was not disrupted by any large earthquakes. The above is depicted in Figure 3 which shows clearly when the earthquake occurred and how it disrupted testing. While it was deemed too unsafe to stay in the laboratory to conduct testing during the period that the University of Canterbury was closed, it was still possible to have access to the laboratory to feed the rats. Therefore, all rats remained on their supplement diet and no effects of previous supplement consumption were lost.

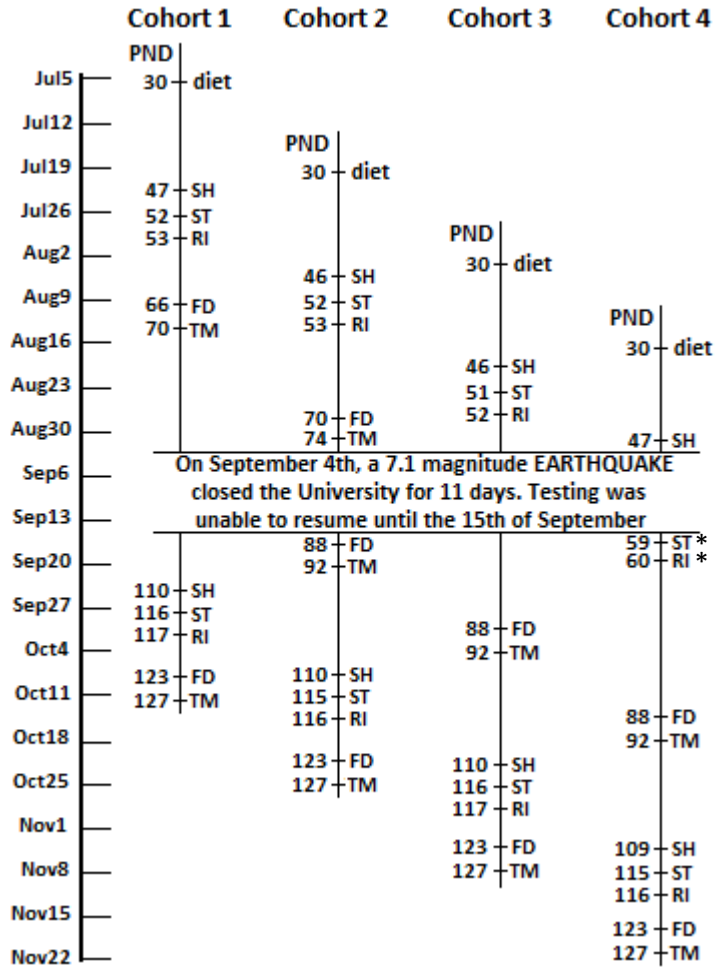

Figure 3. Timeline showing when each cohort underwent each experimental procedure, PND at time of procedure, and occurrence of the earthquake. PND = post natal day, diet = administration of diet (each cohort contained a random mix of males and females, and when possible was comprised of rats from each of the 4 diets – control, 1.25%, 2.5%, or 5%), SH = single housing begins, ST = startle procedure, RI = resident intruder paradigm, FD = food deprivation begins, TM = T-maze training and testing, * = data was excluded due to earthquake interference.

All rats were weaned on PND 30, and experimental rats were introduced to their supplement diet on this day. On PND 46 or 47, experimental rats were housed individually in preparation for the resident-intruder test (specific experimental procedures are detailed in the next sections). On day five or six of being single housed, each rat completed the acoustic startle test, followed by the resident-intruder test the next day. However, due to the earthquake, this was not the case for the fourth cohort of rats. They were housed on PND 46, but the earthquake struck the day after this. Testing was not able to resume for several days so they were startled 12 days after being single housed, followed by the resident-intruder

paradigm on their thirteenth day of being single housed. On PND 88, the experimental rats went on food deprivation for four days in preparation for the T-Maze experiment. The process of food deprivation was necessary for making the rats hungry (but not starving) so that they would eat the chocolate buds that were used as reinforcement. For each of the four days of food deprivation, each cage of rats had access to their food for only 30 minutes each day. This was enough time for each rat to eat sufficient food, but meant that they could not eat again until the next food deprivation day. They then underwent three days of T-Maze training and two days of testing. After each day of training and testing, each cage was again given access to their food for 30 minutes. Ad libitum access to their food was regained after their final testing day. It was believed that the short period of food deprivation would not reduce the levels of EMP+ enough in the rats to significantly affect their performance on the impulsivity task. The initial plan was to have all rats start their food deprivation on PND 66. This was the case for the first cohort of rats. However, the earthquake prevented the second cohort from beginning their food deprivation until PND 88. It was therefore decided that the third and fourth rat cohorts would also start their food deprivation on PND 88.

This whole cycle of testing was completed once more, beginning with single housing on PND 109 or 110. However, this time around, the rats were only exposed to two days of T-Maze training. It was presumed that only two days were needed since they had experience of the T-Maze from the first phase of testing. Testing for phase two was not disrupted by any large earthquakes, meaning that all rats were the same age when they completed each test, give or take a day.

3.3.1 Acoustic Startle Paradigm

The rats were placed in one of four identical startle cages (165mm x 80mm x 90mm) which were located in sound-attenuating melamine chambers (600mm x 340mm x 560mm).

The walls and lid of each startle cage (Med Associates, Fairfield, VT, USA) consisted of horizontal stainless steel rods 2.5mm in diameter while the rods comprising each chamber's floor were 4.5mm in diameter. All rods were spaced 15mm apart. A 2.8W lamp and a 60mm speaker were housed in a metal frame next to each startle cage. The startle cages were mounted on a Med Associates load cell-based startle platform (250mm x115mm x 450mm). The acoustic stimulus was produced by a programmable audio generator and consisted of a 100ms white noise burst with a rise-decay time of 10ms. The ambient noise level in the chambers was 36dB as measured by a Bruel and Kjaer sound level meter (A scale, model 2235; Naerum, Denmark). The Med Associates software (0.5V peak voltage amplitude equals 100 units) used in the startle paradigm recorded startle amplitudes in response to the white noise bursts. This software also controlled the light and white noise.

On the day of testing, rats were placed in the startle chambers. Testing started after a 3 minute adaptation period to the chamber. Rats were exposed to 30 noise trials, each with a 30 second inter-stimulus interval. Each testing session lasted 18 minutes. Rats were removed from the chambers and placed back in their single housed cages where they had access to their food and water in preparation for the resident-intruder test the following day.

3.3.2 Resident Intruder Paradigm

Resident rats were matched with intruder rats for date of birth, sex, and weight. In a large number of the cases, the resident rat was larger than the intruder rat. Rats were matched by weight like this on purpose to increase the expression of aggressive behaviours of the resident rat due to the smaller size of the intruder. Each subject was housed separately in a large polypropylene cage (440mm × 270mm × 210mm) for approximately seven days before testing. On the day of testing, the single housed resident rats' cages were placed in the observation room, food and water were removed from each cage, and an intruder rat was

placed in the cage with the resident rat. The behaviours described in Table 7 were recorded for the resident rat over a 15 minute period. Bite attack latency, i.e. time elapsed until the resident first bites the intruder, was also recorded. Each intruder was used only once during phase one of testing, and only once again during phase two of testing.

Table 7.
Descriptions of the behaviours observed during the resident intruder paradigm.

Behaviour	Description
Anogenital Contact	Contact of the genital/anal area, usually with nose or paw
Rough Paw	Aggressive pawing of the intruders body
Allogrooming	Aggressive rapid nibbling/grooming of intruder, usually of the neck
Bite	Contact with teeth or tugging at intruders body
Mutual Upright Position	Both resident and intruder face each other in an upright position
Upright Boxing	Pushing and pawing with front legs while both rats upright
Nose Off	Both rats stand immobile facing each other with all paws on ground
Wrestle	Both rats wrap around each other rolling around in a tight ball
Push/Nudge	Resident presses against intruder using broadside of body
Pinning	Resident pins the intruder rat on its back
Kick	Resident kicks intruder with its hind foot
Pursuit	Resident runs after intruder
Escape	Resident runs away from intruder
Mount	Resident places front paws on intruders rump from behind
Intruder Shriek	Intruder lets out a noise as a result of resident's attack

3.3.3 Impulsivity in T-Maze

Training and trials for the impulsivity experiments were performed in a T-Maze constructed from 12mm thick wooden panels with 3mm thick plastic hinged openings on top (Figure 4). It consisted of a starting runway (300mm long) and two arms (300mm long) each leading to a rectangular goal-box (150mm × 100mm × 140mm). Each arm was equipped with two removable 25mm thick wooden guillotine doors that could be inserted into the vertical clefts. The right goal-box always contained the large reward (four dark chocolate buds), while the left goal-box always contained the small reward (one dark chocolate bud). The pellets were placed at the far wall of the goal-box before each trial began.

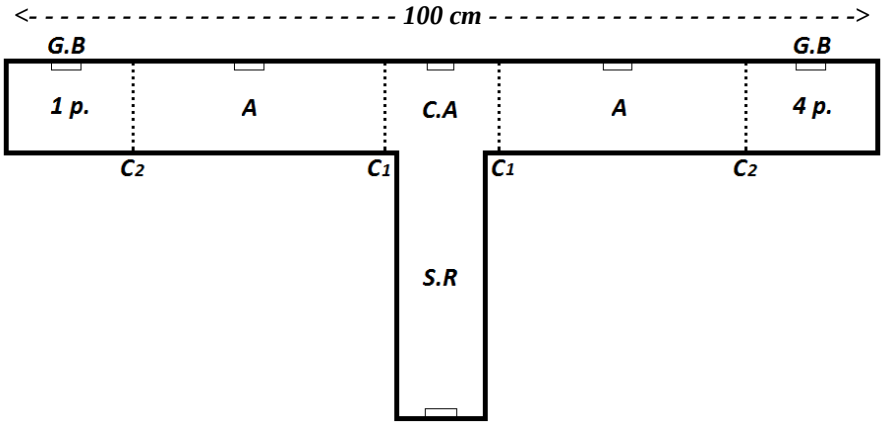

Figure 4. Schema of the T-maze. *A* = arm, *C.A* = choice area, *C1* and *C2* = clefts for guillotine doors, *G.B* = goal-box, *S.R* = starting runway, *p* = pellets of chocolate, *cm* = centimetres.

