University of Reading

The acute effects of anthocyanin-rich wild blueberries on cognition and postprandial glucose response in healthy young adults

Submitted for the degree of Doctor of Philosophy

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August 2017

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Following recent interest in the maintenance of health and wellbeing through dietary intervention, the acute cognitive benefits of wild blueberries were investigated in healthy young adults. Blueberries have shown promise in their ability to mediate postprandial cognition and mood; however the effects of dose, and potential mechanisms of action, are yet to be fully elucidated. My thesis, therefore, aimed to determine whether cognition and mood effects following wild blueberry supplementation were dose dependent, and whether modulation of postprandial glucose response was a likely mechanism of action, through increased availability of glucose to the brain.

The cognitive and physiological effects of wild blueberry doses, containing 129mg-724mg anthocyanins, were investigated across three placebo controlled, crossover experiments. Data were analysed using linear mixed-effects modeling, and key findings were identified through pairwise comparisons. Observed dose-dependent cognitive benefits included the maintenance of immediate recall on a single-trial word list learning task, the improvement of working memory on a serial subtraction task, and the attenuation of negative affect using a self-report questionnaire. In addition, dose-dependent attenuation of postprandial heart rate decline was suggestive of increased glucose availability. Further investigation revealed dose-dependent effects on postprandial blood glucose regulation, including attenuation of postprandial glucose peak, and extended availability of blood glucose. In all cases, the strongest effects were observed following blueberry doses containing 517mg anthocyanins or higher. Few significant effects were observed following lower doses.

With some statistical caveats, this programme of research is the first to demonstrate a dose-dependent effect of anthocyanin-rich wild blueberries on episodic memory, working memory and mood. The research also indicates a dose-dependent glucoregulatory effect that may provide a plausible mechanism of action for observed cognitive benefits. Future research should consider the potential application of wild blueberries as a treatment or preventative intervention for metabolic disorders such as type 2 diabetes, where both cognition and glucoregulation are typically impaired.

Acknowledgements

(In alphabetical order)

Al-Farhan, Nasser – placement student

Bell, Iain – moral, financial & IT support

Bracknell, David – moral & grammatical support

Butler, Professor Laurie – PhD supervisor

Coleman, Emma - placement student

Dent, Richard – IT & facilities support

Ellis, Professor Judi – PhD examiner

Economic and Social Research Council – PhD funding provider

Ferreira, Georgina - placement student

Glennerster, Professor Andrew – PhD monitor

Gray, Dr Katie – PhD monitor

Haskell-Ramsay, Dr Crystal – PhD examiner

Heaton, Paul – E Prime support

Holmes, Dr Nicholas – PhD monitor

Lamport, Dr Daniel – PhD supervisor

Leidi, Alessandro – statistical support

Murayama, Dr Kou – statistical support

Nutritional Psychology Lab Group – preparation of intervention drinks

University of Reading staff and students – study participants

Wild Blueberry Association of North America – supplier of freeze-dried wild blueberry powder

Williams, Professor Claire – PhD supervisor

Woodhatch family – provision of endless childcare

All contributions gratefully received

Thank you!

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Chapter 1
Introduction

An abridged version of this chapter has been published as Bell, L., Lamport, D. J., Butler, L. T., & Williams, C. M. (2015). A review of the cognitive effects observed in humans following acute supplementation with flavonoids, and their associated mechanisms of action. Nutrients, 7, 10290–10306.

1.1 General introduction

Due to the rising cost of healthcare provision in the UK, there is increasing interest in the health benefits of natural foods. Government campaigns such as '5 A Day' and 'Change 4 Life' have focused on the promotion of healthy eating throughout life, with a view to stemming observed rises in rates of obesity, type 2 diabetes, cardiovascular disease, stroke, and mental health problems including depression and dementia. As cognitive deficits have been associated with all of these health conditions, my research sought to investigate the cognitive, mood, and physiological health benefits of supplementation with anthocyanin-rich blueberries. Specifically, this thesis investigated the acute effects of blueberries, in the immediate postprandial period between 0-2 hours after consumption, in a young adult population. Effects were investigated across a range of doses as is recommended in clinical trials in order to gain insights into likely mechanisms of action. The longer term chronic effects of blueberries have already been the subject of much previous research and are not considered here. The thesis starts with a review of recent literature relating to acute intervention with flavonoid-rich food sources. Based on this review, specific research aims were identified and are reported at the end of this introductory chapter. Subsequent chapters report the methods and findings of a series of experiments designed to address each of these research questions. A final general discussion consolidates the findings of this programme of research and considers its impact.

1.2 Introduction to flavonoids

Flavonoids are a class of organic polyphenolic compounds found in varying concentrations in plant-based whole foods such as berries, tea, cocoa, soybeans, and grains. Herbal extracts are also commonly prepared from the leaves, bark, or berries of these plants to provide a more concentrated flavonoid source. There are several subclasses of flavonoid, including flavanols, flavonols, anthocyanidins, flavones, flavanones and isoflavones. Flavonoids often naturally occur in polymer or conjugate form depending on the food type. A detailed overview of flavonoid structure is provided

by Beecher (Beecher, 2003), however a brief description of the main flavonoid forms relevant to this review are contained in Table 1.1.

Table 1.1 Flavonoid subclasses and their naturally occurring forms

Flavonoid subclass	Food source	Additional naturally occurring forms
Anthocyanidins e.g. cyanidin, delphinidin	Berries	Anthocyanidins may occur in methylated form, e.g. malvidin. All anthocyanidins conjugate with saccharide (sugar) groups to form anthocyanins, e.g. chrysanthemin
Flavanols e.g. catechin	Tea, cocoa	All flavanols are isomers, polymers or gallated conjugates of catechin, e.g. epicatechin, epigallocatechin gallate (EGCG)
Flavonols e.g. kaempferol, quercetin	Fruits, vegetables	Flavonols may occur in methylated form, e.g. isorhamnetin and/or conjugate with saccharides
Flavones e.g. apigenin, luteolin	Cereals, herbs	Flavones conjugate with saccharides
Flavanones e.g. naringenin	Citrus fruits	Flavanones may occur in methylated form e.g. hesperetin, and/ or conjugate with saccharides, e.g. hesperidin, narirutin
Isoflavones e.g. daidzein, genistein	Soya beans, peanuts	Isoflavones may occur in methylated form and/or conjugate with saccharides

Flavonoid-rich foods have been well documented to elicit health benefits by reducing the risk factors associated with cardiovascular disease, diabetes and stroke (Basu et al., 2010; Novotny, Baer, Khoo, Gebauer, & Charron, 2015). Over recent years interest has also grown in their ability to elicit cognitive benefits. As such, long term chronic supplementation with flavonoid-rich foods has been investigated extensively, particularly with respect to cognitive ageing and related neurodegenerative disorders, (Blumberg, Ding, Dixon, Pasinetti, & Villarreal, 2014; Cherniack, 2012; Lamport, Dye, Wightman, & Lawton, 2012; Lamport, Saunders, Butler, & Spencer, 2014; Lau, Shukitt-hale, & Joseph, 2006; Macready et al., 2009; Mecocci, Tinarelli, Schulz, & Polidori, 2014; Miller & Shukitt-Hale, 2012; Poulose, Carey, & Shukitt-Hale, 2012; Rendeiro, Guerreiro, Williams, & Spencer, 2012; Scholey & Owen, 2013; Shukitt-Hale, 2012; Solanki, Parihar, Mansuri, & Parihar, 2015; Spencer, 2008, 2010; Vauzour, 2014; R. J. Williams & Spencer, 2012). Less attention has been given to the acute effect of flavonoid-rich interventions on cognitive outcomes i.e. within the immediate 0-6

hours post ingestion. This review focuses on the limited, but increasing body of evidence for the cognitive benefits of acute flavonoid-rich supplementation. Immediate cognitive enhancement is often desirable in academic and work environments, such as during an exam or assessment. Flavonoid-rich foods may, therefore, be useful alternatives to stimulants such as caffeine in these situations. In addition to these potential practical benefits, acute studies are important in understanding the full range of effects that flavonoid-rich foods may elicit, and their mechanisms of action. A number of recent acute supplementation studies in humans are reviewed here.

1.3 Method

A search of Google Scholar, Pubmed, Web of Science and PsychInfo was performed using the keywords flavonoid, polyphenol, memory, and cognition (including truncated forms). The studies selected for inclusion have all been subject to peer and/or editorial review, or form part of a published doctoral thesis. For this reason, conference abstracts have been omitted.

Flavonoids are typically administered in food form rather than as pure compounds, therefore the studies reviewed here have been categorised according to the food source. As shown in Table 1.1 and Table 1.2, the flavonoid subclass or subclasses present often differ according to the food source. This suggests the potential for differences in cognitive effect between food groups, and further supports this method of categorisation. The subclass and dose of flavonoids administered, the cognitive domains affected, and the effect size of the response are discussed, along with associated physiological mechanisms of action. Where possible, Cohen's d effect sizes have been calculated using the method described in Chapter 2. In the majority of studies reviewed, post-intervention scores, or gain scores, have been compared with those obtained for a placebo or control (d), or if this information was not available then post-intervention scores were compared with baseline values (d_b). A summary of all cognitive effects reported in the literature review, including details of participants, intervention type, dose, cognitive domain, and effect size, is shown at the end of the review in Table 1.3.

Table 1.2 Average flavonoid compositions for different food types

Food type	Flavonoid composition (mg/100g)				
	Anthocyanidins	Flavanols	Flavanones	Flavones	Flavonols
Apples (whole)	1.59	9.29	0.00	0.12	4.15
Blackcurrants (whole)	157.78	1.17	-	0.00	11.46
Blueberries (whole, cultivated)	163.30	6.69	0.00	0.20	10.63
Cherries (whole, red)	33.44	4.13	-	0.00	2.43
Cocoa (powdered)	-	52.73	-	-	2.03
Ginkgo biloba (EGb 761)*	-	-	-	-	-
Grapes (whole, Concord)	120.10	2.14	-	-	3.11
Green tea (brewed)	-	132.81	-	0.30	4.82
Oranges(whole)	-	0.00	42.57	0.19	0.73

Data were obtained from the USDA database for the flavonoid content of selected foods (Bhagwat, Haytowitz, & Holden, 2014).*Ginkgo biloba EGb 761 is a standardised extract that contains 24% flavonols in saccharide conjugate form (Clostre, 1999).

1.4 Cognitive effects following acute flavonoid-rich food supplementation

1.4.1 Fruit supplementation

1.4.1.1 Berry anthocyanins (Blackcurrant, Blueberry, Cherry, Cranberry, Grape)

As shown in Table 1.2, berries contain a range of different flavonoid subclasses, but they are typically richest in anthocyanins. Initial berry studies predominantly investigated the cognitive effects of whole fruit. For example, Dodd (2012) demonstrated improved accuracy on a letter memory task (measuring working memory) following freeze dried whole blueberries (200g fresh equivalent, 631mg anthocyanidins), in 19 young adults at a postprandial time point of 5 hours (d=0.57). The study employed a double blind, crossover design with an energy matched control condition. No effects were observed at an earlier time of 2 hours or for other measures of executive function, memory, or mood. For a subset of participants, blood samples taken 1 hour postprandially revealed a trend towards increased plasma levels of brain-derived neurotrophic factor (BDNF) in the blueberry condition. Unfortunately, cognition was not measured at this time point so it is impossible to say whether the neurochemical changes are related to the cognitive outcome. In the same study, older adults' BDNF values decreased from baseline for both blueberry and placebo conditions, but the decrease at the 1 hour time point was attenuated in the blueberry condition. These older adults

(n=18) showed improved performance on an immediate word recognition task at both 2 hours (d=0.44) and 5 hours (d=0.69) postprandially, but no improvements in executive function or mood were observed.

A later study by Whyte & Williams (2015), using fresh whole blueberries (200g, 143mg anthocyanins), investigated cognitive effects in children. They found no effects at 2 hours for a range of executive function tasks, but did observe a significant improvement in delayed word recall using the Rey auditory verbal learning task (RAVLT) (d=0.74). This was a small, crossover study with only 14 participants. As no baseline measures were taken, variations in performance across test days may have reduced the statistical power. Nevertheless, the medium effect size for the RAVLT provides good evidence for positive effects of blueberry flavonoids in children. Whyte, Schafer & Williams (2016) conducted a larger (n=21) double-blind, placebo-controlled, crossover study investigating the cognitive effects of two separate blueberry doses (127mg & 253mg anthocyanins), again in children. The highest dose resulted in significant improvements in immediate word recall after 1.25 hours (d=0.80), and in delayed word recognition after 6 hours (d=0.78). Improved accuracy was observed during a flanker interference task after 3 hours, although only for cognitively demanding incongruent trials (d=0.78). However, reaction times for a Go-NoGo measure of inhibition revealed significantly faster performance following the placebo compared with the blueberry interventions. In a further blueberry study by Khalid et al. (2017) significant effects for mood were observed in similar aged children (7-10) and in young adults (18-21) following a drink made from freeze dried whole blueberry powder (253mg anthocyanins). Mood was measured 2 hours post-intervention using the Positive and Negative Affect Schedule (PANAS). In both age groups positive affect was significantly elevated following the blueberry intervention when compared with a control condition (d=0.28 and d= 0.61 for the children and young adults, respectively), while negative affect remained unchanged.

The positive blueberry effects in older adults and children appear to be focussed on episodic memory, whereas improvements in executive function are more consistent in young adults. The differences in cognitive domains may be an artefact of the small sample sizes, but could also be indicative of age related differences in underlying neuronal structures that affect the capacity for improvement. For example, hippocampal function may be more receptive during development in childhood and decline in old age, whilst frontal regions associated with executive function may be more sensitive in young adulthood. It is noteworthy that neurochemical changes in BDNF were apparent after 1 hour, yet distinct time points for memory effects emerged at 1.25-2 hours and 5 hours, but not at an intervening 3 hour time point. At this stage only BDNF trends have been observed, and not directly in association with cognitive changes. Although it is perhaps premature to

comment on the relationship between acute changes in cognition and BDNF, it has nevertheless been posited that flavonoid induced increases in BDNF may facilitate stronger memory encoding (Whyte et al., 2016). Possible mechanisms of action are discussed later in this chapter. Overall, the timings of cognitive effects are likely to be related to the digestion, absorption and metabolism of flavonoids, but further mapping of cognitive and physiological observations is required in order to resolve inconsistencies within the current observations.

The flavonoid content of blueberries is known to vary widely depending on growing, processing and storage conditions (Rodriguez-Mateos, Cifuentes-Gomez, Tabatabaee, Lecras, & Spencer, 2012; Rodriguez-Mateos, George, Heiss, & Spencer, 2014). The same 200g quantity of whole blueberries used in the first two studies described above (Dodd, 2012; Whyte & Williams, 2015) showed clear differences in flavonoid content (631mg anthocyanidins & 143mg anthocyanins respectively). This highlights the importance of analysing fresh fruits for their flavonoid content when conducting an intervention. It is also important to note that compositional analysis of anthocyanins typically (but not exclusively) involves the removal of saccharide conjugates prior to quantification; therefore anthocyanin content is often reported as anthocyanidin equivalent. This difference is critical when comparing doses between studies. For example, a berry intervention reported to contain 100mg cyanidin may actually contain 156mg chrysanthemin (a saccharide of cyanidin). Some studies reviewed here appear to use the terms anthocyanins and anthocyanidins interchangeably without acknowledging this distinction, making it unclear whether a reported anthocyanin dose is actually referring to an equivalent anthocyanidin dose.

As with blueberries, blackcurrants are a rich source of anthocyanins. Watson et al. (2015) conducted a double-blind, controlled crossover trial of two blackcurrant extracts (cold-pressed juice or freeze-dried powder). Improved attention compared with an energy-matched control was observed in 36 young adults during a 70-minute-long, cognitively fatiguing battery, beginning 1 hour postprandially. Specifically, declining accuracy on a rapid visual information processing (RVIP) task was attenuated after taking the powdered extract (d=0.10, d=0.47, d=0.47, d=0.49, d=0.49, d=0.59, d=0.56, measured for seven task repetitions; once every 10 minutes). Similarly, a slowing of reaction time on a digit vigilance task was attenuated, relative to the control, after taking the juiced extract (d=0.60, d=0.73 & d=0.60 for the 1st, 4th and 7th repetitions of the task, respectively). No effects were observed for the Stroop task (a measure of inhibition and attention), or for subjective measures of mood and mental fatigue. The total polyphenol content of the two extracts were matched at 525mg/60kg bodyweight. However, the anthocyanin content differed slightly; 483mg/60kg bodyweight for the powder and 467 mg/60 kg for the juice. This difference was also reflected in the

analysis of plasma anthocyanin levels, which were observed to be higher following consumption of the powdered extract compared with levels recorded following the juice. Interestingly, the juice but not the powder was observed to inhibit platelet monoamine oxidase (MAO) and to attenuate blood glucose decline over the duration of the 70-minute task battery. This study suggests that the way an extract is prepared may influence cognitive and physiological outcomes, however as different blackcurrant cultivars were used for each extract, the contrasting observations may simply represent compositional differences such as the ratio of flavonoid subclasses present. For the juiced blackcurrant extract, MAO inhibition and blood glucose regulation were observed providing evidence for additional mechanisms of action further to the neurochemical changes observed for blueberries. The significant cognitive effects were observed for tests of executive function (RVIP and to some extent vigilance) which is consistent with the executive function benefits reported in healthy young adults following blueberry anthocyanins.

In a double-blind crossover intervention study, Hendrickson & Mattes (2008) investigated whether an acute dose of grape juice would mitigate deficits in mood and cognition that commonly occur following a large meal. Approximately 600ml (10ml/kg), containing around 580mg anthocyanins, was administered to young adult smokers along with a standardised lunch. Smokers were selected on the rationale that this population have an increased propensity to oxidative stress, and because smoking abstinence can exaggerate the post-meal dip in cognitive or affective state, thus this population may be more sensitive to the effects of flavonoid-rich intervention than healthy non-smokers. This was a large study (n=35) with considerable statistical power, yet no significant effects of grape juice were observed 1 hour postprandially when compared to an energy matched placebo condition. Mood ratings for positive mood states (pleasure, arousal & vigour) were observed to decline under both grape and placebo conditions, similarly ratings of negative mood states (confusion & fatigue) increased under both conditions. Although mood generally declined, word fragment completion task performance did not significantly change over time in either condition. It is unfortunate that performance was examined on only one cognitive domain (implicit memory), which is an area that has not previously been considered with respect to flavonoid-rich intervention. Studies have observed effects on traditional measures of explicit memory and executive function that were not measured here. Indeed, a more recent study by Haskell-Ramsay, Stuart, Okello, & Watson (2017) observed significant cognitive and mood effects following purple grape juice intervention in young adults (n=20), when compared to a sugar-matched white grape juice control. The purple grape juice contained only 27mg anthocyanins, but elicited a significant reduction in a composite measure of speed of attention (d=0.50) at 20 minutes postprandially. The same dose

concurrently increased composite ratings of calmness (d=0.57). Therefore improvements following flavonoid-rich interventions may be both temporally and cognitive-domain sensitive.

Caldwell, Charlton, Roodenrys, & Jenner (2016) published their investigations into the effects of flavonoid-rich cherry. Following administration of 300ml cherry juice (55mg anthocyanins) to younger adults (n=6), older adults (n=5) and older adults with mild cognitive impairment (n=5), tests of executive function, speed of processing, and verbal learning and memory were performed at baseline and 6 hours postprandially. At 6 hours, the older adults displayed improved task switching performance compared to baseline (d_b=0.75). No other cognitive effects were observed. The authors attribute this single effect to type 1 error, citing attrition of participants in that group as a likely cause. However the small sample size in all groups suggests that the whole study is likely to be severely underpowered. The lack of an energy matched, low flavonoid control condition is also cause for concern; a second crossover condition only administered the same juice in three separate 100ml aliquots each consumed 1 hour apart. No cognitive effects were observed relative to baseline following consumption of the juice in these consecutive smaller doses. A further problem may be the intervention itself; the anthocyanin content of the cherry juice appears very low compared to some of the above studies. A considerably larger, controlled study is therefore needed to determine if anthocyanin-rich cherries elicit acute cognitive effects similar to those of other anthocyanin-rich fruits. Indeed more recently, Keane, George, et al. (2016) investigated the effects of 60ml cherry juice concentrate, containing 68mg anthocyanins, on 27 middle aged adults. No effects were observed on any of the cognitive or mood measures deployed at baseline, 1, 2, 3, or 5 hours postprandially, when compared to an energy matched control. Measures included digit vigilance, RVIP, Stroop, and mood scales. Cerebral blood flow (CBF) was measured concurrently using transcranial Doppler imaging. No changes in CBF were evident, although near-infrared spectroscopy (NIRS) revealed a significant increase in oxygenated haemoglobin at the 1 hour time point following the cherry intervention. Significant reductions in systolic blood pressure (SBP) were also observed at 1 hour when compared with the control. It appears, then, that cherries are less effective than other fruits at impacting cognitive function despite evidence for cardiovascular effects.

1.4.1.2 Citrus hesperidin (Orange)

As with berries, citrus fruits contain several different flavonoids, but they are richest in the flavanone hesperidin. In a crossover intervention study, Lamport et al. (2016) supplemented 24 young adults with a commercially available flavanone-rich orange juice (70.5mg total flavonoids; 42.15mg hesperidin, 17.25mg naringin, 6.75mg narirutin) or an energy matched control. An extensive battery of tasks including measures of vision, episodic memory, processing speed, working memory, and

other executive functions were performed 2 hours postprandially. Significant improvements when compared to both control and baseline were observed only for the digit symbol substitution task (DSST), a measure of psychomotor processing speed (d=0.30). An additional group of participants underwent fMRI assessment using arterial spin labelling (ASL). Increased CBF was observed at 2 hours but not 5 hours postprandially. However, as cognition was not measured directly in conjunction with CBF (and not at all at 5 hours) it remains unclear whether the observed improvements in processing speed are causally related to the CBF changes. The flavonoid dose used in this study was low, particularly when compared with the typical doses of other flavonoid subclasses reviewed here. If flavonoid effects are dose-dependent then a higher dose may have elicited increased cognitive benefit. Indeed, a subsequent study by Alharbi et al. (2016) used orange juice fortified with additional flavanone-rich orange pulp, to achieve a greater total flavonoid content (272mg; 220.46mg hesperidin, 34.54mg narirutin). Middle aged adults (n=24, 30-65 years) showed improved psychomotor performance on a finger tapping task at both 2 hours (d=0.87) and 6 hours (d=0.62). Improvements in attention and general executive function, as measured by a continuous performance task (CPT), were observed at 6 hours (d=0.58). The orange juice was observed to attenuate a decline in subjective alertness throughout the testing period compared to a sugar-matched control.

