

**EFFECTS OF CHRONIC VITAMIN C TREATMENT ON RESPONSIVENESS TO
AN ENVIRONMENTAL STRESSOR.**

**A THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE
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Abstract

To investigate if the chronic administration of ascorbic acid (vitamin C) would exert anxiolytic effects when rats are exposed to a stressor in the form of bright light, 40 female and 40 male PVG /c hooded rats were individually treated with approximately 61, 114, or 160 mg/kg of ascorbic acid in their drinking water. Following treatment, each rat was exposed to a 5-min trial in the open-field (OF), the elevated plus-maze (EPM), and the light-dark box (LDB) either with or without the presence of a bright light stressor. A number of dose-related anxiolytic effects were observed that appeared to depend on the measure of anxiety used. This was evident with higher frequencies of walking in the OF for rats treated with 114 and 160 mg/kg of ascorbic acid. Furthermore, 114 mg/kg of ascorbic acid led to rats spending more time in the light compartment of the LDB, but this was true only for rats in the low stress condition. Interestingly, 61 mg/kg and 114 mg/kg produced a sex-related anxiogenic effect for rats tested in the EPM, with 61 mg/kg leading to a decrease in open arm entries for females, and 114 mg/kg leading to an increase in open arm entries for both sexes combined. Furthermore, male rats in the high stress condition made a higher percentage of entries into the open arms and fewer entries into the closed arms of the EPM. Male rats treated with 114 mg/kg also had lighter adrenal gland weights compared to controls. These results suggest that a dose of 1.45 g/day of vitamin C may have the potential of reducing anxiety in humans under certain conditions. However, vitamin C may not be as effective under high stress conditions but rather may be useful for anxiety experienced when low levels of stress are present.

Chapter 1

Introduction

1.1 Overview

Growing evidence has recently emerged that suggests vitamin C (or ascorbic acid), a powerful water-soluble antioxidant, may have significant benefits in regards to psychological functioning - namely emotionality and cognition. Studies so far have found that ascorbic acid has been shown to decrease neophobia and measures of fear in Japanese quail and broiler chickens (Jones, Saterlee & Moreau, 1996; Satterlee, Jones, & Ryder, 1993), attenuate anxiogenic effects of prolonged exposure to loud noise in mice (Angrini & Leslie, 2012), decrease several forms of anxiety related behaviour in rats (Hughes, Lowther, & van Nobelen, 2011), and enhance recognition memory in a novel object recognition test (Hughes, Hancock, & Thompson, 2015). In humans, ascorbic acid has been found to reduce salivary cortisol, blood pressure, and subjective anxiety responses to a psychological stressor (Brody, Preut, & Shurmeyer, 2002).

Vitamin C possesses antioxidant properties through its function as an electron donor and a broad spectrum radical scavenger (Rice, 2000). It is suggested that it is through this mechanism that the vitamin may exert its anxiolytic effects (Bouayed, Rammal, & Soulimani, 2009). Furthermore, vitamin C has also been identified as a neuromodulator of dopamine and glutamate-mediated transmission (Grunwald, 1993; Rebec and Pierce, 1994; Rice, 2000), which may be a further potential mechanism of action for the vitamin's anxiolytic effects and memory enhancing properties.

The aim of this review is to elaborate on the structure and mechanisms underlying vitamin C and its relation to stress and anxiety while simultaneously exploring some of the research which has established a link between the two. Studies examining vitamin C's potential memory-enhancing effects will also be lightly touched upon. Furthermore, common

procedures used to examine anxiety in the rat will be explored in regards to their efficacy and results, and the use of bright light as a stressor will additionally be noted. Finally, observed sex differences between male and female rodents will also be examined due to the use of both sexes in this thesis.

The purpose of this thesis is to add to the current research surrounding vitamin C's potential anxiolytic effects, and to determine if chronic administration of doses of 61mg/kg – 160mg/kg per day of the vitamin will exert anxiolytic effects when rats are exposed to a stressor in the form of bright light. From here, appropriate doses for humans can be suggested to help achieve similar outcomes.

1.2 Vitamin C

Ascorbic acid (vitamin C) is a water-soluble antioxidant that is involved in a vast array of physiological reactions within the human body and required for normal metabolic functioning (Shahidi, Komaki, Mahmoodi, Atrvash, & Ghodrati, 2008). Vitamin C acts as a cofactor for a number of enzymes involved in the synthesis of collagen, carnitine, and catecholamines (Padh, 1990). Furthermore, vitamin C possesses antioxidant properties due to its action as either an electron donor or a broad spectrum radical scavenger (Rice, 2000). Vitamin C has also been identified as a neuromodulator within the brain, modulating dopamine and glutamate-mediated neurotransmission (Grunewald, 1993; Rebec and Pierce, 1994; Rice, 2000).

1.2.1 Vitamin C and oxidative stress

Oxidative stress occurs when there is an imbalance between the oxidative and anti-oxidative systems in the organism, with this imbalance being in favour of the oxidative systems rather than the latter (Sarandol et al., 2007). Furthermore, oxidative damage occurs through the generation of reactive species, which can occur through the presence of

environmental stressors and normal cell processes. Oxidative stress, whether due to increased production of free radicals or inefficient antioxidant systems, leads to lipid peroxidation, along with damage to cell proteins, and nucleic acids in DNA (Schlueter and Johnson, 2011). Supplementation with vitamin C has been shown to reduce oxidative stress levels, in turn reducing damage to tissues (Halliwell & Whiteman, 1997). Vitamin C fights against the oxidation of lipids, proteins, and DNA, in turn protecting their structure and biological function.

Oxidative stress has been implicated in the pathophysiology of many psychiatric disorders, including anxiety disorders. Some animal studies have linked genes glyoxalase 1 (Glo1) and glutathione reductase 1 (GR), both of which protect against oxidative stress, with anxiety in mice (Hovatta et al., 2005). It was found that the over expression of Glo1 and GR in the cingulate cortex was linked to increased anxiety behaviours in mice, while the inhibition of the Glo1 gene reduced anxiety behaviours. In humans, elevated lipid peroxidation products and antioxidant changes have been found in obsessive-compulsive disorder (Ersan, Bakir, Ersan, & Dogan, 2006), panic disorder (Kuloglu, Atmaca, Tezcan, Ustundag, & Bulut, 2002) and social phobia (Atmaca, Tezcan, Kuloglu, Ustundag, & Tunckol, 2004).

It has also been shown that patients with Generalised Anxiety Disorder (GAD) and depression have significantly lower plasma levels of vitamins C, A, and E in comparison to controls. After 6 weeks of treatment with both vitamin A, C, and E a significant reduction in anxiety and depression scores was observed along with a significant increase in the blood levels of vitamin C and A, but not vitamin E (Gautam, Agrawal, Gautam, Sharma, & Gautam, 2012). When taken together with the antioxidant changes reported in some psychiatric disorders, the above may suggest a link between vitamin C, oxidative stress, and such disorders.

To add to this, a number of studies have demonstrated the potential antioxidant effects of antidepressant medications. Bilici et al. (2001) found that patients who were majorly depressed had higher levels of antioxidant enzyme activities and lipid peroxidation compared to those of healthy controls. After 3 months treatment with selective serotonin reuptake inhibitors (SSRIs), antioxidant enzyme activities and lipid peroxidation levels were significantly decreased to normal levels.

Furthermore, Khanzode, Dakhale, Khanzode, Saoji, and Palasodkar (2003) found that patients with depression showed significant increases in serum superoxide dismutase (SOD) and serum MDA levels, and a significant decrease in plasma ascorbic acid levels compared to controls. After treatment with citalopram and fluoxetine this trend was significantly reversed.

Moreover, Moretti et al. (2013) demonstrated that oral administration of vitamin C or fluoxetine one hour before restraint stress in mice prevented the stress-induced increase on immobility time in the forced swimming test, a marker of depressive behaviour in mice. In addition to this, vitamin C reduced lipid peroxidation to normal levels and restored the activity of superoxide dismutase, glutathione reductase, and glutathione peroxidase.

Despite the above, not all studies have found a link between oxidative damage and antidepressant treatment. Sarandol et al. (2007) found that 6 weeks of antidepressant treatment did not have an impact on antioxidant systems in depressed patients. While evidence does vary, it appears that some link is present between psychiatric illness and the presence of oxidative stress, implicating vitamin C an antioxidant vitamin in the potential treatment of some psychiatric disorders including major depression and anxiety disorders.

1.2.2 Vitamin C and neuromodulation

In addition to its function as an antioxidant, vitamin C is believed to be a neuromodulator of both glutamate and dopamine mediated neurotransmission (Rice, 2000). Dopamine and glutamate have both been found to be implicated in the release of ascorbate

and in turn appear to be regulated by this release (Rebec & Pierce, 1994). Rebec and Pierce (1994) suggest that ascorbic acid in particular brain regions may act as a type of signalling molecule, regulating the postsynaptic efficacy of these neurotransmitters. Behavioural, electrophysical, and pharmacological evidence for ascorbic acid-related modulation of neuronal function currently exists. Ascorbate has been shown to block amphetamine induced turning behaviour in rats with unilateral nigrostriatal lesions (Tolbert, Thomas, Middaugh, & Zemp, 1979). Furthermore, intrastriatal infusions of ascorbate have been shown to attenuate the behavioural response to amphetamine (White, Maurer, Kraft, Oh, & Rebec, 1990).

In addition to the above, systemic administration of ascorbic acid has been shown to change neuronal firing rates during single unit recording, which may be due to the modulation of the N-methyl-D-aspartate (NMDA) receptor function (Majewska, Bell & London, 1990). The modulation of the above neurotransmitters by a readily available vitamin, ascorbic acid, has important clinical implications as dopamine and glutamate play a critical role in severe neurological and psychiatric disorders such as Parkinson's disease, schizophrenia, and anxiety disorders.

1.2.3 Vitamin C concentrations, synthesis and dose

Ascorbic acid is most highly concentrated in the brain, spinal cord and adrenal glands with these areas also holding the greatest retention capacities (Hornig, 1975). In the rat brain, the highest concentrations of ascorbic acid are found in the amygdala, hippocampus, and hypothalamus; while for humans the highest concentrations are found in the nucleus accumbens and hippocampus. Furthermore, in the human brain, the lowest levels of ascorbic acid are found in the substantia nigra, and a gradient of ascorbic acid concentration has been found in some brain nuclei, such as the thalamus (Grunewald, 1992).

There are two Na^+ - dependent vitamin C (ascorbate) transporters SVTC1 and SVTC2. Both facilitate electrogenic Na^+ - dependent uptake of ascorbate with similar

affinities for this substrate. The main difference between the two transporters appears to be their localisation within the body. SVCT1 is present in the kidneys, liver, and lungs while SVCT2 is present in neural, neuroendocrine, exocrine, and endothelial tissues meaning SVCT2 is only found in the brain. Human SVCT2 shares a 95% sequence homology with rat SVCT2 and is also found in the brain (Rice, 2000).

Unlike most animals, humans are unable to synthesise vitamin C, so must obtain it solely from diet. Humans are unable to synthesise vitamin C because they lack the last enzyme in the biosynthetic pathway (Levine, Rumsey, Daruwala, Park, & Wang, 1999). Humans consuming 200-300 mg of vitamin C per day maintain steady-state fasting plasma concentrations of 70 to 80 $\mu\text{mol/L}$ (Levine, Conry-Cantilena, & Wang, 1996).

The recommended daily intake of vitamin C is 90 milligrams per day and no more than 2 grams (2,000 milligrams) per day (Food and Nutrition Board, Institute of Medicine, 2000). Vitamin C has few toxic effects, and any adverse effects experienced are directly related to dose. Diarrhoea and abdominal bloating can occur when several grams are taken at once (Levine et al., 1999). Furthermore at doses of 1000 mg daily, urine acid and oxalate have been found to become elevated. If high doses were administered for longer periods of time it was unclear whether renal calculi would result (Levine et al., 1996). Levine et al., (1996) have therefore suggested that upper safe doses of vitamin C in healthy people are <1000 mg daily.