For training, the rats were placed one at a time into the starting runway. As soon as they entered one of the goal-boxes, a guillotine door was inserted behind them (*C2*). The rat was removed once all chocolate pellets had been eaten and returned to its home cage for an intertrial interval of three minutes. Their choice was recorded, as well as the time it took them to choose a goal-box, and the total time for each trial (usually less than two minutes once trained). Each rat underwent this procedure ten times a day (five in the morning, five in the afternoon). Within three days of training, most rats were choosing the larger reward 80% of the time for phase one of testing, which suggested they were ready for testing (Thiébot et al., 1985). Training lasted for two days for phase two of testing.

After training, rats underwent two days of trials where increasing delays were introduced before receiving the large reward. On the first day of these trials, rats were exposed to a 15 sec delay for five trials, followed by a 30 sec delay for five trials. On the second day of these trials, rats were exposed to a 60 sec delay for five trials, followed by a 90 sec delay for five trials. This procedure was the same for phase one and phase two of testing. A door was placed in *C2* near each goal-box before the rat was introduced to the starting runway. Once the rat entered an arm, a second door was inserted behind it in the closest *C1* (see Figure 4) so that the rat could be detained for the appropriate delay. At the end of the waiting period, the door preventing access to the goal-box was removed and the rat was

allowed to eat the pellets. If the animal selected the arm associated with the small reward, *C2* was removed immediately after *C1* was inserted so that no delay occurred between manipulations of the doors.

3.4 Rationale for Behavioural Measures

Anxiety was measured using the acoustic startle reflex (ASR) paradigm. The startle reflex is characterised by exaggerated or increased behavioural activity in response to an intense sensory stimulus (Li et al., 2009). Therefore, the ASR paradigm seemed particularly suitable for measuring anxiety in the current study. Furthermore, the paradigm has been widely used as a reliable nonverbal index of fear and anxiety in humans and animals (Bradley et al., 2001; Rosen and Schulkin, 1998). A great benefit of the ASR paradigm is that the response is purely reflexive and easily elicited (Li et al., 2009). Additionally, the test is widely used in behavioural research, giving it considerable face validity as a measure of anxiety in animals (van Nobelen and Kokkinidis, 2006). Also, the potentiated startle response (a test very similar to the simpler startle response paradigm) has shown substantial construct and predictive validity as a model for measuring anxiolytic processes i.e. those that prevent or reduce anxiety (Hijzen et al., 1995). The ASR paradigm is less invasive than tests that involve measuring the adrenal hormones or the weight of the adrenal glands, and are more reliable than tests relying on muscle tone or tail-pinch responses.

Aggression was measured using the resident-intruder (R-I) paradigm, which is simple to use and doesn't require the use of aversive stimulation or conditioning to elicit aggressive behaviours (Miczek, 1979). A range of alternative defense test batteries use a recently killed or anaesthetised rat to elicit the aggressive behaviours in the subject (Blanchard et al., 1998), while others involve cats or humans as the intruder (Blanchard et al., 2003). Previous experience in this laboratory has shown that the type of rat used in the current study is well-

habituated to humans and does not seem to react aggressively toward cats. Furthermore, the R-I paradigm does not require the use of recently killed or anaesthetised rats. Therefore, the R-I intruder paradigm is a much more suitable and humane method for assessing aggression than these other methods. An earlier study using a similar test to the R-I paradigm, the rat exposure test (which involves an intruder rat being placed behind a mesh barrier), showed that all strains of rat tested exhibited defensive behaviours thereby indicating the value of this test in assessing aggressive and defensive behaviours (Yang et al., 2004). The removal of this mesh barrier in the R-I paradigm used in the current study allows for additional and more aggressive contact behaviours to be recorded, making it a more appropriate method for assessing aggression. Significant decreases in attack frequency after administration of tetrahydrocannabinol (THC), the psychoactive compound in marijuana, in a dose dependent manner in resident mice, rats, and squirrel monkeys has been found which shows that the resident-intruder paradigm has a degree of species generality (Miczek, 1978). Additionally, the test's predictive validity enables us to predict outcomes when applied to humans (Olivier and Young, 2002).

There are fewer choices of tests for impulsivity but most are generally a variation of the T-maze procedure used in the current study. They typically vary with how the cost involved to attain the greater of two rewards (or the tolerance to delay of reward) is produced. The 5-Choice Serial Reaction Time Task requires rats to detect brief flashes of light presented in a pseudorandom order in one of five spatial locations (Bari et al., 2008). Impulsivity is apparent when the rat responds before the onset of the stimulus. The training for this task takes 30-40 daily sessions. Due to time restrictions, and availability of the apparatus for this test, it was deemed not suitable for the current study. The T-maze apparatus however was readily available, and training takes only three daily sessions. A study using a T-maze to test for impulsivity found that all animals chose the high reward arm on the

majority of trials when the delay was equal to both the high and low reward arms (Denk et al., 2005). This shows that the rat does value the larger reward, and is likely to behave as expected when delays to the larger reward are introduced. The spontaneously hypertensive rat (SHR) is believed to be a valid model of ADHD and therefore impulsivity. Illustrating this are results from choice procedures showing that SHR's are more impulsive than the Wistar-Kyoto rats as defined by preference for the smaller, immediate reinforcer over larger, delayed ones (Fox et al., 2008). Sensitivity to delay of reinforcement was also found for the SHR and the genetically hypertensive rat, while not as much in their respective genetic control strains Wistar-Kyoto and Wistar (Sutherland et al., 2009). These studies effectively measured sensitivity to delay of reinforcement and their findings suggest that the T-Maze procedure used in the current study is a valid measure of impulsivity in the rat.

4.0 Statistical Analyses

All raw data were analysed by a repeated measures analysis of variance (ANOVA) using the statistical programme *SPSS v19*. Analyses were repeated using the statistical programme *Statisctica v9* to ensure that values were computed correctly. Separate 4 (treatment) x 2 (testing phase/age) x 2(sex) factorial repeated measures ANOVAs were used on each measure. These were followed by one-way ANOVAs of each sex at each testing phase and post hoc Fisher PLSD tests. Statistical significance was assumed at values of $p \leq 0.05$. All values reported were rounded to two decimal places. Differences in behaviour across each testing phase were examined to see if the supplement had a greater effect on anxiety, aggression, and impulsivity over time. Sex differences in effects of EMP+ on behaviour were tested because so far, it is unknown whether the supplement affects males and females differently.

5.0 Results

Each animal was tested once at both testing phases for anxiety, aggression, and impulsivity. For the startle and resident-intruder paradigm, subjects were 29 males and 34 females, due to the exclusion of cohort four's data from these analyses (see Table 7). No data was excluded from the T-maze analyses as this data was not affected by the earthquake. Even though the first cohort completed the T-maze test earlier than the other three cohorts, this difference in testing age and any effect on their responses was not significant or considered an issue. Due to time restraints, only males were tested for impulsivity in the T-maze. Therefore, in this case, the subjects were 40 male rats.

Body weights were analysed to see if the diet concentration had a significant effect on the weights of the rats in each diet group. This is important because if the supplement caused a particular diet group to put on weight, testing results, particularly startle results, may have been affected. Mean ± S.E.M body weights (g) of the rats in the control, 1.25%, 2.5%, and 5% supplement groups respectively were 186.91 ± 11.21, 191.07 ± 11.86, 182.32 ± 9.84 and 192.14 ± 10.50. The treatment effect was not significant [$F(3,59) = 0.17$, $p=0.92$]. Not surprisingly, the male rats were significantly heavier (mean ± S.E.M = 232.03 ± 2.30) than the females [150.12 ± 1.26, $F(1,61) = 1057.86$, $p<0.001$].

There was either a significant main sex effect, or a sex × treatment interaction effect for startle amplitude and most resident-intruder behaviours. Therefore, separate graphs for each sex were displayed for each measure. As each sex had obviously reacted significantly differently in these cases, it was not appropriate to combine and average their responses. Furthermore, results from each testing phase were also represented separately in all graphs for the startle, resident-intruder, and T-Maze results to display what occurred at each phase because again, there was often a significant main effect of testing phase. Treatment group means were not averaged for the graphs. Because supplement treatment was the main

variable of interest, any nonsignificant trends could still be of interest with regard to suggesting possible future lines of research.

Initially, repeated measures ANOVAs were carried out for all variables. Due to the significant interactions between sex and treatment for several of the measures, one-way ANOVAs were performed for each sex at each testing phase to see if there were suggestions of any specific effects of treatment on each sex at each testing age. While it is not standard practice to do so, particularly if there is not a significant treatment effect or interaction, this approach was adopted due to the exploratory nature of this research. It was also guided by the view that the primary objective of a study should determine which statistical tests are made to arrive at scientifically meaningful inferences (Winer, 1962). As there is abundant evidence that the two sexes can respond differently to pharmacologically active agents (Hughes, 2007) and that the effects of such agents can change over time (e.g., Beluzzi et al., 2004; Anderson and Hughes, 2008), the approach adopted seemed justified as it was felt that any suggestive findings in this hitherto unexplored area might help guide future research. Therefore, no firm conclusions should be drawn from the post hoc analyses following the one-way ANOVAs. They should be considered purely suggestive rather than conclusive. Also, one does not generally include statistical results of non-significant main and interaction effects. They have been included in the current study due to the nature of this research. Some of the non-significant effects were clearly far from being significant, which is still informative. However, the few that were close to significance may have been significant if testing had not been interrupted by a 7.1 magnitude earthquake. Also, because this was the first study of its kind, reporting these near significant and not so significant results might be helpful for directing future studies.

5.1 Startle Results

Data from cohort four was excluded from all startle analyses due to the effects of the September earthquake on their startle amplitudes. The startle amplitudes of cohort four were significantly higher than those of the other three cohorts, while the other three cohorts' were not significantly different from each other. Furthermore, as can be seen in Table 5, cohort four is not an exact average representation of all treatment groups or each sex. Therefore, the effects of the earthquake on the startle amplitudes of cohort four are not represented equally in each diet group or sex, giving more reason to remove cohort four from data analyses. Unfortunately, due to the removal of cohort four's data from the startle analyses, an unequal number of rats in each treatment group resulted (Table 8). Group numbers were reduced to as low as seven, particularly for males, for some of the concentration groups.

Table 8.
Reduced numbers of rats in each treatment group as a result of removing cohort four due to the September earthquake.