From this limited research, orange juice appears to benefit psychomotor performance across age groups at a time point also associated with increased CBF. Higher doses appear to elicit greater effect sizes for psychomotor performance and provide additional benefits in executive function, tentatively suggesting that flavanone effects may be dose-dependent.

1.4.1.3 Quercetin & Epicatechin (Apple)

Bondonno et al. (2014) conducted a crossover intervention study investigating the cognitive effects of whole fresh apple (184mg quercetin & 180mg epicatechin), spinach or a combination of the two. It was hypothesised that the nitrate content of spinach and the flavonoids in apple would both augment plasma nitric oxide (NO), although through distinct mechanisms. Increased NO status has been previously associated with vasodilatory related increases in blood flow, which the authors predicted would lead to a beneficial cognitive outcome following all three intervention conditions. Cognition and mood were measured 2.5 hours postprandially, using the Cognitive Drug Research (CDR) assessment battery and Bond-Lader mood scales. Even though significant increases in plasma NO were observed for the apple, spinach and combined conditions when compared with a control condition, all cognitive outcomes remained non-significant. This included factors for working memory and attention derived from the CDR results. This was a reasonably well powered study with

30 middle aged participants (mean age 47 years), so the lack of cognitive findings suggests that augmented NO status may not affect cognition. If alternative mechanisms are responsible for the previously observed cognitive effects of flavonoid-rich foods, then it may be that the flavonoids present in apple are not particularly effective, or that the peak time for observing apple flavonoid effects was missed by the study. The rationale for testing 2.5 hours postprandially is not given in the paper, but may relate to previous NO observations rather than the absorption and metabolism of flavonoid subclasses present in apples.

1.4.2 Cocoa supplementation (Epicatechin)

To date, much of the flavonoid-related literature has focussed on the effects of cocoa. Cocoa is a rich source of the flavanol epicatechin; however it also contains caffeine and theobromine that have known psychoactive properties. Not all of the cocoa studies reviewed here have matched for these potential confounds across experimental conditions. Scholey et al. (2010) carried out a double-blind crossover study of 30 young adults (age 18-35), comparing two acute doses of chocolate milk with a fully matched control. Beginning 90minutes postprandially, participants performed 6 consecutive repetitions of a 10minute task battery, designed to be cognitively fatiguing. A 520mg dose of cocoa flavanol resulted in significant improvements in working memory on a serial threes (3s) subtraction task at all six time points (d=0.57, d=0.71, d=0.50, d=0.64, d=0.50 and d=0.41 respectively) relative to the control. A 994mg dose resulted in significant improvements for the first four repetitions on the same serial 3s task (d=0.44, d=0.52, d=0.41 and d=0.67 respectively). However, no improvements were observed whilst performing the more difficult serial sevens (7s) task. Only the high dose was reported to improve reaction time on an RVIP attention task, with significant improvements observed during the 3rd and 4th repetitions (d=0.35 for each repetition). Conversely, only the low dose improved self-reported levels of mental fatigue, with significant attenuation of fatigue across all but the 3rd repetition (d=0.39, d=0.37, d=0.30, d=0.27 and d=0.30 respectively). Overall the low dose was observed to be effective over more time points than the high dose for serial 3s and conveyed greater benefits in terms of counteracting mental fatigue. The high dose resulted in some additional reaction time benefits but also incurred cognitive costs with an increased error rate observed during the serial 7s task. The authors conclude that lower doses of cocoa flavanol may be more effective but do not offer an explanation as to why this might be the case.

In contrast, a similar study of older adults by Pase et al. (2013) failed to observe any acute effects of a water based chocolate drink on cognition or mood. Doses containing 250mg and 500mg cocoa flavanol were compared with a fully matched control. The study used a broad spectrum of tasks from the CDR assessment battery and a range of Bond-Lader mood visual analogue scales. Testing

was performed at 1 hour, 2.5 hour, and 4 hour time points. The inconsistencies between the two studies in population, testing time point, and dose make it difficult to draw conclusions from the opposing outcomes. However, a notable methodological difference was that Pase et al. (2013) provided a light lunch 1.5 hours after the intervention. The effects of flavonoids may have been masked by stronger macronutrient effects, such as the neuronal energy provided by carbohydrate ingestion.

In support of Scholey et al. (2010), Field, Williams, & Butler (2011) observed improved performance in visual spatial working memory (VSWM) (d=0.35) and choice reaction time (CRT) (d=0.16) in 30 young adults (age 18-30), following a 773mg high dose of cocoa flavanols relative to a low flavanol white chocolate control. Testing was performed 2 hours postprandially. Visual function also benefited; significant improvements in visual contrast sensitivity and time to detect motion direction were observed following the high dose. However, caffeine and theobromine were not matched across the two conditions and baseline measurements were not recorded before administering either of the interventions. These methodological limitations are reflected in the lower effect sizes obtained relative to the previously reviewed flavanol-rich cocoa studies. Massee et al. (2015) investigated the effects of a cocoa extract containing 250mg cocoa flavanol in a between-subjects study of 40 young adults. At a postprandial time point of 2 hours, mental fatigue was observed to be significantly lower following the cocoa intervention when compared to a control (d=0.80). Improvement from baseline on a serial 7s subtraction task was also observed to be significantly improved at 2 hours for the cocoa condition when compared to the control (d=0.52). Again however, the control was not matched for caffeine so it is unclear whether the outcome was confounded by the small amounts of caffeine (5.56mg) also present.

Most recently, Boolani, Lindheimer, Loy, Crozier, & O'Connor (2017) investigated the effects of cocoa and caffeine on mood and cognition in 23 young adults. Compared to placebo, fewer commission errors were observed on the Bakan task (similar to RVIP) at a postprandial time point of 98 minutes (d=0.76), however the large effect size observed here may be due to the confounding influence of caffeine and theobromine that were not matched in the placebo. A significant lowering of anxiety was observed at the same time point when cocoa was compared with a caffeine-matched control (d=0.84). However no other cognitive effects were reported. The authors comment that an absence of calories compared with other cocoa research may have influenced the outcome; the cocoa interventions were sweetened using artificial sweetener in this case.

Findings following cocoa intervention appear mixed. However, when combined, these studies suggest that moderately high doses of cocoa flavanol provide benefits for cognition and mood up to two hours after ingestion in young adult populations. Enhanced visual functioning accompanied some of the cognitive improvements and as the majority of cognitive tasks are visual, it is possible that this concurrent effect may also play a role in the facilitation of cognitive improvement. Further cocoa flavanol studies are required to clarify the effects of dose, the effects on different populations, and the role of vision as a potential mechanism of action.

1.4.3 Green tea supplementation (Epigallocatechin gallate)

The main flavonoid constituent of green tea is the flavanol epigallocatechin gallate (EGCG). Dietz, Dekker, & Piqueras-Fiszman (2017) investigated the acute cognitive and mood effects of green tea using a tea powder intervention containing 280mg EGCG. Twenty-three young adults consumed the tea in drink and snack bar form. At a postprandial time point of 1 hour no significant changes in mood were observed compared with the drink or bar control conditions. Cognitive measures included immediate and delayed recall, simple and choice reaction tasks, digit vigilance, and spatial and numeric working memory tasks. Choice reaction time was observed to be significantly faster following green tea when compared with performance following control conditions, irrespective of the delivery form of the intervention (drink or bar) (d=0.59). However, the authors attribute the finding to other active constituents in the intervention, including L-theanine (67mg), and caffeine (136mg) that were not matched in the control conditions. No other cognitive effects were observed.

To avoid stimulant confounds, green tea is more typically administered in caffeine-free extract form. In a double-blind, placebo controlled crossover study, Wightman, Haskell, Forster, Veasey, & Kennedy (2012) administered 135mg and 270mg doses of EGCG extract, but observed no cognitive or mood effects for either dose. Cognitive tests included simple reaction time (SRT), serial subtraction, RVIP and Stroop, and were measured at baseline and 45 minutes after supplementation. Near-infrared spectroscopy (NIRS) revealed a lowering of CBF during cognitive testing following the lower dose. The authors attribute this CBF effect to previously observed vasoconstrictor properties of EGCG seen at low doses. This vasoconstrictor effect appears to contrast with vasodilatory properties previously observed for higher doses. In support of the latter, a study by Scholey et al. (2012) observed increased alpha, beta and theta electroencephalography (EEG) activity in the frontal gyrus 2 hours after a high dose of 300mg EGCG extract. This change in brain activity was accompanied by increased feelings of calm (d=0.55) and a reduction in ratings of stress (d=0.64). Scholey et al. (2012) reported that similar EEG activity has been observed in studies of meditation and mindfulness, suggesting a correlational link with mood. However, attention

outcomes measured using the RVIP task were reported as non-significant (in a separate publication; Camfield, Stough, Farrimond, & Scholey, 2014). The acute effects of EGCG therefore appear restricted to mood at this stage, and then only for higher doses. Some modulation of brain activity is apparent, but further investigation is needed to understand dose-related differences in vasoreactivity, and whether or not these are likely to lead to behavioural benefits on cognitive tasks.

1.4.4 Ginkgo biloba supplementation (Quercetin, Kaempferol & Isorhamnetin)

Ginkgo biloba is a flavonol-rich leaf extract that is typically standardised to contain 24% flavonols and 6% terpenoids (a group of bioactive plant lipids). A popular herbal supplement, it is reported anecdotally to improve blood circulation, relieve anxiety and enhance memory e.g. (Mahadevan & Park, 2008). Despite these claims, in a double-blind crossover trial, Warot et al. (1991) failed to observe any cognitive improvements in 12 young adults 1 hour after an acute dose of 600mg (containing approx. 144mg flavonols). The task battery included choice reaction time (CRT), picture recognition, the Sternberg memory scanning task, and critical flicker fusion frequency (CFF) – a measure of vigilance. However, the small sample size may have impacted the statistical power of the study. Similarly, Subhan & Hindmarch (1984) found no effect on either CFF or CRT at the same time point for doses of 120, 240 or 600mg (containing approx. 29, 58 & 144mg flavonols respectively). Significant improvements were however observed for the Sternberg task, with a decrease in reaction time observed after the 600mg dose, and decreases in memory scanning rates observed after the 120mg and 600mg doses. These observations were in comparison with a placebo in a double-blind crossover design; however baseline measurements were not recorded prior to administering each intervention and only 8 participants were tested. Unfortunately there was insufficient data reported in the paper to allow calculation of effect size. Again, the small sample size may have impacted the statistical power of the study.

Nathan et al. (2002) tested at the slightly later postprandial time of 90 minutes but found no significant improvements in older adults following 120mg supplementation (containing approx. 29mg flavonols) using the CDR battery and RAVLT. Yet again this was a small study (n=11) with a small dose size and as such the conclusions may be statistically unreliable. A larger double-blind crossover study of young adults (n=20) conducted by Kennedy, Scholey, & Wesnes (2000) observed dose- and time-related improvements, in a 'speed of attention' factor derived from the CDR task battery. Effects were not apparent at 1 hour postprandially but became evident at 2.5, 4 and 6 hours. Cohen's d values were d=0.45, d=0.45 & d=0.50, respectively for a 240mg dose (containing approx. 58mg flavonols), and d=1.29, d=0.86 & d=0.80 for a 360mg dose (approx. 86mg flavonols) across the time period. No effect was observed for a lower 120mg dose (approx, 29mg flavonols).

The higher effect sizes for the 360mg dose compared with the 240mg dose, and the lack of any significant effect for the lowest 120mg dose, are indicative of a dose-related response for attention. This was not the case for memory; improvements in a 'quality of memory' factor were observed at 1 hour and 4 hours for the 120mg dose only (d=0.57 & d=0.52), although trends were evident for the higher doses. A follow up study by the same authors (Kennedy, Scholey, & Wesnes, 2002) comparing ginkgo effects with panax ginseng (a plant extract rich in bioactive saponins) failed to replicate the 'speed of attention' finding for an identical 360mg ginkgo biloba dose. This inconsistency was attributed to a procedural change in the second study. They did however observe serial 7s improvements at 4 hours (d=0.65) and 6 hours (d=0.28). Immediate recall (d=0.83) and delayed recall (d=0.67) were also observed to improve at 6 hours, as did a 'Quality of memory' factor (d=0.59).

Elsabagh, Hartley, Ali, Williamson, & File (2005) administered a low dose of 120mg ginkgo biloba extract (containing approx. 29mg flavonols) and after 4 hours observed improved attention relative to a placebo condition, using a paced auditory serial addition test (PASAT) (d=0.58). Improved pattern recognition (d=0.55) and a trend towards improved delayed recall were also observed. This was a well powered between-subjects study (n=52) but with only a single post-ingestion time point and no baseline. The two participant groups did not differ significantly in terms of age, gender, BMI, verbal IQ, or habitual caffeine and alcohol intake, however without cognitive baseline measures, the possibility remains that group differences in cognition may simply be due to differences in the participants.

In order to consolidate the ginkgo biloba data from three smaller studies, Kennedy, Jackson, Haskell, & Scholey (2007) combined their data into a meta-analysis. In young adults (combined n=78), a general decline in 'quality of memory' observed following the placebo was attenuated at 1 hour and 4 hours following 120mg ginkgo biloba supplementation. However 'speed of attention' performance slowed at 1 hour and 6 hours relative to both baseline and placebo. The authors were unable to explain this unexpected outcome, particularly as higher doses have been shown to improve speed of attention relative to placebo. Such inconsistencies in the data highlight the need for further investigation. Overall, memory findings following ginkgo biloba supplementation appear to be relatively consistent across the larger, well powered crossover studies and mimic the timings of memory effects observed in the blueberry supplementation studies, occurring at 1 hour and 4-6 hours postprandially. Attention effects following ginkgo biloba appear dose-dependent and seem to occur at later time points than some other flavonoid subclasses (from 2.5-6 hours). The effects of ginkgo biloba also appear to span several cognitive domains including attention, working memory,

and episodic memory. Some of the largest cognitive effect sizes, as summarised in Table 1.3, are observed following ginkgo biloba. This may reflect the additional effects of terpenoids also present in the extract.

Table 1.3 Statistically significant cognitive outcomes in decreasing order of effect size

Study	Age (years) n	Flavonoid dose (mg)	Cognitive measure	Postprandial time point	Effect size (d)
Kennedy et al. (2000)	19-24 20	ginkgo 360	Speed of attention	2.5-6h	(Average) 0.98
Boolani et al. (2017)	20.3(2.2) 23	cocoa 499	Mood	1.6h	0.84
Kennedy et al. (2002)	21.2(3.9) 20	ginkgo 360	IR	6h	0.83
Massee et al. (2015)	24.1(4.5) 40	cocoa 250	Mental fatigue	2h	0.80
Whyte et al. (2016)	7-10 21	berry 253	IR	1.25h	0.80
Whyte et al. (2016)	7-10 21	berry 253	Flanker	3h	0.78
Whyte et al. (2016)	7-10 21	berry 253	Word recognition	6h	0.78
Boolani et al. (2017)	20.3(2.2) 23	cocoa 499	Bakan	1.6h	0.76
Alharbi et al. (2016)	30-65 24	citrus 272	Finger tapping	2-6h	(Average) 0.75
#Caldwell et al. (2016)	74.1(7.9) 5	berry 55	Task switching	6h	(d_b) 0.75
*Whyte & Williams (2015)	8-10 14	berry 143	RAVLT	2h	0.74
Kennedy et al. (2002)	21.2(3.9) 20	ginkgo 360	DR	6h	0.67
Watson et al. (2015)	18-34 36	berry 467	Digit vigilance	1-2.5h	(Average) 0.64
Scholey et al. (2012)	27.7(9.3) 31	tea 300	Mood	2h	0.64
Khalid et al. (2017)	18-21 21	berry 253	Mood	2h	0.61
Dietz et al. (2017)	20-35 23	tea 280	CRT	1h	0.59
Kennedy et al. (2002)	21.2(3.9) 20	ginkgo 360	Quality of memory	6h	0.59
Alharbi et al. (2016)	30-65 24	citrus 272	CPT	6h	0.58
*Elsabagh et al. (2005)	18-26 52	ginkgo 120	PASAT	4h	0.58
Dodd (2012)	18-25 19	berry 631	Letter memory	5h	0.57

Table 1.3 continued

Study	Age (years) n	Flavonoid dose (mg)	Cognitive measure	Postprandial time point	Effect size (d)
Dodd (2012)	62-73 18	berry 631	Word recognition	2-5h	(Average) 0.57
Haskell-Ramsay et al. (2017)	18-35 20	berry 27	Mood	0.3h	0.57
Scholey et al. (2010)	18-35 30	cocoa 520	Serial 3s	1.5-2.5h	(Average) 0.56
Kennedy et al. (2000)	19-24 20	ginkgo 120	Quality of memory	1-4h	(Average) 0.55
Scholey et al. (2012)	27.7(9.3) 31	tea 300	Mood	2h	0.55
*Elsabagh et al. (2005)	18-26 52	ginkgo 120	Pattern recognition	4h	0.55
Massee et al. (2015)	24.1(4.5) 40	cocoa 250	Serial 7s	2h	0.52
Scholey et al. (2010)	18-35 30	cocoa 994	Serial 3s	1.5-2.5h	(Average) 0.51
Haskell-Ramsay et al. (2017)	18-35 20	berry 27	Speed of attention	0.3h	0.50
Kennedy et al. (2000)	19-24 20	ginkgo 240	Speed of attention	2.5-6h	(Average) 0.47
Kennedy et al. (2002)	21.2(3.9) 20	ginkgo 360	Serial 7s	4-6h	(Average) 0.47
Watson et al. (2015)	18-34 36	berry 483	RVIP	1-2.5h	(Average) 0.45
Scholey et al. (2010)	18-35 30	cocoa 994	RVIP	1.5-2.5h	(Average) 0.35
*Field et al. (2011)	18-25 30	cocoa 773	VSWM	2h	0.35
Scholey et al. (2010)	18-35 30	cocoa 520	Mental fatigue	1.5-2.5h	(Average) 0.33
Lamport et al. (2016)	18-30 24	citrus 71	DSST	2h	0.30
Khalid et al. (2017)	7-10 52	berry 253	Mood	2h	0.28
*Field et al. (2011)	18-25 30	cocoa 773	CRT	2h	0.16

*Studies with no baseline measurements. #Studies with no control condition. (Average)Average of effect sizes for the same dose & cognitive measure recorded across multiple time points.

1.5 Mechanisms of action

1.5.1 Absorption and metabolism

In vitro and in vivo evidence suggests that flavonoids and their metabolites are readily absorbed into the blood stream and are able to cross the blood brain barrier (Jäger & Saaby, 2011; Youdim, Shukitt-Hale, & Joseph, 2004). Although it is not possible to directly measure flavonoid concentrations in the human brain, the timings of peak levels in blood plasma have been observed to correspond with the timings of cognitive effects. For example, whole anthocyanins are observed in plasma 1-4 hours postprandially (Mazza, Kay, Cottrell, & Holub, 2002), and their metabolites are observed to peak at 1-2 hours and 6 hours following berry supplementation (Kay, Mazza, Holub, & Wang, 2004; Rodriguez-Mateos et al., 2013), which seems to correspond with the two distinct timings of cognitive benefits observed for blueberry. Plasma flavanols following cocoa ingestion peak at 2 hours only (Holt et al., 2002; Rein et al., 2000; Schroeter et al., 2006; Wang et al., 2000), and this is reflected by a number of positive cognitive effects at 2 hours but an absence of cognitive effects at later time points. Conversely, the cognitive findings for ginkgo biloba are mainly observed between 4-6 hours postprandially (Elsabagh et al., 2005; Kennedy et al., 2000, 2002). Bioavailability studies for ginkgo were not apparent in the literature, however bioavailability of some conjugates of quercetin, the main flavonoid present in ginkgo, have been observed to peak at 4-6 hours postprandially (Graefe et al., 2001). This may also explain the lack of cognitive findings for quercetin-rich apple where cognitive testing was carried out at 2.5 hours (Bondonno et al., 2014). Similarly, late peak plasma timings have also been observed for the citrus flavonoid hesperidin (Manach, Williamson, Morand, Scalbet, & Remesy, 2005). The reviewed cognitive effects following orange juice consumption support this, but also show an earlier effect on psychomotor performance that may relate to mechanisms of action such as improved blood glucose regulation or increased cerebral/peripheral blood flow. In general however, the timings of cognitive effects appear closely related to the absorption and metabolism rates of the supplemented flavonoid compounds.

Broad individual differences have been noted in the absorption profiles of a range of flavonoids and their associated metabolites (Bresciani et al., 2017; Mennen et al., 2008; Rodriguez-Mateos et al., 2015); not only in the quantities of metabolites present, but also in the timings of their appearance (Bresciani et al., 2017). Intact flavonoids are generally only absorbed in very small quantities via the stomach and small intestine, large quantities therefore enter the colon where they are metabolised by gut microbiota before being absorbed into the blood stream. Specific bacteria have been identified that act on different flavonoids (Braune & Blaut, 2016), for example *Bifidobacterium* and *Lactobacillus–Enterococcus* catalyse the metabolism of anthocyanins (Aura et al., 2005; Hidalgo et

al., 2012) and *Eggerthella lenta* and *Flavonifractor plautii* catalyse the metabolism of catechins (Jin & Hattori, 2012; Kutschera, Engst, Blaut, & Braune, 2011). Individuals have varying polyphenol metabolising phenotypes, largely dependent on their gut microbiota profile (Tomás-Barberán, Selma, & Espín, 2016). The effectiveness of a flavonoid intervention is therefore dependent on the phenotype of the subject which is typically unclear at the onset of testing. Interestingly, a diet habitually rich in flavonoids can have a positive impact on the profile of gut microbiota (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; M. Hidalgo et al., 2012; Valdés et al., 2015), and so subjects who regularly consume flavonoids are likely to propagate a gut microbiota profile specifically suited to the metabolism of flavonoids. If cognitive health benefits observed following a single acute dose of flavonoids are dependent upon the metabolism of the flavonoid compounds by such gut microbiota, individuals who habitually consume greater quantities of flavonoids might therefore be expected to experience greater acute cognitive health benefits in the immediate postprandial period, particularly at later time points, due to the presence of greater quantities of flavonoid metabolising bacteria in the intestinal tract (Laparra & Sanz, 2010). This idea is contrary to the commonly posited theory that interventions are more effective in those with a low habitual intake of flavonoid-rich foods and requires further investigation.