There seems to be no clear benefit of taking doses >1000 mg daily as no benefit has been found from excess excreted or unabsorbed vitamin C. Furthermore, plasma concentrations appear to reach saturation at 400 mg daily and there is the possibility of adverse effects of doses over 1000 mg daily. Despite this, the National Academy of Sciences sets the tolerable upper limit of vitamin C intake at 2000 mg/d and cited that the main concern in regards to adverse effects was osmotic diarrhoea (Schlueter & Johnston, 2011).

1.2.4 Vitamin C, stress and the adrenal glands

Due to vitamin C being highly concentrated in the adrenal glands, with concentrations as high as 10 mmol/L, vitamin C and stress have long been associated. Furthermore, an increase in the concentration of vitamin C in human plasma has been related to corticotrophin (Stewart, Horn, & Robson, 1953). The pituitary gland, which coordinates the physiologic response to stress, secretes tropic hormones in response to central nervous system input from the hypothalamus. Adrenocorticotrophic hormone (ACTH) is secreted by the pituitary glands and acts to stimulate the adrenal glands aiding in the synthesis and secretion of cortisol. In animals, ACTH also causes the loss of vitamin C from the adrenal glands (Padayatty et al., 2007). While direct evidence is limited to support the association between stress and vitamin C, a study by Padayatty et al. (2007) found that stimulation increased adrenal vein vitamin C concentrations, suggesting that adrenal vitamin C secretion in humans is an integral part of the stress response.

Vitamin C is implicated in the functioning of many systems within the human body including enzyme activation, oxidative stress reduction and has even been associated with the neuromodulation of neurotransmitters dopamine and glutamate. As oxidative stress has been implicated in the pathophysiology of a number of psychiatric disorders including anxiety, vitamin C may have potential merit in the treatment of anxiety and stress related disorders.

1.3 Anxiety

Anxiety is described as a negative mood state that is characterised by a number of bodily symptoms including physical tension and apprehension about future events (Barlow, 2002). Fear, a seemingly similar construct to anxiety, is the emotional response to real or perceived imminent threat (American Psychiatric Association, 2013). Evidence supports the notion that fear and anxiety reactions differ psychologically and physiologically (Barlow,

2002; Bouton, 2005; Craske et al., 2010). Anxiety is a future-oriented mood state that is characterised by apprehension around the inability to predict or control upcoming events. In contrast, fear is an immediate emotional reaction to current danger that is characterised by strong escapist action tendencies and often an increase in the sympathetic nervous system (Barlow, Brown & Craske, 1994). While anxiety and fear responses are normal and adaptive to certain degrees, in humans anxiety becomes maladaptive when the anxiety and fear response becomes excessive and leads to related behavioural disturbances (American Psychiatric Association, 2013). Excessive anxiety and the behavioural disturbances that follow can manifest in a number of different ways, presenting as various different psychological disorders.

One of the most common anxiety disorders is Generalized Anxiety Disorder (GAD) with a 12-month prevalence rate of 2.9% in the general adult US population and prevalence rates in other countries ranging from 0.4% to 3.6% (American Psychiatric Association, 2013). GAD is characterised by excessive anxiety and worry about everyday life circumstances, which is difficult to control. This excessive anxiety and worry is also accompanied by a number of other symptoms, which include muscle tension, restlessness, being easily fatigued, difficulty concentrating, disturbances in sleep, and irritability (American Psychiatric Association, 2013). In regards to differences in the prevalence of anxiety disorders between the two sexes, it appears that females receive the majority of the diagnoses for specific phobias and that more women than men suffer from social anxiety. Furthermore, epidemiological studies have shown that twice as many females suffer from generalized anxiety disorder compared to males (American Psychiatric Association, 2013).

1.3.1 Anxiety in the rat

According to Darwin (as cited in Palanza, 2001) the defensive behaviour of other species can be viewed as evolutionary precursors to human fear and anxiety reactions. For

this reason, the fear-like reactions displayed in animals can be viewed as analogous to the anxiety-related behaviours in humans (Palanza, 2001). From here it may be suggested that human and animal responses share common substrates. This suggests that in animal models of anxiety, the response being observed shows clinical significance for the disorder being modelled (Palanza, 2001).

Palanza (2001) suggests that human anxiety disorders can be considered disorders of defence as they involve the inappropriate activation of defensive behaviour arising from the erroneous assessment of danger. In human anxiety, behavioural disturbances commonly occur. These include: avoidance, escape, non-verbal vocalisation, and/or hypervigilance. For animals, when anxiety is experienced, similar behavioural disturbances can be observed. An example of this is when animals are exposed to unfamiliar environments. At the beginning of such an exposure the animal may display behaviours such as inhibition of exploratory behaviour, freezing, flight, risk assessment, increases in heart rate, urination, defecation, and an increase in plasma corticosterone levels (Blanchard, Blanchard, & Rodgers, 1991; Rodgers, Cao, Dalvi, & Holmes, 1997). From here, Palanza (2001) suggests that these reactions may be interpreted as the activation of the animal's defensive system, which occurs when the animal is placed in potentially dangerous situations.

1.3.2 Stress

Stress, anxiety and depression are commonly believed to be interrelated phenomena. It has been suggested that stress is implicated in the etiology of both depressive and anxiety disorders, or as a consequence. Animal models of anxiety and depression are commonly based on the exposure of animals to a stressful condition (Palanza, 2001).

Stress can be defined as an imbalance in physiological systems that activate both physiological and behavioural responses to restore balance (Buckworth & Dishman, 2002). Stress is provoked by stressors, the human body responds to stressors by the activation of the

central nervous system and the release of specific hormones. One of these hormones is ACTH which is secreted by the pituitary gland that in turn stimulates the adrenal glands to synthesize and secrete cortisol. ACTH also causes the loss of vitamin C from the adrenal glands (Padayatta, 2007). Depletion of vitamin C from the adrenal glands has been associated with increased secretion of corticosteroids and it has been suggested that this is due to an inhibitory effect of ascorbic acid on the hydroxylations involved in the biosynthesis of steroid hormones (Bjorkhem, Kallner & Karlmar, 1978).

1.3.3 Current treatments for anxiety

Anxiety disorders are commonly treated with anxiolytic medications including Selective Serotonin Reuptake Inhibitors (SSRIs) and benzodiazepines. SSRIs work by preventing the reuptake of serotonin from the synapse, allowing the neurotransmitter to remain in the area of activity for longer. Similarly, benzodiazepines stimulate GABA receptors, mimicking the calming effects of the neurotransmitter and thus reducing anxiety. While anxiolytic medication is useful in the treatment of anxiety; many negative side effects can be experienced. These side effects can include: addiction, depression, suicide, seizures, sexual dysfunction, headaches and more. Furthermore, the brain often becomes accustomed to these anxiolytic medications resulting in the medication losing its effectiveness and higher doses or different drugs being required (Alramadhan, 2012). In contrast to the above, nutrients such as vitamin C have also been found useful in the treatment of anxiety, with the absence of many of the side effects experienced with anxiolytic medications, potentially offering a much safer alternative.

1.4 Vitamin C and anxiety

A number of studies have begun to display a link between vitamin C and psychological functioning. Such a link has suggested that chronic treatment with vitamin C

may have significant benefits in terms of emotionality and cognition. These benefits are suggested to be derived from the vitamin's potent antioxidant properties.

One such study was conducted by Hughes et al. (2011), which found that 90 days of exposure to a single dose of vitamin C (approximately 80mg/kg/day) in drinking water markedly reduced anxiety in rats. Following this research, a dose-response study was conducted where anxiolytic effects of vitamin C were also evident with ascorbic acid treatment (Hughes et al., 2015). These effects however depended on the measure of anxiety and were only displayed in higher frequencies of walking (with doses of 114mg/kg and 160mg/kg), higher frequencies of rearing (with 61mg/kg) in the open-field, and more frequent occupation of the open arms in the elevated plus-maze (61mg/kg).

Further animal studies have shown that broiler chickens, treated with 1200 ppm of vitamin C in their drinking water, displayed a reduction in the stress response to slaughter preparation in comparison to controls (Saterlee, Aguilera-Quintana, Munn, & Krautmann, 1989). Vitamin C supplementation has also been shown to reduce underlying fearfulness in Japanese quail. Individually-housed Japanese quail treated with vitamin C showed less home-cage avoidance of a novel object and shorter tonic immobility fear reactions compared to controls (Jones et al., 1996). Furthermore, Japanese quail chicks, genetically selected for high stress, were treated with either 1200 ppm of vitamin C or untreated drinking water and exposed to a stressful cooping procedure. Vitamin C supplementation significantly attenuated the tonic immobility fear reactions of stressed and unstressed quail (Saterlee et al., 1993).

It is also apparent that vitamin C is able to reduce underlying fearfulness in Japanese quail despite genetic differences (Jones, Saterlee, & Cadd, 1999). In this study, a decrease in latency to emerge from a sheltered compartment (into a light compartment) in Japanese quail treated with vitamin C was found. Quail were from two genetic lines selected for low or high adrenocortical responses to mechanical restraint. As the vitamin C effect was common in

quail of both genetic lines, this study found that a decrease in fearfulness was evident regardless of genetic differences in this particular behavioural trait.

Choi et al. (2011) investigated whether vitamin C influences the stress response system of the adrenal gland. Adrenalectomized rats and non-adrenalectomized rats were supplemented with vitamin C and subjected to electroshock stress for 5 days. Vitamin C supplementation was found to decrease corticosterone levels in non-adrenalectomized rats. Stress decreased the mean level of rearing in both groups of rats; however, vitamin C supplementation only partially enhanced rearing in the non-adrenalectomized rats. Furthermore, vitamin C decreased mean ACTH levels in both groups, and significantly decreased freezing time augmented by stress. The results of this study suggests that the vitamin C effect on stress-related rearing may be exerted via modulation of corticosterone, while its effect on freezing behaviour may act via modulation of ACTH.

Further in regards to stress, another study by Choi et al. (2010) sought to determine whether or not vitamin C affects the stress response in mice. The stressors used in this study included electroshock and restraint stress. They found that supplementation with 25 and 100 mg/kg of vitamin C partially blocked stress-related behaviours such as freezing, sniffing, burrowing, face washing, and grooming. Vitamin C also acted to increase staying time in the closed arms of the elevated plus-maze. Stress responses that were induced by electroshock (but not restraint stress) were also reduced by vitamin C administration, with 25mg/kg and 100 mg/kg decreasing corticosterone levels that were increased by electroshock. Additionally, 25 mg/kg of vitamin C increased crossing frequency to drink water in an electrified field after mice were deprived of water for 24 hours. This suggests that vitamin C supplementation reduced anxiety or fear.

Along a similar vein to Choi et al. (2010), Angrini and Leslie (2012) used noise as a stressor when testing vitamin C's effect on the resulting physiological and behavioural

changes induced by such a stressor. Mice were exposed to 100Db of noise for 2 months, 5 days a week, 4 hours daily. Prior to the noise exposure mice were pre-treated with 100 mg/kg or 200 mg/kg of vitamin C. The anxiogenic and behavioural effects of noise exposure were explored using the open-field and elevated plus-maze, and by measuring the neutrophils-to-lymphocytes ratio (which is a physiological stress measure).

The study found that in the open-field, noise increased grooming, defecation and immobility time, while both doses of vitamin C reduced the effects of noise on grooming and defecation. Furthermore, noise was shown to increase immobility time; however, vitamin C did not appear to reduce this effect. Prior administration of the higher dose of vitamin C also reduced the effects of noise on the peripheral and central movements. In the elevated plus-maze, mice exposed to noise spent significantly more time in the closed arms in comparison to controls. Both doses of vitamin C decreased time spent in the closed arms, which is analogous to a reduction in anxious behaviour. The results from the open-field and elevated plus-maze suggest that vitamin C can reduce the anxiogenic effects of noise exposure in mice.