Sex	Diet	N
F	0%	10
(N=34)	1.25%	7
	2.5%	10
	5%	7
M	0%	7
(N=29)	1.25%	8
	2.5%	7
	5%	7

No significant startle amplitude differences were found between any of the four startle chambers, indicating that each chamber was calibrated correctly [$F(3,59) = 0.17$, $p=0.91$]. Of the 30 startle amplitudes collected for each rat, the first ten trials were discarded to reduce overall variance and the remaining 20 trials were reported (van Nobelen, 2006). Therefore, the variable of interest that was used for data analyses was an average of the last 20 startle amplitudes for each rat.

A repeated measures ANOVA found no significant main effect of supplement treatment on startle amplitude (p=0.680). There was a significant main effect of testing phase [F(1,55) = 11.38, p<0.001]. On average, startle amplitudes were higher at testing phase two (mean ± S.E.M=306.09 ± 21.74) than at testing phase one (241.83 ± 14.64). Ages of the rats at each testing phase and their corresponding developmental stage for each test can be found in Table 4. There was also a significant main effect of sex [F(1,55) = 10.83, p<0.01]. On average, males (361.72 ± 22.20) produced larger startle amplitudes than females (258.63 ± 19.48). There was a significant interaction between sex and treatment [F(3,55) = 3.40, p<0.05]. With increasing diet concentration, male startle amplitudes decreased while female startle amplitudes increased. No other interaction effects were significant. There was not a significant interaction between testing phase and treatment [F(3,55) = 1.29, p=0.29], testing phase and sex [F(1,55) = 0.03, p=0.86], or between testing phase, treatment, and sex [F(3,55) = 0.76, p=0.52].

Due to the main effect of sex and the significant interaction effect between sex and treatment, further one-way ANOVAs were carried out to see if supplement treatment was significant for each sex at each phase. There was not a significant treatment effect for males at phase one [F(3,25) = 2.13, p=0.12] or phase two [F(3,25) = 1.06, p=0.38], or for females at phase one [F(3,30) = 0.73, p=0.54] or phase two [F(3,30) = 2.04, p=0.13]. Fisher PLSD post hoc comparisons revealed three differences between supplement treatment groups, which are illustrated in Figure 5. Specifically, the male control group produced significantly larger startle amplitudes than the male 1.25% group (p<0.05) and the male 5% supplement group (p<0.05) at phase one, meaning they were more anxious. Therefore, the supplement reduced anxiety for males on the supplement. The female 5% diet group produced significantly larger startle amplitudes than the controls (p<0.05) at phase two, showing they were more anxious. Unlike the males, a larger dose of EMP+ increased anxiety.

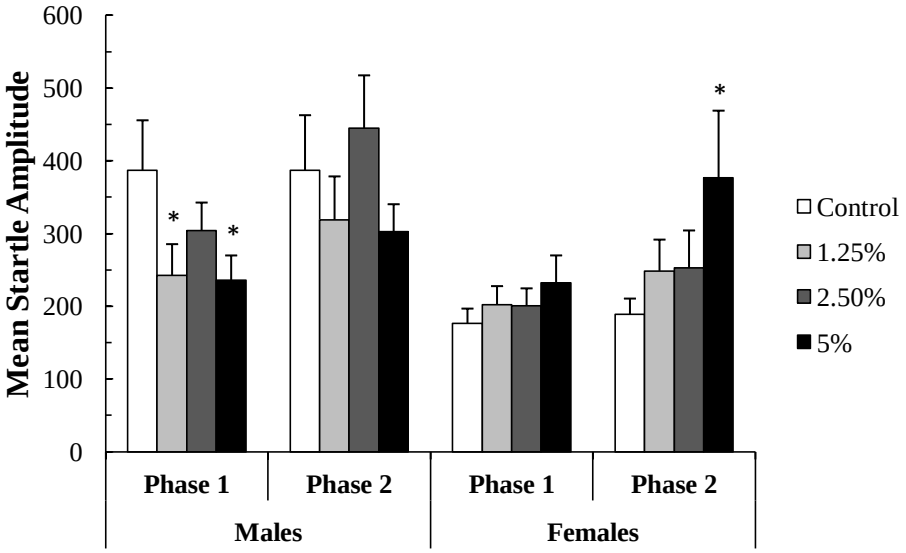

Figure 5. Mean (±S.E.M) startle amplitude responses for males and females showing the differences in responses between each diet group at phase one and phase two of testing. * = significantly different from the control group (p<0.05).

Even though there were no main effects of supplement treatment on startle amplitude, it is clear that each sex reacted differently to the supplement. Males appeared to benefit from the supplement when tested at phase one (PND 52-53), which is illustrated by their lower startle amplitudes, while females were more anxious on the highest supplement strength when tested at phase two (PND 116-117), therefore producing higher startle amplitudes.

5.2 Resident Intruder Results

Cohort four's resident-intruder data was also excluded from all analyses because testing of cohort four happened very soon after the September earthquake (see Table 8). Also, they were housed for a prolonged period of time due to the earthquake i.e. 13 days compared to six or seven days for the other three cohorts. The difference in testing conditions that cohort four experienced compared to the other three cohorts justified their removal. A repeated measures ANOVA was carried out for the remaining subjects, and the results are presented in Table 9. For the main effect of treatment, values shown were averaged across sex and testing phase. For the main effect of sex, the values shown were averaged across

treatment groups and testing phase. For the main effect of testing phase, values shown were averaged across treatment groups and sex. Several behaviours that occurred so rarely in any treatment group were not analysed. These included mutual upright position, upright boxing, nose off, wrestle, push/nudge, pinning, kick, pursuit, escape, mount, and intruder shriek which left five behaviours for analysis (Table 9).

Table 9.
Means (±S.E.M) for resident intruder behaviour measures for each diet concentration for male and female rats at phase one and two of testing, and results of* F *tests from repeated measures ANOVA.

Treatment Group	Control (n=17)	1.25% (n=15)	2.50% (n=17)	5% (n=14)	*F* (3,55)	*p*
Latency to bite (sec)[a]	715.88(45.14)	642.77(54.21)	713.47(51.89)	710.25(50.16)	0.59	=0.626
Anogenital Contact	14.41(1.21)	10.67(1.21)	11.21(1.03)	10.68(1.08)	2.32	=0.085
Rough Paw[b]	10.35(1.47)	10.83(1.66)	10.35(1.35)	8.54(1.45)	0.77	=0.515
Allogrooming[ab]	2.12(0.75)	2.63(0.82)	2.12(0.65)	2.11(0.63)	0.30	=0.829
Bite[c]	1.76(0.56)	2.40(0.69)	2.06(0.68)	2.14(0.66)	0.20	=0.894
Sex		Male (n=29)	Female (n=34)		*F* (1,55)	*p*
Latency to bite (sec)		650.12(40.49)	736.19(30.46)		3.36	=0.072
Anogenital Contact		8.79(0.62)	14.41(0.82)		28.11	<0.001
Rough Paw		9.38(0.97)	10.65(1.09)		1.04	=0.312
Allogrooming		1.50(0.37)	2.87(0.57)		5.00	<0.05
Bite		3.09(0.60)	1.22(0.27)		10.65	<0.01
Testing Phase		Phase One (n=63)	Phase Two (n=63)		*F* (1,55)	*p*
Latency to bite (sec)		551.51(37.89)	841.63(20.37)		58.28	<0.001
Anogenital Contact		12.62(0.81)	11.03(0.83)		2.75	=0.103
Rough Paw		15.19(1.01)	4.94(0.56)		123.54	<0.001
Allogrooming		3.83(0.62)	0.65(0.22)		41.87	<0.001
Bite		3.75(0.54)	0.41(0.18)		40.35	<0.001

[a] Phase × treatment × sex interaction significant (see text)
[b] Treatment group × sex interaction significant (see text)
[c] Phase × sex interaction significant (see text)

In summary, there were no significant main effects of supplement treatment for any of the behaviours recorded (see Table 9). There were significant main effects of sex for anogenital contact, allogrooming, and bite. There were also significant main effects of phase for all variables except for anogenital contact. Significant interaction effects between

treatment and sex were found for rough paw and allogrooming, and were found between phase and sex for bite, but none were found between phase and treatment. An overall significant phase × treatment × sex interaction effect was found for latency to bite and allogrooming. These findings are explained in more detail in the following sections. As was done for the startle results, due to the main effects of sex and the significant interaction effects between sex and treatment, further one-way ANOVAs were carried out to see if supplement treatment was significant within each sex at each phase. As mentioned earlier, the results from the one-way ANOVAs and their post hoc results should be interpreted with caution, and are only indicative of possible treatment effects.

5.2.1 Latency to Bite (sec)

There was not a significant treatment effect for latency to bite, nor was there a significant main effect of sex (see Table 9 for further references to main effect statistics). There was however a significant main effect of testing phase. The time taken to perform a bite attack increased significantly from phase one (PND 52-53) to phase two (PND 116-117), suggesting decreased aggression. Additionally, there was a significant phase × treatment × sex interaction effect [$F(3,55) = 2.84$, $p<0.05$]. No significant interactions were found between treatment and sex [$F(3,55) = 2.42$, $p=0.076$], testing phase and treatment [$F(3,55) = 1.13$, $p=0.34$], or between testing phase and sex [$F(1,55) = 2.30$, $p=0.14$].

A one-way ANOVA for males at phase one showed a suggestive treatment main effect [$F(3,25) = 2.78$, $p=0.062$], but no significant main effects of treatment were found for males at phase two [$F(3,25) = 0.10$, $p=0.96$], females at phase one [$F(3,30) = 1.39$, $p=0.27$], or for females at phase two [$F(3,30) = 0.53$, $p=0.67$]. Fisher PLSD post hoc comparisons showed that bite attack latencies for the male control group were significantly longer than

those of the male 2.5% supplement group at phase one (p<0.01) meaning that controls were less aggressive because they took longer to initiate a bite attack.