1.5.2 Endothelial function

1.5.2.1 Vasodilation

The vasoactive properties of flavonoid-rich foods as demonstrated by flow-mediated dilation of the brachial artery following ischemia (FMD) (Alexopoulos et al., 2008; Alqurashi, Galante, Rowland, Spencer, & Commane, 2016; Dohadwala et al., 2011; Monahan et al., 2011; Rendeiro et al., 2017; Rodriguez-Mateos et al., 2013; Schroeter et al., 2006; Widlansky et al., 2007), peripheral arterial tonometry (PAT)(Dohadwala et al., 2011), and Laser Doppler Flowmetry (LDF)(Morand et al., 2011), are known to result in increased peripheral blood flow. Peak vasodilatory effects have been observed at 1-2 hours and 6 hours postprandially for blueberry (Rodriguez-Mateos et al., 2013) and acai berry supplementation (Alqurashi et al., 2016), at 2 hours for cocoa (Monahan et al., 2011; Schroeter et al., 2006), at 30 minutes (Alexopoulos et al., 2008) and 2 hours (Widlansky et al., 2007) for green tea, at 4 hours for cranberry (Dohadwala et al., 2011) and at 6 hours for orange juice (Morand et al., 2011), thereby covering the full range of time points at which cognitive effects have been observed. Meta-analysis of these FMD effects has revealed that they may be non-linearly dose-dependent, following an inverted U shaped curve (Kay, Hooper, Kroon, Rimm, & Cassidy, 2012; Rodriguez-Mateos et al., 2013). Blood pressure reductions have also been associated with the consumption of flavonoid-rich foods, which may again be related to their vasoactive properties.

Although more commonly observed in chronic intervention studies (Kay et al., 2012), the effects have also been observed following acute supplementation. For example, acute reductions in systolic blood pressure (SBP) have been observed 1-3 hours following cherry juice concentrate (Keane, George, et al., 2016; Keane, Haskell-Ramsay, Veasey, & Howatson, 2016).

These endothelial effects are not restricted to peripheral systems; flavonoids have also been observed to result in increased CBF 1 hour after acute blueberry supplementation (Dodd, 2012), and 2 hours after cocoa (Francis, Head, Morris, & Macdonals, 2006) and orange juice supplementation (Lamport et al., 2016). Selective increases have been observed in the dorsolateral prefrontal cortex (DLPFC) following green tea supplementation (Borgwardt et al., 2012), however time of testing and exact dose of EGCG were not stated in the paper which makes interpretation of the observation difficult. It is likely that CBF increases will occur across the range of time points observed in peripheral blood flow studies, but currently there are insufficient published findings to support this fully. In particular, little work appears to have been reported regarding CBF modulation following acute ginkgo biloba supplementation in humans. However, CBF has been shown to be positively correlated with cognitive performance, particularly in epidemiological assessment of dementia risk, where CBF is reduced in patients with dementia, and greater CBF velocity is associated with a lower rate of cognitive decline and lower risk of dementia in healthy ageing (Ruitenberg et al., 2005; Spencer, 2010). Cognitive training during healthy ageing has also been observed to increase both CBF and cognitive performance (Mozolic, Hayasaka, & Laurienti, 2010). Therefore, vasodilatory mechanisms of action may account for at least some of the cognitive improvements observed in acute supplementation studies. Similarly, increased CBF has been posited to account for cognitive benefits observed following acute exercise, although findings are not equivocal (McMorris, 2015). Cognitive improvements following flavonoid ingestion have yet to be directly matched with acute increases in CBF, making this an important area for further research.

1.5.2.2 Nitric oxide synthesis

There are a number of different chemical mediators for vasodilation including nitric oxide (NO). Flavonoids have been associated with acute augmentation of NO status. Bondonno et al. (2014) demonstrated a significant increase in plasma NO in response to flavonoid-rich apple, although no cognitive benefits were observed for a battery of tasks performed immediately after the blood samples were taken (2.5 hours postprandially). Similar enhancement of NO status has been observed at 1 hour for cocoa supplementation (Schroeter et al., 2006), and at 2 hours following pure epicatechin and quercetin (Loke et al., 2008). In addition to its role in endothelial function, NO has also been implicated in the regulation of the transcription factor CREB; an important factor in

neuron survival and synaptic plasticity (Ciani, Guidi, Bartesaghi, & Contestabile, 2002). A proposed mechanism for flavonoid induced augmentation of NO status is through enhancing the expression or activity of endothelial nitric oxide synthase (eNOS) (Stoclet et al., 2004). eNOS itself has been implicated in the regulation of BDNF expression (Chen et al., 2005). This may account for the increased plasma BDNF levels observed after acute blueberry supplementation (Dodd, 2012), although this connection is speculative at this stage. Depending on the isoform present, NOS has also been implicated as a causal agent in neurodegenerative disease (Cárdenas, Moro, Hurtado, Leza, & Lizasoain, 2005; Chung & David, 2010). Although eNOS is considered to be neuroprotective, neuronal and inducible isoforms (nNOS and iNOS) are thought to be neurotoxic through mechanisms of oxidative stress. So flavonoid regulatory effects on NO systems may be beneficial over long term supplementation, in addition to the acute mechanisms considered here.

1.5.3 Blood glucose regulation

Attenuation of peak postprandial plasma glucose followed by a more gradual post-peak decline observed following blackcurrant (Törrönen et al., 2010; Watson et al., 2015) and cranberry (Wilson et al., 2008) suggests that altered blood glucose regulation may provide an additional mechanism of action for the executive function effects observed in young adults. Irrespective of the presence of flavonoids, a postprandial glucose profile characterised by a low peak and gradual decline is associated with better cognitive function than a poor glucose profile with a high peak and rapid decline (Benton & Nabb, 2003; Sunram-Lea & Owen, 2017), thus this mechanism could account for cognitive benefits following consumption of flavonoid-rich foods. In particular, Watson et al. (2015) observed both higher blood glucose levels and improved attention 1 hour following blackcurrant supplementation relative to a sugar matched control. There is some evidence to suggest that the absorption of sugar may be slowed when consumed in conjunction with flavonoids (Hanhineva et al., 2010; Sancho & Pastore, 2012; Williamson, 2013); mechanisms of action are discussed in more detail in Chapter 5. Therefore a flavonoid-rich intervention may result in greater availability of glucose over a longer period relative to a low-flavonoid sugar-matched control. As glucose is necessary for all human cell function, and glucose supplementation has been directly linked to cognitive improvement (Benton, Owens, & Parker, 1994; Jones, Sünram-Lea, & Wesnes, 2012; Kennedy & Scholey, 2000), this mechanism offers a plausible explanation for the observed cognitive benefits of flavonoid-rich interventions (relative to sugar matched controls) in the immediate postprandial period. Here, reported effects are for sweet berry interventions. Similar effects may be observed following consumption of carbohydrate rich foods such as dark chocolate, or for low carbohydrate

drinks such as green tea if consumed alongside carbohydrate based foods, however further research is needed.

1.5.4 Neuronal enhancement

1.5.4.1 Monoamine oxidase inhibition

Monoamines comprise a class of neurotransmitters that are responsible for the regulation of many cognitive processes. Monoamine levels, particularly for dopamine, have been observed to increase during working memory and attention tasks, correlating positively with task performance (Cox, Pipingas, & Scholey, 2015). Therefore inhibition of monoamine oxidase (MAO)), an enzyme responsible for the breakdown of monoamines, may be beneficial to monoaminergic neurotransmission during cognitive performance, through increased concentrations of monoamine neurotransmitters. Watson et al. (2015) observed MAO inhibition in association with improved attention after blackcurrant supplementation. None of the other studies reviewed here have measured MAO levels, but in vitro studies and animal studies using chronic supplementation have demonstrated MAO inhibitory effects for all flavonoid subclasses (Jäger & Saaby, 2011). Consideration should be given to the inclusion of such measures in future research.

1.5.4.2 BDNF synthesis

BDNF is a complex protein that has been implicated in the regulation of multiple neuronal processes including synaptic plasticity and neurogenesis. Expression of BDNF is regulated by the transcription factor CREB. Trends towards increased plasma BDNF observed after 1 hour (Dodd, 2012) are a slightly unexpected finding in an acute study. BDNF regulated protein synthesis mechanisms of flavonoid action have been more consistently associated with chronic supplementation (Spencer, Vauzour, & Rendeiro, 2009). However as mentioned above, eNOS has been implicated in the regulation of BDNF expression (Chen et al., 2005). Acute exercise has been observed to result in rapid increase in BDNF (Szuhany, Bugatti, & Otto, 2015). Therefore, vasodilation following flavonoid ingestion might reasonably have a similarly rapid effect on BDNF availability. Assuming BDNF levels can increase over such short time periods following flavonoid ingestion, it seems unlikely that subsequent facilitation of neuronal functioning would occur in time to explain cognitive effects apparent at only 1-2 hours postprandially, but may provide a possible mechanism for the cognitive improvements observed at later time points. BDNF has been implicated in both short term memory formation and long term memory formation (Bekinschtein, Cammarota, Izquierdo, & Medina, 2008) through a permissive role in the facilitation of early long term potentiation (LTP) (Bramham &

Messaoudi, 2005), and so increases in BDNF might be responsible for acute enhancement of episodic memory.

1.5.5 Visual function

Flavonoids have been extensively associated with improvements in visual function both in vitro and in vivo (for a review see Kalt, Hanneken, Milbury, & Tremblay, 2010). Improvements in visual contrast sensitivity and detection of motion direction after acute cocoa flavanol supplementation in young adults have been described above (Field et al., 2011). Additionally, similar contrast sensitivity improvements have also been seen in older adults (Field, Bell, Mount, Willliams, & Butler, 2014). Flavonoids have also been shown to influence the focussing ability of the eyes; improvements in accommodative facility have been observed in young adults following acute cocoa, and improvements in convergence facility have been observed after acute blueberry supplementation (Field et al., 2014). Of the studies reviewed here only three have reported significant findings for non-visual tasks: RAVLT (Whyte & Williams, 2015), PASAT (Elsabagh et al., 2005), Simple & complex finger tapping (Alharbi et al., 2016). All other significant findings have been for visually presented tasks; therefore improved vision may account for at least some of these findings. The link between enhanced vision and improved cognitive performance following flavonoid supplementation is only speculative at this stage. However, epidemiological research has identified clear associations between visual acuity and cognitive performance across multiple age groups (Baltes & Lindenberger, 1997). This is likely to reflect a common underlying variable such as general health. But as a possible mechanism, enhanced visual function warrants further investigation.

1.6 Literature summary and Conclusions

Table 1.3 summarises the cognitive effects observed across all reviewed studies. Acute flavonoid-induced cognitive effects have been found across multiple cognitive domains. In particular, a number of studies report improvements for attention tasks and factors. Therefore, it is possible that improvements over a range of cognitive domains may be facilitated by general improvements in attention. The evidence suggests that the effects of flavonoids on cognitive outcomes are mediated by age. For example, executive function, working memory and psychomotor processing speed effects are apparent in young and middle aged adults. Episodic memory effects appear more prevalent in children and older adults, particularly following blueberry supplementation. This may reflect the relatively lower episodic memory performance generally observed in the very young or old when compared with adults in their cognitive prime. While the hippocampus has been shown to have the potential for improved neuronal connectivity and even neuronal growth throughout life,

those at critical developmental stages may simply have greater potential for episodic memory improvement. Conversely, developmental differences in the structure and functioning of the prefrontal cortex may mean that the potential for improvement in executive function is more limited in these extreme age groups.

The studies reviewed have been primarily concerned with young adults and therefore more work is needed to determine true differences between age groups, ideally using mixed designs that allow direct comparison of different participant groups within the same study. Whatever the age group, cognitive effects are likely to be dose-dependent, as evidenced by the increasing cognitive effect sizes observed with increasing doses of ginkgo biloba (Kennedy et al., 2000) and the similar dose-dependent vascular effects of blueberry (Rodriguez-Mateos et al., 2013).These potential effects of dose clearly need further investigation across all flavonoid subclasses and using a wider range of doses than those currently investigated.

The timings of cognitive effects vary depending on the flavonoid-rich food source, which may reflect differences in rates of absorption and metabolism for individual flavonoid types, or simply for different food types. The majority of observed cognitive improvements match peaks in plasma metabolite concentrations and also peaks in peripheral and cerebral blood flow. Although vasodilation is often cited as the most likely mechanism for acute flavonoid induced improvements in cognition, cognitive improvements have not yet been observed directly in conjunction with vascular-related effects. This suggests a need for studies incorporating concomitant cognitive and vascular measurements. Cognitive improvements have been observed alongside other physiological changes such as altered rates of glucose absorption (Watson et al., 2015), inhibition of MOA (Watson et al., 2015), and improved vision (Field et al., 2011), suggesting that these factors may also be influential.

Observed physiological responses to flavonoid-rich supplementation such as vasodilation have been consistently replicated, but cognitive findings are not as robust despite the number of moderate to large effect sizes apparent in Table 1.3. As discussed, methodological differences between studies are likely to partially explain the inconsistencies in cognitive observations. Comparisons between studies are often difficult due to differences in dose and flavonoid source. The design of a study may also impact on the size of any observed cognitive effects. For example, from Table 1.3 it can be seen that effect sizes in studies without a control (Caldwell et al., 2016) tend to be large, whereas those with no baseline measurements (Elsabagh et al., 2005; Field et al., 2011; Whyte & Williams, 2015) tend to exhibit small or moderate effect sizes or even no effects at all (Bondonno et al., 2014). From

Table 1.3 it can also be observed that studies with very small numbers of participants tend to exhibit greater effect sizes. This may be indicative of a type 1 error (false positive outcome) as such studies have a lower statistical power to determine a true cognitive effect, and other larger studies reviewed here have generally not observed similarly large effect sizes. In crossover studies, it is also important to reduce the impact of practice-related improvements in cognitive performance that is often associated with repeated testing. It is not always clear whether this has been adequately addressed in some of the studies reviewed here (Caldwell et al., 2016; Whyte & Williams, 2015). Additionally, extensive batteries of tasks are often deployed at several different time points, but often only one or two measures prove significant. This increases the potential for type 1 error considerably. Future studies therefore also need to address these methodological issues, to improve their statistical power to reliably observe small changes in cognition. Finally, it should be noted that many of these studies rely on whole foods as their source of flavonoids. While the flavonoid subclasses typically associated with each food type are the most abundant flavonoid forms within that food type, other flavonoids, and indeed other polyphenols may be present in varying concentrations. Therefore, any observed effects cannot be fully attributed to a single subclass.

In conclusion, the evidence so far suggests that research into the cognitive benefits of flavonoid-rich foods is a promising area that demands further investigation. However, in terms of design, current studies are a long way from the large scale randomised controlled trials (RCTs) that are required to build a strong and robust evidence base supporting beneficial effects of flavonoid ingestion for cognitive outcomes in the immediate postprandial period.

1.7 Thesis objective & research questions

1.7.1 Objective

From the literature review it is apparent that flavonoid-rich foods have the potential to elicit cognitive benefits following acute supplementation, however the findings of human studies remain mixed. There is some evidence to suggest that both cognitive and physiological effects may be dose-dependent, and therefore differences in dose may be responsible for many of the inconsistencies between studies. Following this observation, the objective of my PhD thesis was to determine whether acute, dose-dependent cognitive effects were evident following anthocyanin-rich blueberry consumption.

1.7.2 Research questions

Specific research questions addressed in this thesis are:

- **In crossover intervention studies, what is the best strategy for minimising the impact of repetition on cognitive task performance (addressed by Experiment 1, reported in Chapter 3)?**
  Rationale & Hypotheses: In studies with repeated cognitive testing, practice-related improvements in performance may confound effects attributed to an intervention. Indeed it is unclear from the literature whether such practice effects have been taken into account in a number of flavonoid-rich intervention studies reporting significant effects. It is clearly important to minimise practice effects where possible in order to validate intervention research findings. Following a review of cognitive practice effects and strategies for their minimisation, a pilot study investigated practice effects for the cognitive tasks to be used in this thesis. It was hypothesised that practice effects would be evident for all cognitive tasks investigated, but that their impact would be attenuated through the provision of appropriate methodology, to be determined from the data collected in this study.

- **Following acute blueberry intervention in a young adult population, are cognitive or mood changes evident and, if so, are they dose-dependent (addressed by Experiments 2 & 4, reported in Chapters 4 & 6, respectively)?**
  Rationale & Hypotheses: Evidence from the literature review suggested that acute blueberry intervention might elicit cognitive and mood benefits; however some of the flavonoid literature findings were mixed. Published studies typically varied in their choice of dose or population, making comparison between studies difficult. The evidence from Rodriguez-Mateos et al. (2013) suggested that the metabolism and vasodilatory response to anthocyanin-rich blueberry may be dose-dependent in a young adult population. In a series of experiments, this thesis explored whether cognitive and mood outcomes followed a similar dose-dependent trajectory. It was hypothesised that the strongest cognition and mood effects would coincide with doses that elicited the greatest vasodilatory response in the previous work, through a mechanism of increased CBF. As with the Rodriguez-Mateos et al. (2013) study, these effects were investigated in a young adult population.

A final research question addressed observations from Experiment 2:

- **Do blueberries impact postprandial blood glucose response in a young adult population, and is this a plausible mechanism of action for observed cognition or mood changes (addressed by Experiments 3 & 4, reported in Chapters 5 & 6, respectively)?**
  Rationale & Hypotheses: Findings from the literature review, further supported by the

outcome of Experiment 2, suggested that blood glucose regulatory effects may play a significant role in cognitive outcome in blueberry intervention studies. The final two experiments of the thesis investigated changes in postprandial glucose response and cognition following varying doses of blueberry. The aim was to determine whether there was a dose-dependent effect of blueberry on postprandial glucose response, and whether there was a relationship between blood glucose level and cognitive outcome. It was hypothesised that dose-dependent changes to postprandial blood glucose regulation would be associated with similarly dose-dependent cognition and mood outcomes, through mechanisms of increased availability of glucose to the brain combined with vasodilatory increases in CBF.

1.7.3 Methodological considerations

Anthocyanin-rich blueberry was selected for investigation due to the strong evidence that the immediate postprandial vasodilatory response following blueberry is dose-dependent (Rodriguez-Mateos et al., 2013). As data for metabolism and FMD response have already been reported for fixed doses of freeze-dried blueberry in previous work, identical doses were used here, after sourcing the same blueberry powder. In order to control for individual differences, a within-subjects design was used, whereby each participant took part in all experimental conditions, effectively acting as their own control. Within-subjects designs increase the statistical power of a study by eliminating between-subjects variance between conditions. This was considered particularly important in light of recent research indicating that individual responses to flavonoid-rich interventions may vary according to gut microbiota. The thesis also aimed to address some of the methodological issues previously identified in the literature review. Therefore, the design of all intervention experiments incorporated measures to improve statistical power such as testing sufficiently large numbers of participants, minimising the effects of repeated cognitive testing, incorporating baseline measurements, including a suitable control condition, and using appropriate statistical analyses.

Chapter 2
Materials & methods

As highlighted in Chapter 1, a number of critical research questions were identified and used as the focus for my thesis. Firstly, what is the most effective methodology for investigating cognitive changes in response to multiple doses, whilst minimising practice-effects? Subsequently, are cognition and mood changes evident following acute blueberry supplementation, and are such effects dose-dependent? Finally, in relation to possible mechanisms of action, do blueberries influence postprandial glycaemic response, and might such glucoregulatory effects underlie observed changes to cognition and mood?

2.1 Experimental design

In order to answer these research questions a series of experiments were designed:

- Experiment 1: Piloting of cognitive tasks and an examination of the effects of repeated cognitive testing on task performance (Chapter 3).
- Experiment 2: A dose-response study of cognitive and blood pressure changes following acute anthocyanin-rich blueberry supplementation in healthy young adults (Chapter 4).
- Experiment 3: A dose-response study of glycaemic response following acute anthocyanin-rich blueberry supplementation in healthy young adults (Chapter 5).
- Experiment 4: A dose-response study of cognitive and blood glucose changes following acute anthocyanin-rich blueberry supplementation in healthy young adults (Chapter 6).

The majority of intervention studies reviewed in the previous chapter opted for a crossover design. In this type of design, a single group of participants take part in each experimental condition of the study. Participants effectively act as their own controls, thereby minimising the impact of between-subjects variance and so improving statistical power. Similarly here, a crossover design was implemented for each blueberry intervention study. As cognitive practice effects are known to be an issue in experimental designs with a repeated testing component, the magnitude of these effects was fully investigated during the piloting of the cognitive tasks and appropriate methodology was implemented for reducing their impact (discussed in detail in Chapter 3). Methodological limitations, identified in several of the reviewed intervention studies, were their lack of an appropriate control condition or an omission of baseline testing. In each of the three intervention studies described in this thesis (Chapters 4, 5 & 6), appropriate matched control conditions were included alongside the

blueberry interventions. Baseline measurements were recorded at each test visit before interventions were administered in order to control for random variations in performance across multiple test visits. In accordance with a further standard of good practice in intervention studies, all testing was performed double-blind in order to prevent potential experimenter effects or demand characteristics from influencing participant performance.

2.1.1 Power analysis

A priori power analysis was performed using GPower 3.1 to determine the minimum number of participants required for each experiment in order to achieve a statistical power of 0.8 with an alpha level of 0.05 (Cohen, 1992). For Experiment 1, assuming an effect size of d=0.47 (Bartels, Wegrzyn, Wiedl, Ackermann, & Ehrenreich, 2010; Donovan & Radosevich, 1999), 30 participants were determined as sufficient to detect an increase in cognitive performance between repeat test sessions. For Experiments 2 & 4, assuming an effect size of d=0.45 (Bell, Lamport, Butler, & Williams, 2015), 32 participants were sufficient to detect an increase in cognitive performance between the control and blueberry conditions. For Experiment 3, assuming an effect size of d=0.71 (Törrönen et al., 2010), 14 participants were deemed sufficient to detect a reduction in peak glycaemic response between the control and blueberry conditions. Extra participants were recruited where possible to allow for attrition/drop outs throughout the course of testing.

2.2 Ethical approval

All studies were approved for ethical conduct by either the School of Psychology Research Ethics Committee (SREC), or the University of Reading Research Ethics Committee (UREC), in accordance with University of Reading guidelines. Evidence of ethical approval for all studies can be found in Appendix A.