As a number of anxiety disorders and Major Depressive Disorder are highly comorbid (American Psychiatric Association, 2013) it is important to note vitamin C's potential antidepressant effects. Vitamin C has been shown to have an antidepressant like effect in animals, and reactive oxygen species (ROS) has been shown to play a role in the pathophysiology of depression. Studies have suggested that oxidative stress may contribute to the neuropathology of neurological and psychiatric diseases (Ng, Berk, Dean, & Bush, 2008). In addition, studies have shown that chronic stress can have a substantial impact on ROS in the brain, which in turn leads to oxidative damage in the central nervous system (Fontella et al., 2005; Lucca et al., 2009; Madrigal et al., 2001).

As vitamin C is an antioxidant showing antidepressant like effects in animals, Moretti et al. (2012) set out to explore the effects of vitamin C on depressive like behaviour induced by chronic stress, along with corticosterone levels and markers of oxidative stress in the cerebral cortex and hippocampus of mice. They found a significant reduction in depressive-like behaviours in stressed mice treated with ascorbic acid or fluoxetine. Furthermore, ascorbic acid and fluoxetine both promoted beneficial effects against stress induced changes in oxidative stress-related parameters in the mouse brain. From here the authors of the study concluded that vitamin C may be an alternative approach for the management of depressive symptoms.

While the studies on the effects of vitamin C on anxiety and stress responses in humans appears to be quite scant, two studies conducted by Brody et al. (2002) and Brody (2002) have produced significant effects in terms of mood and physiological responses to a stressor with the administration of vitamin C. In a study of human subjects by Brody et al. (2002), two 500 mg sustained release vitamin C capsules four times a day for 14 days, has been shown to lower blood pressure significantly in people subjected to a stressful situation (public speaking). The amount of vitamin C found in the blood of the subjects receiving vitamin C supplementation was higher than what would be expected from diet alone. Reported stress and anxiety after the stressful situation was also significantly lower in the vitamin C group compared to controls. Furthermore, a study by Gautam et al. (2012) found that patients with GAD had significantly lower levels of vitamin C compared to controls. After 6 weeks of vitamin C supplementation, a significant reduction in anxiety scores of patients was observed. These results suggest that vitamin C supplement therapy is useful in patients with stress-induced psychiatric disorders.

The second study by Brody (2002) explored the effect of vitamin C on intercourse frequency and mood in human subjects. Participants receiving 3000 mg/day of ascorbic acid

supplementation reported greater frequency of penile-vaginal sexual intercourse (FSI) and also experienced a decrease in Beck Depression Scores. Within their study they found that women appeared to be more responsive to ascorbic acid supplementation compared to men in regards to an increase in FSI. While they suggest it is possible that woman may have benefited differently to men due to a sex difference in receptor sensitivity, it is more likely that sociobiological factors were at work; wherein young women who experience an increase in sexual motivation may be more rapidly able to fulfil their desires compared to young men (Brody, 1997).

1.5 Vitamin C and memory

In addition to vitamin C's apparent anxiolytic effects, some research has also suggested a potential memory-enhancing effect of the vitamin. Furthermore, it is suggested that similar mechanisms may be at play in terms of the vitamin's anxiolytic and memory-enhancing properties, namely its antioxidant effects and status as a neuromodulator. Arzi, Hemmati and Razian (2004) looked at the role of vitamins C and E on the cognitive function of young (aged 3 months) and aged mice (aged 15 months). Young and aged mice received either 250 mg/kg of vitamin E, 300 mg/kg of vitamin C or a combination of both. It was found that, in aged mice only, vitamin E alone or in combination with vitamin C compared to the control group caused significant improvement in memory (as measured by a passive avoidance task). As the aged mice that only received vitamin C did not show significant improvements in memory compared to the control group, but did when this was paired with vitamin E, it was suggested that it may be due to vitamin C's ability to regenerate α -tocopherol from tocopherol radical.

Parle and Dhingra (2003) have found that 60 and 120 mg/kg of ascorbic acid injected for either 3 or 8 consecutive days into aged mice improved their learning and memory, as

displayed by transfer-latency in the elevated plus-maze and increased step-down latency in the passive-avoidance test. In addition to these findings, it was also shown that ascorbic acid acted to protect against scopolamine-and diazepam-induced impairment of memory in young mice.

Studies have also explored the effects of ascorbic acid on spatial memory in the mouse. For instance, a study by Kennard and Harrison (2014) showed that a single intravenous treatment of ascorbic acid reversed short-term age-related spatial memory deficits in 9 month old (middle aged) mice. However, no such effect was found in 20 month old (old) mice.

Furthermore, Harrison, Hosseini, Daws, Weaver, and May (2009) have previously shown that scopolamine-induced spatial learning deficits (shown by Morris water maze performance) were partially attenuated when young mice were administered 125mg/kg of ascorbate intraperitoneally 1 hour before testing. They suggest that, as ascorbate was effective against the amnesic effects of scopolamine, it is likely that cholinergic function was modulated in some way. It is possible that an overall upregulation of cholinergic function, including AChE, took place which resulted from 14 days of ascorbate treatment. Furthermore, Hughes et al. (2015) recently found that rats treated with 160 mg/kg of ascorbic acid explored a novel versus familiar object in the open-field novel recognition test (NOR) to a significantly greater extent than controls, suggesting an enhancement of recognition memory.

Human studies have also been conducted exploring the association between vitamin C and cognitive impairment. A study conducted by Paleologos, Cummin and Lazarus (1998) found that supplementation with vitamin C (assessed four years before assessment of cognitive function) was associated with a lower prevalence of severe cognitive impairment (based on scores on the Mini-Mental State Examination). It is suggested that vitamin C's

association with a decreased prevalence in cognitive impairment may be due to the vitamin's antioxidant effects. Antioxidant vitamins protect against free radical oxidation and free radicals have been implicated in the etiology of Alzheimer's disease and vascular dementia. This suggests that vitamin C having a protective effect against cognitive impairment is biologically plausible. While ascorbic acid's memory-enhancing effects, like its anxiolytic effects, have been related to its antioxidant properties (Castagne, Rougemont, Cuenod, & Do, 2004), it is also possible that the vitamin's function as a neuromodulator of brain neurotransmitters (such as dopamine, acetylcholine, glutamate and GABA) is also implicated in its memory-enhancing effect (Harrison et al. 2009).

In light of the above review, it appears that an increasing amount of evidence has surfaced to suggest that vitamin C may exert anxiolytic and memory-enhancing effects through its antioxidant properties and status as a neuromodulator. Thus far studies have displayed vitamin C's ability to affect indices of fear in Japanese quail and broiler chickens (Satterlee, 1989; Jones, 1996), reduce the anxiolytic effects of stressors such as noise, restraint stress, and electroshock in rodents (Choi, 2010; 2012; Angrini & Leslie, 2012), and lead to lower blood pressure, faster cortisol recovery, and reductions in state anxiety in humans (Brody et al., 2002). Findings have also displayed the vitamin's ability to protect against memory impairment and enhance learning and memory (Parle & Dhingra, 2003).

1.6 Apparatus and sex differences

Common types of apparatus used to assess anxiety in rodents include the open-field, light-dark box and elevated plus-maze. Furthermore, research (in particular concerning the elevated plus-maze) has suggested that bright light illumination acts as a stressor, and for this reason is commonly used to elicit an anxiety response in rodents.

1.6.1 The open-field

The open-field test is used to assess emotionality, anxiety, and responses to stressors in the rat. In the open-field test, the animal is placed into the centre of an illuminated arena from which it cannot escape, and for a brief period of time (i.e., 5 minutes) the animal's patterns of ambulation and behaviours such as rearing, grooming, immobility and defecation are observed and recorded (Stout & Weiss, 1993).

Peripheral movements, and immobility are regarded as related to attempts to escape (Archer, 1973), central movements however, are more related to exploratory behaviour. Like central movements, rearing is also considered an exploratory behaviour in some studies (Hine, 1995; Johansson & Ahlenius, 1989), whereas other studies have suggested that anxiolytic agents decrease rearing (Hughes, 1972; Stout & Weiss, 1994).

Grooming increases with fear in rodents, and a number of studies have demonstrated a decrease in grooming behaviour when antianxiety drugs are administered (Barros, Tannhauser, Tannhauser, & Tannhauser, 1994; Dunn, Guild, Kramarcy, & Ware, 1981). Furthermore, defecation is regarded as an indicator of high emotionality (Archer, 1973). However, as Archer (1973) suggests, defecation may be influenced by other factors besides emotionality (e.g., time of last meal).

1.6.2 The light-dark box

The light-dark box test is a test based on a rodent's innate aversion to brightly lit areas and on its spontaneous exploratory behaviour in response to mild stressors (i.e. novel environment and light; Crawley & Goodwin, 1980). When an animal is exposed to an unfamiliar environment or novel object, a natural conflict occurs between the tendency to explore and the initial tendency to avoid the unfamiliar.

From here the exploratory activity reflects the combined results of these tendencies in novel situations. Therefore, in the light-dark box, drug-induced increases in behaviours in the white illuminated part of the two-compartment box (in which a white compartment is

illuminated and a black compartment is darkened) are suggested to reflect anxiolytic activity (Bourin & Hascoet, 2003).

1.6.3 The elevated plus-maze

The elevated plus-maze is a widely used behavioural assay for rodents, which has been validated to display the anxiolytic effects of pharmacological agents (Pellow, Chopin, File, & Briley, 1985). The test consists of a plus-shaped maze that is elevated above floor level. It contains two arms closed in by walls and two opposite open arms. A rat is placed into the centre of the plus-maze and is allowed to explore the apparatus freely. Rats will explore the maze and enter both arms, but will generally prefer to enter the closed arms and remain inside them for longer periods. Due to this, there is a negative correlation between anxiety levels and the exploration of the open arms. From here, the plus-maze can be used to define the anxiolytic or anxiogenic properties of substances or behavioural procedures (Garcia, Cardenas & Morato, 2005).

It has been suggested however, that differing light levels can affect the behaviour of rats tested within the apparatus. Garcia et al. (2005) found that rats tested in the elevated plus-maze under 0 and 1x illumination levels explored the open arms more frequently compared to other levels of light (3, 10, 30, 100 and 300 1x). In contrast, locomotor activity in the closed arms was not influenced by illumination.

It is therefore suggested that vision is important in triggering aversion to the open arms of the elevated plus-maze and that the threshold for such aversion is between 1 and 3 1x of illumination. Furthermore, this suggests that animals tested under low light levels behave as expected of animals with low anxiety, and that animals tested under bright light levels behave as expected of animals with high anxiety, suggesting that bright light acts a stressor.

Further support for bright light's ability to increase anxiety in the rat has been shown in a recent study conducted by Hughes, Hancock, Henwood, and Rapley (2014). Here it was

found that rats tested in the open-field under bright light levels (averaging around 600 lx) had increased anxiety levels as evidenced by longer latencies of emergence into the open-field and decreased ambulation. In addition to this, male rats showed decreased centre occupancy and increased corner occupancy. Females also showed fewer entries into the open arms of the elevated plus-maze under bright light conditions suggesting increases in anxiety also.

Other tests of anxiety frequently use more aversive methods to elicit the desired response, such as noxious stimuli including electric shock to condition fear in the animal and procedures such as food or water deprivation. It is likely that the latter procedures alter the animal's response to drugs and often require additional testing to control for the effects on water and food consumption (Pellow et al., 1985).

1.6.4 Sex differences in the rat

In rodent behavioural research, particularly research exploring drug effects, male rats are commonly used in the absence of females. A survey conducted by Hughes (2007), which explored the use of male and female rats and mice in published behavioural pharmacological studies, found that between 80-90% of studies examined within the survey used male rodents only. The use of both female and male rodents was very rare. Of the studies that did involve the use of both female and male rodents, over half showed sex-dependent drug effects, thereby supporting the use of both male and female rodents in such studies, especially when a reason does not appear to exist to expect a sex-dependent drug effects in the first place.