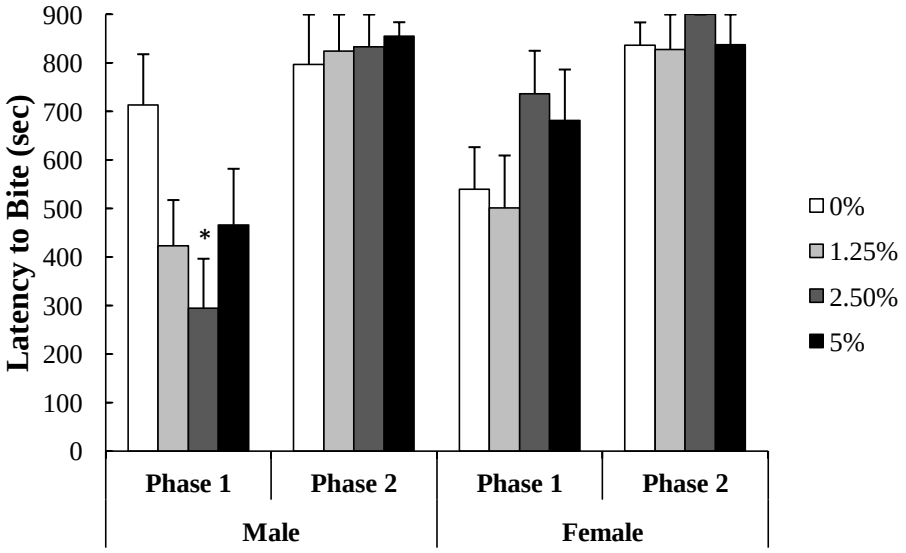

Figure 6. Mean (±S.E.M) bite attack latency for males and females at phase one and phase two for all treatment groups. * = significantly different from control group (p<0.01).

It is clear from Figure 6 that bite attack latencies were significantly longer at phase two than phase one, and that supplement treatment did not significantly affect these, particularly at phase two.

5.2.2 Anogenital Contact

There was a significant main effect of sex for anogenital contact (see Table 9). Females performed anogenital contact significantly more often than males at both testing phases, which is clearly displayed in Figure 7. There were no significant main effects of treatment or testing phase. There were no significant interactions between treatment and sex [F(3,55) = 0.69, p=0.56], testing phase and treatment [F(3,55) = 0.39, p=0.76], testing phase and sex [F(1,55) = 0.08, p=0.78], or between testing phase, treatment, and sex [F(3,55) = 0.66, p=0.58].

One-way ANOVAs revealed a near significant main effect of treatment for males at phase one [$F(3,25) = 2.51$, $p=0.08$], but no significant main effects of treatment were found for males at phase two [$F(3,25) = 0.42$, $p=0.74$], females at phase one [$F(3,30) = 0.56$, $p=0.65$], or for females at phase two [$F(3,30) = 1.12$, $p=0.36$]. Fisher PLSD post hoc comparisons for the one-way ANOVA for males at testing phase one showed that the control group performed anogenital contact significantly more often than the 1.25% supplement group ($p<0.05$), which can be seen in Figure 7.

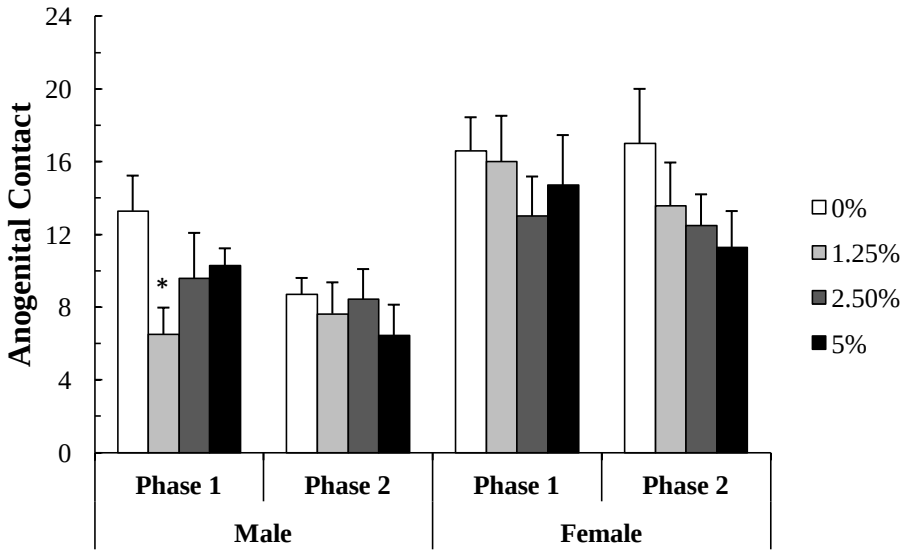

Figure 7. Mean (±S.E.M) anogenital contact showing male and female responses for all treatment groups at both testing phases. * = significantly different from the control group ($p<0.05$).

Although a main effect of supplement treatment was not found, it seems that being on any strength of the supplement reduces anogenital contact in males and females compared to controls.

5.2.3 Rough Paw

While there was not a significant main effect of treatment or sex, there was a significant main effect of testing phase (see Table 9). Rough paw occurred significantly more

frequently at phase one than phase two. There was also a significant interaction between sex and treatment [$F(3,55) = 4.65$, $p<0.01$]. Being on the supplement seemed to increase rough paw frequency for males, and decrease it for females, although this pattern was not perfectly linear. No significant interactions were found between testing phase and treatment [$F(3,55) = 1.28$, $p=0.29$], or testing phase and sex [$F(1,55) = 1.77$, $p=0.19$]. There was a near significant interaction between testing phase, treatment, and sex [$F(3,55) = 2.68$, $p=0.056$].

A one-way ANOVA for females at phase one revealed a significant main effect of treatment [$F(3,30) = 3.34$, $p<0.05$]. This was likely due to the significant post hoc differences revealing that the 1.25% supplement group performed rough paw significantly more often than the 2.5% supplement group ($p<0.05$) and the 5% supplement group ($p<0.05$). There were no significant main effects of treatment for females at phase two [$F(3,30) = 0.74$, $p=0.54$], males at phase one [$F(3,25) = 2.19$, $p=0.11$], or males at phase two [$F(3,25) = 1.76$, $p=0.18$]. Post-hoc Fisher PLSD comparisons revealed some differences between treatment groups for the males. At phase one, the males in the 2.5% supplement group performed rough paw significantly more often than the males in the control group ($p<0.05$). Additionally, at phase two, the males in the 2.5% supplement group performed rough paw significantly more often than the males in the 1.25% supplement group ($p<0.05$). That is, for males, being on the supplement increased rough paw in the 2.5% group. These post hoc differences are illustrated on the graph in Figure 8.

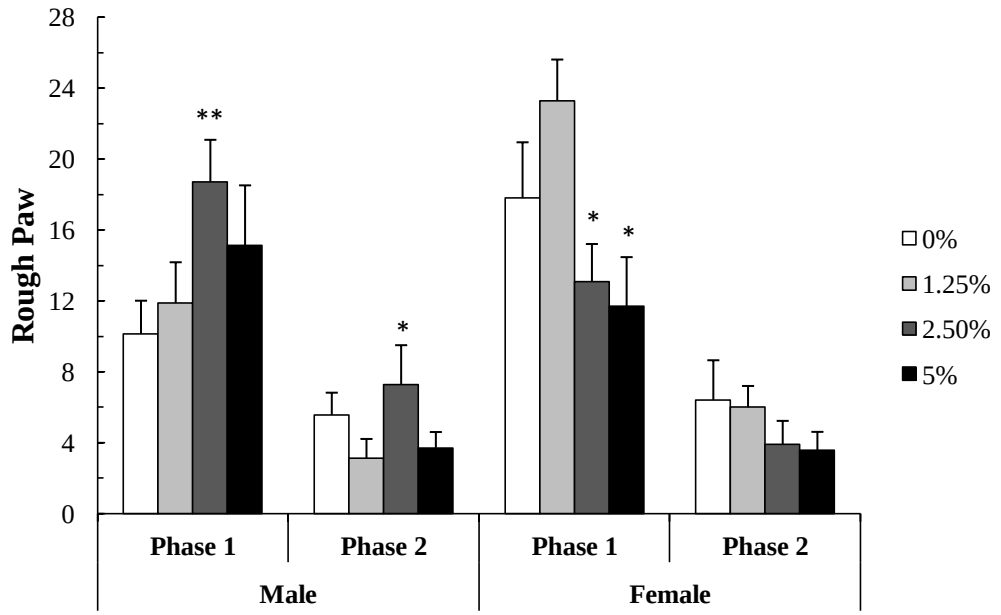

Figure 8. Mean (±S.E.M) rough paw showing the differences between each diet concentration for testing phases one and two, males and females. * = significantly different from the 1.25% supplement group ($p<0.05$). ** = significantly different from the control group ($p<0.05$).

5.2.4 Allogrooming

There was not a significant main effect of treatment for allogrooming but there was a significant main effect of sex (see Table 9). Females performed allogrooming significantly more often than males. There was also a significant effect of phase. Rats performed allogrooming more often at phase one than phase two. The interaction between sex and treatment was significant [$F(3,55) = 4.65$, $p<0.01$] due to the opposite patterns of responding of each sex at phase one. There was not a significant interaction between testing phase and treatment [$F(3,55) = 0.41$, $p=0.74$] or between testing phase and sex [$F(1,55) = 2.48$, $p=0.12$], but the interaction between testing phase, treatment, and sex was significant [$F(3,55) = 7.46$, $p<0.001$].

A one-way ANOVA for males at phase one revealed a significant main effect of treatment [$F(3,25) = 5.23$, $p<0.01$]. This was likely due to the high level of allogrooming by the 2.5% supplement group. Post hoc analyses showed that the allogrooming levels of the male 2.5% supplement group at phase one were significantly higher than the control group

(p<0.01), the 1.25% supplement group (p<0.01), and the 5% supplement group (p<0.05). A one-way ANOVA for females at phase one also showed a significant main effect of treatment [F(3,30) = 3.18, p<0.05]. This was likely due to the significantly higher levels of allogrooming by the 1.25% group than the 2.5% supplement group (p<0.01) for females at phase one. These findings are illustrated in Figure 9. There was not a significant main effect of treatment for males at phase two [F(3,25) = 0.81, p=0.50] or for females at phase two [F(3,30) = 0.03, p=0.99].