2.3 Participants

2.3.1 Recruitment

Participants were healthy young adults, aged 18 to 40, recruited from staff and student populations at the University of Reading. This population was selected as data outlining bioavailability (Rodriguez-Mateos, Feliciano, Cifuentes-Gomez, & Spencer, 2016) and vasoreactivity (Rodriguez-Mateos et al., 2013) following acute doses of blueberry anthocyanins have already been published for this age group. Similarly, the literature review in Chapter 1, identified cognitive and mood effects in young adults following blueberry intervention. Recruitment was carried out via postings to group

email listings, campus notice boards, and the Psychology Department Undergraduate Research Panel.

2.3.2 Screening

Aside from smoking status, no screening restrictions were in place for Experiment 1. However, all participants were screened for suitability before inclusion in any of the blueberry intervention studies. Participants were required to be non-smokers, free from pregnancy, food allergies, diabetes mellitus (Type 1 & Type 2), and any conditions requiring hypotensive or anticoagulant medication. Full eligibility criteria are reported in Appendix B. A copy of the health questionnaire used for screening can be found in Appendix C.

2.3.3 Participant demographic data

Health and lifestyle demographic data comparing participants for all experiments are shown in Table 2.1. The participant profile remained consistent between all experiments.

2.3.4 Informed consent

Before participation in any of the experiments reported here, prospective participants were emailed an information sheet providing detailed information about the study. If after reading the information participants were happy to continue, then they were required to sign a consent form at the beginning of their first test visit. Information sheets for all experiments can be found in Appendix D. Consent forms for all experiments can be found in Appendix E.

Table 2.1 Participant demographic data

Demographic		Experiment				
		1.1	1.2	2	3	4
N		29	33	45	17	41
Gender	male	9	3	12	4	11
	female	20	30	33	13	30
Age(years)	Mean	25.6	20.8	20.9	24.1	23.5
	SD	7.7	4.9	3.6	4.9	5.1
BMI (kg/m2)	Mean	N/R	N/R	N/R	23.8	23.4
	SD				3.6	5.0
Fruit & veg (daily portions)	Mean	N/R	N/R	4.1	5.3	4.4
	SD			1.4	2.3	1.9
Tea & coffee (daily cups)	Mean	N/R	N/R	1.7	2.2	1.3
	SD			1.8	1.5	1.2
Alcohol (weekly units)	Mean	N/R	N/R	5.1	3.6	3.3
	SD			6.8	5.0	5.0
Exercise (weekly hours)	Mean	N/R	N/R	2.9	3.0	3.3
	SD			3.3	2.3	3.5

N/R Not recorded

2.3.5 Low polyphenol diet

Before participation in any of the blueberry intervention experiments reported here, participants were required to follow a low polyphenol diet for the 24 hours prior to each test visit. Similar methodology has been employed by studies investigating bioavailability (Rodriguez-Mateos et al., 2016), vasodilatory response (Rodriguez-Mateos et al., 2013), cognition (Dodd, 2012), and mood (Khalid et al., 2017) following acute wild blueberry intervention. In addition to food and supplement restrictions, participants were asked to abstain from alcohol, fruit juices, and caffeine containing beverages such as energy drinks, cola, tea, coffee or cocoa. Participants were also asked to fast (no food or drink) for 2 hours immediately prior to attending each test visit. Participants were asked to

eat the same breakfast before each test visit and to ensure that breakfast was consumed before the 2 hour fast, with baseline cognitive testing commencing at 9am. This methodology was introduced in order to minimise the interfering effects of habitual dietary polyphenols on each of the measured outcomes following blueberry intervention, and to minimise the impact of dietary changes on test performance between visits. The 2 hour fast ensured that participants were thirsty enough to consume the intervention beverage, whilst minimising the confounding effects of any breakfast food intake. A copy of the dietary requirements can be found in Appendix F. At each test visit participants retrospectively documented their 24 hour food intake using the record sheet found in Appendix G. Minor transgressions were noted and, where necessary, participants were asked to modify their pre-test diet.

2.3.6 Payment

Participant payments were standardised in line with current University of Reading guidelines. Psychology undergraduates received 1 course credit per hour of testing completed. All other participants received expenses payments of £5 per visit.

2.4 Blueberry interventions

2.4.1 Freeze dried blueberry powder

Rather than use fresh berries, which may vary in their composition according to growing and storage conditions, freeze dried wild blueberry powder derived from the Vaccinium angustifolium cultivar was used in all intervention experiments. This cultivar has been shown to have a high total polyphenol content and is a rich source of anthocyanins (Rodriguez-Mateos et al., 2012). Bioavailabilty data has similarly been published for this cultivar (Rodriguez-Mateos et al., 2013, 2016). In freeze-dried form each batch of blueberry powder is homogenous; the anthocyanin content remains constant throughout the batch, and is reportedly stable when stored at -20 degrees Celsius (Lohachoompol, Srzednicki, & Craske, 2004). The powder was provided free of charge by the Wild Blueberry Association of North America (WBANA). Due to limited availability, two separate harvest batches were used during the course of this research. The second batch was derived from a harvest that had experienced more favourable ripening conditions and subsequently was found to contain higher anthocyanins levels than the initial batch. Adjustments were made to the quantities used in each experiment in order to match the anthocyanins content of the interventions. The composition of each batch can be found in Table 2.2.

Table 2.2 Wild blueberry powder composition

Compound	Batch 1[a] (Experiments 2 & 3)	Batch 2[b] (Experiment 4)
Total polyphenols (mg/100g)	2239	2900
Anthocyanins (mg/100g)	905	1900
Procyanidins (mg/100g)	400	Not quantified
Vitamin C (mg/100g)	12	335
Total sugars (g/100g)	50	70
Fructose (g/100g)	26	36
Glucose (g/100g)	24	34
Dietary fibre (g/100g)	Not quantified	16

[a]Polyphenol, vitamin and sugar analyses conducted by Food & Nutritional Sciences Department, University of Reading, UK
[b]Polyphenol analyses conducted by FutureCeuticals, Illinois, USA and vitamin, sugar and dietary fibre analyses conducted by RSSL, Reading, UK

2.4.2 Drink preparation

All blueberry intervention doses were administered in drink form by mixing appropriate quantities of blueberry powder with water. The exact doses are specified in each separate experimental chapter. Within each experiment, all blueberry doses, including a blueberry-free control, were matched for sugars and Vitamin C by the addition of food grade fructose, glucose, and Vitamin C powders (Sports Supplements Ltd, UK). All drink doses were blind-coded and prepared by a third party not involved with the testing component of the study. Drinks were served in opaque, lidded cups, and consumed through black straws. The participants and the researcher therefore remained blind to the intervention dose at any given test visit.

2.4.3 Drink palatability

In order to determine how well the different dose conditions were matched for taste, participants were asked to judge the palatability of each drink. Using the questionnaire found in Appendix H, participants rated their general liking for each drink as well as specific taste dimensions such as sweet or sour. Likert scale anchor points for each rating dimension were 1 ‘Not at all’ and 9 ‘Extremely.’

2.4.4 Counterbalancing

To reduce the confounding influence of order effects, the control and all blueberry doses were administered in counterbalanced order, determined using Williams matrices (Williams, 1949).

2.4.5 Washout

During each intervention study participants were required to attend multiple visits due to the crossover nature of the study design. The majority of participants attended regular weekly sessions with a 7 day washout period between visits. However in some instances adjustments to the testing schedule were required in order to accommodate participant availability. In previous research, after an acute blueberry anthocyanins dose of 348mg, anthocyanins were no longer detectable in plasma after a period of 24 hours (Del Bo' et al., 2013). Similarly, metabolites of anthocyanins from elderberries showed no evidence of accumulation in plasma when comparing chronic daily supplementation with acute supplementation (de Ferrars, Cassidy, Curtis, & Kay, 2014). However, anthocyanin metabolites have been reported in urine after a five day anthocyanin-free diet preceded only by habitual consumption of anthocyanin-rich foods (Kalt, Liu, Mcdonald, Vinqvist-tymchuk, & Fillmore, 2014). This observation suggests that baseline metabolite levels may persist for extended periods of time. Therefore any washout period following acute consumption will, at best, only minimise acutely elevated circulating metabolites. Metabolites may not be completely eliminated unless participants are restricted from following their habitual diet for the entire duration of a study. In light of the combined evidence, on the rare occasions that a 7 day washout was not possible, a minimum washout period of 3 days between test visits was considered sufficient for the minimisation of significantly elevated levels of anthocyanins or metabolites following an acute dose. It was acknowledged that circulating metabolites were likely to remain throughout the testing period; however these baseline levels were considered unlikely to significantly impact cognitive data.

2.5 Cognitive measures

2.5.1 Task selection & piloting

The cognitive tests selected for inclusion in this thesis have all shown positive results in previous flavonoid or similar nutrition intervention studies. All tasks were programmed using E-Prime 2.0 (Psychology Software Tools, Inc.). All tasks were piloted in Experiment 1 before being used in the later blueberry intervention studies. Piloting of the tasks served a multiple purpose; to identify any potential running problems with the tasks, to determine alternate form reliability, and to investigate

the occurrence of practice effects resulting from repeated cognitive testing. Issues with the tasks were rectified as they arose, and appropriate methodology was subsequently adopted in order to minimise the influence of cognitive practice effects in the later blueberry intervention experiments. Full descriptions of each task are given below.

2.5.2 Neural correlates

The selected cognitive tasks (described below) each contain an attention, working memory or episodic memory retrieval component, or a combination of the three. Neural correlates have been described individually for each of the cognitive tasks, and it is apparent from these descriptions that there is large degree of overlap between the neural correlates for each of these cognitive functions; an observation also confirmed by a previous review study (Naghavi & Nyberg, 2005).

2.5.3 Sternberg memory scanning task

This was a modified, computerised version of the original task developed by Sternberg (Sternberg, 1966, 1969). This short term memory task is a measure of working memory and serial attention (Corbin & Marquer, 2013; Garavan, 1998). Successful performance on the task has been associated with activation in the dorsolateral prefrontal cortex (dPFC) (D'Esposito, Postle, & Rypma, 2000). The task measures how fast participants can scan through a list of items held in their short term memory. Previous research has shown this is a fixed time per item, therefore the task has been described as resistant to practice (Kristofferson, 1972, 1977; Sternberg, 1975); although underlying reaction times from which this measure is derived are still subject to practice related improvements.

During the Sternberg task, participants were presented with a sequential series of one to six digits, the order of which they were required to memorise. A new set of digits was randomly generated on each trial. The appearance of a fixation point indicated the end of the sequence. The digits and fixation were each presented for 1200ms at a rate of one every 1200ms. Participants were required to indicate as quickly as possible, with a labelled yes/no key press ('b' &'n' respectively), whether or not a final digit, presented 2000ms after the fixation point, was present in the original memory set. Participants completed 12 familiarisation trials and 96 test trials at each test session. The dependent variables (DVs) were accuracy, scanning rate, and extrapolated RT. These latter variables were, respectively, the slope and intercept from the regression model for predicting RT from memory set size (Böcker et al., 2010; D'Esposito et al., 2000; Grattan-Miscio & Vogel-Sprott, 2005; Subhan & Hindmarch, 1984; Vinkhuyzen, van der Sluis, Boomsma, de Geus, & Posthuma, 2010). Accuracy rates

were important in order to provide sufficient power for the regression analysis, therefore performance feedback was given after each trial.

2.5.4 Stroop task

This was a modified, computerised version of the original executive function task (Stroop, 1935) and is a measure of inhibition, or selective attention . The words “Purple”, “Green”, “Blue”, and “Red” were displayed individually in randomised, counterbalanced order on a computer screen, with each word being displayed in either a congruent or an incongruent ink colour. The words were presented at a rate of one every 2000ms and each word remained on screen for 1250ms. Participants were instructed to respond to the ink colour as quickly as possible by pressing the corresponding coloured button on the keyboard (coloured stickers were placed over the keys ‘1’,’2’,’3’,&’4’ on the main keyboard). An equal number of congruent and incongruent trials were presented. Twelve familiarisation trials and 96 test trials were presented at each sitting of the task. DVs included accuracy and the interference effect of the semantic meaning of the word; calculated by subtracting the mean reaction times (for correct responses only) for congruent trials from incongruent trials. Neural correlates for this interference effect include the left posterior parietal cortex (PPC) (Galer et al., 2015).

2.5.5 Serial 3s and 7s subtraction task

Using a previously published method for this working memory task (Scholey et al., 2010, 2013; Scholey, Harper, & Kennedy, 2001), a random number between 800 and 999 was presented on screen and participants counted backwards, at first in 3s, entering their answers via the computer number pad as quickly as possible for a total of two minutes. The task was then repeated subtracting 7s instead of 3s. A 20 second familiarisation trial was completed immediately before each 2 minute test. The DVs in both cases were the total number of correct responses, the total number of errors, and the mean reaction time for correct responses (ms). The accuracy of the response was determined relative to the previous response, irrespective of whether or not the previous response was correct. No studies have reported neural correlates for this task directly, but a meta-analysis of similar working memory tasks revealed the involvement of an extensive fronto-parietal network (Rottschy et al., 2012).

2.5.6 Immediate & delayed word recall task

This episodic memory task was a modified, computerised version of a single-trial word list learning task originally developed by Édouard Claparède (Boake, 2000; Lezak, Howieson, Bigler, & Tranel,

2012). For the immediate recall, 15 words were visually presented in sequence on a computer screen at a rate of one word every 2500ms. Each word remained on screen for 2000ms. Participants were instructed to read and remember each word. At the end of the presentation participants were asked to recall as many of the words as possible, writing them on a piece of paper. Participants were allowed as much time as they required to perform the recall. This task was the first task administered during each test session. After all other tasks had been completed, participants were again asked to recall the words. Delayed recall occurred approximately 30 minutes after the initial presentation of the word lists. Different word lists, matched for concreteness and familiarity, were used at each testing time point (Whyte et al., 2016). The full lists can be found in Appendix I. The DVs for each recall task were the total number of correct words and the total number of interference words recalled. Due to the crossover nature of the study design, multiple word lists were presented across different test sessions. Interference words were considered to be any words previously presented at an earlier test session. Neural correlates of episodic memory function involve complex interaction between the hippocampus and PFC areas during encoding and retrieval (Ranganath, Heller, Cohen, Brozinsky, & Rissman, 2005), and a large degree of overlap has been observed between the PFC correlates of working memory and long term memory (Ranganath, Cohen, & Brozinsky, 2005; Ranganath, Johnson, & D'Esposito, 2003).

2.5.7 Rapid visual information processing task (RVIP)

This working memory and sustained attention task correlates with both right and left fronto-parietal activation networks (Coull, Frith, Frackowiak, & Grasby, 1996; Lawrence, Ross, Hoffmann, Garavan, & Stein, 2003; Neale, Johnston, Hughes, & Scholey, 2015). Using a previously published method (Watson et al., 2015), participants were shown a continuous string of single digits, presented at a rate of 100 digits per minute in the centre of a computer screen. Participants were required to continuously monitor the digits for specific target strings of three consecutive odd or three consecutive even digits, pressing the space bar as quickly as possible when a target string was observed. A short familiarisation phase consisting of 30 single digits, including four target strings, was performed at each presentation of the task. The main test phase of the task lasted for 5 minutes with 501 single digits presented, including a total of 40 target strings. The dependant variables were accuracy score (correct out of 40), mean reaction time (ms) (for correct responses), and commission error score.

2.5.8 Digit vigilance task

This task is a measure of sustained attention, a cognitive function which is reported to correlate with neural activation in right fronto-parietal brain regions (Sarter, Givens, & Bruno, 2001). Using a previously published method (Watson et al., 2015), a continuous string of single digits were displayed in the centre of a computer screen at a rate of 80 digits per minute. Participants were required to press the spacebar as quickly as possible when the digit matched a randomly selected, static, target digit, displayed to the right of the screen. A short familiarisation phase consisting of 25 single digits, including 5 targets, was performed at each presentation of the task. The main test phase of the task lasted for 3 minutes, with 240 digits presented, including 45 target matches. The dependant variables were accuracy score (correct out of 45), mean reaction time (ms) (for correct responses), and commission error score.

2.5.9 Positive & negative affect schedule (PANAS) & subjective mental fatigue

The PANAS-Now (Watson, Clark, & Tellegen, 1988) mood questionnaire and a subjective Likert scale measure of mental fatigue (Scholey et al., 2010) were administered at the end of each cognitive test session. The PANAS questionnaire asked participants to rate their current mood in relation to a series of 20 mood related adjectives (10 positive & 10 negative items). The instrument was scored according to published criteria (Watson et al., 1988) by summing the Likert scale ratings (out of 5) for positive questionnaire items to create a Positive Affect score (out of 50). Similarly, ratings for negative questionnaire items were summed to create a Negative Affect score. The wording of the questionnaire asked participants to 'Indicate to what extent you are feeling this way right now.' Anchor points were 1 'Very slightly/Not at all' and 5 'Extremely'. Therefore, high scores indicated a high level of positive or negative affect, respectively. The mental fatigue questionnaire asked participants 'How mentally fatigued do you feel at this moment?' Anchor points were 1 'Not at all' and 9 'Extremely.' Therefore a high rating indicated a high level of mental fatigue.

2.5.10 Subjective motivation & subjective perception of task difficulty

During the piloting of the cognitive tasks in Experiment 1, measures of task difficulty and motivation were recorded using a nine point Likert scale questionnaire immediately after completing each cognitive task. The wording on each scale was as follows: 'How difficult did you find the <task name> task?' and 'How motivated were you to do well during the <task name> task?' Anchor points were 1 'Not at all' and 9 'Extremely'. Therefore, high scores indicated a high level of difficulty or a high level of motivation respectively. These measures were repeated each time a particular task was performed. Example questionnaires can be found in Appendix J.

2.5.11 Alternate forms & counterbalancing

All cognitive tasks used alternate forms for multiple presentations of the same task in order to minimise learning. In the case of the Sternberg, Stroop, serial subtraction, and digit vigilance tasks, new forms were randomly generated within E-Prime each time the task was run. For RVIP and word recall, separate versions of the tasks were created and presented in counterbalanced order. Within each cognitive test battery, the order of presentation of the different cognitive tasks was also counterbalanced, such that different participants experienced the cognitive battery in a different order. This was deemed appropriate as order effects have previously been reported within a cognitive test battery (Collie, Maruff, Darby, & McStephen, 2003). Williams matrices (Williams, 1949) were used for all counterbalancing.

2.6 Other measures

2.6.1 Fruit & vegetable consumption

For Experiments 2, 3 & 4, participants were each asked to estimate their average daily consumption of fruits and vegetables. The measure was recorded using a questionnaire that gave portion size definitions for a range of different fruit and vegetable types, in an effort to standardise the responses between participants. A copy of the questionnaire can be found in Appendix K.

2.6.2 Body mass index (BMI)

For each participant in Experiments 3 & 4, weight (kg) and height (m) was measured. Body mass index was calculated using the formula: $BMI = \frac{weight\ (kg)}{(height\ (m))^2}$

2.6.3 Blood pressure (BP)

Blood pressure measurements were recorded in Experiment 2, using a clinically validated Omron M6 Comfort automatic digital upper-arm blood pressure monitor (Omron Healthcare UK Ltd). Three repeat readings were taken at 2 minute intervals and average values recorded, in accordance with the manufacturer's instructions. To maintain consistency between repeat test sessions, blood pressure measurements were recorded in a seated position, with the cuff placed on the left arm, and with the arm resting on an adjacent desk throughout the measurement.

2.6.4 Blood glucose

Blood glucose levels were recorded in Experiments 3 & 4 by capillary sampling using the clinically validated Accu-Chek Aviva Blood Glucose Meter System (Roche Diagnostics UK). The units of measurement were millimoles per litre (mmol/l). World Health Organisation guidelines on capillary sampling (World Health Organisation, 2010) were strictly adhered to. Blood was sampled from the edge of the finger pad. Prior to sampling, the area was cleansed using an alcohol wipe. Disposable lancets were used for each finger prick. For comfort, a different finger tip was used for successive sampling time points. All waste including alcohol wipes, lancets and test strips, were disposed of in appropriate contaminated waste containers.

2.7 Testing procedure

All testing was carried out at the Nutritional Psychology Unit at the University of Reading. On arrival at the unit, all baseline cognitive and physiological measures were recorded. Cognitive testing was performed in individual testing cubicles in order to minimise distraction; however cubicles were found not to be completely sound proof. In Experiment 4 participants were additionally required to wear noise cancelling headphones in order to reduce noise from adjacent offices; a problem that had been identified during Experiment 2. When baseline tests were complete, the appropriate intervention drink was administered (Experiments 2, 3, & 4). Participants were required to fully consume the drink within 10 minutes (Rodriguez-Mateos et al., 2013). In order to maintain precise timings between interventions and testing time points, digital timers were used. Repeat physiological and cognitive testing occurred at fixed postprandial time points. These timings are specified in each separate experimental chapter. At the end of each visit, with the exception of the final visit, a return appointment date was confirmed.

2.8 Data analysis

All data cleaning and analysis procedures were performed using Microsoft Excel 2007 and IBM SPSS Statistics 21.

2.8.1 Outlier procedures

All raw data were systematically screened for outliers prior to statistical analysis using a published data cleaning protocol (Tabachnick & Fidell, 2013). For raw RT data collected across multiple test trials at each test session, only RT values for correct responses were included in subsequent analyses. RTs less than 200 milliseconds were immediately removed. Z scores were calculated for all

remaining data points for each participant, and RTs with a corresponding z score of greater than 3.29 were removed. This procedure was then repeated before calculating mean RTs for each participant at each test session. It was necessary to repeat the procedure due to the presence of some extremely slow RTs which biased the initial z distribution and prevented appropriate removal of all outliers. A similar procedure was followed to remove outlier means (RTs or test scores) across participants; Z scores were calculated according to session (Experiment 1) or dose (Experiments 2, 3, & 4), and mean values with a corresponding |z score| of greater than 3.29 were removed from the dataset.

2.8.2 Sternberg regression analysis

As described in the cognitive task section above, linear regression analysis of the Sternberg data was performed in order to obtain two of the DVs for the task. For each participant, and each repetition of the task, coefficients were determined for the regression line predicting RT from memory set size. The regression analysis was performed using data points for all correct responses, rather than using mean RTs obtained by averaging multiple trials with the same memory set size. A simulation of the analysis performed by Dr Kou Murayama (personal communication, February 13, 2015) at the University of Reading revealed that this method would better preserve statistical power.