It appears that one of the most common reasons that is given for the exclusion of female rats and mice from behavioural pharmacological research is the widely held belief that the effects of an experimental manipulation may confound their oestrous cycle events. This is because it is believed that these oestrous cycle events may cause variability between subjects and in turn interfere with the effects of the experimental manipulation (Hughes, 2007). While

studies have shown that such variability does exist between female rats (D'Souza, Harlan, & Garcia, 1999), there are also studies that suggest that there is a lack of oestrous cycle-based fluctuations in activity (Sell, Scalzitti, Thomas, & Cunningham, 2000).

Interestingly, some studies have shown that male rats may in fact have greater inter-subject variability than females (Camp & Robinson, 1988). Furthermore, human males tend to show greater variability compared to women in a number of behavioural and intellectual characteristics (Archer & Mehdikhani, 2003; Feingold, 1992, 1994). It has also been shown that male hormones can interact with drug effects. This has been illustrated by the testosterone-mediated increase in aggression seen in male mice with the administration of ethanol (DeBold & Miczek, 1985).

In regards to the exploration of anxiety responses in rats with the administration of a substance, in this case vitamin C, it would appear that the inclusion of female rodents would be an important factor. This is due to the prevalence of anxiety disorders in the human female population being greater than that of the male population (Cameron & Hill, 1989; Blehar, 1995).

Furthermore, it has been acknowledged that other disorders are more prevalent in men compared to women. Alcoholism, drug abuse, antisocial personality, attention deficit disorders, Tourette's syndrome, and completed suicide are most common in men; whereas women are commonly over represented in depression, anxiety, eating disorders, and attempted suicide (American Psychiatric Association, 2013). Furthermore, sex differences in emotional behaviour in addition to sex-linked differences in neurotransmitter and neuromodulatory systems (which are potentially related to stress or fear eliciting behaviours) have been documented in a variety of mammals (Palanza, 2001).

In addition to the above, adrenocortical activity differences have been found between female and male rats. Female rats show higher baseline corticosterone levels (Kitay, 1961;

Critchlow, Liebelt, & Bar-Sela, 1963) and larger corticosterone secretion after ACTH injections (Kitay, 1961; Hess & Riegle, 1970).

Furthermore, females appear to show enhanced responses to stressors such as handling, restraint, shocks, or high conflict situations (Weinberg, Gunnar, & Brett, 1982; Ader & Grota, 1969; Dunn, Scheving, & Millet, 1972; Kant, Bunnell, & Mougey, 1983; Heinsbroek, Van Haaren, & Feenstra, 1991). Enhanced adrenocortical activity in female rats correlates with sex steroid levels and gonadectomy in female rats' results in corticosterone profiles that are similar to males (Critchlow, Liebelt, & Bar-Sela, 1963; Weinberg, Gunnar, & Brett, 1982).

In humans, the literature is less clear cut and appears to contradict the findings in rodents. Some studies have reported higher baseline cortisol levels in men (Zumoff, Fukushima, & Weitzman, 1974), however, no differences have been found between the sexes in other studies (Jonkman, Reinberg, & Oosterhuis, 1988; Friedmann & Kindermann, 1989).

Despite fear responses in animals being similar to anxiety reactions in humans (in terms of biological and environmental substrates; Palanza, 2001) it would appear that most studies exploring anxiolytic drug effects in rodents have used males only (Hughes, 2007). Therefore, in light of the above research, it would seem pertinent to include both male and female rats in the current study.

1.7 The current study

The recent study which found that 90 days of exposure to an approximate single dose of 80 mg/kg of vitamin C in drinking water markedly reduced anxiety in rats (Hughes et al., 2011) was subsequently followed by a dose-response study of vitamin C as ascorbic acid (Hughes et al., 2015). The results of this study have shown a dose of approximately 114mg/kg/day to be more effective as an anxiolytic than 61mg/kg/day, but a dose of

160mg/kg/day to be either as effective or less effective than 114mg/day. Following on from this, the present study was intended to determine if 61-160mg/kg/day of ascorbic acid will exert anxiolytic effects when rats experience mild stress in the form of exposure to bright light, which is a common way of inducing anxiety in rats (e.g., Henry, Kabbaj, Simon, Le Moal, & Maccari, 1994). It would then be possible to recommend appropriate doses for humans to achieve similar outcomes. The doses used were equivalent to a daily vitamin C intake of about 0.8-2g/day for an average 75.6kg North American and European human (Walpole et al., 2012). This reflects the upper tolerable limit set by the National Academy of Sciences, which cites osmotic diarrhoea as the main concern in regards to adverse effects of vitamin C (Schlueter & Johnston, 2010).

Chapter 2

Method

2.1 Subjects

The subjects comprised 40 male and 40 female PVG/c hooded rats. The rats were housed in same sexed groups of four in 560 x 350 x 215-mm cages. Each group of rats was provided with two drinking bottles and ad libitum food, and were kept in 12-h light: 12-h dark conditions with an ambient temperature of 20 ± 1 degree Celsius. There were 20 rats in each group (males, n = 10, females, n =10) as per the design outlined in the table below.

Table 1: Group design

	Control		Vitamin C1 (Low Level)		Vitamin C2 (Medium Level)		Vitamin C3 (High Level)	
	Low	High	Low	High	Low	High	Low	High
	Stress	Stress	Stress	Stress	Stress	Stress	Stress	Stress
Males (n)	5	5	5	5	5	5	5	5
Females (n)	5	5	5	5	5	5	5	5

2.2 Vitamin C treatment

All rats were maintained on normal drinking water (males, n = 10, females, n = 10), a low level of vitamin C (males, n = 10, females, n = 10), a medium level of Vitamin C (males, n = 10, females, n = 10), or a higher level of vitamin C (males, n = 10, females, n = 10) for several weeks before testing and during testing. Vitamin C (ascorbic acid) was administered in the form of a soluble powder which was added to the rat's drinking water.

All rats continued their treatment with ascorbic acid, which was begun for an earlier study by Hughes et al. (2015). Specifically: (a) 10 males and 10 females received

unadulterated drinking water, (b) 10 males continued receiving 0.90 mg/ml and 10 females continued receiving 0.66 mg/ml ascorbic acid in water, (c) 10 males continued receiving 1.80 mg/ml and 10 females continued receiving 1.32 mg/ml ascorbic acid in water, (d) 10 males continued receiving 2.70 mg/ml and 10 females continued receiving 1.98 mg/ml ascorbic acid in water.

The above amounts roughly equalled consumption of 61, 114 or 160 mg/kg a day of vitamin C. Different concentrations of ascorbic acid for the two sexes were necessary because females drink more solution/bodyweight than males (Hughes et al., 2011). Each week the rat's water bottles were topped up once, and then replaced later in the week with sterilised bottles containing freshly prepared fluids.

2.3 Behavioural testing

After a total of 8 months of vitamin C treatment when the rats were approximately 12 months old they all experienced each of the following tests once with an interval of at least 24 hours between them. At the end of each testing session the apparatus was wiped down with a 70% ethanol solution and dried. Within each group (control, low level vitamin C, medium level vitamin C, and high level vitamin C) half of the rats experienced each test in a dim light condition (males, n = 5, females, n = 5) or a bright light condition (males, n = 5, females, n = 5). The order of the tests varied in a non-systematic way for individual rats. Vitamin treatment was continued throughout the testing period.

2.3.1 Elevated plus-maze

This test of anxiety/fear involves placing an individual rat into the centre of a 4-armed plus-maze of which two arms are open (clear Perspex walls) and the other two are closed (wooden walls). The whole maze was elevated 1m above the floor. For 5 minutes the rat's entries into and time spent in each arm were recorded on a prepared data sheet. This enabled

calculation of preferences for entering and occupying open vs. closed arms (more anxious rats prefer closed arms, [Pellow et al., 1985]). Furthermore, a circular 22-watt fluorescent lamp was positioned 350 mm above the floor of the distal end of each arm, acting as a bright light stressor for the rats in the high stress condition.

2.3.2 Light-dark box test

This test of anxiety/fear involves placing an individual rat into a wooden box which is comprised of two compartments separated by a wooden partition containing a 10x10 cm opening which can be closed by means of a removable horizontal slide. The dark compartment was covered by a hinged lid made from wood and clear Perspex for the light compartment.

Each individual rat was placed into the dark compartment of the box for 30 seconds with the slide separating the two compartments in place. The slide was then removed and for 5 minutes it was noted which side of the box the rat was occupying and the number of times it entered the light side. Furthermore, the time it took each rat to emerge from the dark compartment after the wooden slide was removed was also noted (in seconds). In addition to this, a circular 22-watt fluorescent lamp was positioned 350 mm above the floor of the light side of the box, acting as a bright light stressor for the rats in the high stress condition.

2.3.3 Open-field test

This test was used to assess activity and emotionality. Each rat was individually placed into a 900cm x 900 open-field and over 5 minutes it was noted which square the rat was located in every 3 seconds. It was also noted whether the rat is rearing up on its hind legs, walking, or grooming itself. At the end of the trial the number of faecal boluses left in the apparatus was also counted. The open-field sat on a 700–mm-high table with a circular 22-watt fluorescent lamp positioned directly above each half of the apparatus 255 mm from the floor, acting as a bright light stressor for the rats in the high stress condition.

2.3.4 Bright light stressor

For the dim light condition (low stress), the fluorescent lamps were not turned on and each type of apparatus was illuminated only by low level fluorescent overhead room lighting. This has been previously shown to give an average reflected light level within the apparatus of approximately 22 lx.

For the bright light condition (high stress), illumination additional to that from the room lighting was provided by the fluorescent lamps being turned on. As previously shown, this should give an average light level of 600 lx. Recent research has confirmed the stressful nature of bright light provided in this way (Hughes et al., 2014).

2.4 Relative adrenal gland weights

After behavioral testing was completed, five rats of each sex from the control and vitamin C treatment groups were euthanized and both their adrenal glands removed. Connective tissue and fat was cleaned from the glands which were then weighed and their paired weights (relative to body weight namely, mg/100g) were recorded. Relative adrenal gland weights are positively related to stress experienced over extensive periods of time (Hatch, Wiberg, Balazs, & Grice, 1963).

2.5 Statistical analyses

All behavioural responses were subjected to separate 3 (vitamin C) x 2 (light stress) x 2 (sex) ANOVAs, followed by Fisher PLSD post hoc tests ($p < 0.05$) for significant main effects and interactions. Due to a lack of normality in the distributions of relative adrenal weights, differences between the control group and each of the vitamin C groups for relative adrenal weights were assessed nonparametrically with Mann-Whitney U tests for each sex separately.

Chapter 3

Results

3.1 Open-field treatment effects

Although the treatment main effect was not significant [$F(3,64) = 1.63, P > 0.1$], there was a significant treatment X sex interaction [$F(3,64) = 2.82, P < 0.05$]. As outlined in Figure 1, significant vitamin C treatment effects occurred for open-field ambulation for female rats only. While significantly more of their open-field ambulation occurred with each of the three treatment groups compared to controls, there were no significant differences between the three vitamin groups.