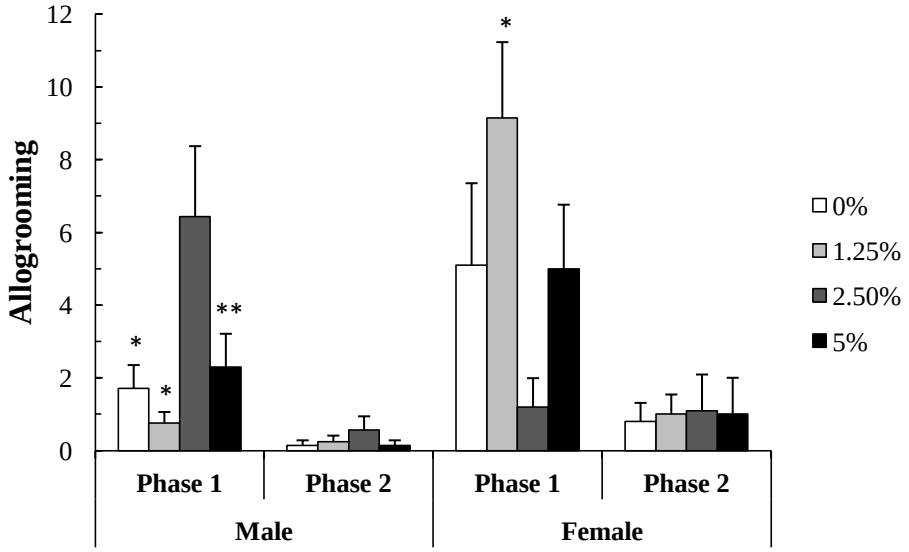

Figure 9. Mean (±S.E.M) allogrooming showing the differences between each diet concentration for testing phases one and two, males and females. * = significantly different from the 2.5% supplement group (p<0.01). ** = significantly different from the 2.5% supplement group (p<0.05).

Allogrooming levels at phase two were quite low, which probably contributed to the overall lack of a main treatment effect in males and females at phase two.

5.2.5 Bite

There was not a significant main effect of supplement treatment for bite (see Table 9). There was a significant main effect of sex and a significant main effect of testing phase. Males performed a bite significantly more often than females, and bites occurred significantly

more often at phase one than phase two. These findings are illustrated in Figure 10. There was also a significant interaction between testing phase and sex [$F(1,55) = 7.73$, $p<0.01$]. Significant interactions were not found between treatment and sex [$F(3,55) = 1.07$, $p=0.37$], testing phase and treatment [$F(3,55) = 0.23$, $p=0.87$], or between testing phase, treatment, and sex [$F(3,55) = 0.25$, $p=0.86$].

One-way ANOVAs revealed no significant treatment main effects for males at phase one [$F(3,25) = 0.31$, $p=0.82$], males at phase two [$F(3,25) = 0.60$, $p=0.62$], females at phase one [$F(3,30) = 0.52$, $p=0.67$], or females at phase two [$F(3,30) = 0.72$, $p=0.55$].

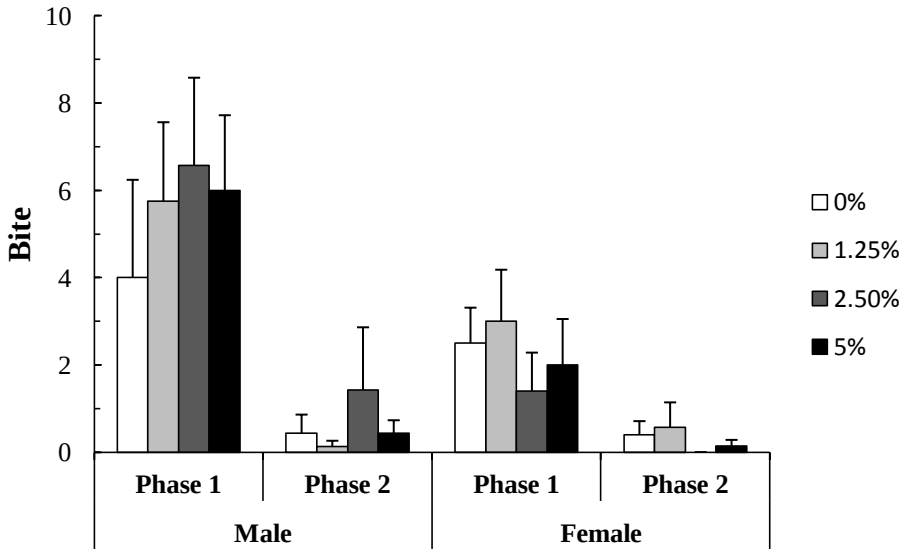

Figure 10. Mean (±S.E.M) bite showing the differences between each diet concentration for testing phases 1 and 2, males and females.

As for allogrooming, bite levels were particularly low at testing phase two, possibly contributing to the lack of treatment effects found.

5.3 T-Maze Results

A repeated measures ANOVA was carried out for the T-maze data and the results are presented in Table 10. For the main effect of supplement treatment, values shown are averaged across testing phase. For the main effect of phase, values shown are averaged across

treatment groups. Values represent the percentage of times that a rat chose the small immediate reward over the larger delayed reward. Therefore, higher percentage values represent higher levels of impulsivity. Only males were tested for impulsivity, so there were no main or interaction effects involving sex for this measure

Table 10.
Means (±S.E.M) for all impulsivity test delays for each diet concentration at phase one and two of testing, and results of* F *tests from repeated measures ANOVA.

Treatment Group	Control (n=10)	1.25% (n=10)	2.50% (n=10)	5% (n=10)	*F* (3,36)	*p*
15sec Delay	36.00(6.09)	42.00(7.24)	30.00(4.92)	40.00(4.81)	0.90	=0.451
30sec Delay	59.00(6.88)	54.00(6.17)	44.00(4.72)	67.00(6.03)	2.29	=0.095
60sec Delay	72.00(4.21)	68.00(5.31)	70.00(4.70)	72.00(5.69)	0.14	=0.938
90sec Delay	83.00(5.48)	81.00(4.70)	75.00(5.78)	82.00(4.57)	0.42	=0.741
Testing Phase		Phase One (n=40)	Phase Two (n=40)		*F* (1,36)	*p*
15sec Delay		27.00(3.89)	47.00(3.76)		13.95	<0.01
30sec Delay		47.00(4.45)	65.00(3.84)		13.35	<0.01
60sec Delay		65.00(4.04)	76.00(2.60)		5.56	<0.05
90sec Delay		75.50(3.00)	85.00(4.04)		4.07	=0.051

There were no significant main effects of treatment for any length of delay (see Table 10). Testing phase was significant for all lengths of delay, except for the 90sec delay, which was very close to being significant. Rats were significantly more impulsive at phase two (PND 127-130) than phase one (PND 92-96). There were no significant interactions between testing phase and treatment for the 15 second delay [$F(3,36) = 0.56$, $p=0.65$], the 30 second delay [$F(3,36) = 1.13$, $p=0.35$], the 60 second delay [$F(3,36) = 0.54$, $p=0.66$], or the 90 second delay [$F(3,36) = 0.31$, $p=0.82$].

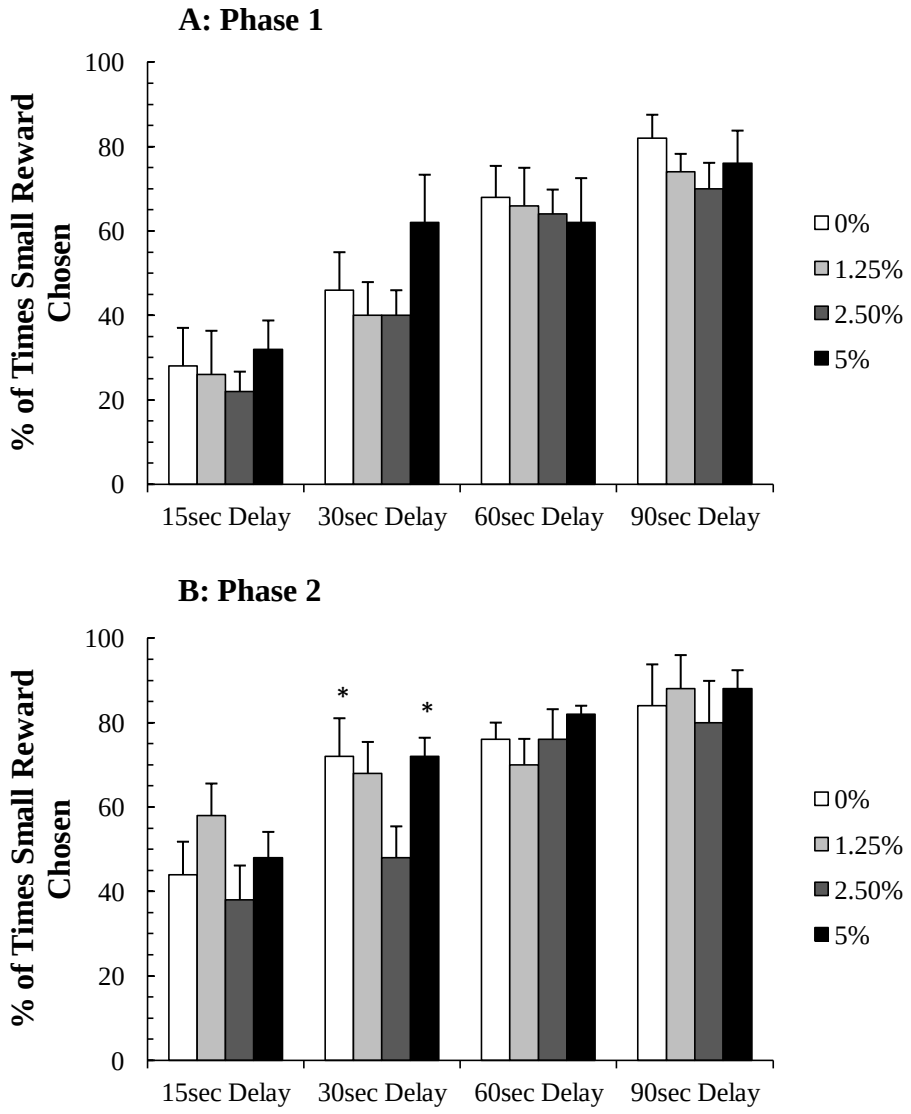

Figure 11. Mean (±S.E.M) of the % of times the small immediate reward was chosen at (A) testing phase one and (B) testing phase two. All rats were males. Higher responses indicate impulsivity. * = significantly different from the 2.5% supplement group (p<0.05).

As expected, rats became more impulsive with increasing delays. However, this was not moderated by the strength of supplement that the rats were receiving. One-way ANOVAs at each phase revealed some differences in responses between supplement groups, which are displayed on the graph in Figure 11. Specifically, for the 30sec delay at phase two, post-hoc analyses showed that the 2.5% supplement group was significantly less impulsive than the control group (p<0.05) and the 5% supplement group (p<0.05). No other differences were found. Because close to no differences were found, the responses were averaged across all

delays to create a summary graph for testing phase one and two, allowing for easier comprehension of the effects of diet concentration on impulsivity (Figure 12).