2.8.3 Repeated measures data

On the recommendation of an independent statistician (University of Reading Statistical Services) all repeated measures data were analysed using Linear Mixed-effects Models (LMMs) in place of repeated-measures Analysis of Variance (repeated ANOVA). LMM analysis is based on a complex linear regression procedure. The technique can be used to model variance relating to both fixed parameters such as experimental doses, and random parameters such as individual differences between subjects, within multiple layers of the same model (Hoffman & Rovine, 2007). LMM has a number of advantages over repeated ANOVA (Field, 2009; Hoffman & Rovine, 2007; Magezi, 2015; Shek & Ma, 2011). Repeated ANOVA requires the assumption that all data observations are independent, however in repeated designs this is generally not the case. In LMM the true covariance structure of the data can be more accurately modelled, increasing the power of the analysis. Additionally, LMM does not require balanced data, thereby further increasing the power of the analysis as subjects with missing data points need not be excluded. As with all regression analysis, there is no requirement for dependent variables to be normally distributed. In general, regression analysis does assume that regression residuals are normally distributed; however the distribution of residuals has been reported not to influence the outcome of LMM, making it a robust method

(Gelman & Hill, 2007). The method also permits the inclusion of repeated covariates within the model, not possible in repeated ANOVA.

Throughout this thesis LMMs have been applied in a systematic way to retain consistency between analyses. In all analyses, subjects have been included as a random factor as a way of controlling for non-independence of data within subjects (Sweet & Grace-Martin, 2011). Additionally, a repeated factor has been included to control the covariance structure for each subject. In LMM the covariance structure is modelled using a covariance matrix of all repeated data observations, with variances defined along the diagonal and covariances in off-diagonal positions. A selected structure can be imposed on relative positions within the matrix. In an unstructured matrix, as suggested by the name, the covariances are assumed to be unpredictable and not have any fixed underlying structure. This general matrix suits all data; however it has some practical limitations. In analyses with many repeated time points and a relatively small number of participants there can be insufficient degrees of freedom available to determine a solution for the model. In these circumstances alternative covariance structures that make some assumptions can be specified. The most appropriate alternatives in respect of this doctoral research data were a heterogeneous, autoregressive structure (ARH1) or a diagonal structure. The ARH1 matrix assumes that variances are heterogeneous, and that covariances increase with greater proximity within the matrix i.e. repeated measurements are more closely correlated with an adjacent measurement than one recorded much earlier or later in the sequence of repeats. The diagonal matrix is a simpler model that assumes heterogeneous variances but a covariance of zero. This is the matrix assumed in repeated ANOVA. Where possible, an unstructured covariance matrix has been specified. LMM reports a measure proportional to the level of any variance not accounted for by the model. These '-2 log linear' (-2LL) values may be compared across related models, with the smallest value indicating the best fit. Taking these values into consideration, the best available model has been used in all analyses and the exact model is clearly described in each set of reported results.

2.8.4 Reporting of statistical results

When reporting LMM outcomes a similar convention to the reporting of ANOVA has been used, with degrees of freedom, F value, and p value reported. However some of the terminology used is slightly different. In ANOVA outcomes are reported as significant (or non-significant) main effects and interactions of factors. LMM is a more complex regression procedure, therefore factors are described as significant (or non-significant) predictors of the dependent variable (Field, 2009). Where significant covariates have been identified, the LMM model has been extended to include covariate interactions. Regression coefficients have been reported for all significant covariates and

covariate interactions. These beta values describe the slope of the regression line. Therefore participants displaying a single unit difference in their covariate measurement are predicted to display a difference in the dependant variable equal to the value of beta.

Where significant fixed factors have been identified, and for all intervention interactions, pairwise comparisons have been used to further interpret the findings. Convention proscribes that pairwise comparisons should only be performed following a significant F-test outcome. However, if multiple pairwise comparisons are appropriately corrected for the increased likelihood of type 1 error (false positives) then a prior F test, significant or otherwise, is not required (Howell, 2010; Huck, 2015; Wilcox, 1987). The F-test is methodologically conservative, particularly in designs akin to those used in this thesis comparing multiple, highly similar means. Under such circumstances, non-significant interactions may still yield significant pairwise comparisons, therefore it is statistically appropriate to investigate these regardless of the F-test outcome (Huck, 2015). Pairwise comparisons, with correction for multiple comparisons, have therefore been applied in the interpretation of all intervention interactions throughout this thesis. Bonferroni correction was selected as the most appropriate correction method as it guarantees the greatest control over type 1 error (Field, 2009).

For clarity, only significant findings have been reported in full in each experimental chapter. All other LMM results for each experiment have been tabulated in Appendix L.

2.8.5 Cohen's d effect sizes

Cohen's d effect sizes were calculated for literature findings reviewed in Chapter 1, and for significant effects observed in each of the experimental chapters. Cohen's d is a standardised measure that describes effect size in terms of the number of standard deviations between group means. Values of d equal to 0.2, 0.5 and 0.8 correspond to small, medium and large effect sizes, respectively (Cohen, 1988). A published meta-analysis method was used for all effect size calculations (Lipsey & Wilson, 2001).

Chapter 3
Experiment 1: Piloting of cognitive tasks and an examination of the effects of repeated cognitive testing on task performance

3.1 Introduction

As an initial step, the cognitive tasks described in Chapter 2 were piloted before inclusion in any blueberry intervention work. Piloting was carried out in order to identify and rectify any technical problems with the tasks themselves, to determine alternate form reliability for each of the cognitive tasks deployed, and importantly to determine the most appropriate methodology for minimising the impact of cognitive practice effects, which are a known problem in studies involving repeated cognitive testing. Previous research on practice effects (described below) was not found to reflect the designs typically used in nutrition intervention studies, and so a new investigation was carried out. The findings are presented in this chapter.

Repeated cognitive testing is often necessary in order to determine the efficacy of a nutrition intervention over time, particularly when determining a dose response curve. Equally this situation may arise in clinical drug trials or with other intervention types where repeated cognitive testing occurs over time. In such studies, multiple time points are used for comparing test values with baseline values and, in both acute and chronic crossover studies, testing may be performed multiple times by the same participant over a period of many weeks or months. However, the effects of repeatedly practising cognitive tasks are known to be problematic for studies using a repeated-measures design (Bartels et al., 2010; Basso, Bornstein, & Lang, 1999; Collie et al., 2003; Falleti, Maruff, Collie, & Darby, 2006; Hausknecht, Halpert, Paolo, & Gerrard, 2007; Lamport et al., 2012; McCaffrey, Ortega, & Haase, 1993; McClelland, 1987). Each time a participant is asked to perform a task they become more familiar with both the procedure and stimuli presented, and a process of learning takes place, often leading to enhanced performance. This becomes increasingly problematic if performance approaches or reaches a ceiling. Additionally, practice may result in a change in the participant's strategy for performing the task (Lowe & Rabbitt, 1998), which may in turn modify the brain network being utilised e.g. (Iaria, Petrides, Dagher, Pike, & Bohbot, 2003; Petersen, van Mier, Fiez, & Raichle, 1998), calling into question the validity of the result. Practice effects also add additional error variance that may impact on the statistical power of the study (McCaffrey, 2001). In order to ensure the validity of cognitive research, the European Food Safety Authority (EFSA)

guidelines on psychological health claims currently specify that practice effects must be addressed in behavioural intervention studies with a repeated testing component (EFSA, 2012). At least one methodological review paper (de Jager et al., 2014) has identified practice to be a problem for cognitive tasks across a number of cognitive domains. Many research groups adopt a range of strategies for dealing with practice; however there is no current consensus on the best methodology to address this issue.

Conceptually, very few cognitive tasks were developed with repeated-measures testing specifically in mind. Instead of seeking practice-resistant tasks, which are likely to be difficult or even impossible to create due to human adaptive behaviour, it has become almost standard practice for studies using multiple testing points to adopt the use of alternative forms of a task. Using this method, the same task is used but different equivalent forms of stimuli are presented across the multiple testing points. However although this strategy has previously been shown to be effective at attenuating practice effects, for some tasks significant residual practice effects may still be evident (Beglinger et al., 2005); it is thought that for many tasks participants are able to develop strategies to enhance their performance over time irrespective of the specific stimuli presented. The use of alternate forms cannot fully counteract this procedural learning process (Roebuck-Spencer, Sun, Cernich, Farmer, & Bleiberg, 2007). A number of studies attempt to reduce practice effects by incorporating an additional task familiarisation session before the test sessions, where participants familiarise themselves with the tasks, either on a prior visit or immediately before data collection, with a view to raising performance to a more stable level before beginning data collection. Indeed, this technique was historically advocated by McClelland (McClelland, 1987), who recommended a minimum of four familiarisation sessions prior to data collection for some tasks, although it is unclear from the paper whether these four sessions should be spread across one or more visits. The addition of this number of sessions has significant time and cost implications and has not been adopted due to its impracticality. Typically, many studies include upwards of one familiarisation session e.g. (Alharbi et al., 2016; Cox et al., 2015; Kennedy et al., 2002; Rigney, Kimber, & Hindmarch, 1999; Wightman et al., 2012), but it has been acknowledged that others entirely forgo adequate training (Wesnes & Pincock, 2002). The effectiveness of different strategies, including the most appropriate time for conducting familiarisation sessions (separate visit or immediately before testing) have not been fully investigated.

The effect of practice on performance has previously been investigated for short term repeated testing on a single visit e.g. (Collie et al., 2003; Falleti et al., 2006), where participants performed four repetitions of a battery of tasks at 10 minute intervals, and for repeated testing over a longer

term with single performances of a task across multiple visits e.g. (Bartels et al., 2010; Basso et al., 1999; Beglinger et al., 2005; Lemay, Bedard, Rouleau, & Tremblay, 2004; McCaffrey, 2001; McCaffrey et al., 1993). A few studies have combined within visit and multiple visit sessions of testing e.g. (McClelland, 1987); however in this example the majority of tasks were conducted using pencil and paper and were observed to be relatively unaffected by practice. Subsequent studies suggest that significant practice effects are observed for most modern computerised tasks, with the strongest practice effects typically observed between the first and second testing time points (Bartels et al., 2010; Collie et al., 2003; Falleti et al., 2006), although significant practice related improvements have been observed beyond a second session of testing (Bartels et al., 2010; Beglinger et al., 2005; Lemay et al., 2004). Practice effects have also been observed both within individual tasks, and within a test battery depending on the temporal positioning of individual tasks (Collie et al., 2003). Overall there is some suggestion that the rate of practice-related improvement may slow after two or more sessions irrespective of the testing interval. However, the timing of the repeated sessions used by these studies generally do not reflect those typically used in crossover nutrition studies or similar clinical intervention trials, where it is common to measure performance at baseline and again at one or more post- intervention time points at each visit, over a number of weekly visits e.g. (Alharbi et al., 2016; Cox et al., 2015; Kennedy et al., 2002; Nathan et al., 2002; Rigney et al., 1999; Watson et al., 2015; Wightman et al., 2012). This is important as practice effects have been observed to differ depending on the testing interval (Dikmen, Heaton, Grant, & Temkin, 1999). Similarly, in the parallel field of learning and memory, the timing of practice sessions is known to be important, as distributed practice spread across days, weeks or months has been observed to facilitate differential learning compared with massed practice performed multiple times on a single occasion (Anderson, 1992).

This experiment aimed to investigate practice effects for the cognitive tasks to be used in later blueberry crossover intervention work; such crossover designs incorporate a larger repeated testing component than parallel designs as participants are actively involved in all control and intervention arms of the study. Practice effects were examined within this crossover design framework; in Experiment 1.1 the design included three test days and two testing points within each day (a 3x2, Visit x Session design); in Experiment 1.2 the design included three test days and three testing points within each day (a 3x3, Visit x Session design). In line with current standard practice, alternate forms of all cognitive tasks were used, and familiarisation trials immediately prior to data collection were also incorporated in the study design. The intention was to identify the extent of practice effects within the cognitive batteries used in this thesis. This data was used to elucidate an effective

strategy for minimising the impact of practice effects in the later blueberry intervention experiments, as required by EFSA.

3.2 Methods

Methodological detail common to all experiments in this thesis can be found in Chapter 2. Methodology specific to the piloting of the cognitive tasks is reported here.

3.2.1 Participants

The participants in Experiment 1.1 were 29 young adults, aged 18-42 years (M 25.6, SD 7.7, 9 male). Thirty participants were recruited, however one participant failed to attend any test visits. The participants in Experiment 1.2 were 33 young adults aged 19-44 years (M 20.8, SD 4.9, 3 male). One participant attended only the first test visit. All participants were apparently healthy, non-smokers.

3.2.2 Design

The study design is illustrated in Figure 3.1. In Experiment 1.1, each participant completed the cognitive test battery six times following a crossover design. The six test sessions were split over three visits, with each visit separated by approximately one week (M 7.02, SD 1.12, Range 3-11 days). Within each visit there were two test sessions which were separated by 1 hour. The cognitive battery lasted 40 minutes. In Experiment 1.2, each participant completed a shorter 15 minute cognitive battery a total of nine times. Within each of the three visits there were three test sessions which were all separated by 1 hour breaks. Again, each visit was approximately one week apart (M 7.09, SD 0.39, Range 6-8 days). In both experiments participants were tested at the same time of day on each visit to minimise diurnal effects. Exceptions were two participants (one participant in each experiment) who attended both morning and afternoon sessions as their circumstances changed after testing had begun.

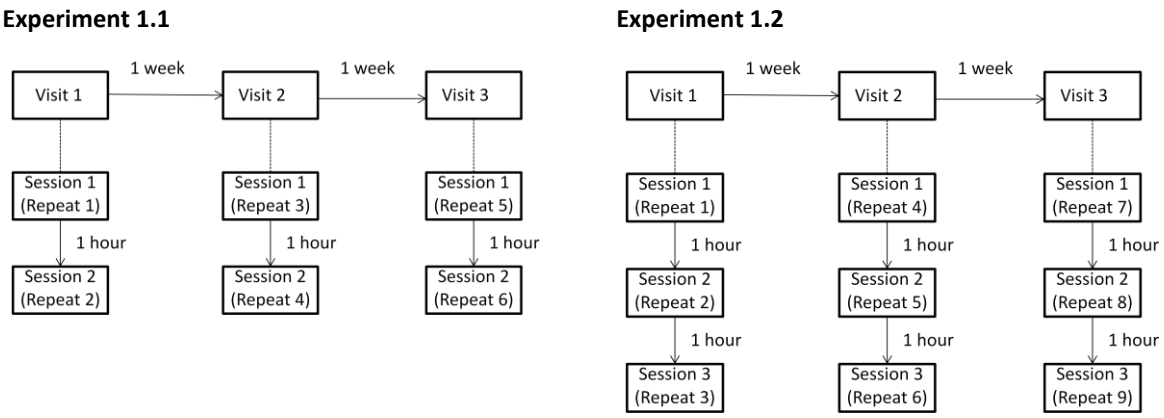


Figure 3.1 Study design for Experiments 1.1 & 1.2

Each pilot experiment was structured to mimic a shortened version of the design used in later blueberry experiments.

3.2.3 Procedure

In both experiments, participants attended the lab for a total of three visits. On arrival they immediately completed the battery of cognitive tasks and subjective measures of mood outlined below. Participants then waited in the lab for 1 hour before repeating the testing. In Experiment 1.2 a further repeat of the test battery was performed after another hour. During all breaks participants were supplied with magazines to read. Participants tested in the morning were asked to record their breakfast intake on the first visit, and were asked to eat the same breakfast prior to all subsequent visits. Participants tested in the afternoon were asked to eat the same lunch before all visits. The two participants who attended both morning and afternoon sessions ate the same meal before attending each visit, regardless of time of day. During all visits only consumption of water was permitted. After testing had been completed a return appointment was arranged for the following week. Participants spent a total of 2 ½ hours in the lab at each visit. All were informed from the outset that the aim of the study was to investigate the effects of repeated practice on cognitive task performance.

3.2.4 Cognitive & subjective measures

The cognitive tasks used in Experiment 1.1 were broadly representative of three main cognitive domains: working memory (serial 3s & 7s subtraction; Sternberg memory scanning), executive function (Stroop) and episodic memory (immediate & delayed recall), all tasks also required varying

degrees of attentional processing. The cognitive tasks used in Experiment 1.2 focussed on working memory (serial 3s & 7s subtraction; RVIP) and sustained attention (RVIP & digit vigilance). Full task descriptions can be found in Chapter 2. In Experiment 1.1, with the exception of the immediate and delayed recall tasks, which were respectively presented first and last, the order of the remaining three cognitive tasks was counterbalanced between participants. In Experiment 1.2 the order of all cognitive tasks was similarly counterbalanced. Short practice trials were incorporated at the beginning of each of the executive function, working memory and sustained attention tasks, for which data was not collected. This was to ensure that participants had fully familiarised themselves with the task directly before each administration. Alternate forms were used at each repeat presentation of any given task.

Subjective measures of task difficulty and motivation were recorded each time a particular task was performed (Chapter 2). In addition, the PANAS-Now (Watson et al., 1988) mood questionnaire and a measure of mental fatigue (Scholey et al., 2010) were administered at the end of each session of cognitive tasks.

3.3 Data analysis

3.3.1 Sternberg regression analysis

Regression analysis was performed on all raw Sternberg data in accordance with the procedure outlined in Chapter 2. Coefficient of determination values were low (R^2<0.380), but were similar to those observed in previous studies e.g. (Corbin & Marquer, 2009), and were typical of behavioural data.

3.3.2 Linear mixed models

LMM using a first-order autoregressive heterogeneous covariance structure (ARH1) to model successive repeat test sessions was used to analyse data for all cognitive tasks. Visit, Session and the Visit x Session interaction were included as fixed factors in the model, and subjects were included as random effects (Model 1.1). Motivation was subsequently included in the model as a repeated covariate (Model 1.2). In order to confirm the validity of motivation as a covariate -2 Log Likelihood (-2LL) values for Model 1.2 were compared with corresponding values for Model 1.1. The addition of motivation improved the fit of the model as evidenced by a reduction in -2LL (Shek & Ma, 2011). The analysis aim was to determine whether practice related improvements in cognitive performance were evident following the use of same day familiarisation trials and alternate forms of cognitive tasks, whilst accounting for potentially confounding changes in motivation. Pairwise comparisons

were used to investigate any significant effects of Visit, Session, or any Visit x Session interaction. A Bonferroni correction was applied to all multiple comparisons. The same LMM procedure (Model 1.1) was used to determine the effects of repeated testing on mood and mental fatigue.

For the analysis of the interference errors DV for the immediate and delayed recall tasks, the random effects component was removed from the LMM procedure (Model 1.3). This modification was necessary in order to counter the large number of zero scores present in the data set. Some participants showed no variation at all in their scores, causing an error in the modelling of the covariance matrix when including random effects (West, Welch, & Galecki, 2015). Motivation was also excluded as a covariate. It was judged impossible for motivation to consistently influence the number of interference errors across all test sessions. By definition, the possibility of making such an error increases with increasing presentations of the task.

Difficulty ratings recorded at each time point and for each cognitive task were analysed using a separate LMM (Model 1.4). ARH1 covariance structure was used to model successive repeat test sessions. Visit, Session, Cognitive Task, and Task x Visit, Task x Session, Visit x Session, and Task x Visit x Session interactions were included as fixed factors in the model. Subjects were included as random effects. The same model was also applied to the motivation ratings recorded at each time point for each cognitive task.

Serial 3s & 7s, positive & negative affect, and mental fatigue data obtained during Experiments 1 & 2 were subsequently combined. This larger dataset was analysed to determine whether time of testing (am or pm) played a significant role in predicting performance. The same LMM procedure was used as for the individual experiment analyses, but with the addition of a Time of Day factor, and all interactions between Time of Day, Visit and Session (Model 1.5). Motivation was again included as a repeated covariate for cognitive outcomes (Model 1.6).

3.3.3 Cohen's d effect sizes

Cohen's d values were calculated to compare cognitive practice effect sizes between all visits and sessions, with a view to determining whether the introduction of a familiarisation visit on a separate day prior to data collection would reduce practice effects in a typical nutrition intervention study design.

3.3.4 Intraclass correlation coefficients

Alternate-form reliability was determined for each of the cognitive tasks through the calculation of Intraclass Correlation Coefficients (ICCs). ICC scores for consistency between alternate test forms were calculated using a two-way mixed effects model. Scores between 0.4-0.75 were judged to be adequate and scores greater than 0.75 were considered good, according to published criteria (Cicchetti, 1994; Weintraub et al., 2014).

3.4 Results

All raw data collected during these experiments are included in the supplementary Excel files 'Experiment 1.1.xls' and 'Experiment 1.2.xls' on the accompanying CD. Means and standard deviations for all recorded variables are tabulated under the relevant sections of the chapter. The findings of Experiments 1.1 and 1.2 are reported individually under each section heading. All LMM results are presented in full in Appendix L. For clarity, only statistically significant effects are reported in the text.

3.4.1 LMM analysis of the changes in cognitive performance across all task repetitions

3.4.1.1 Experiment 1.1

Means and standard deviations for all cognitive variables recorded in Experiment 1.1 are shown in Table 3.1.