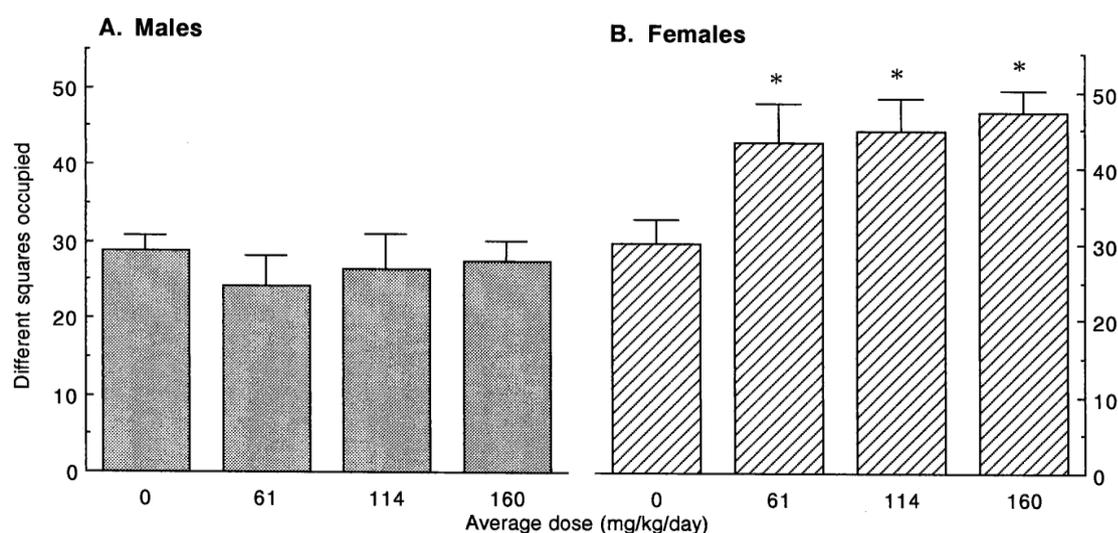


Figure 1: Mean (\pm SEM) percentages for open-field ambulation for a. males and b. females treated with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid. *Significantly different ($p < 0.05$) from control.

There was no significant treatment effect for rearing [$F(3,64) = 0.21, P > 0.10$], grooming [$F(3,64) = 1.19, P > 0.10$], immobility [$F(3,64) = 0.48, P > 0.10$] (Figure 2), centre

occupancy [$F(3,64) = 0.26, P > 0.10$], or corner occupancy [$F(1,64) = 0.99, P > 0.10$] (Figure 3). However, this effect was significant for walking [$F(3,64) = 2.98, P < 0.05$]. Rats given dose 3 and dose 4 displayed significantly more walking behaviour than controls (see Figure 2 ‘Walking’).

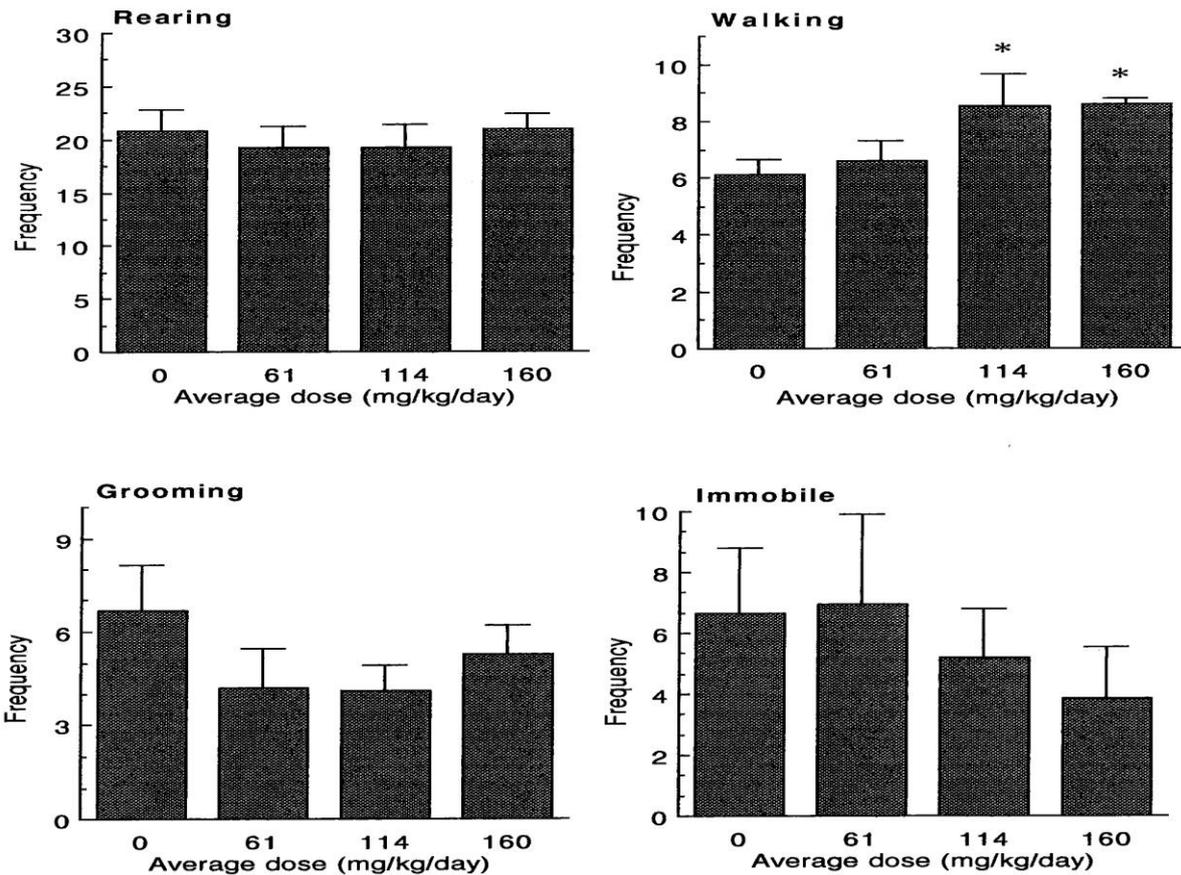


Figure 2: Mean (\pm SEM) percentages for frequency of rearing, walking, grooming and immobility in the open-field for rats (both high and low stress conditions and sexes combined) treated with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid. *Significantly different ($p < 0.05$) from control.

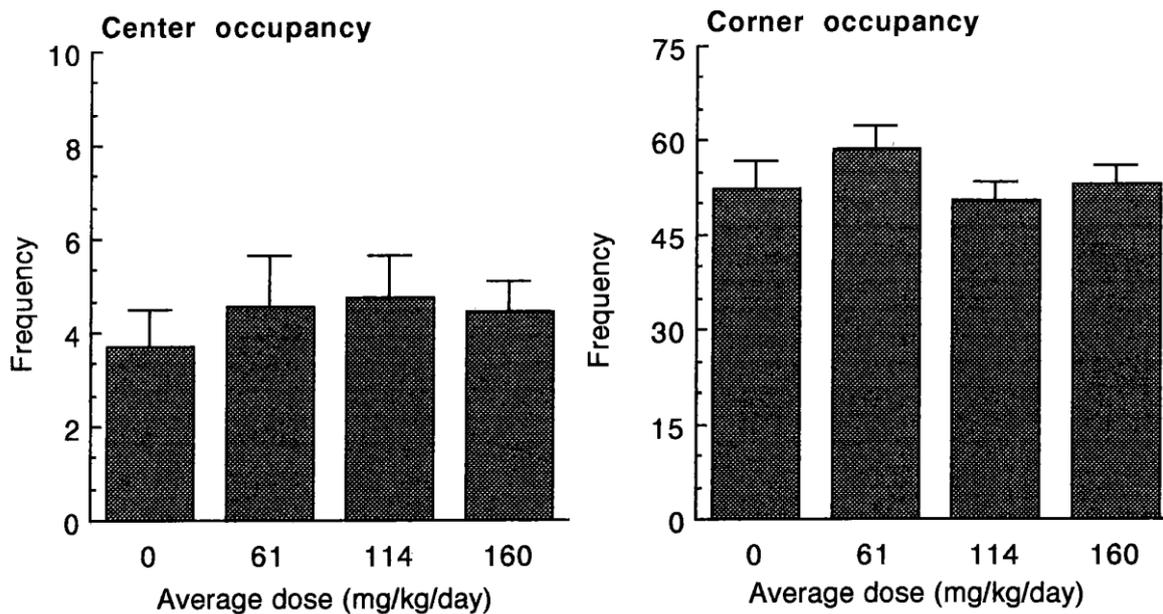


Figure 3: Mean (\pm SEM) percentages for frequency of centre occupancy and corner occupancy in the open-field for rats (both high and low stress conditions and sexes combined) treated with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid

3.2 Open-field stress and sex effects

As shown in Table 3, female rats displayed significantly more ambulation and walking, and less immobility than male rats. In the high stress condition both sexes combined displayed significantly less grooming and more immobility than rats in the low stress condition.

3.3 Elevated plus-maze treatment effects

Significant vitamin C treatment effects occurred for elevated plus-maze percentage of entries into the open arms [$F(3,64) = 4.43, P < 0.01$]. However, this result is more appropriately considered in the light of a significant treatment X sex interaction outlined in

Figure 4 [$F(3,64) = 4.86, P < 0.005$]. The interaction revealed that only females were affected by the treatment, wherein females given dose 1 made significantly fewer entries into the open arms than controls, dose 2, or dose 3 (Figure 4B).

In addition to the above, significant vitamin C treatment effects occurred for the number of entries into the closed arms of the elevated plus-maze for rats treated with dose 2 compared to controls [$F(3,64) = 2.96, P < 0.05$] (Figure 5). As is evident in Figure 6, there was no significant treatment effect for the percentage of time rats spent in the open arms [$F(3,64) = 1.28, P > 0.10$].

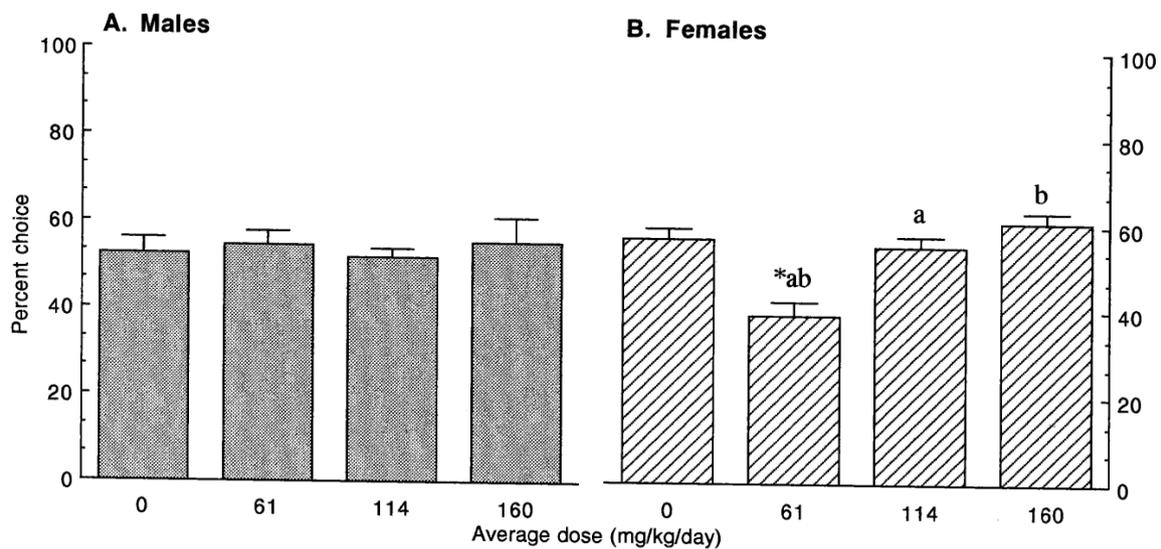


Figure 4: Mean (\pm SEM) percentages of entries into the open arms of the elevated plus-maze for a. males and b. females treated with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid.

*Significantly different ($p < 0.05$) from control. ab Difference between groups with superscript in common significant ($p < 0.05$).

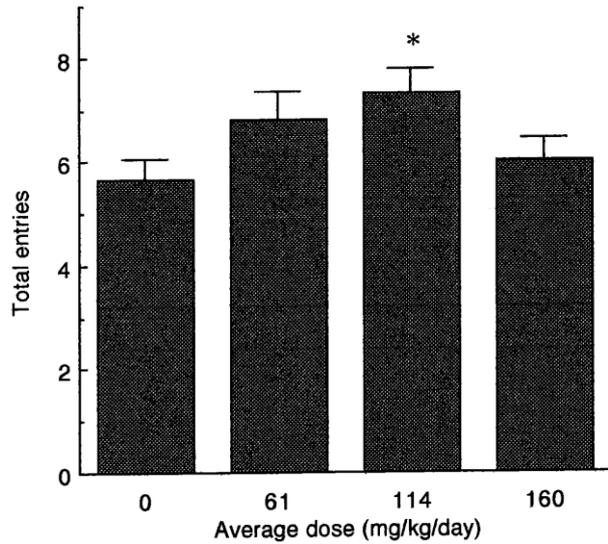


Figure 5: Mean (\pm SEM) number of entries into the closed arms (both low and high stress conditions and sexes combined) of the elevated plus-maze following treatment with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid.