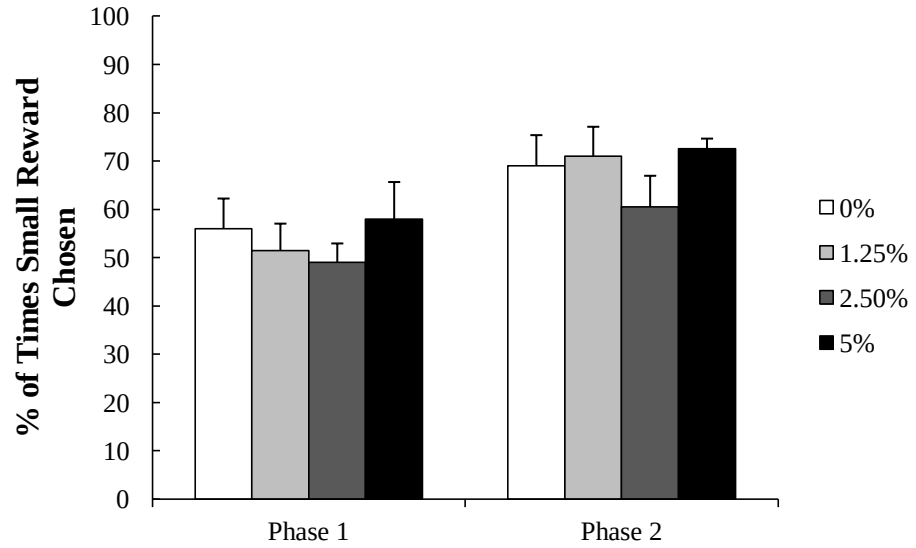

Figure 12. Mean (S.E.M) of the % of times the small immediate reward was chosen at testing phases one and two, averaged over delay to reinforcement. All rats were males. Higher responses indicate impulsivity.

6.0 Discussion of Results

In this study, rats were exposed to differing concentrations of a supplement diet of EMP+. Rats had constant access to a diet that contained 0%, 1.25%, 2.5% or 5% EMP+. Rats were first tested on measures of anxiety and aggression during adolescence (PND 52-53) and for impulsivity during early adulthood (PND92-96). They were tested again for anxiety and aggression during mid adulthood (PND 116-117) and for impulsivity during late adulthood (PND 127-130). Male and female rats were tested for anxiety and aggression to allow for possible sex differences in the effects of the supplement, and testing was carried out at two ages to see if the supplement had a cumulative effect over time as well as to assess the effects of EMP+ at different stages of development.

6.1 Summary of Results

The results revealed a select number of significant behavioural differences across the measures as a result of supplement treatment. A summary of the main effects and interactions from the repeated measures ANOVAs are presented in Table 11. This table clearly shows the different effects found across the three behaviours investigated. It is clear that no main effects of treatment were found for any of the measures when repeated measures ANOVAs were carried out. For nearly all measures, there was a significant effect of phase, but this never interacted with treatment. Sex differences were quite often found, and this interacted with treatment half of the time. These findings will be explained in more detail in the following sections.

Table 11.
Summary of main effects and interactions from repeated measures ANOVAs for anxiety, aggression, and impulsivity measures.

		T	S	P	T × S	P × T	P × S	P × S × T
Anxiety	Startle Amplitude	-	✓	✓	✓	-	-	-
Aggression	Latency to Bite (sec)	-	-	✓	-	-	-	✓
	Anogenital Contact	-	✓	-	-	-	-	-
	Rough Paw	-	-	✓	✓	-	-	-
	Allogrooming	-	✓	✓	✓	-	-	✓
	Bite	-	✓	✓	-	-	✓	-
Impulsivity	15sec Delay	-	n/a	✓	n/a	-	n/a	n/a
	30sec Delay	-	n/a	✓	n/a	-	n/a	n/a
	60sec Delay	-	n/a	✓	n/a	-	n/a	n/a
	90sec Delay	-	n/a	-	n/a	-	n/a	n/a
	Trials Averaged	-	n/a	✓	n/a	-	n/a	n/a

Note: A tick signifies that the result was significant, and a dash signifies that the result was not significant.
T = treatment, S = sex, P = phase, n/a = not applicable (only males were tested for impulsivity, so results that included sex main or interaction effects were not gathered for this measure).

Exposure to the diet did not produce uniform effects on the anxious, aggressive, or impulsive behaviours of the rats. Instead, the supplement produced opposite behavioural effects in each sex for some of the aggression measures, showing that males and females reacted to the supplement in different ways. Supplement treatment increased aggression in one case as shown by a decrease in bite attack latencies in males on the supplement, but decreased aggression in another as shown by a decrease in frequency of rough paw in females on the supplement. Furthermore, while there were no significant main effects of treatment on startle amplitudes, the interaction between sex and treatment was significant, confirming that the supplement causes different behavioural outcomes in each sex. There were no main effects of treatment for impulsivity, nor were there any interaction effects. The results of the three tests are covered in more detail as follows.

For the startle paradigm, there was not a significant supplement treatment effect on mean startle amplitudes when data was averaged across sex (see Figure 5 and the corresponding text). Supplement treatment also did not have a significant effect on mean startle amplitudes when each sex was assessed separately. However, the interaction between

treatment and sex was significant, showing that each sex reacted to the supplement in a different manner. Males tended to become less anxious, while females tended to become more anxious. For both sexes, startle amplitudes increased significantly from testing phase one to two. This was not expected as it was thought that rats would habituate to the testing apparatus over time (Koch, 1999). It is unclear why this happened, and possible reasons, such as prolonged anxiety as a result of the September earthquake and its aftershocks, are discussed later.

There were no main effects of supplement treatment for any of the aggression behaviours measured when data was averaged across sex. When each sex was assessed separately, supplement treatment did not have a statistically significant effect on bite attack latency, bite frequency or anogenital contact for any sex at either testing phase, although it did have a near significant effect on males' bite attack latencies and anogenital contact at phase one (see previous text). Supplement treatment had a significant effect on males' and females' allogrooming at phase one, and in both cases this was not linear (see Figure 9). A significant treatment effect for rough paw was found for females at phase one (see Figure 8). No other significant treatment effects were found when each sex was assessed separately.

No significant main or interaction effects were found for supplement treatment for any of the delays tested in the T-maze. The supplement treatment did not influence the male rats' impulsivity behaviour. Rats were significantly more impulsive at testing phase two (PND 127-130) than testing phase one (PND 92-96). Similar to the patterns found with the anxiety and aggression measures, the effects of the strength of EMP+ diet on impulsivity were not linear. For nearly every single delay, the rats on the 2.5% diet were least impulsive, as shown by them choosing the small immediate reward less often at both testing ages (see Figure 11). This is more clearly illustrated in Figure 12. Therefore, a diet strength of 2.5% seems most suitable for reducing impulsivity in the male rat during early adulthood (PND 92-

96) and late adulthood (PND 127-130). It should be noted however that these differences were not significant, and are only suggestive of a trend.

6.1.1 Sex Differences

There were significant sex differences found for startle and aggression measures, as well as some interaction effects with treatment. For startle amplitudes, there was a significant main effect of sex. On average, males produced larger startle amplitudes than females, which is not surprising since male body weights were significantly higher than those of females. Strangely, some studies have not found significant main effects of sex for startle amplitudes (Hughes et al., 2011; Lacy, Mactutus, and Harrod, 2011), even though the males in the first of these were significantly heavier than the females. It doesn't seem likely that this is a result of diet consumption due to the fact that startle amplitudes for controls were significantly higher for the males (386.80 ± 68.89) than the females (176.23 ± 20.53) at the first testing age [$F(1,55) = 17.01$, $p<0.001$] and were also higher for the males (387.14 ± 75.57) than the females (189.27 ± 21.27) at the second testing age [$F(1,55) = 6.16$, $p<0.05$].

As outlined earlier, each sex reacted in a different manner to the supplement when tested for anxiety. Females showed a nearly perfect linear increase in startle amplitudes with increasing diet concentration, while males generally showed a decrease in startle amplitudes with increasing diet concentration at both testing ages (see Figure 5). This suggests that any strength of EMP+ is not appropriate for reducing anxiety in a female rat population in adolescence (PND 52-53) or mid adulthood (PND 116-117). Additionally, startle amplitudes for the females in the 5% diet group at the second testing age were unexpectedly high. It is possible that the large dose of EMP+ and the stress resulting from the earthquake compounded, therefore causing further anxiety, or that the high strength of EMP+ itself had a toxic effect. For males, startle amplitudes were the lowest at both testing ages when they

were on the 5% diet strength. Strangely, when on the 1.25% diet, their startle amplitudes were nearly equally as low as the 5% group, while amplitudes were much higher for the controls and the 2.5% diet group at both testing ages. There are a few explanations for this non-linear pattern. Firstly, each diet group may have been characteristically different before they started the diet, suggesting that the high startle amplitudes of the control and the 2.5% diet group were due to individual differences of those groups. Secondly, the earthquake may have had an impact on some rats more than others, resulting in varied responses across groups when tested for anxiety. A third explanation is simply that differing strengths of EMP+ have a selective non-linear effect on startle amplitudes in a normal rat population. Specifically, during adolescence (PND 52-53), a diet strength of 1.25% EMP+ results in significantly lower startle amplitudes than controls, with amplitudes increasing for those on the 2.5%, and resulting in the lowest startle amplitudes in those rats on the 5% diet. A similar pattern resulted in the males during mid adulthood (PND 116-117), with highest startle amplitudes in the 2.5% diet group instead of controls.

Several sex differences were found for the aggression measures (see Table 9 for further references to sex differences in aggression measures). Females performed anogenital contact and allogrooming significantly more often than males. They also performed rough paw more often than males, but this difference was not significant. Males performed a bite attack significantly more often than females, and while their bite attack latency was shorter than the females', it was not significantly shorter. It is thought that male rats are more aggressive than female rats, particularly when male rats are with other male rats, and that male rats engage in more play-fighting behaviour than female rats (Pellis et al., 1996). This is evidenced by the fact that males performed a bite attack significantly more often than the females, which is the most extreme form of aggression measured. However, from these findings it is impossible to conclude that one sex is more aggressive than the other due to the

range of main effects found. Particular aggression-related behaviours may be sex-specific. The literature testing aggression using the R-I paradigm is limited to male subjects, which makes it difficult to speculate further on this.