Table 3.1 Cognitive data for Experiment 1.1 (n=29)

Cognitive variable	Visit	Testing time point			
		Session 1		Session 2	
		Mean	SD	Mean	SD
Immediate recall score (correct/15)	Visit 1	7.52	2.01	8.00	2.27
	Visit 2	9.03	1.97	8.59	2.11
	Visit 3	10.21	2.51	9.24	2.61
Immediate recall interference errors	Visit 1	0.03	0.19	0.07	0.26
	Visit 2	0.07	0.26	0.03	0.19
	Visit 3	0.10	0.41	0.03	0.19

Table 3.1 continued

Cognitive variable	Visit	Testing time point			
		Session 1		Session 2	
		Mean	SD	Mean	SD
Delayed recall score (correct/15)	Visit 1	6.00	2.38	5.45	2.64
	Visit 2	6.69	2.38	5.41	2.95
	Visit 3	7.90	2.94	5.79	4.14
Delayed recall interference errors	Visit 1	0.03	0.19	0.31	0.66
	Visit 2	0.14	0.44	1.41	1.50
	Visit 3	0.07	0.26	1.45	1.74
Serial 3s score (correct in 2 minutes)	Visit 1	32.79	14.37	40.07	16.23
	Visit 2	41.62	18.45	46.72	16.78
	Visit 3	49.59	19.31	51.21	18.80
Serial 3s RT (ms)	Visit 1	3515.13	1457.62	3094.01	1412.59
	Visit 2	2935.73	1165.08	2512.30	997.39
	Visit 3	2621.67	1229.22	2446.37	1120.99
Serial 3s errors (incorrect in 2 minutes)	Visit 1	2.24	2.44	2.36	2.33
	Visit 2	2.62	2.01	2.33	1.98
	Visit 3	2.10	1.45	2.86	2.50
Serial 7s score (correct in 2 minutes)	Visit 1	20.28	11.59	22.45	10.56
	Visit 2	23.97	13.68	25.10	12.66
	Visit 3	26.72	13.76	28.10	13.89
Serial 7s RT (ms)	Visit 1	5644.86	2609.60	5086.12	2157.89
	Visit 2	4910.95	2413.92	4484.32	1670.49
	Visit 3	4601.17	2085.15	4505.79	2017.93
Serial 7s errors (incorrect in 2 minutes)	Visit 1	3.14	2.47	3.34	3.14
	Visit 2	2.57	2.39	3.17	2.90
	Visit 3	2.86	2.31	2.69	2.59
Sternberg accuracy (correct/96)	Visit 1	91.38	3.52	91.24	3.57
	Visit 2	91.50	3.39	89.66	4.70

Table 3.1 continued

Cognitive variable	Visit	Testing time point			
		Session 1		Session 2	
		Mean	SD	Mean	SD
	Visit 3	89.90	5.23	90.68	4.42
Sternberg scanning rate (ms/item)	Visit 1	37.67	15.17	35.59	26.13
	Visit 2	26.98	18.15	28.42	14.62
	Visit 3	27.05	21.30	25.55	21.65
Sternberg extrapolated RT (ms)	Visit 1	668.81	189.31	595.49	180.10
	Visit 2	616.15	169.75	583.18	153.24
	Visit 3	538.86	147.41	561.33	162.40
Stroop accuracy (correct/96)	Visit 1	90.82	3.27	90.55	4.64
	Visit 2	91.52	4.08	91.14	3.85
	Visit 3	89.86	4.47	90.90	3.30
Stroop incongruent RT (ms)	Visit 1	783.37	90.70	729.09	93.62
	Visit 2	743.47	93.88	734.31	97.31
	Visit 3	725.15	109.73	715.61	89.13
Stroop congruent RT (ms)	Visit 1	714.54	97.83	661.10	101.75
	Visit 2	681.62	99.68	663.20	109.07
	Visit 3	673.58	119.38	640.12	96.09
Stroop interference effect (ms)	Visit 1	68.84	46.16	62.39	38.26
	Visit 2	61.85	51.41	71.11	44.20
	Visit 3	51.56	47.76	75.48	44.19

3.4.1.1.1 Motivation

As a repeated covariate in the model, Motivation significantly predicted IR score [F(1,136.77)=13.56, p<0.001, beta=0.400], DR score [F(1,127.22)=7.64, p=0.007, beta=0.364], serial 7s score [F(1,135.85)=19.05, p<0.001, beta=1.186], serial 7s errors [F(1,82.72)=16.49, p<0.001, beta=-0.433], Sternberg accuracy [F(1,131.06)=44.25, p<0.001, beta=0.949], Stroop accuracy [F(1,102.34)=6.35, p=0.013, beta=0.477], and Stroop RT for congruent trials [F(1,140.24)=5.82, p=0.017, beta=-6.734],

such that higher motivation was associated with better performance in all cases. Means and standard deviations for all motivation ratings collected during Experiment 1.1 are shown in Table 3.2.

3.4.1.1.2 Visit

Fixed effects of Visit were significant for IR score [$F(2,54.20)=16.82$, $p<0.001$], serial 3s score [$F(2,49.95)=70.48$, $p<0.001$], serial 3s RT [$F(2,53.89)=35.30$, $p<0.001$], serial 7s score [$F(2,46.26)=32.86$, $p<0.001$], serial 7s RT [$F(2,66.14)=11.43$, $p<0.001$], Sternberg scanning rate[$F(2,60.89)=6.87$, $p=0.002$], and both incongruent [$F(2,52.41)=13.62$, $p<0.001$] and congruent RTs on the Stroop task.

Pairwise comparisons showed performance improvements between Visits 1 & 2 for IR score [$p=0.004$], serial 3s score [$p<0.001$], serial 3s RT [$p=0.001$], serial 7s score [$p<0.001$], Sternberg scanning rate [$p=0.010$], and Stroop incongruent RT [$p=0.018$]. Continued improvements between Visits 2 & 3 were observed for IR score [$p=0.010$], serial 3s score [$p<0.001$], serial 7s score [$p=0.020$], and Stroop incongruent RT [$p=0.029$]. Therefore, 8 of 15 DVs were predicted by the Visit factor, with 6 DVs showing significant improvements between Visits 1 & 2 but only 4 showing significant improvements between Visits 2 & 3. Accuracy and error scores were observed to remain stable across repeated visits, irrespective of the type of cognitive task.

Table 3.2 Motivation data for Experiment 1.1 (n=29)

Motivation rating (out of 9)	Visit	Testing time point			
		Session 1		Session 2	
		Mean	SD	Mean	SD
Immediate recall rating	Visit 1	6.76	1.57	5.72	2.05
	Visit 2	6.62	1.63	6.03	2.18
	Visit 3	6.55	1.94	6.10	2.09
Delayed recall rating	Visit 1	6.24	1.77	5.97	2.13
	Visit 2	6.00	2.00	5.83	2.05
	Visit 3	6.31	2.09	6.14	2.33
Serial 3s rating	Visit 1	6.10	1.95	6.31	1.91
	Visit 2	5.69	2.16	5.69	2.38
	Visit 3	6.38	1.90	5.97	2.11
Serial 7s rating	Visit 1	6.03	2.13	5.76	2.12
	Visit 2	5.38	2.23	5.45	2.35
	Visit 3	5.86	2.13	5.79	2.38
Sternberg rating	Visit 1	5.45	1.97	5.45	2.16
	Visit 2	5.14	2.00	5.05	2.10
	Visit 3	5.72	2.25	5.48	2.15
Stroop rating	Visit 1	6.34	1.74	6.14	1.68
	Visit 2	6.14	1.85	5.97	1.90
	Visit 3	6.24	1.94	6.07	2.17

3.4.1.1.3 Session

Fixed effects of Session were significant for DR score, serial 3s score, serial 3s RT, serial 7s score, serial 7s RT, Sternberg extrapolated RT, Stroop incongruent RT, Stroop congruent RT, and Stroop interference effect. Significant improvements between Sessions 1 & 2 were observed for serial 3s score [$F(1,76.87)=42.98$, $p<0.001$], serial 3s RT $F(1,86.05)=16.59$, $p<0.001$], serial 7s score [$F(1,69.02)=10.74$, $p=0.002$], serial 7s RT [$F(1,67.33)=17.04$, $p<0.001$], Sternberg extrapolated RT

[F(1,55.60)=9.37, p=0.003], Stroop incongruent RT [F(1,57.19)=24.57, p<0.001], and Stroop congruent RT [F(1,42.89)=50.08, p<0.001]. Conversely, significant decreases in performance between Sessions 1 & 2 were observed for DR [F(1,71.43)=17.08, p<0.001], and Stroop interference effect [F(1,61.19)=6.50, p=0.013]. Therefore, 9 of 15 DVs were predicted by the Session factor, with 7 DVs showing improvement, and 2 DVs showing a decline in performance. Accuracy and error scores were observed to remain stable across repeated sessions, irrespective of the type of cognitive task.

3.4.1.1.4 Visit x Session interactions

A significant Visit x Session interaction observed for IR score [F(2,81.91)=4.36, p=0.016] was explained by a significant increase in performance between Sessions 1 & 2 at Visit 1 [p=0.041], but not at any other visits. For Serial 3s score [F(2,76.20)=3.87, p=0.025] improvements were observed between Sessions 1 & 2 on Visit 1 [p<0.001] and Visit 2 [p<0.001], but not on Visit 3 [p=0.179]. Similarly for Serial 7s RT [F(2,73.47)=4.39, p=0.016] the significant interaction was explained by improvement in performance between Sessions 1 & 2 at Visit 1 [p=0.001] and Visit 2 [p=0.029], but not at Visit 3 [p=0.503]. A significant interaction for Stroop incongruent RT [F(2,74.68)=8.70, p<0.001] was explained by improvements between Sessions 1 & 2 on Visit 1 only [p<0.001]. For Stroop congruent RT [F(2,83.05)=3.52, p=0.034], larger improvements were observed between Sessions 1 & 2 on Visit 1 [p<0.001], although moderate improvements remained evident between Sessions on Visit 2 [p=0.016] and Visit 3 [p=0.001]. In all interaction cases, therefore, practice effects between Sessions 1 & 2 were attenuated or eliminated altogether at later visits.

3.4.1.2 Experiment 1.2

Means and standard deviations for all cognitive variables recorded in Experiment 1.2 are shown in Table 3.3.

Table 3.3 Cognitive data for Experiment 1.2 (n=33)

Cognitive variable	Visit	Testing time point					
		Session 1		Session 2		Session 3	
		Mean	SD	Mean	SD	Mean	SD
Digit vigilance score (correct/45)	Visit 1	43.52	1.68	42.91	2.79	43.25	2.71
	Visit 2	43.29	2.72	42.22	4.12	41.97	3.50
	Visit 3	43.03	3.35	42.35	3.33	42.40	4.01
Digit vigilance RT (ms)	Visit 1	425.65	36.69	436.72	39.38	434.76	39.11
	Visit 2	429.53	39.19	430.89	36.03	440.81	40.71
	Visit 3	433.35	39.38	438.05	38.57	441.21	43.72
Digit vigilance commission errors	Visit 1	1.48	1.68	1.56	1.32	1.64	1.90
	Visit 2	1.48	1.75	1.75	1.59	2.16	1.92
	Visit 3	2.23	1.89	2.34	1.84	2.00	1.60
RVIP score (correct/40)	Visit 1	17.91	7.65	19.64	6.61	20.18	7.09
	Visit 2	21.59	7.49	24.34	7.04	22.97	7.25
	Visit 3	24.45	7.47	24.13	7.64	24.50	7.56
RVIP RT (ms)	Visit 1	395.26	39.03	384.65	46.28	375.48	35.28
	Visit 2	377.91	44.34	378.06	46.89	372.67	44.82
	Visit 3	386.77	47.20	381.23	41.81	379.42	45.54
RVIP commission errors	Visit 1	23.39	17.09	19.27	17.56	17.91	17.59
	Visit 2	18.25	18.14	17.94	18.88	19.50	20.04
	Visit 3	19.81	21.60	18.69	19.18	20.22	22.47
Serial 3s score (correct in 2 minutes)	Visit 1	26.12	12.62	31.39	12.35	35.03	14.82
	Visit 2	39.32	14.22	40.35	16.83	42.32	16.92
	Visit 3	43.72	16.35	45.19	19.09	46.41	17.69
Serial 3s RT (ms)	Visit 1	3935.75	1490.55	3612.64	1393.70	3444.88	1505.46
	Visit 2	3031.82	1119.44	3045.28	1248.74	2766.70	1056.58
	Visit 3	2900.23	1285.32	2771.11	1174.39	2709.68	1202.84

Table 3.3 continued

Cognitive variable	Visit	Testing time point					
		Session 1		Session 2		Session 3	
		Mean	SD	Mean	SD	Mean	SD
Serial 3s errors (incorrect in 2 minutes)	Visit 1	2.97	2.38	2.97	2.35	2.48	2.43
	Visit 2	2.35	2.46	2.29	2.33	2.90	2.26
	Visit 3	1.66	2.74	2.31	2.12	2.06	2.23
Serial 7s score (correct in 2 minutes)	Visit 1	15.38	7.25	17.79	6.41	19.44	7.49
	Visit 2	21.38	9.54	23.28	9.85	23.74	9.91
	Visit 3	23.74	10.63	24.56	11.05	24.39	8.85
Serial 7s RT (ms)	Visit 1	6844.93	2861.18	6170.24	2859.86	5428.16	2394.88
	Visit 2	5637.11	2751.33	5376.46	2555.78	5156.44	2277.00
	Visit 3	5339.91	2348.87	4852.81	2305.07	4864.63	2005.26
Serial 7s errors (incorrect in 2 minutes)	Visit 1	2.03	1.45	2.31	1.86	2.69	2.35
	Visit 2	1.72	1.46	2.42	2.39	2.23	2.05
	Visit 3	1.33	1.71	2.69	1.96	2.19	2.06

3.4.1.2.1 Motivation

As a repeated covariate in the model, Motivation significantly predicted RVIP score [F(1,243.60)=9.49, p=0.002, beta=0.584], digit vigilance score [F(1,172.34)=6.54, p=0.011, beta=0.236], digit vigilance RT [F(1,237.80)=8.14, p=0.005, beta=-2.736], digit vigilance commission errors [F(1,224.24)=10.48, p=0.001, beta=-0.186], serial 3s score [F(1,197.14)=17.64, p<0.001, beta=1.311], serial 7s score [F(1,209.03)=11.28, p=0.001, beta=0.705], and serial 7s RT [F(1,219.22)=4.45, p=0.036, beta=-92.460], such that higher motivation was associated with better performance. Means and standard deviations for all motivation ratings collected during Experiment 1.2 are shown in Table 3.4.

Table 3.4 Motivation data for Experiment 1.2 (n=33)

Motivation rating (out of 9)	Visit	Testing time point: Session 1 Mean	Session 1 SD	Session 2 Mean	Session 2 SD	Session 3 Mean	Session 3 SD
Digit vigilance rating	Visit 1	5.58	2.02	5.03	1.93	4.52	2.22
	Visit 2	5.38	2.01	4.88	2.25	4.53	2.29
	Visit 3	4.70	2.14	4.41	2.01	4.34	1.68
RVIP rating	Visit 1	5.03	2.02	4.94	1.84	4.38	2.15
	Visit 2	4.87	2.00	4.47	2.16	4.26	2.32
	Visit 3	4.84	2.03	3.94	2.03	3.91	1.94
Serial 3s rating	Visit 1	5.67	1.73	5.39	1.85	5.36	1.78
	Visit 2	5.69	1.58	5.47	2.06	5.41	2.11
	Visit 3	5.97	1.96	5.50	2.02	5.50	1.97
Serial 7s rating	Visit 1	5.97	1.79	5.64	1.60	5.27	1.84
	Visit 2	5.53	1.57	5.06	1.81	5.00	2.21
	Visit 3	5.58	1.71	5.03	2.01	5.06	1.81

3.4.1.2.2 Visit

The Visit factor was a significant predictor of RVIP score [$F(2,71.74)=26.88$, $p<0.001$], RVIP RT [$F(2,82.56)=3.59$, $p=0.032$], digit vigilance commission errors [$F(2,93.92)=4.94$, $p=0.009$], serial 3s score [$F(2,35.86)=49.65$, $p<0.001$] and serial 3s RT [$F(2,64.06)=30.25$, $p<0.001$], serial 7s score [$F(2,63.84)=32.34$, $p<0.001$], and serial 7s RT [$F(2,79.61)=44.95$, $p<0.001$].

Pairwise comparisons showed performance improvements between Visits 1 & 2 for RVIP score [$p<0.001$], RVIP RT [$p=0.026$], serial 3s score [$p<0.001$], serial 3s RT [$p<0.001$], serial 7s score [$p<0.001$], and serial 7s RT [$p<0.001$]. Significant improvements between Visits 2 & 3 were only observed for serial 3s score [$p=0.002$], serial 3s RT [$p=0.012$], and serial 7s RT [$p=0.002$]. Therefore, 6 of 12 DVs showed practice effects across test visits with 6 DVs showing significant changes between Visits 1 & 2 but only 3 showing significant changes between Visits 2 & 3. Error scores were generally

observed to remain stable across visits, irrespective of the type of cognitive task. The only exception was for commission errors produced during the digit vigilance task, where an increase in errors was observed between Visits 1 & 3 [p=0.007].

3.4.1.2.3 Session

The Session factor was a significant predictor of performance for the majority of DVs. Performance improvements across sessions were observed for RVIP score [F(2,138.85)=5.51, p=0.005], RVIP RT F(2,133.46)=5.38, p=0.006], RVIP commission errors [F(2,116.65)=3.06, p=0.050], serial 3s score [F(2,105.34)=22.49, p<0.001], serial 3s RT [F(2,104.83)=12.12, p<0.001], serial 7s score [F(2,114.35)=16.54, p<0.001], and serial 7s RT [F(2,110.43)=28.16, p<0.001]. Conversely, significant decreases in performance across sessions were observed for digit vigilance score [F(2,117.55)=5.87, p=0.004], digit vigilance RT [F(2,127.82)=4.21, p=0.017], and serial 7s errors [F(2,133.11)=8.63, p<0.001].

Pairwise comparisons showed performance improvements between Sessions 1 & 2 for RVIP score [p=0.009], serial 3s score [p<0.001], serial 3s RT [p=0.008], serial 7s score [p=0.001], and serial 7s RT [p<0.001]. Significant improvements between Sessions 2 & 3 were only observed for serial 3s score [p=0.019], serial 3s RT [p=0.037], and serial 7s RT [p=0.020]. Therefore, 7 of 12 DVs showed practice effects across test sessions with 5 DVs showing significant performance improvements between Sessions 1 & 2 but only 3 showing continued improvements between Sessions 2 & 3. Decreases in performance between Sessions 1 & 2 were evident for digit vigilance score [p=0.019], and serial 7s errors [p=0.001], however this decline did not persist between Sessions 2 & 3 [p=0.996 & p>0.999 respectively]. With the exception of serial 7s then, error scores were observed to remain stable across repeat sessions, irrespective of the type of cognitive task. Practice effects were more likely to persist across all repeat test sessions for the serial subtraction tasks, compared with RVIP and digit vigilance.

3.4.1.2.4 Visit x Session interactions

A significant Visit x Session interaction observed for RVIP commission errors [F(4,86.94)=3.22, p=0.016] was explained by a significant increase in the number of errors between Sessions 1 & 3 at Visit 2 [p=0.012], but not at any other visits. For serial 3s score [F(4,82.51)=2.87, p=0.028] improvements were observed between Sessions 1 & 3 on Visit 1 [p<0.001] but not at any other visits, suggesting an attenuation of practice related improvement.

3.4.2 LMM analysis of the incidence of interference errors in the immediate & delayed recall tasks (Experiment 1.1 only)

Mean interference errors with standard deviations, recorded at each time point, are presented in Table 3.1. IR interference errors were not predicted by any of the factors included in the model: Visit [$F(2,73.11)=0.07$, $p=0.934$]; Session [$F(1,61.39)=0.38$, $p=0.538$]; Visit x Session [$F(2,71.07)=0.67$, $p=0.517$], indeed the incidence of interference errors remained consistently low for the IR task throughout repeated testing. Conversely, DR interference errors were significantly predicted by all factors: Visit [$F(2,54.91)=11.31$, $p<0.001$]; Session [$F(1,67.05)=44.29$, $p<0.001$]; Visit x Session [$F(2,71.07)=9.16$, $p<0.001$]. Pairwise comparisons revealed a significant increase in the number of errors between Visits 1 & 2 [$p=0.001$] and Visits 1 & 3 [$p=0.005$], but not between Visits 2 & 3 [$p>0.999$]. Increases in the number of errors at Session 2 relative to Session 1 were marginally significant at Visit 1 [$p=0.032$] and highly significant at Visits 2 & 3 [both $p<0.001$]. As expected by their nature, interference errors for the DR task were less prevalent at Visit 1 compared with later visits and occurred more often at the second session of testing on any given visit.

3.4.3 Comparison of Cohen's d effect sizes between visits and sessions for all cognitive tasks

3.4.3.1 Experiment 1.1

Cohen's d effect sizes for cognitive changes observed in Experiment 1.1 are presented in Table 3.5. By comparing Cohen's d effect sizes between visits, it can be seen that practice effect sizes between Visits 2 & 3 were reduced for 11 of 15 DVs when compared with effect sizes between Visits 1 & 2. The only exceptions were DR score, Serial 3s errors, Sternberg accuracy, and Stroop interference, where slight increases in practice effect sizes were observed. Between visit effect sizes for accuracy and error rates were typically lower than for other DVs reflecting overall stability in these measures. Practice effect sizes between same-day Sessions 1 & 2 were reduced when comparing Visits 2 & 3 with Visit 1. For IR score, DR score and Stroop interference, where overall performance decreases were observed between same-day sessions, the magnitude of these negative Cohen's d values increased at subsequent visits.

3.4.3.2 Experiment 1.2

Cohen's d effect sizes for cognitive changes observed in Experiment 1.2 are presented in Table 3.6. By comparing Cohen's d effect sizes between visits, it can be seen that practice effect sizes between Visits 2 & 3 were reduced for 8 of 12 DVs when compared with effect sizes between Visits 1 & 2. The

only exceptions were Serial 3s errors, where a slight increase in practice effect size was observed; and all of the digit vigilance DVs, where consistent decreases in performance were observed across visits. Between-visit effect sizes for error rates were typically lower than for other DVs reflecting overall stability in these measures. Practice effect sizes between same-day test sessions were typically reduced when comparing performance increases between Sessions 2 & 3 with increases between Sessions 1 & 2. Similarly, practice effect sizes between sessions were typically attenuated at Visits 2 & 3 relative to Visit 1. For RVIP, serial 3s, and serial 7s error scores, overall performance decreases were observed between same-day sessions. Similarly, consistent decreases in performance were observed between test sessions for all of the digit vigilance DVs.