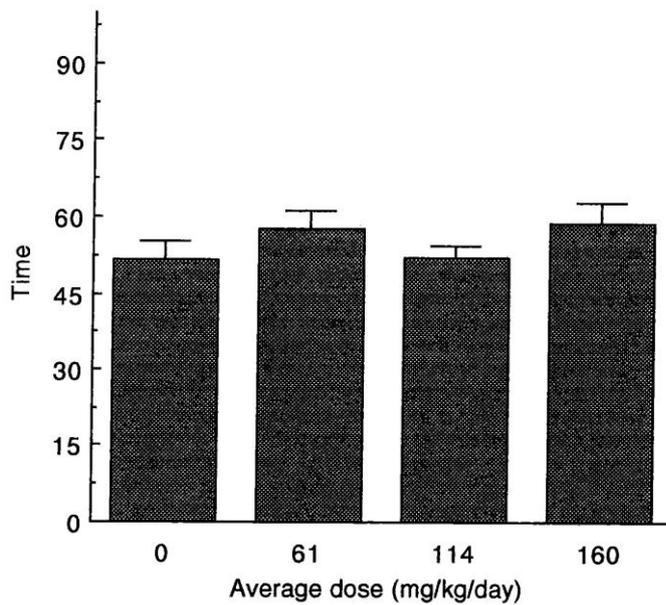


Figure 6: Mean (\pm SEM) percentages of time spent in the open arms (both low and high stress conditions and sexes combined) of the elevated plus-maze following treatment with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid.

3.4 Elevated plus-maze stress and sex effects

Overall, rats in the high stress condition displayed a significantly higher percentage of entries into the open arms compared to rats in the low stress condition (Table 3). However, a significant sex X stress interaction [$F(1,64) = 6.24, P < 0.05$] revealed that this difference only applied to male rats (Figure 7).

While male rats made significantly fewer entries into the closed arms of the elevated plus-maze compared to female rats overall (Table 3), a significant sex X stress interaction [$F(1,64) = 4.04, P < 0.05$] revealed that this sex difference only typified rats tested in the high stress condition.

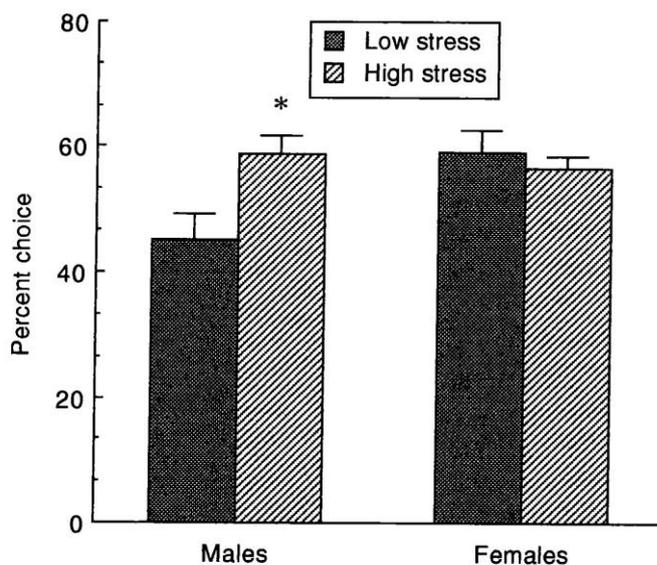


Figure 7: Mean (\pm SEM) percentages of time spent in the open arms of the elevated plus-maze for males and females in the low stress and high stress conditions. *Significantly different ($p < 0.05$) from low stress.

3.5 Light-dark box treatment effects

Significant vitamin C treatment effects occurred for time spent in the light side of the light-dark box [$F(3,64) = 3.29, P < 0.05$] (Figure 8). However, a significant dose X stress interaction [$F(3,64) = 3.59, P < 0.05$] showed the effect to only apply to rats in the low stress condition, where low stress rats given dose 3 spent significantly more time in the light side compared to dose 2 and dose 4 (Figure 8a). There were no significant treatment effects for emergence latency [$F(3,64) = 0.12, P > 0.10$] or entries into the light side [$F(3,64) = 1.35, P > 0.10$] (Figure 9).

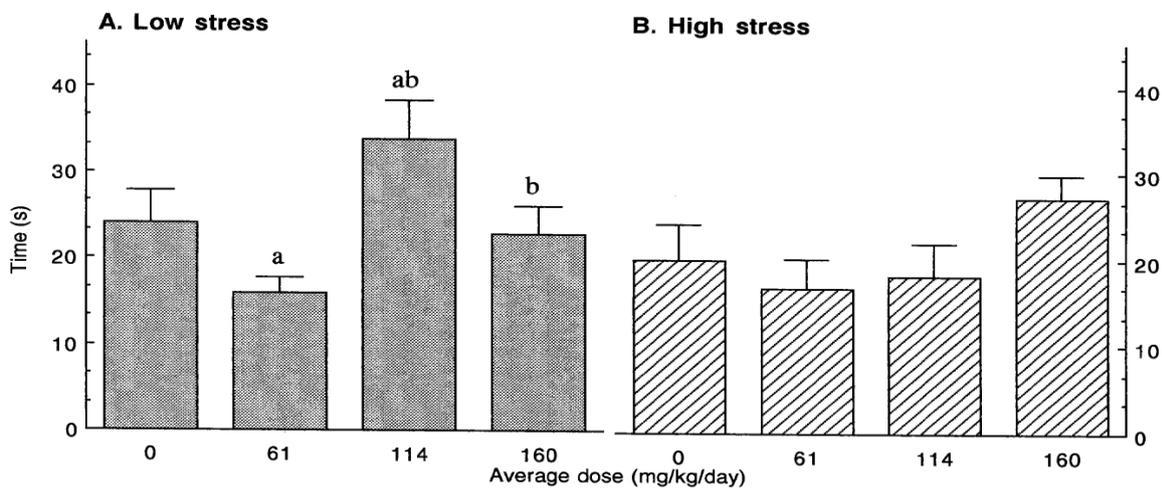


Figure 8: Mean (\pm SEM) of time spent in the light side of the light-dark box for a. low stress and b. high stress following treatment with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid. abDifference between groups with superscript in common significant ($p < 0.05$).

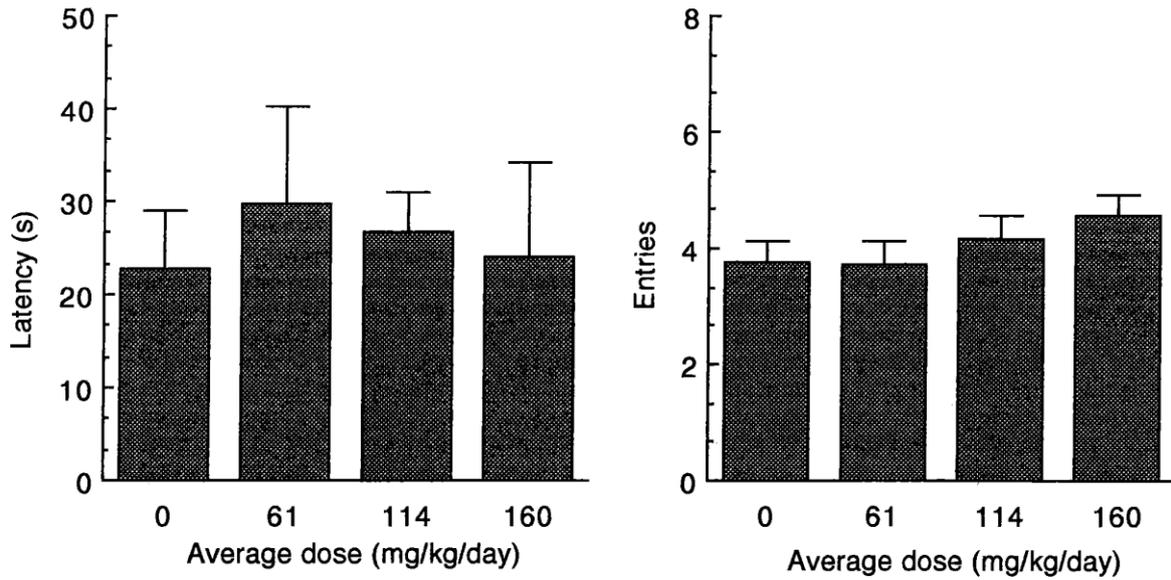


Figure 9: Mean (\pm SEM) percentage of emergence latency and entries into the light side of the light-dark box for males and females in the low stress and high stress conditions treated with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid.

3.6 Light-dark box stress and sex effects

As shown in Table 3, female rats spent significantly more time in the light side and made significantly more entries into the light side compared to male rats (Table 3).

3.7 Relative adrenal gland weights

Adrenal weights are outlined in Figure 10 for males and females separately, and results of the Mann-Whitney Tests are shown in Table 2. Male rats given dose 3 had significantly lighter adrenals weights compared to controls (Figure 10a). No significant effects were found for the female rats (Figure 10b).

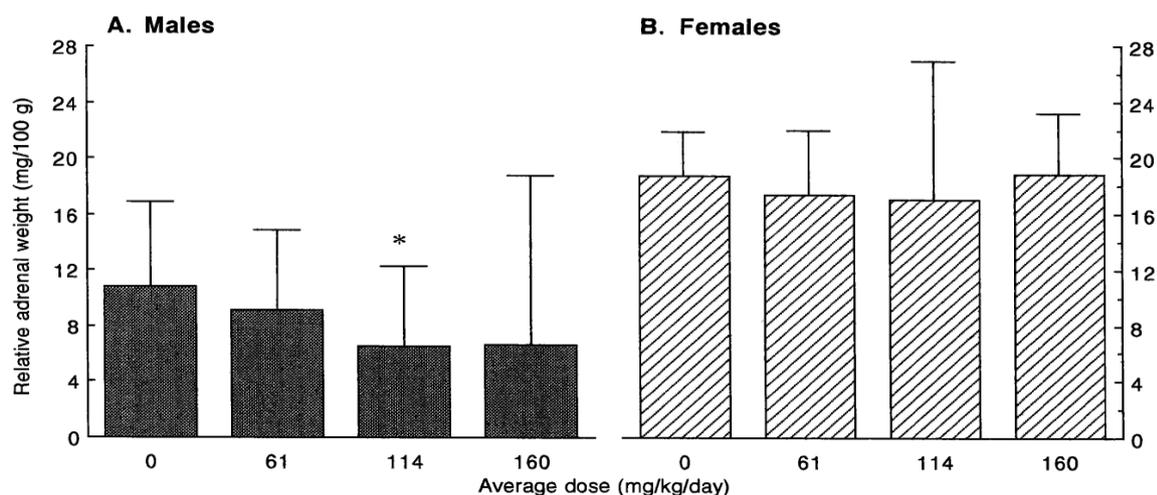


Figure 10: Medians and ranges for relative adrenal weights in a. males and b. females treated with 0 (control), 61, 114 or 160 mg/kg of ascorbic acid. *Significantly different ($p < 0.05$) from control.

Table 2: Results of effects of vitamin C treatment on relative adrenal weights as determined by Mann-Whitney U tests i.e., values of U, z scores and probabilities for each comparison.

Comparison:	0/61	0/114	0/160	61/114	61/160	114/160
Males						
U	8.0	3.0	6.0	7.0	9.0	14.0
z	0.94	1.98	1.36	1.15	0.73	0.31
p	>0.3	=0.047	>0.1	>0.1	>0.2	>0.3
Females						
U	9.5	8.0	12.0	11.0	7.0	8.8
z	0.63	0.94	0.10	0.31	1.15	0.94
p	>0.2	>0.1	>0.4	>0.3	>0.1	>0.1

Table 3: Main effects of sex and stress on all behavioural measures, and results of ANOVAs.