For the males, most allogrooming occurred in those on the 2.5% diet. The male rats on the 1.25% and 5% diet were relatively similar to the controls. For the females, most allogrooming occurred amongst those on the 1.25% diet, significantly less amongst those on the 2.5% diet (which was completely opposite to the males), whereas the 5% diet group performed allogrooming to the same extent as the controls. While both patterns are not linear, the allogrooming behaviour of each sex basically mirrored each other. It is possible that a lower dose of EMP+, i.e. 1.25%, is more optimal for males because they allogroomed less when on this dose, and that a slightly higher dose, i.e. 2.5%, is optimal for females because they performed allogrooming the least when on this dose. However, this was only found at phase one of testing when rats were PND 52-53, so the result can be generalised no further than this age group. Allogrooming levels were too low at testing phase two (PND 116-117) for both sexes to come to any conclusions. The only other main effect found in a particular sex for an aggression measure was on females' rough paw frequency at testing phase one (see Figure 8). The highest frequency of rough paw occurred in those females at PND 52-53 that were on the 1.25% diet, which was consistent with the allogrooming finding just mentioned. While not significant, bite also occurred most frequently and bite attack latencies were shorter in females at PND 52-53 when on the 1.25% diet, which also suggested increased aggression. Therefore, it seems that a diet consisting of 1.25%, which is the dose suggested to help with more general mental health problems, is not appropriate when aggression is the target problem for females (at least for rats).

Significant interactions involving supplement treatment were also found for most of the aggression measures. As described earlier, significant interactions between sex and

treatment were found for allogrooming and rough paw, suggesting that the supplement worked differently for each sex. Additionally, there was a significant interaction effect between sex and phase for bite frequency, and a significant interaction between sex, treatment and phase for allogrooming and latency to bite. The interpretation of these interactions is difficult due to the low frequency of responding at the second testing age, the non-linear pattern of responding with increasing diet concentration, and any individual differences present in each treatment group. Furthermore, any interpretation of these is complicated by the impact that the earthquake likely had on responding.

It is worth noting that some studies using EMP+ in human populations have not found sex differences, suggesting that both males and females benefit from the supplement. A database analysis using self-report data from 120 males and 238 females with a diagnosis of bipolar disorder showed a 41% reduction of symptoms from baseline after three months of consuming EMP+ (Gately and Kaplan, 2009). This decrease in symptoms was significant for both males and females. An unpublished study looking at the effects of the 7.1 magnitude September 2010 earthquake on anxiety in a normal healthy population found beneficial effects of EMP+ on anxiety in males and females (Julia Rucklidge, personal communication, October 17, 2011). Additionally, Schlebusch and colleagues (2000) found significant reductions in stress in a large population of males and females who consumed Berocca, showing that both sexes benefit from natural supplementation. These findings are in opposition to the finding of the current study that female rats' anxiety increase with higher EMP+ concentrations, particularly after an earthquake. However, many factors need to be taken into consideration when interpreting the results of the current study, such as the efficiency of the diet administration, lack of baseline behavioural measurements, and low sample sizes. These issues are covered in more detail in the limitations section of this study.

6.1.2 Testing Age Differences

Significant testing age differences were found for the majority of the tests. For anxiety, startle amplitudes were higher when the rats were older (PND 116-117) than when they were younger (PND 52-53). This is inconsistent with the idea that rats' startle amplitudes decrease over time due to habituation as a result of repeated exposure to the startle apparatus (Koch, 1999). It is likely that the magnitude 7.1 September earthquake and the repeated exposure to frequent aftershocks had a prolonged effect on the rats' anxiety levels, producing higher amplitudes at the second testing time. This observation was consistent with the idea that a more anxious rat will produce higher startle amplitudes (Koch, 1999). There was not a significant interaction effect of testing age with treatment. Each sex's pattern of responding remained the same from the first testing phase to the second, but was elevated during the second. This is consistent with the hypothesis that the effects of EMP+ would become more pronounced over time, although it was expected that anxiety would be reduced in both sexes for all treatment groups compared to controls, not increased.

All aggression-related behaviours decreased significantly in frequency from testing phase one (PND 52-53) to testing phase two (PND 116-117) apart from anogenital contact which remained basically unchanged. This is not necessarily a result of continued exposure to the EMP+ diet, but may be due to decreased activity of rats as they get older (Scimonelli et al., 1999), and an increased tendency to engage in hyperactive play behaviours in early adolescence compared to older rats (Spear and Brake, 1983). There were no significant interaction effects of testing age with concentration for any of the five aggression behaviours. Therefore, similar to that of anxiety, the effect of diet strength on aggression was consistent over time.

On average, rats were significantly more impulsive during late adulthood (PND127-130) than early adulthood (PND 92-96). This is in contrast with the idea that impulsivity is at

its highest during early adolescence and declines steadily from the age of 10 onwards (Steinberg et al., 2008). It is unlikely that the earthquake caused an increase in impulsivity during the second testing phase due to the length of time since the earthquake that this was carried out. By the second testing phase, the rats had been on the supplement for approximately 100 days, so it is possible that taking EMP+ for an extended period of time increases the likelihood of impulsivity. It is also possible that after prolonged consumption of the EMP+ and pellet mix, the rats found the chocolate chips particularly satiating by the second phase of testing. The rats had also learned from testing at phase one that their food source would not return to free feeding until the end of testing, so they were more inclined to rush for the smaller reward, resulting in what looked like higher impulsivity when it may have been more of a "binge-eating" mentality due to the unreliability of their food source during testing. The effect that diet strength had on impulsivity over time was consistent, as evidenced by the lack of a significant interaction effect between testing age and treatment.

6.2 Relationship to Previous Findings

There is no published literature on the effects of EMP+ on any types of behaviour in a rat population so there are no directly relevant studies to compare the results of the current study to. Even if there were, the occurrence of the 7.1 magnitude earthquake would make any comparisons difficult because there is no literature on how rats behave after earthquakes when on EMP+. However, it is known how a human population with ADHD behaves when on EMP+ after a large earthquake. The Christchurch 2011 7.1 magnitude earthquake provided a unique opportunity for an investigation into its effects in a population of adults with ADHD taking EMP+ in Christchurch at the time. Baseline measures of anxiety had already been collected before the earthquake and it was found that two weeks (but not one week) post-quake, the group taking EMP+ reported significantly less anxiety than controls

(Rucklidge et al., 2011). Therefore, it would be expected that rats on the supplement would have responses lower than those of controls. Surprisingly, this was not the case for the female rats whose control group produced the lowest startle amplitudes while the 5% diet groups startle amplitudes were particularly high.

The studies assessing the effects of EMP+ in human populations that have a disorder have proven promising. Specifically, EMP+ successfully reduced symptoms associated with these disorders, including anxiety, aggression, and impulsivity. Similarly, there were some instances in the current study where EMP+ produced desirable effects on the rats' anxiety, aggression, and impulsivity responses. Males benefited significantly more than females when it came to anxiety, which was illustrated by their decreased startle amplitudes when on the supplement compared to the increased startle amplitudes of the females. While the populations of studies assessing the effects of EMP+ in humans with disorders were often made up of males and females, no reference to sex differences in outcomes have been made, and it is not possible to identify the sex of the participants and their particular outcomes from the articles. Therefore, even though desirable effects were found in the studies on humans, no conclusions can be made about the efficacy of the supplement for each sex. This makes it difficult to discuss and support the finding of the current study that females' anxiety levels did not benefit from the supplement.

When each sex was looked at separately, significant treatment effects for some of the aggression behaviours were found. Females performed rough paw less often than controls when on higher doses of the supplement during the first testing phase (PND 52-53), suggesting decreased aggression. Conversely, males were quicker to perform a bite attack when on any strength of diet at the first testing phase (PND 52-53) compared to controls, with those in the 2.5% supplement group performing a bite attack significantly faster than the control group, which suggests increased aggression as a result of taking the supplement.

Again, the consistency of this finding with previous research cannot be commented on because studies that found beneficial effects of EMP+ on aggression measures in human populations did not make reference to sex differences.

There are no studies on the effects of EMP+, let alone any of its ingredients, on impulsivity in a rat population. Studies of human populations have consistently shown improvements in impulsivity after taking EMP+. However, the current study found no main effect of treatment for impulsivity.

It is possible to compare the results of the current study to previous research looking at the effects of single nutritional elements of EMP+ on anxiety, aggression, or impulsivity. The current study found no significant main effect of treatment on anxiety. Similarly, Dopheide and Morgan (2008) found no change in anxiety levels after administration of vitamin A. However, other studies do not support this finding. Hughes and colleagues (2011) found a decrease in anxiety after vitamin C and E consumption, while another study found increased anxiety in rats after vitamin E consumption (Kolosova et al., 2006), suggesting that the effects on anxiety are dependent on the vitamin studied. This has important implications for the use of EMP+ because it shows that not all of its ingredients are necessarily beneficial for reducing anxiety.

The literature looking at the effects of specific vitamins and minerals on aggressive behaviour indicates decreased aggression in most cases. However, the current study found mixed effects on aggression, with some aggressive behaviour increasing and some decreasing in frequency. It is important to remember that the current study evaluated a multi-ingredient formula, and that results from single nutrient studies are not necessarily indicative of what their effects will be when combined.

7.0 General Discussion

Up until this point, any discussion has been specifically based on the results of the current study and their relationship to previous results. This section aims to evaluate the current study with regards to its quality, and to discuss the implications of this study.

7.1 Methodological Strengths

Firstly, as clearly stated in section 3.4 Rationale for Behavioural Measures, the validity and reliability of the three measures used were of a high standard. Several methods for testing anxiety, aggression, and impulsivity were considered, and the most suitable were chosen for this study. Consequently, there can be confidence in the methods used.

Another major strength of this study was the use of rats as subjects. The use of rats allowed for control over the experimental conditions of the study, reducing the confounding variables associated with testing humans. Therefore, substance abuse, missed appointments, and not taking the supplement were not an issue for this study. All of the rats' experiences were the same because they were handled, weighed, treated, and tested in the same manner. Consequently, significant results could be attributed to the effects of the supplement with a higher degree of confidence. An additional advantage of using rats is their faster rate of development compared to humans. Therefore, time differences could be assessed more efficiently and the implications of the results found can be applied to humans before clinical trial results are available. While direct extrapolation of results to humans should be done with caution, animal models have been proven useful in predicting responses in humans.

The use of a control group and two testing phases allowed for a more in-depth investigation into whether the supplement was responsible for the effects on the rats' behaviour in each experimental group. Having a control group meant that comparisons could be made between the groups on the supplement and a group that has never taken the

supplement. Differences in behaviour over time due to age would show up in the control group, which could be taken into account when drawing conclusions about changes in treatment groups. If the behaviour of the control group did not change over time, then it was more likely that any changes over time found in treatment groups were solely attributable to the treatment. This is the ideal scenario, but the impact of the earthquake further confounded any interpretation of treatment effects, particularly over time.