Table 3.5 Cohen's d effect sizes for cognitive changes in Experiment 1.1

Cognitive Measure	Mean practice effect size between Visits			Mean practice effect size between Sessions		
				Visit1	Visit2	Visit3
	Visit1-Visit2	Visit2-Visit3	Visit1-Visit3	Session1-Session2	Session1-Session2	Session1-Session2
Immediate recall score	0.368	0.333	0.686	0.395	-0.104	-0.354
Delayed recall score	0.117	0.188	0.304	-0.173	-0.440	-0.650
Serial 3s score	0.351	0.258	0.608	0.434	0.311	0.113
Serial 3s RT	0.273	0.149	0.450	0.324	0.150	0.167
Serial 3s errors	-0.051	-0.013	-0.063	-0.091	0.116	-0.238
Serial 7s score	0.250	0.157	0.409	0.236	0.097	0.133
Serial 7s RT	0.198	0.100	0.321	0.371	0.305	0.048
Serial 7s errors	0.183	-0.018	0.166	-0.034	-0.246	0.087
Sternberg accuracy	-0.160	-0.133	-0.322	-0.046	-0.153	0.043
Sternberg scanning rate	0.382	0.081	0.438	0.100	-0.088	0.069
Sternberg extrapolated RT	0.159	0.117	0.259	0.395	0.221	0.091
Stroop accuracy	0.053	-0.036	0.020	0.131	-0.269	0.250
Stroop incongruent RT	0.148	0.146	0.293	0.619	0.112	0.114
Stroop congruent RT	0.125	0.112	0.235	0.557	0.208	0.356
Stroop interference effect	0.022	0.058	0.080	0.002	-0.217	-0.528

Decreases in performance are prefixed with a minus sign

Table 3.6 Cohen's d effect sizes for cognitive changes in Experiment 1.2

Cognitive measure	Mean practice effect size between Visits		Mean practice effect size between Sessions					
			Visit1		Visit2		Visit3	
	Visit1-Visit2	Visit2-Visit3	S1-S2	S2-S3	S1-S2	S2-S3	S1-S2	S2-S3
RVIP score	0.346	0.136	0.226	0.106	0.380	0.183	0.006	0.054
RVIP RT	0.160	0.069	0.243	0.239	0.040	0.114	0.124	0.047
RVIP commission errors	0.045	-0.009	0.213	0.032	-0.019	-0.226	0.012	-0.086
Digit vigilance score	-0.097	-0.045	-0.136	-0.086	-0.239	-0.049	-0.305	-0.128
Digit vigilance RT	-0.059	-0.030	-0.255	-0.035	0.012	-0.249	-0.132	-0.032
Digit vigilance commission errors	-0.141	-0.141	0.037	-0.011	-0.077	-0.218	-0.022	0.141
Serial 3s score	0.389	0.190	0.408	0.260	0.133	0.076	0.139	0.08
Serial 3s RT	0.317	0.091	0.297	0.129	0.018	0.128	0.119	0.052
Serial 3s errors	0.078	0.177	0.017	0.186	0.061	-0.359	-0.254	0.11
Serial 7s score	0.370	0.092	0.274	0.173	0.250	0.139	0.116	0.039
Serial 7s RT	0.196	0.109	0.361	0.233	0.201	0.120	0.210	0.023
Serial 7s errors	0.123	0.040	-0.188	-0.130	-0.349	0.103	-0.859	0.266

Decreases in performance are prefixed with a minus sign

3.4.4 LMM analysis of the changes in subjective mood across all test sessions

3.4.4.1 Experiment 1.1

Mental fatigue was significantly predicted by the factor Visit [F(2,58.82)=5.18, p=0.008] with pairwise comparisons revealing decreased ratings of fatigue at Visit 3 compared with both Visit 1 [p=0.077] and Visit 2 [p=0.008]. Session number was also a significant predictor of mental fatigue [F(1,69.09)=7.24, p=0.009], with increased mental fatigue observed at Session 2 relative to Session 1.

Positive affect was significantly predicted by Visit [F(2,53.70)=5.18, p=0.009], with pairwise comparisons revealing decreased positive affect at Visit 2 compared with Visit 1 [p=0.006]. Significant decreases in positive affect across same-day sessions were also evident [F(1,61.90)=13.24, p=0.001]. Moreover, the interaction between Visit x Session was also marginally significant [F(2,71.46)=3.13, p=0.050]. Significant decreases in positive affect between sessions were found at Visit 1 [p=0.001] and Visit 2 [p<0.001] only. Expected decreases in positive affect at the second session of testing on the final visit were likely to have been negated by positive feelings associated with completing the study. Reassuringly, no significant changes in negative affect were observed throughout testing.

Means and standard deviations for all mood ratings recorded during Experiment 1.1 are shown in Table 3.7.

Table 3.7 Mood data for Experiment 1.1 (n=29)

Mood variable	Visit	Testing time point			
		Session 1		Session 2	
		Mean	SD	Mean	SD
Mental fatigue (rating/9)	Visit 1	5.31	1.91	6.10	1.74
	Visit 2	5.48	1.70	6.00	2.04
	Visit 3	4.79	2.19	5.03	2.04
Positive affect (score/50)	Visit 1	24.66	7.59	21.97	8.35
	Visit 2	23.24	9.28	19.55	7.55
	Visit 3	22.76	7.86	23.00	8.50
Negative affect (score/50)	Visit 1	13.34	3.87	12.72	4.10
	Visit 2	12.86	4.56	12.76	5.12
	Visit 3	12.72	5.65	12.38	2.88

3.4.4.2 Experiment 1.2

Mental fatigue was not significantly predicted by any of the factors. Positive affect was significantly predicted by the Visit factor [$F(2,73.67)=3.48$, $p=0.036$], with pairwise comparisons revealing decreased positive affect at Visit 2 compared with Visit 1 [$p=0.039$]. Significant decreases in positive affect across same day test sessions were predicted by the Session factor [$F(2,126.73)=8.93$, $p<0.001$]. Significant decreases in positive affect were found between Sessions 1 & 2 only [$p<0.001$]. Unexpectedly, negative affect was also significantly predicted by Session [$F(2,136.12)=6.35$, $p=0.002$]. Post hoc comparisons revealed a reduction in negative affect between Sessions 1 & 2 [$p=0.012$].

Means and standard deviations for all mood ratings recorded during Experiment 1.2 are shown in Table.3.8.

Table 3.8 Mood data for Experiment 1.2 (n=33)

Mood variable	Visit	Testing time point					
		Session 1		Session 2		Session 3	
		Mean	SD	Mean	SD	Mean	SD
Mental fatigue (rating/9)	Visit 1	5.00	1.94	4.55	1.87	5.10	2.04
	Visit 2	5.00	2.05	4.28	2.23	4.81	2.26
	Visit 3	4.56	2.26	4.81	2.26	4.91	2.32
Positive affect (score/50)	Visit 1	24.76	7.07	22.30	7.92	22.13	9.27
	Visit 2	22.59	7.25	20.34	7.29	20.77	6.85
	Visit 3	22.06	7.11	20.47	6.69	21.09	7.71
Negative affect (score/50)	Visit 1	13.38	3.97	12.31	3.82	12.27	3.23
	Visit 2	13.59	4.17	12.31	3.24	11.74	2.44
	Visit 3	13.26	3.69	13.03	3.61	13.09	3.97

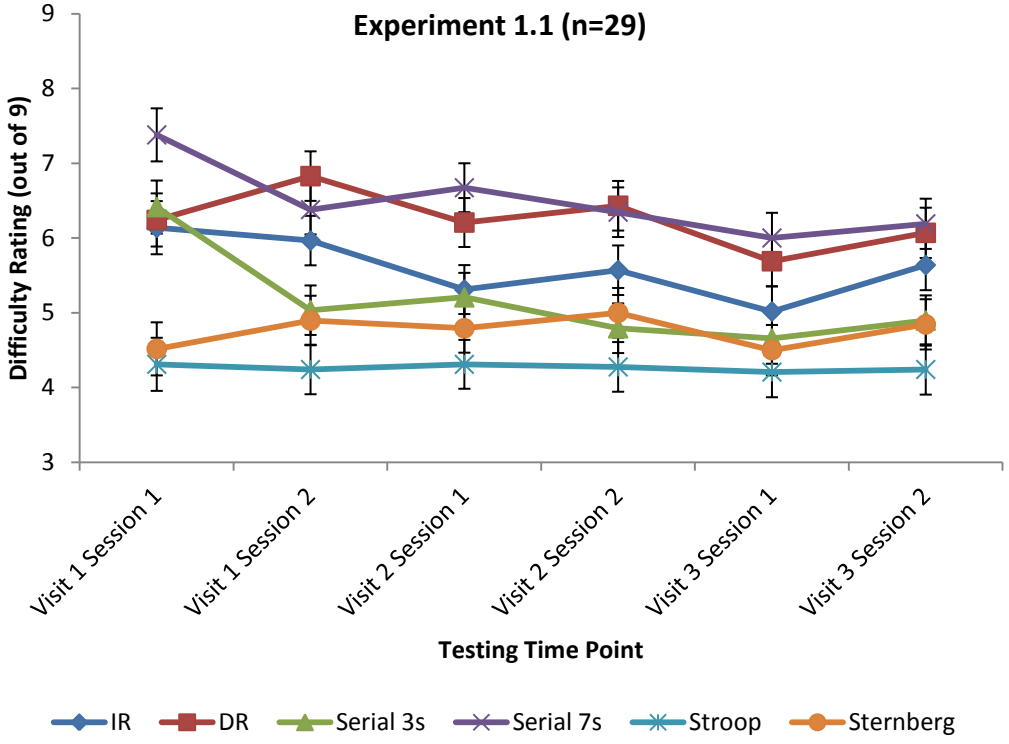
3.4.5 LMM analysis of the changes in perception of task difficulty for all cognitive tasks across all task repetitions

3.4.5.1 Experiment 1.1

Cognitive task type was a significant factor in predicting difficulty ratings [$F(5,186.29)=20.12$, $p<0.001$]. In task order, serial 7s was rated the most difficult (M 6.49, SD 1.89), followed by DR (M 6.24, SD 1.79), IR (M 5.61, SD 1.64), serial 3s (M 5.17, SD 2.12), Sternberg (M 4.76, SD 2.06) and Stroop (M 4.26, SD 1.62). Difficulty ratings were also predicted by the Visit factor [$F(2,595.30)=7.38$, $p=0.001$], although not by Session [$F(1,602.72)<0.01$, $p=0.947$]. Tasks were perceived to become significantly easier between Visits 1 & 2 [$p=0.030$] and Visits 1 & 3 [$p<0.001$], but not between Visits 2 & 3 [$p=0.069$]. Variations in ratings of difficulty were indicated by significant interactions: Session x Cognitive Task [$F(5,602.72)=7.22$, $p<0.001$]; Visit x Session [$F(2,54.08)=6.54$, $p=0.002$]; Visit x Session x Cognitive Task [$F(10,544.08)=1.87$, $p=0.047$]. The Visit x Cognitive Task interaction was not significant [$F(10,595.30)=1.07$, $p=0.387$]. Due to their complex nature, these interactions have not been fully interpreted here but are shown in Figure 3.2.

3.4.5.2 Experiment 1.2

Cognitive task type was a significant factor in predicting difficulty ratings [F(3,204.29)=142.51, p<0.001]. In task order, RVIP was rated the most difficult (M 5.77, SD 2.03), followed by serial 7s (M 5.72, SD 1.89), serial 3s (M 4.22, SD 1.86), and digit vigilance (M 2.84, SD 1.73). Difficulty ratings were also predicted by Visit [F(2,452.45)=22.98, p<0.001], and by Session [F(2,586.29)=37.75, p<0.001]. Tasks were perceived to become significantly easier between Visits 1 & 2 [p<0.001], and continued to do so between Visits 2 & 3 [p=0.003]. Similarly, tasks were perceived to become significantly easier between Sessions 1 & 2 [p<0.001], and this trend continued between Sessions 2 & 3 [p=0.001]. Variations in difficulty rating patterns across cognitive tasks were indicated by significant interactions: Visit x Cognitive Task [F(6,585.91)=2.83, p=0.010]; Session x Cognitive Task [F(6,449.48)=2.72, p=0.013], and Visit x Session x Cognitive Task [F(16,447.23)=3.11, p<0.001]. Due to their complex nature, these interactions have not been fully reported here but are evident in Figure 3.2.



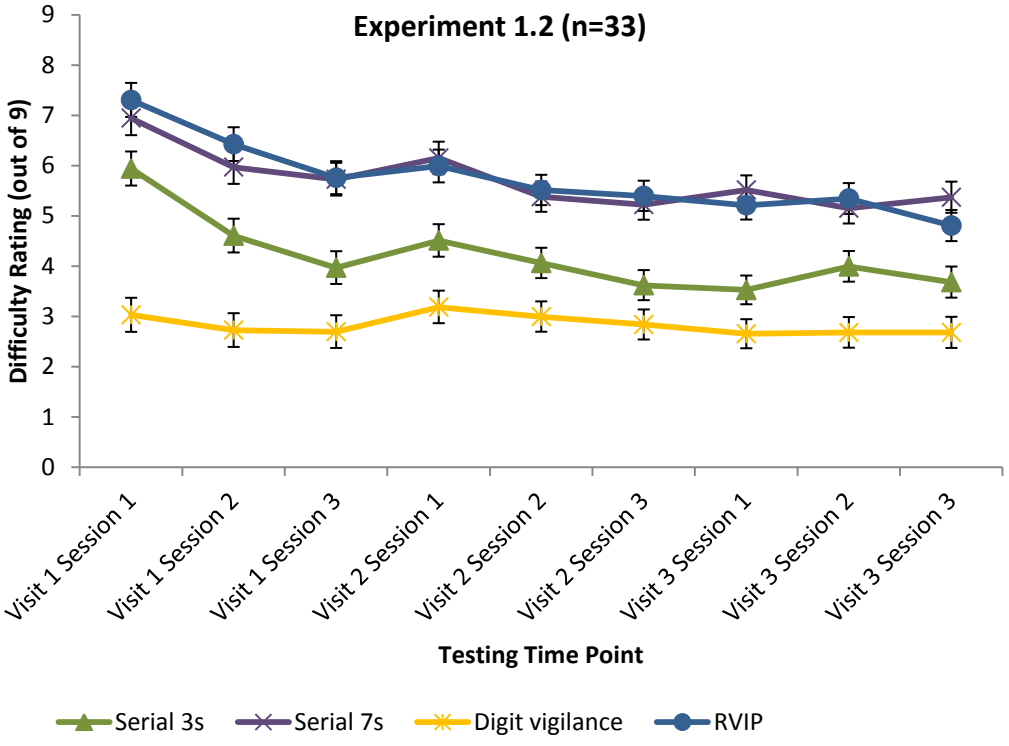


Figure 3.2 Subjective ratings of task difficulty for each cognitive task in Experiments 1.1 & 1.2

Reported values are estimated marginal means. Error bars represent standard error of the mean

3.4.6 LMM analysis of the changes in motivation for all cognitive tasks across all task repetitions

Mean motivation ratings for all cognitive tasks used in Experiments 1.1 & 1.2 can be found in Table 3.3 and Table3.4, respectively.

3.4.6.1 Experiment 1.1

Cognitive task type was a significant factor in predicting motivation ratings [F(5,262.17)=10.42, p<0.001]. In task order, motivation was rated highest for IR (M 6.30, SD 1.93), followed by Stroop (M 6.15, SD 1.86), DR (M 6.08, SD 2.04), serial 3s (M 6.02, SD 2.06), serial 7s (M 5.71, SD2.21) and finally Sternberg (M 5.38, SD 2.09). Motivation ratings were also predicted by Visit [F(2,537.87)=8.03, p<0.001], and by Session [F(1,481.32)=15.14, p<0.001]. Motivation dropped significantly between Visits 1 & 2 [p=0.007], but increased again between Visits 2 & 3 [p=0.001]. Motivation also dropped between Sessions 1 & 2 [p<0.001]. Variations in motivation patterns across cognitive tasks were indicated by a significant interaction between Session x Cognitive Task [F(5,481.32)=2.72, p=0.019]; motivational differences apparent between cognitive tasks at Session 1 were less apparent at Session 2. No other significant interactions were evident.

3.4.6.2 Experiment 1.2

Cognitive task type was a significant factor in predicting motivation ratings [F(3,225.84)=21.04, p<0.001]. In task order, motivation was rated highest for serial 3s (M 5.55, SD 1.88), followed by serial 7s (M 5.35, SD 1.83), digit vigilance (M 4.82, SD 2.08), and RVIP (M 4.52, SD 2.07). Motivation ratings were also predicted by Visit [F(2,414.59)=5.28, p=0.005], and by Session [F(2,558.39)=27.76, p<0.001]. Motivation significantly decreased between Visits 1 & 3 [p=0.004]. Similarly, motivation decreased between Sessions 1 & 2 [p<0.001], and this trend continued between Sessions 2 & 3 [p=0.048]. There were no significant interactions between Cognitive task, Visit or Session.

3.4.7 LMM analysis of time of day effects

After combining serial subtraction and mood data from Experiments 1.1 & 1.2 in order to increase statistical power, LMM analysis revealed that Time of Day was a significant predictor of serial 3s errors [F(1,71.49)=6.66, p=0.012]. More errors were made in the morning (M 2.92, SE 0.24) compared with the afternoon (M 2.07, SE 0.23). For serial 7s score, the Time of Day x Visit interaction was significant [F(2,111.67)=5.80, p=0.004]; at Visit 2 participants scored higher in the morning (M 25.92, SE 1.64) compared with the afternoon (M22.47, SE 1.60), however statistical significance of this pairwise comparison was not maintained following Bonferroni correction

[p=0.087]. For positive affect, the Time of Day x Visit interaction was similarly significant [F(2,107.87)=4.32, p=0.016]. At Visit 1 [p=0.053] & Visit 2 [p=0.056] participants rated positive affect higher in the mornings (M_{V1} 24.79 SE_{V1} 1.15; M_{V2} 22.66, SE_{V2} 1.13) than in the afternoons (M_{V1} 21.93 SE_{V1} 1.12; M_{V2} 19.89, SE_{V2} 1.10). Time of day was not a significant predictor of performance for any other DV included in the analysis.

3.4.8 Intraclass correlation coefficients for the determination of alternate-form reliability

ICCs for all cognitive tasks are reported in Table 3.9.

Alternate-form reliability was acceptable for all cognitive DVs with the exception of error scores for the serial 3s & 7s and digit vigilance tasks, and the accuracy score for the Stroop task. However, in light of the good scores for the other DVs determined for each of these tasks, the poor ICC scores were more likely due to low between-subjects variability in the data rather than an indication that alternate forms were not suitably equivalent (Weir, 2005).

Table 3.9 ICC values for all cognitive tasks in Experiment 1.1 & Experiment 1.2

Cognitive Measure	ICC
Experiment 1.1:	
Immediate recall score	0.44
Delayed recall score	0.42
Serial 3s score	0.90
Serial 3s RT	0.83
Serial 3s errors	0.25
Serial 7s score	0.90
Serial 7s RT	0.77
Serial 7s errors	0.38
Sternberg accuracy	0.53
Sternberg scanning rate	0.41
Sternberg extrapolated RT	0.67
Stroop accuracy	0.27
Stroop incongruent RT	0.86
Stroop congruent RT	0.86
Stroop interference effect	0.54
Experiment 1.2:	
Serial 3s score	0.83
Serial 3s RT	0.88
Serial 3s errors	0.05
Serial 7s score	0.77
Serial 7s RT	0.89
Serial 7s errors	0.35
Digit Vigilance correct	0.56
Digit Vigilance RT	0.60
Digit Vigilance commission errors	0.31
RVIP correct	0.67
RVIP RT	0.61
RVIP commission errors	0.82

3.5 Discussion

The purpose of this experimental chapter was to pilot the selected cognitive tasks before including them in subsequent blueberry intervention work; in particular, the aim was to investigate the effects of repeated exposure to a task on cognitive performance, within and between multiple test days. By gaining a better understanding of the patterns of cognitive practice effects it was hoped to reduce this confound by adopting appropriate methodology. Practice effects were evident for all cognitive tasks investigated in Experiment 1.1 and Experiment 1.2, either across weekly visits or between same-day sessions despite the use of alternate forms of each task and familiarisation trials immediately before the start of each test performance. However, in accordance with previous findings (Bartels et al., 2010; Collie et al., 2003; Falleti et al., 2006) the strongest practice effects appeared between Visits 1 & 2, such that Cohen's d effect sizes would be attenuated in all cases by discarding data from the first visit. Specifically, significant practice effects remained evident between visits 2 & 3 for IR score, serial 3s score, serial 7s score and Stroop incongruent RT in Experiment 1.1; and for serial 3s score and RT, and serial 7s RT in Experiment 1.2. But effect sizes were attenuated in all cases when compared with performance increases observed between Visits 1 & 2. Therefore a separate familiarisation visit followed by a period of consolidation before data collection, is likely to aid in addressing the impact of practice related improvements on test performance between visits.

In both experiments, practice effect sizes between same day sessions were similarly reduced at Visits 2 & 3 compared with Visit 1. In Experiment 1.1 significant improvements evident between Sessions 1 & 2 at Visit 1 for IR score, serial 3s score, serial 7s RT, and Stroop congruent & incongruent RT, were greatly attenuated by Visit 3. Interestingly in Experiment 1.2, where three test sessions were performed per visit, it was evident that performance increases were also attenuated between Sessions 2 & 3, when compared with increases observed between Sessions 1 & 2. However significant increases in performance across Sessions 2 & 3 still remained evident for serial 3s score & RT, and serial 7s RT. The results suggest that familiarisation trials, whether on a separate visit or immediately prior to data collection, are likely to be ineffective at actually eliminating practice effects between same day test sessions. A similar observation was made by Lemay et al (Lemay et al., 2004). Indeed, the lack of a *Visit x Session* interaction for the majority of the DVs reported here suggests that performance increases across multiple testing points on a single day remain relatively constant between visits, with only slight attenuation of effect sizes at subsequent visits. After excluding Visit 1, residual between-session practice effects were evident only as small Cohen's d effect sizes (d=0.01-0.35). Nevertheless in nutrition intervention studies, nutrient effect sizes typically range from small to moderate. For example, effect sizes following acute flavonoid

intervention have been reported to range upwards from d=0.16 (Bell et al., 2015). Therefore even small practice effect sizes may impact statistical power in a nutrition intervention study. Future consideration of how to take practice effects into account in *a priori* power calculations may provide a solution to the problem of underpowered nutrition intervention studies.