Response	Males	Females	F(1,64)	Low stress	High stress	F(1,64)
Open-field:						
Ambulation ^a	26.77 (± 1.67)	41.33 (± 2.18)	29.60***	32.42 (± 2.05)	35.67 (± 2.43)	1.48
Rearing	19.20 (± 1.50)	20.90 (± 1.24)	0.74	21.20 (± 1.37)	18.90 (± 1.37)	1.36
Walking	6.12 (± 0.43)	8.80 (± 0.65)	12.60***	7.32 (± 0.51)	7.60 (± 0.66)	0.13
Grooming	5.22 (± 0.90)	4.90 (± 0.72)	0.09	6.18 (± 0.98)	3.95 (± 0.56)	4.04*
Immobile	7.85 (± 1.79)	3.47 (± 1.10)	4.48*	3.58 (± 1.00)	7.75 (± 1.85)	4.08*
Centre Occupancy	4.32 (± 0.67)	4.40 (± 0.55)	0.01	4.32 (± 0.61)	4.40 (± 0.62)	0.01
Corner Occupancy	52.55 (± 2.87)	54.62 (± 2.18)	0.35	53.25 (± 2.64)	53.92 (± 2.47)	0.04
Elevated plus-maze:						
%open arm entries ^a	53.33 (± 1.95)	52.85 (± 1.76)	0.04	50.69 (± 1.74)	55.48 (± 1.89)	4.52*
%time in open arms ^b	52.12 (± 2.93)	57.81 (± 1.64)	3.05	52.07 (± 2.62)	57.87 (± 2.10)	3.17
Closed arm entries ^b	5.65 (± 0.35)	7.22 (± 0.29)	13.08***	6.65 (± 0.29)	6.22 (± 0.39)	0.95
Light-dark box:						
Emergency latency	33.15 (± 8.32)	18.42 (± 3.48)	2.64	24.17 (± 6.31)	27.40 (± 6.65)	0.13
Entries of light side	3.24 (± 0.22)	4.85 (± 0.26)	24.96***	4.30 (± 0.24)	3.78 (± 0.31)	2.49
Time in light side ^c	19.79 (± 2.11)	24.75 (± 1.65)	4.64*	24.15 (± 1.95)	20.42 (± 1.84)	2.71

^aVitamin C dose x sex interaction significant (p <0.05, see text).

^bSex x stress interaction significant (p <0.05, see text).

^cVitamin C dose x stress interaction significant (p <0.05, see text).

Chapter 4

Discussion

The aim of the current study was to determine if the chronic administration of doses of 61, 114, or 160 mg/kg of vitamin C will exert anxiolytic effects when rats are exposed to an environmental stressor in the form of a bright light. The purpose being to add to the current research surrounding vitamin C's potential anxiolytic effects and to suggest appropriate doses for humans to help achieve similar outcomes.

In summary, it appears that doses of 114 mg and 160 mg/kg of vitamin C were able to exert an anxiolytic effect on rats, with both doses leading to significantly more walking in the open-field. For females only, all doses (including 61 mg/kg of vitamin C) led to an increase in ambulation compared to controls. These results however, were irrespective of the presence of an environmental stressor. Similarly, in the light-dark box 114 mg/kg of vitamin C resulted in rats spending significantly more time in the light compartment than rats treated with either 61 or 160 mg/kg. This however, was only true for rats in the low stress condition.

Interestingly, 61 mg/kg and 114 mg/kg of vitamin C appeared to produce a sex-related anxiogenic effect for rats tested in the elevated plus-maze, with 61 mg/kg leading to a decrease in open arm entries for females only, and then 114 and 160 mg/kg leading to an increase in this response. Another interesting finding was that males (but not females) in the high stress condition spent more time in the open arms. It was also found that male rats given 114 mg/kg of vitamin C had significantly lighter adrenal gland weights compared to controls.

4.1 Overall findings

4.1.1 Open-field

It was found that all doses of vitamin C increased ambulation in female rats compared to

controls, and both sexes given doses of 114 and 160 mg/kg of vitamin C displayed more walking in the open-field suggesting an anxiolytic effect for the above doses. These findings are in line with Hughes et al. (2015) who similarly found that 114 mg/kg and 160 mg/kg of vitamin C increased walking frequency in the open-field. In the current study, female rats in the open-field displayed more ambulation and walking and less immobility than males. There were no significant treatment effects found for rearing, grooming, immobility, centre occupancy, or corner occupancy.

Generally speaking, female rats ambulate more in an open-field compared to male rats, which is related to their overall increased activity level (Archer, 1975) and findings that they are generally the less anxious of the two sexes (Gray, 1971). In light of this, it would be expected that female rats in both the high stress and low stress condition would show an overall elevated rate of ambulation compared to males. However this was not the case. For both sexes combined, rats in the high stress condition also showed significantly less grooming and more immobility compared to rats in the low stress condition in the open-field. Immobility is indicative of anxiety in rats (Archer, 1973). However, more grooming (as some findings suggest) rather than less is indicative of higher anxiety in the rat (Barros et al., 1994; Dunn et al., 1981). This suggests a somewhat counterintuitive finding in which the rats' levels of grooming and immobility suggest both less and more anxiety. However, it could be assumed that increased levels of immobility may have an impact on grooming behaviour, meaning that the more time spent immobile the less time there is to engage in grooming behaviour.

4.1.2 Elevated plus-maze

Interestingly 61 mg/kg of vitamin C decreased the percentage of open arm entries in the elevated plus-maze in females and 114 mg/kg increased the number of entries into the closed arms for both sexes combined, suggesting an anxiogenic effect of 61 mg/kg and 114

mg/kg of vitamin C for rats tested in the elevated plus-maze. Furthermore, males in the high stress condition displayed a higher percentage of entries into the open arms of the elevated plus-maze and fewer entries into the closed arms.

The above results, particularly the finding that 61 mg/kg of vitamin C decreased entries into the open arms, are contradictory to findings found by Hughes et al. (2015). In this study by Hughes et al. (2015) it was found that 61 mg/kg of vitamin C resulted in more frequent occupation of the open arms of the elevated plus-maze, suggesting an anxiolytic effect. In the current study no significant treatment effects were found for the percentage of time spent in the open arms.

4.1.3 Light-dark box

In the light-dark box, 114 mg/kg of vitamin C resulted in rats in the low stress condition making significantly more entries into the light compartment of the apparatus compared to rats given doses of 61 mg/kg or 160 mg/kg. This suggests an anxiolytic effect of the vitamin in absence of an environmental stressor. Irrespective of dose or the presence of an environmental stressor, female rats made significantly more entries into the light compartment of the light-dark box and spent significantly more time there compared to males. The latter effect likely reflects a sex difference in fearfulness between male and female rats. For both sexes and doses combined, there were no significant treatment effects for emergence latency or entries into the light side of the light-dark box.

The data suggests that female rats are less fearful than male rats. This is evidenced through measures of exploratory behaviour. For example, female rats show increased speed of emergence from familiar to novel ground (Meyers, 1962) and greater amounts of exploration of novel territory compared to males (Zimbardo & Montgomery, 1957).

Gray (1971) suggests that these differences in exploratory behaviour between male and female rats likely do not reflect differences in exploratory behaviour per se, as wild male

rodents usually have larger home ranges compared to females. Therefore, it is likely that the sex differences in rodent exploratory behaviour in the lab are due to differences in fearfulness. This sex difference is thought to depend in part on levels of circulating oestrogen, as spontaneous oestrus in the rat is associated with lower defecation and higher ambulation in the open-field and a shorter emergence latency into novel territory (Burke & Broadhurst, 1966).

It has also been suggested that the sex difference observed in exploratory behaviour may be due to an increased olfactory sensitivity at oestrus. This sensitivity is likely to induce a greater tendency to actively explore novel surroundings (Archer, 1975). However, it is unlikely that this explains the full extent of such a sex difference as males generally show lower olfactory thresholds than females but without any cyclic changes (Petras & Moulton, 1974).

Within the current study the nature of the testing regime (which was carried out over a number of weeks) would have made it very unlikely that all of the female rats were tested at the same stage of their oestrous cycle. This means that the sex differences found between male and female rats may not be due to differences in fearfulness caused by oestrous, but rather may be due to an overall greater level of circulating oestrogen in female rats.

4.1.4 Adrenal glands

Male rats given 114mg/kg of vitamin C had significantly lighter adrenal weights compared to controls. This likely suggests that for males, 114 mg/kg of vitamin C was able to attenuate the effect of stress on the adrenal glands, as these rodents had a lower base-line anxiety level due to the vitamin C dose.

In male rats, stress has been shown to increase adrenal weights. It has been found that crowding produces a greater increase in the weight of the adrenal glands in males. This

increase in weight, under such conditions, is taken to indicate the degree to which the rat is susceptible to the stress that comes with high population density (Christian, 1963).

Vitamin C is highly concentrated in the adrenal glands, and it has been found that vitamin C supplementation can lead to a decrease in corticosterone levels in rats and decrease the mean level of adrenocorticotropin-releasing hormone (Choi et al., 2011). This same study found that vitamin C significantly decreased freezing time that is typically increased by stress, and also motivated rats to cross over an electric field more frequently than controls. These results suggest that the ameliorating effect of vitamin C on these stress-related behaviours displayed by the rats within the above study may have been exerted via modulation of corticotrophin or adrenocorticotropin.

As adrenal weight is a crude index of anxiety (i.e. the more the glands have to work pushing out adrenalin and stress hormones the bigger they become) it is possible that for the rats within the current study, vitamin C (at a dose of 114 mg/kg) was able to exert its effects through the modulation of CORT or ACTH, in turn protecting against the effects of stress on the adrenal glands (i.e. meaning they did not enlarge).

Female rats, compared to males, have higher resting levels of both plasma and adrenal corticosterone (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963) and in turn have larger adrenal gland weights (Malendowicz, 1974). The finding that there were no significant differences between vitamin C doses and controls in females may be due to a ceiling effect. This means that for females, the dosages used within this study were unable to affect adrenal gland size due females possessing larger adrenal glands, which may have been at their maximal size to begin with.

A number of potential mechanisms of action can be put forward for the anxiolytic effects of vitamin C. Among these is a reduction in oxidative stress (Halliwell & Whiteman, 1997; Bouayed et al., 2009) neuromodulation of dopamine and glutamate (Rice, 2000;

Majewska et al., 1990), or in humans, an attenuation of the stress hormone cortisol (Brody et al., 2002). However, none of these have been reliably established as a likely mechanism.

Within this study, the result that male rats treated with 114 mg/kg of ascorbic acid had significantly lighter adrenal glands compared to controls suggest that vitamins C may have been able to exert its anxiolytic effects through the modulation of corticotrophin or adrenocorticotrophin, thus leading to a reduction in anxiety behaviours displayed in the testing apparatus. However, as only the adrenals of male rats treated with 114 mg/kg of vitamin C showed lighter adrenal glands (despite other doses also exerting anxiolytic effects), the above does not fully explain the vitamin's underlying mechanism for its anxiolytic effect.

4.1.5 Summary

In view of the above results, it would appear that 114 mg/kg of vitamin C exerted the greatest anxiolytic effect on the rats; however, 114 mg/kg also appeared to produce an anxiogenic effect for rats tested in the elevated plus-maze. Overall, the anxiolytic, and similarly the anxiogenic effect of the vitamin appeared to be dependent on the particular response measured, and no linear dose-response relationship was found.

Results also suggest that the presence of an environmental stressor was likely to buffer against the anxiolytic effects of vitamin C, with rats in the low stress condition given 114 mg/kg of the vitamin making more entries into the light compartment of the light-dark box and spending more time there. Male rats given 114 mg/kg of vitamin C showed lower adrenal weights compared to controls, suggesting a lower stress response. Female rats did not display any significant differences, which may have been due to a ceiling effect related to their overall larger adrenal size.