It is quite common for experiments to use only male rats as subjects. The use of female as well as male rats in this study allowed for investigation of the different effects of the supplement on each sex. This proved beneficial because a number of sex differences and interactions with treatment were found. This is important because the supplement is intended for use in both sexes in a human population. Females are fundamentally different from males and it is just as vital to know whether the supplement works for or produces any harmful effects in females.

Finally, but of no less importance, this study is the first of its kind. No studies have looked at the effects of EMP+ in a normal rat population. This study was able to provide important preliminary findings to guide future research. Furthermore, the identification of any limitations as a result of the current study will help improve the quality of future research.

7.2 Methodological Limitations

The most disappointing and disrupting limitation of this study was the 7.1 magnitude earthquake that struck during the middle of testing (see Figure 3). This produced changes in the rats' behaviour that confounded the results of the current study. As a result of the earthquake, data from cohort four was removed from analyses due to significantly higher startle responses that were measured soon after the earthquake resulting in smaller sample

sizes (see Table 7) and reduced strength and power of the results. It is also difficult to interpret the findings at the second testing time because by this stage, all rats had been exposed to several frequent large aftershocks. Therefore, it is hard to be sure whether significant effects found over time were due to testing age differences, the supplement, or the earthquake itself. The most obvious effect of the earthquake was the resulting significant and unexpected increase in startle amplitudes. It is likely that the aggression and impulsivity findings were affected similarly. It is difficult to evaluate the extent of the effects of the earthquake on these behaviours due to the uncontrolled nature of its occurrence and the abundance of aftershocks following the initial earthquake.

Rats in each experimental group were not tested on each measure before going on the supplement. Therefore, there are no baseline results for each experimental group. It is possible that the cohorts were significantly different to each other on measures of anxiety, aggression, and impulsivity before going on the supplement due to individual differences in the characteristics of each cohort. This may explain strange results due to characteristics of the groups that were not established before the rats went on the supplement such as the non-linear pattern of allogrooming frequency by males and females. Establishing baseline measures of anxiety, aggression, and impulsivity would have allowed for the control of individual differences in each cohort. Additionally, time constraints meant that female rats were not tested for impulsivity. Therefore, comparisons between sexes for impulsivity were not able to be made.

It was difficult to measure how much each rat ate because they were housed in cages of two to four rats, and they threw their food around. Therefore, it was difficult to establish whether each rat was getting the appropriate amount of EMP+. Housing the rats in their own cage and providing the EMP+ in their water supply would have allowed for closer monitoring of their EMP+ consumption.

Finally, there was little control over the number of rats that were supplied in each cohort. Newborn rats were available bi-weekly, and numbers were dependent on how many were born and how much demand there was from other projects. Therefore, it was not possible to gain a representative number of each sex from all four diet concentrations in each cohort. To allow for this, it would have been necessary to receive 24 rats each time, with half being male and half being female, and three of each sex being allocated to each of the four diet concentrations. Doing so would have allowed for the inclusion of the data from cohort four that was excluded from the current study because the cohort would have been an average representation of the other cohorts i.e., each sex and each diet group would have been affected similarly and any effects of the earthquake could have been averaged across all treatment groups.

7.3 Implications

This is the first study known to assess the effects of a multinutrient supplement on rat behaviour. The lack of available data makes the results of this study important. They add to the body of research that isn't particularly straightforward, and currently only looks at single vitamins and minerals on rat behaviour. This research is a valuable stepping stone because it has helped identify gaps in the literature that need addressing, and has highlighted some important considerations that need attention when planning future research. The findings of the current study are important due to the quickening pace of the use of EMP+ in a human population. They have highlighted a need to establish a complete understanding of the effects of EMP+.

The most important implication was the finding that each sex exhibited significantly different anxiety and aggression behaviours when on the supplement. This has important clinical implications because the results suggest that the use of EMP+ is less suitable for

females due to heightened anxiety responses. It is unfortunate that several of the published studies on the effects of EMP+ in populations of humans that have disorders do not put much emphasis on sex differences, even though both males and females have been tested. It is therefore difficult to tell how much each sex benefited from the supplement compared to the other sex in these studies. However, as mentioned earlier, studies have found benefits of EMP+ in humans with bipolar disorder and on anxiety in a normal population in both males and females, suggesting that the supplement is beneficial for both sexes.

A few other negative effects of EMP+ on behaviour were found in the current study in addition to increased anxiety in females, as evidenced by increased aggression in some cases. Specifically, there was a near significant decreasing trend of bite attack latency with increasing diet strength for adolescent males (PND 52-53), suggesting they were quicker to perform a bite attack when on higher concentrations of EMP+. Additionally, males on a diet of 2.5% EMP+ performed allogrooming significantly more often than the control group during adolescence, again suggesting increased aggression as a result of taking the supplement. It is important to remember that the current study was affected by a severe earthquake during testing, and that increases in any of the behaviours measured may be a result of this. However, negative results were still found and it is necessary to see whether or not future research will replicate these. This could be important, particularly when EMP+ is given to a human population.

Overall, it is likely that a diet strength of 1.25% is most suitable for a population without a mental health disorder. Apart from the high frequency of the female rat's rough paw and allogrooming at testing phase one when on the 1.25% diet, behaviour when on this dose strength was in the most part reduced compared to other diet strengths. In contrast, startle amplitudes were notably higher when on the 2.5% diet strength for males and the 5% diet strength for females. Additionally, males consistently performed rough paw,

allogrooming, and a bite attack more frequently when on the 2.5% diet than any other diet strength, and also performed these behaviours more frequently when on the 5% diet than the 1.25% diet. They were also quicker to perform a bite attack when on the 2.5% diet at testing phase one. Therefore, a diet strength of 2.5% (15 capsules a day) and 5% (30 capsules per day) may not be suitable for a human population that does not have a disorder. Further research is necessary to confirm this.

8.0 Future Directions

This was the first study of its kind, so there is a definite need for future research. Most importantly, although not under our control, future research needs to take place in the absence of earthquakes. The best research is research that has minimal confounding variables affecting the outcomes of the study. The effect of earthquakes on those taking the supplement is a whole other avenue of research, and is not at the forefront of the current research at this stage.

The limitations of this study need to be addressed in future research. It is clearly important to test both sexes for all measures because it is apparent that EMP+ produces different effects on anxiety and aggression in male and female rats. Sex differences would probably have been found in impulsivity also, and testing both sexes would have shown the direction of these differences. Future research should ensure that baseline data are gathered, and that cohorts are representative of the whole population, to avoid the problems encountered by the current study.

If similar studies are to be carried out again, it would be appropriate to mix the EMP+ into the rats' drinking water to solve the problem of food being thrown around the cage. Additionally, each rat should be provided with their own drinking water. This can be achieved by having two rats in each cage separated by a mesh barrier to still allow for socialisation. Doing both of these would have allowed for direct measurement of the amount of EMP+ consumed by each rat in the current study. However, this method still involves self-administered consumption so, while the water would contain either 1%, 1.25%, 2.5% or 5% of EMP+, each rat will not necessarily receive the same amount of EMP+ as another rat in the same diet group. Injection of the EMP+ would allow for further control over administration, meaning that each rat could receive the exact amount of EMP+ as each other rat in its diet group on a regular basis. It would be necessary to convert the EMP+ powder

into a substance that can be injected. Also, since the research on supplementation using EMP+ is based on there being an underlying nutritional deficiency, it would be worthwhile including pre- and post-supplementation blood tests to check if any deficiencies are ameliorated, in addition to symptom assessments. It is likely that all of these ideas would have been considered if time had allowed for it. Future research would need to be planned well ahead to ensure that all of these limitations are considered and addressed. Additionally, larger sample sizes allow for larger power and therefore confidence in results. Larger samples also act as a defense against having to eliminate data, and would have been beneficial in the current study due to lost data as a result of the earthquakes.

There is scope for assessing the effects of EMP+ on a range of other behaviours such as sleeping problems or low mood, whether the population has a disorder or not. Also, even though EMP+ has already been tested in people with disorders such as ADHD, OCD, and bipolar disorder, it would be suitable to test the supplement in a "disordered" rat population such as the SHR, which is an animal model of ADHD. As explained previously, rat studies provide controlled results in which we can have more confidence in than those of human studies.

Gathering inter-observer reliability data for the aggression measures should be considered because if reliability is found to be high, more confidence could be placed in the results found. This was not done in the current study, and the descriptions and recordings of the aggression measures may have been subject to bias. However, it was ensured that all behaviours were defined appropriately because they were based on previous valid and reliable definitions. It was not possible to gather inter-observer reliability data for the startle response or the impulsivity T-maze test because there were no subjective influences in these paradigms.

A large body of research should be aimed at establishing the effects of each individual ingredient of EMP+ on the behaviours it is tested on because this research is incomplete, particularly in a rat population. Also, identification of the mechanisms of action of the ingredients of EMP+ and how they specifically affect behaviours such as anxiety, impulsivity, and aggression is paramount. While this will be particularly difficult, doing so will allow us to move on from speculated hypothesis to a more in depth and confident understanding of exactly how multinutrient supplements such as EMP+ are producing their effects on behaviour.

9.0 Conclusions

Earthquakes aside, there is still more than a glimmer of hope for EMP+. The current study has shown that males and females behave differently on the supplement, and that in some cases it has beneficial effects on anxiety, aggression, but not necessarily impulsivity in a normal healthy rat population. It is unfortunate that the earthquake has made it difficult to come to solid conclusions. With the earthquake in mind, and in light of the evidence from human studies, it would be foolish to dismiss the findings of the current study.

This study is the first of its kind and has highlighted some important limitations that need addressing such as the administration of the supplement, and gathering baseline data to help rule out individual differences in responding. The main thing to get from this research is that the supplement is doing something, and it is necessary for structured research to continue in order to establish exactly what this is.

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Appendix A

AEC Ref: 2010/09R

18 June 2010

Sarah Dymond
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Sarah

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Effects of micronutrients on stress, impulsivity and aggression in a rat population"

Approval has been granted:

(a) for the use of 160 Rattus Norvegicus
(b) for your research project to be undertaken from 18 June 2010 to 30 June 2011. If you require an extension of this period please contact the AEC Secretary.

As part of the 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 collecting 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, whichever 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