4.2 Practical implications

While a number of studies have found an anxiolytic effect of vitamin C in various animal models of anxiety (Satterlee et al., 1989; 1993; Jones et al., 1996; 1999; Hughes et al., 2015) studies by Choi et al. (2010) and Angrini and Leslie (2012) have found anxiolytic effects with similar doses to those used in the current study.

Choi et al. (2010) used a dose of 100 mg/kg in their study with mice and found that this dose was able to partially block stress-related behaviours such as freezing, sniffing, burrowing, and grooming while also decreasing staying time in the closed arms of the elevated plus-maze. Doses of 25 mg/kg of vitamin C were also able to exert such effects. A lower dose within the current study however, only exerted an anxiolytic effect in regards to ambulation in female rats, while also managing to decrease entries into the open arms suggesting an anxiogenic effect. In the Choi et al. (2010) study both doses were also able to alleviate stress responses induced by electric shock and decrease corticosterone levels augmented by electric shock, while 25 mg/kg alone was able to increase crossing frequency across an electrified field.

Angrini and Leslie (2012) found that noise when used as an environmental stressor led to increases in grooming, defecation, and immobility. Vitamin C at doses of 100 mg/kg and 200 mg/kg were able to reduce the effects of noise on grooming and defecation but not immobility. While a dose of 114 mg/kg of vitamin C within the current study affected measures of anxiety in the open-field and light-dark box, the presence of an environmental stressor, unlike the Angrini and Leslie (2012) study, appeared to work against the potential anxiolytic effects of the vitamin, as suggested by findings from the light-dark box.

The current study suggests that a dose of 114 mg/kg may be effective in alleviating anxiety. An equivalent dose in humans would work out to be 1.45 g/day. The tolerable upper limit of vitamin C set by the National Academy of Sciences is 2000 mg/d, with few (if any)

adverse side-effects identified (Schlueter & Johnston, 2010). The results suggest however, that under particularly stressful conditions the anxiolytic effects of the vitamin may be less effective. This is evidenced by the finding that rats given 114 mg/kg of the vitamin made more entries into the light compartment of the light-dark box in the low stress condition, but the same was not true for rats in the high stress condition.

While 1.45 g/day of vitamin C may have benefits for humans suffering from anxiety, not only in terms of the vitamins anxiolytic effects but also its lack of side effects compared to pharmacological medications (Schlueter & Johnston, 2010), there are a number of factors that may get in the way of the vitamins utility as an anxiolytic medication. Firstly, the attitudes of medical practitioners towards the use of a vitamin supplement as an anxiolytic medication may discourage patients from using vitamin C for such a reason. Lakhan and Vieira (2008) claim that there is a huge resistance to the use of supplements as treatments from clinicians, which they suggest mostly stems from a lack of knowledge and a preference to prescribe pharmacological medications.

This is evident in the case of omega-3 fatty acids in the treatment of depression. The effect of omega-3 fatty acids for the treatment of depression is well documented. A Cochrane review found that there was a significant effect of omega-3 fatty acids on reducing bipolar depression (Montgomery & Richardson, 2008). Furthermore, a deficit in omega-3 fatty acids has been shown to be a potential contributing factor in the development of mood disorders (Parker, Gibson, Brotchie, & Heruc, 2006). Despite the positive evidence and the fact that omega-3 fatty acids have other general health benefits and adjunctive use has a low risk (Freeman, 2009) they are rarely recommended for patients.

Johannessen, Skagestad and Bergkaasa (2011) found in a sample of 50 nursing students, 20 tutor nurses and 6 psychiatrists that there was a lack of knowledge and interest in nutrition and fatty acids as a supportive treatment for depression. Furthermore, the

psychiatrists within the sample did not know or believe that omega-3 fatty acids could have a positive effect on depression. It is likely that vitamin C used for its anxiolytic effects may be met with a similar fate, despite growing evidence to support its efficacy.

Another factor, which may influence the practicality of using vitamin C as an anxiolytic medication, includes adherence and cost. Selective Serotonin Reuptake Inhibitors (SSRIs), commonly used to treat anxiety disorders, have been associated with a failure to reach therapeutic outcomes primarily because of premature treatment discontinuation and nonadherence (Lin, Von Korff, & Katon, 1995). A study by Eaddy, Bramley and Regan (2003) found that of a sample of 76,107 patients taking SSRIs, 28% discontinued therapy within the first month.

It could be argued that adherence to vitamin C over a chronic course may suffer a similar fate, however, Lin, Von Korff and Katon (1995) who found similar SSRI discontinuation rates, found that 60% of the patients who discontinued treatment early cited adverse effects as the primary reason for ending therapy. As vitamin C supplementation has very little (if any) adverse effects, it has potential as an alternative to SSRI's when side effects make adherence difficult.

In regards to cost, for a one month supply of 1000 mg of vitamin C in chewable tablet form, the average regular retail price is around \$15.00 NZD. While for some this may be a manageable expense, for others it may be a significant cost, especially over a long course. Alternatively, adding more foods rich in vitamin C to the diet may be another option, but keeping up with consistent doses may be difficult.

Sex differences in response to the vitamins anxiolytic effects and the sex differences in the rates of anxiety disorders is another area to consider. Anxiety disorders are more common among women, with an approximately 2:1 ratio (APA, 2013). From the results of this study, it could be suggested that male and female humans may respond differently to

differing doses of vitamin C, and some doses may even lead to increased anxiety under certain conditions (related to the finding that 61 mg/kg led to an anxiogenic effect in female rats and 114 mg/kg in both sexes).

In summary, attitudes towards the use of a vitamin supplement for the treatment of anxiety, adherence, cost, and potential sex differences in response to the vitamin's effects, are all factors that may affect the practicality of using vitamin C as an anxiolytic medication. Despite this, the lack of side effects associated with vitamin C supplementation may have pulling power for many patients who use SSRIs and suffer from unpleasant side effects.

4.3 Limitations and future research

4.3.1 Habituation

It is important to note, that the rats within the current study were previously used in a similar study conducted by Hughes et al. (2015) and were tested in the elevated plus-maze and open-field. A feature of the elevated plus-maze is a marked attenuation, and in some cases a complete abolition of the anxiolytic effects of drugs such as chlordiazepoxide (File, 1990) and diazepam (Rodgers, Lee, & Shepherd, 1992). This phenomenon has been described as 'one trial tolerance'. A study by Rodgers et al. (1992) found that, in the elevated-plus maze, daily treatment with 2-4 mg/kg of diazepam for 8 days increased the percentage of open arm entries and the percentage of time spent in the open arms in maze-naïve rats, but not maze-experienced rats. The time between testing in the elevated plus-maze spanned 9 days, while other research has reported the 'one trial tolerance' effect between 48 h to 14 days (Lister, 1987; Rodgers et al., 1992).

While the rats within the current study experienced the elevated plus-maze and other tests more than 14 days after their initial experience, a 'one trial tolerance' effect could be one way to explain the differences between current and previous findings.

The Hughes et al. (2015) study used the same rats as the current study, and found that 61 mg/kg and 114 mg/kg of vitamin C had an anxiolytic effect in the elevated plus-maze, but the same doses within the current study appeared to have the opposite effect. Perhaps for rats given 114 mg/kg, such a tolerance took place, where prior knowledge of the maze (i.e. escape is not possible through the open arms), reduced the likelihood that these rats would explore the area, thereby eliminating a positive response to the vitamin. However, this does not explain why only rats given 114 mg/kg were affected. Similarly, this could be said for rats given 61 mg/kg of vitamin C, but again this does not explain why only females were affected.

As previous research has discovered a potential memory enhancing effect of vitamin C (Parle & Dhingra, 2003; Kennard & Harrison, 2014; Harrison et al., 2009; Hughes et al., 2015), this may also help explain the results of the current study. As vitamin C has led to greater recognition memory, effects of 'one trial tolerance' took place due to the rat's prior knowledge of the maze. However, in the Hughes et al. (2015) study using the same rats, 160 mg/kg of vitamin C enhanced recognition memory, and not 114 mg/kg, so this suggestion does not explain why rats treated with 160 mg/kg in the current study did not show similar results. Future research may consider the use of rats who are naïve to the apparatus used to eliminate the possibility of such a phenomenon as 'one trial tolerance' taking place.

4.3.2 Age of the rats

The rats tested within the current study were over 140 days old and therefore age-related changes in behaviour within the apparatus used should be considered. Research has shown that as rats age they become more fearful. For example, Imhof, Coelho, Schmitt, Morato and Carobrez (1993) found that up to 60 days of age, both sexes of rats showed a higher number of entries and time spent in the open arms of the elevated plus-maze, whereas for rats that were 120 days of age or more, there was a reduction in the number of entries and time spent in the open arms.

A significant sex difference was also found, with male rats showing a reduction in entries and time spent in the open arms sooner than females (at 90 days rather than 120 days). Another study by Masur et al. (1980) found similar results but with the sex difference becoming apparent at 60 days rather than 90 days for males. Motor activity has also been shown to decline with age, so concerns could be raised surrounding the possibility that a lack of mobility is mistaken for heightened anxiety in the open-field and elevated plus-maze in aged rats.

A factor analysis conducted by Boguszewski and Zagrodzka (2002) suggests that the above two factors (anxiety and motor activity) both influence spontaneous behaviour in the open-field and elevated plus-maze and are both independent of one another. This suggests that the increased level of anxiety in older rats does not result from the decreased level of their motor activity.

Similarly to the 'one trial tolerance' effect discussed above, perhaps the age of the rats affected the ability of doses of 61 mg/kg in female rats, and 114 mg/kg in both sexes to attenuate anxiety in the elevated plus-maze, leading to fewer open arm entries (for female rats given 61 mg/kg) and more closed arm entries (for rats given 114 mg/kg). However again, this does not explain why only rats of a certain sex given certain doses experienced such an effect, as it would be assumed that the age of the rats would show a general effect over conditions.

From here future research may consider the use of younger rats. A comparison with the current research may be able to shed some light on the findings that as rats age they become more fearful. Furthermore, the finding that as rats' age they become less mobile could also be explored.

4.4 Conclusion

Overall the current study found that 114 mg/kg was able to exert the most consistent anxiolytic effects amongst the measures used, but interestingly also appeared to exert an anxiogenic effect in rats tested in the elevated plus-maze along with 61 mg/kg of vitamin C. It has been suggested the phenomena of 'one trial tolerance' may have a role in this finding, as the rats used within this study had been tested in the elevated plus-maze once before. However, this was not a general effect over conditions and therefore does not fully explain such an occurrence.

As rats in the low stress condition given 114 mg/kg of vitamin C made more entries into, and spent more time in, the light compartment of the light-dark box, it has been suggested that perhaps the absence of an environmental stressor rendered the vitamins anxiolytic effects more potent. Alternatively, it may be that the presence of an environmental stressor acts to limit the anxiolytic effects of the vitamin. Male rats within the study given 114 mg/kg had lighter adrenal glands compared to controls. No significant effects were observed in females, potentially due to a ceiling effect.

The findings of this thesis suggest that a dose of 1.45 g/day of vitamin C may have the potential of reducing anxiety in humans under certain conditions, while offering a safer alternative to psychopharmacological medications with known side effects. The findings suggest that vitamin C may not be as effective under high stress conditions but rather may be useful for anxiety experienced when low levels of stress are present.

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Appendix A



ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz

Ref: 2014/04R

13 June 2014

Nalita Naidu
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Nalita

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Effects of chronic vitamin C treatment on responsiveness to an environmental stressor"

Approval has been granted:

- (a) for the use of 80 *Rattus norvegicus*
- (b) for your research project to be undertaken from 11 June 2014 to 31 December 2014. If you require an extension of this period please contact the AEC Secretary.

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

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

Yours sincerely

A handwritten signature in cursive script that reads 'James Briskie'.

Associate Professor Jim Briskie
Chair
University of Canterbury Animal Ethics Committee