The statistically significant improvements in task performance between test days shown here remained apparent when the variance accounted for by motivation was included in the model. By including motivation, this supports the notion that the observed effects are likely related to practice, although other variables such as mood or fatigue cannot be ruled out. As a covariate, motivation was found to significantly predict episodic memory (IR and DR) recall, Stroop, RVIP, digit vigilance and serial 7s task performance. With the exceptions of Stroop and digit vigilance, these tasks were also rated to be the most difficult tasks overall. Therefore, when tasks were particularly difficult motivation was a strong predictor of performance, independently of practice effects. Motivation was reported retrospectively and so it is possible that participants reported high motivation when they felt they had performed well during the task, even though direct feedback was not given. However if this was the case, motivation might be expected to have been a significant predictor of performance for all of the cognitive tasks investigated. Fatigue is also likely to be a major contributing factor to task performance. As expected following an extended period of cognitive testing, mental fatigue was observed to increase at session 2 relative to session 1 and may also have impacted on subjective ratings of motivation. Perceptions of task difficulty were observed to decrease over time for all cognitive tasks, suggestive of a general practice effect. After repetition, the tasks were rated as becoming easier and performance on the tasks improved. Minor fluctuations in this downward trend in perceived difficulty are again likely to result from the impact of fatigue on participants' retrospective, subjective ratings. Interestingly, accuracy or error scores were observed to remain relatively unaffected by repeat performance for the Stroop, Sternberg, RVIP, digit vigilance and serial subtraction tasks. With the exception of the RVIP task, participants were performing near ceiling levels on each of these measures. Although appearing resistant to practice, these measures may have little practical application in nutrition intervention work as high accuracy scores leave little room for cognitive improvement following an intervention. Nevertheless, task parameters were not altered at this stage as the tasks were designed to replicate those used in previous research (Chapter 2) and RT measures are more commonly reported for these tasks.

Depending on the cognitive task, different performance related effects of repeated exposure were observed. It has been demonstrated that practice related effects are evident across separate visits and same day sessions of testing, particularly for tasks measuring working memory (RVIP, serial

subtraction). For attention based DVs including Stroop interference effect and digit vigilance score & RT, and episodic memory DVs such as immediate and delayed recall score, practice-related improvements observed across visits were slight or not present at all, but apparent decreases in performance were observed between same day test sessions. For the IR & DR episodic memory tasks there is some suggestion of interference at the second session of each visit, that counters practice related improvements. The LMM analysis revealed that motivation significantly predicted memory performance and so motivational decreases are likely to account for some of the observed decreases in performance between these same day sessions, although as discussed above, motivation may be confounded with mental fatigue. Word list interference from the previous session is also likely to be a major contributing factor here (Greenberg & Underwood, 1950; Underwood, 1957). Indeed, analysis of the interference error scores revealed the stable presence of low level interference for the IR task, and significant increases in interference across Visits and Sessions for the DR task.

Performance decreases on the Stroop interference measure are more complex. In fact, overall reaction times decreased between sessions, but more so for the congruent trials, resulting in an increased interference effect. This is a potentially interesting observation that warrants further investigation, particularly as previous research has often, though not always, shown the Stroop interference effect to be reduced by practice (Macleod, 1991).Therefore, it is important to interpret Stroop interference effect changes with reference to the underlying reaction times. For the digit vigilance task, where sustained attention was required for a sustained period on a relatively simple task, decreases in performance appear simply to reflect increased fatigue. Interestingly, where interference related performance decreases were observed between same day sessions (Stroop, IR, DR), Cohen's d effect sizes increased at all subsequent visits. This appears consistent with the notion that practice related improvements had been attenuated and therefore interfering effects now demonstrated greater magnitude in the opposing direction.

The Sternberg task appears relatively robust to practice compared to the other cognitive tasks investigated here; an observation shared by previous research (Kristofferson, 1972, 1977; Sternberg, 1975). Overall, extrapolated RT performance did speed up slightly due to practice, which may be explained by faster visual processing, decision making or motor output. However the main DV for the task measures how fast participants can scan items in their STM, and the above cited research shows this to be a fixed time per item that cannot be changed by practice, reflecting a fundamental cognitive process. Despite this, the observational data here suggest some practice related improvement between visits 1 & 2 but performance appeared to stabilise after this. Similarly, task performance appeared to stabilise after Visit 1 for the RVIP task, although this is likely to reflect the

attainment of a ceiling level of performance imposed by task parameters rather than a cognitive process.

For the subjectively reported mood measures, as might reasonably be expected after an extended period of cognitive testing, higher ratings of mental fatigue were observed at Session 2 relative to Session 1, within visits. However this effect was observed in Experiment 1.1 only, possibly due to the longer duration of the cognitive battery in this experiment. A decrease in positive mood was also observed at Session 2, this time in both experiments. However it was evident from Experiment 1.2 that no further decreases in positive mood occurred between Sessions 2 & 3. In general, positive affect scores remained just below mid-range. No corresponding increases in negative affect were observed, in fact negative affect actually decreased between Sessions 1 & 2 in Experiment 1.2. This may reflect a reduction in anxiety initially present when facing the prospect of an extended period of cognitive testing. Together, these measures imply no serious negative consequences for participants in terms of fatigue, or mood over prolonged periods of repeated testing.

Times of day effects were limited to serial 3s errors, serial 7s score, and positive affect. With the exception of serial 3s errors, performance was generally better in the mornings. However, any observed differences were minimal and no longer reached statistical significance following corrections for multiple comparisons. This suggests that diurnal effects may have little impact on cognition or mood. However, the cognitive findings were based on serial subtraction data only and may not generalise to other cognitive domains. Regardless of time of day or cognitive domain, alternate-forms reliability analysis revealed acceptable consistency between the different forms of each task used at successive time points.

There are some shortcomings to this study. The effects of practice have not been investigated beyond a period of three weeks; the findings are exclusive to the tests examined; and the effects of including dual baselines (McCaffrey, Ortega, Orsillo, Nelles, & Haase, 1992), such as those used by Scholey et al. (Scholey et al., 2010), have not been investigated. Dual baseline designs discard the first session of data collected on each visit. The findings of Experiment 1.2 support the theory that between session practice effects may be attenuated, but not eliminated by this method. The findings of the study demonstrate that practice cannot be assumed to have been adequately addressed through the use of alternate forms of stimuli or familiarisation trials immediately prior to data collection, and suggest that dual baseline designs may be an effective addition to crossover study methodology in their ability to attenuate practice effects. But the method is likely to be better suited

to short cognitive test batteries, and the optimal length of time between the first and second performance of the test battery needs further investigation.

Contrary to previous findings (Bartels et al., 2010; Falleti et al., 2006) but consistent with theories of learning and memory, within a nutrition intervention design framework the effects of practice have been observed to persist well past the first two repeat sessions of cognitive testing on the majority of tasks investigated here. The only exceptions were the Sternberg memory scanning task and RVIP task that appeared to become relatively resistant to practice-related improvements after an initial test visit. Practice can interact with a nutrition intervention such that more learning takes place in the active condition and then what is learned is retained and causes elevated performance in the control condition, masking intervention effects for half the participants in a typical counterbalanced crossover design. Conversely, care must be taken to ensure that improvements in performance arising from repeated practice of the task when the control condition is performed first are not confused with those associated with the intervention. Therefore the use of practice resistant tasks or appropriate methodology, as outlined below, for minimising the influence of practice effects is paramount. Motivation and fatigue clearly also contribute to cognitive performance, particularly for more difficult cognitive tasks. The relationship between motivation, fatigue and practice requires further investigation.

The findings of this experiment are likely to generalise as similar memory, reaction time and accuracy components are inherent in all commonly used cognitive tasks. In future intervention studies, whether that be nutritional or pharmacological, it is recommended that methodologies take practice effects into account; a summary of recommendations is included in the final paragraph. For studies which have already reported effects of nutrition interventions, care should be taken when retrospectively calculating effect sizes. For example in the absence of an appropriate control condition, effect size may be inflated by practice. On the other hand, a study may be underpowered as a result of the additional variance introduced by practice (McCaffrey, 2001). In the absence of good design and/or replication, effect sizes for nutrition interventions should be accepted with caution (Bell et al., 2015).

3.5.1 Conclusion

Methodological recommendations emerging from the experiment include the importance of an initial familiarisation visit which is on a separate day to subsequent testing. Indeed, further investigation is required to determine the impact of the length of time between familiarisation and test days. The inclusion of at least one baseline at each visit within a counterbalanced framework

including the control, and the use of tasks with a reduced susceptibility to practice such as RVIP and Sternberg is also recommended. Alternate forms and familiarisation trials are also recommended at each task performance, along with a between participants counterbalanced order of tasks within a battery. As these measures will only minimise, not eliminate practice effects, residual practice effects should always be taken into account in the design stage when selecting the number of participants required to adequately power a crossover intervention study. Additionally practice effects may be taken into account in the data analysis stage. For example, using LMM analysis it is possible to include testing order as a factor within the analysis model. Parallel designs, with a separate control group of participants, may be used to mitigate practice effects, but will add between-subjects error variance and increase the number of participants needed. An active intervention may also interact with practice, for example through improved strategy selection. Therefore it is still recommended to address practice effects in a parallel design if there is a repeated testing component, such as with longitudinal designs. In combination, these measures will aid in improving the reliability and validity of repeated cognitive testing in nutritional intervention designs and, as such, have been included in the methodology of all blueberry experiments in this thesis.

Chapter 4
Experiment 2: A dose-response study of cognitive and blood pressure effects following acute anthocyanin-rich blueberry supplementation in healthy young adults

4.1 Introduction

In order to fully understand the acute effects of anthocyanin-rich blueberries on human cognition, it is critical to determine the most effective doses and their mode of action. Preclinical trials carried out in animal models have clearly demonstrated cognitive benefits from berry flavonoids e.g. (Carey, Gomes, & Shukitt-hale, 2014; Kumar, Arora, Kuhad, & Chopra, 2012; Rendeiro et al., 2013; Shukitt-Hale et al., 2015). However, human data is more mixed. Indeed, as shown in Chapter 1, acute studies are often not consistent in their choice of flavonoid-rich food type, population, cognitive outcome or dose, making direct comparison between studies difficult.

Some studies have sought to directly link cognitive outcomes following flavonoid-rich intervention with concurrently measured physiological changes in order to determine a likely mode of action. Francis et al. (2006) observed increased cerebral blood flow (CBF) in healthy females one to two hours after acute supplementation with 450mg cocoa flavanol. However, no corresponding behavioural improvements were observed using a cognitive switching task paradigm. The study tested only four participants, so it may have been lacking the statistical power necessary to observe such behavioural effects. A larger, more recent study by Brickman et al. (2014) developed a pattern recognition task that demonstrated functional localisation to the dentate gyrus region of the hippocampus. After a 3 month period of daily supplementation with 900mg cocoa flavanol, 19 older adults showed significant improvements in both task performance and regional BOLD activation compared to a control group of 18 participants that consumed a lower daily dose of 10mg cocoa flavanol. However, although the study provides strong, domain-specific evidence for a connection between increased CBF and cognitive task performance, the study did not investigate acute intervention and it is also unclear from the paper whether cognitive performance and CBF were measured concurrently.

The above studies relate only to flavanol-rich cocoa. Dodd (2012) reported an acute effect of blueberries on human cognitive performance in young adults in a task measuring executive function after administering a freeze-dried blueberry powder containing 631mg anthocyanins. The observed

improvement in cognitive performance was also accompanied by a trend towards increased plasma BDNF levels. And the same dose of anthocyanin-rich blueberry was also found to increase cerebral blood flow to the precentral and middle frontal gyrus in the frontal lobe, and the angular gyrus in the parietal lobe, 1 hour post-consumption, when compared to a control. The study provides evidence that anthocyanin-rich blueberries are associated with acute changes in cognition and CBF, although again these were not observed concurrently. The study also suggests that acute supplementation may result in chemical changes in the brain normally associated with longer term supplementation. A similar acute intervention study (Watson et al., 2015) observed cognitive improvements following acute blackcurrant supplementation that were accompanied by significant inhibition of monoamine oxidase (MAO) activity and sustained elevation of plasma glucose levels.

It is likely, then, that increases in cognitive performance after acute supplementation with blueberries are, at least in part, due to cerebral blood flow increases, although this has only been demonstrated indirectly, but other mechanisms including BDNF synthesis, MAO inhibition and blood glucose regulation may play a role. However, the limited aforementioned evidence demonstrates that it is difficult to directly match behavioural effects with physiological changes such as increases in cerebral blood flow; these measures often involve expensive, complex or lengthy procedures that cannot be easily combined with cognitive testing at the same time points, or for a large sample of participants.

With regard to the effects of dose, as identified in Chapter 1, few studies have investigated the impact of dose on cognition and those that do typically investigate only two or three doses e.g. (Kennedy et al., 2000; Pase et al., 2013; Scholey et al., 2010; Whyte et al., 2016; Wightman et al., 2012). The majority of these studies investigated cocoa and ginkgo biloba effects. The cognitive effects of dose are therefore yet to be fully elucidated, particularly following anthocyanin-rich berry intervention. However, dose-dependent vascular effects following acute blueberry supplementation have been observed; Rodriguez-Mateos et al (2013) measured changes in flow mediated dilation (FMD) of the brachial artery over time and in response to five doses of blueberry and a control. FMD measures the level of naturally occurring dilation by an artery in response to shear stress, and is a measure of cardiovascular health. Maximum increases in FMD were observed 1-2 hours after ingestion. Significant dose-dependent increases in FMD response were observed across doses ranging from 319-766mg of total polyphenols (129-310mg anthocyanins). Peak FMD response was observed following a polyphenol dose of 766mg (310mg anthocyanins). At higher doses, up to 1791mg total polyphenols (724mg anthocyanins) the FMD response was observed to plateaux. FMD and CBF responses are likely to be synonymous as evidenced by a correlation found between FMD

and coronary blood flow (Teragawa et al., 2005), and a correlation found between FMD and BOLD response for a working memory task (Gonzales et al., 2010). As reported in Chapter 1, the timings of observed FMD, CBF and cognitive effects following acute supplementation with flavonoid-rich foods appear similar. Timings, of course, vary according to the flavonoid subclasses present in the food source; however following anthocyanin-rich berry interventions FMD, CBF and cognitive effects have each been independently observed around 1-2 hours postprandially. As FMD effects have been shown to be dose-dependent (Rodriguez-Mateos et al., 2013), beneficial cognitive effects may also be dose-related.

The current experiment aimed to determine a dose-response for the cognitive effects of blueberry flavonoids, using doses matched exactly to those used by Rodriguez-Mateos et al. (2013) for which vascular changes have already been documented. In this way, it was hoped to link dose-related cognitive effects with known dose-related vascular effects whilst avoiding the inherent difficulty of measuring the two concurrently. Blood pressure measurements were included in the design as an alternative indicator of vasoreactivity. As described in Chapter 1, a lowering of blood pressure has been observed following both acute and chronic flavonoid supplementation, likely due to their vasodilatory properties. Blood pressure measurements and cognitive testing were performed pre- and 1 hour post-intervention in order to coincide with the maximum increases in FMD observed by Rodriguez-Mateos et al. (2013). The tasks incorporated into this study were those previously identified in the literature review as sensitive to flavonoid-rich intervention, and that were subsequently investigated for practice effects in the previous chapter. The methodology used here reflected the findings of that study, in order to comply with EFSA guidelines on addressing practice effects in studies utilising repeated cognitive testing.

4.2 Methods

Methodological detail common to all experiments in this thesis can be found in Chapter 2. Methodology specific to this intervention study is reported here.

4.2.1 Participants

The participants were 45 healthy adults, aged 18-36 years (M 20.87, SD 3.63 years). All participants were screened according to criteria outlined in Chapter 2.

4.2.2 Design

A double-blind crossover intervention study design was used. Independent variables were blueberry dose (6 levels; 5 doses and a matched control) and test session (2 levels; pre- and 1 hour post-intervention). All testing took place in the morning. Participants attended a total of 7 regularly spaced visits. The mean number of days between visits was 7, but ranged from a minimum of 3 days to a maximum of 14 days where occasional rescheduling was required to compensate for missed appointments. In order to minimise the impact of practice on cognitive task performance, in accordance with the findings of Chapter 3, the first visit was treated as a familiarisation visit. Cognitive and physiological data collected during this visit were not included in subsequent analyses. Participants were not made aware of this and followed the same procedure as for all other visits, with the exception that participants were permitted as much practice as they required in order to feel comfortable with the cognitive tasks. The cognitive battery lasted 40 minutes. The different blueberry doses were administered in drink form. The drink consumed at the familiarisation visit was identical to the control. At subsequent visits, the control and all blueberry doses were administered in counterbalanced order, determined using Williams matrices (Williams, 1949).

4.2.3 Procedure

At each visit participants arrived 2 hours fasted having previously followed the prescribed 24 hour low polyphenol diet (Chapter 2). They completed a pre-intervention battery of cognitive tasks and blood pressure measurements. Participants then consumed a blueberry or control drink. Participants were required to finish the drink within a ten minute time period (Rodriguez-Mateos et al., 2013), after which they were asked to remain in the lab waiting room. During this time participants completed a 24 hour retrospective food diary to check for compliance with the low polyphenol diet. A post-drink palatability questionnaire was also completed to determine opinions on the drinks and to establish whether participants were remaining blind to the intervention. Participants resumed testing, repeating the cognitive task battery and blood pressure measurements, at the post-intervention time point of 1 hour. A return appointment was then booked for the following week, at the same start time. Participants were asked to eat the same breakfast prior to fasting before each attendance.

4.2.4 Blueberry intervention drinks

The blueberry doses were aligned with those used in the previous vascular study (Rodriguez-Mateos et al., 2013). In the vascular study the drinks were not matched for sugars or vitamin C. In the current study they were matched to the highest dose on these constituents to avoid potential

confounding effects on cognition. As the same source/batch of freeze-dried wild blueberry powder was used for both studies, the compositional analysis of the drinks was taken from the previous study and is shown in Table 4.1. The drinks were prepared immediately prior to consumption by adding the appropriate amounts of freeze-dried blueberry powder, sugars and vitamin C to 500ml of water. These were mixed until smooth using opaque coloured protein shaker cups commonly used in sports nutrition. Participants consumed the drinks through a black straw, before completing a palatability questionnaire.

Table 4.1. Compositional analysis of the control and blueberry drinks in Experiment 2

		Freeze-dried wild blueberry powder (g)				
Composition	Control	14	28	34	57	80
Anthocyanins (mg)	0	129	258	310	517	724
Procyanidins (mg)	0	57	114	137	228	320
Total polyphenols (mg)	0	319	639	766	1278	1791
Vitamin C (mg)	9.5	9.5	9.5	9.5	9.5	9.5
Fructose (g)	21	21	21	21	21	21
Glucose (g)	19	19	19	19	19	19

4.2.5 Cognitive & subjective measures

Full details of the selected cognitive tasks are given in Chapter 2. The order of the tasks was: immediate word recall (IR); Stroop task, Sternberg memory scanning task, and serial 3s & 7s subtraction tasks, in between-subjects counterbalanced order; delayed word recall (DR); PANAS Now mood questionnaire.

4.2.6 Physiological measures

Blood pressure and resting heart rate were measured after all cognitive tasks had been completed. Participants had therefore been seated for a minimum of 30 minutes prior to the measurements. Equipment details and measuring procedure are reported in Chapter 2.

4.3 Data analysis

Data collected during Visit 1 were discarded prior to analysis and any outliers were removed from the remaining data for Visits 2-7 according to criteria previously described in Chapter 2.

4.3.1 Linear mixed models

All cognitive and mood data were analysed by LMM using an unstructured covariance matrix to model successive repeat measurements. Visit, Session, Dose, and Session x Dose interaction were included as fixed factors in the model. The factor Visit was included in the LMM model for each dependent variable in order to identify and account for the presence of any order effects. Subjects were included as random effects (Model 2.1). The analysis aim was to determine whether blueberry dose was a significant predictor of cognition or mood performance. However, for the analysis of the interference errors DV for the immediate and delayed recall tasks, the random effects component was removed from the LMM procedure (Model 2.2). This modification was necessary in order to counter the large number of zero scores present in the data set. Some participants showed no variation at all in their scores, causing an error in the modelling of the covariance matrix when including random effects (West et al., 2015). Pairwise comparisons were used to investigate significant effects and all interactions (see Chapter 2 for rationale). A Bonferroni correction was applied to all multiple comparisons.

Blood pressure measurements were analysed using the same model as above (Model 2.1). The analysis aim was to determine whether blueberry dose had a mediating effect on postprandial blood pressure and heart rate. Palatability ratings for the intervention drinks were analysed by LMM using an unstructured covariance matrix to model successive ratings. Dose was the only fixed factor in the model (Model 2.3). The analysis aim was to determine whether the intervention drinks were adequately matched for flavour across a number of different taste dimensions.

4.3.2 Cohen's d effect sizes

Cohen's d effect sizes (see Chapter 2 for calculation method) were determined where pairwise comparisons revealed significant changes from baseline at 1 hour postprandially following blueberry intervention, but not following the control intervention (or vice versa).

4.4 Results

Raw data for all measured variables can be found in the supplementary data accompanying this thesis. Means and standard deviations for all recorded variables are tabulated under the relevant sections of the chapter. Tabulated LMM results can be found in Appendix L. For clarity, only significant F-statistics are reported in full in the text.

4.4.1 Cognitive analysis

4.4.1.1 Order effects and baseline differences

For the episodic memory tasks, Visit was a significant predictor of delayed recall score [F(5,219.14)=2.98, p=0.013] and a trend was evident for immediate recall score [F(5,219.21)=1.88, p=0.098]. Pairwise comparisons revealed a significant improvement in delayed recall score between Visit 2 (M 5.91, SE 0.43) and Visit 7 (M 6.93, SE 0.44) [p=0.006]. Slight improvements in immediate recall score were found not to be statistically significant [p>0.1].

For the serial subtraction tasks, Visit was a significant predictor of 3s score [F(5,219.20)=80.02, p<0.001], 3s RT [F(5,218.15)=109.92, p<0.001], 7s score [F(5,218.11)=27.62, p<0.001], and 7s RT [F(5,192.26)=20.45, p<0.001]. Pairwise comparisons revealed a significant increase in 3s score between Visit 2 (M 38.74, SE 2.74) and Visit 7 (M 55.90, SE 2.74) [p<0.001]. Significant 3s score improvements were maintained between consecutive Visits 2 & 3, 4 & 5, and 5 & 6 [p<0.05]. Similarly, for 3s RT, a significant improvement was observed between Visit 2 (M 2878.72, SE 102.52) and Visit 7 (M 2050.25, SE 102.77) [p<0.001]. Significant improvements were observed between all consecutive, intermediate test visits up to Visit 6 [p<0.05]. Pairwise comparisons for 7s score revealed a significant increase between Visit 2 (M 21.97, SE 1.70) and Visit 7 (M 29.78, SE 1.71) [p<0.001]. However, consecutive improvements were observed between Visits 6 & 7 only [p=0.002]. For 7s RT, a significant improvement was again observed between Visit 2 (M 5226.32, SE 245.47) and Visit 7 (M 4164.13, SE 246.60) [p<0.001]. Marginally significant gains were observed between Visits 2 & 3 [p=0.065], however no further significant gains were observed between later consecutive test visits.

For the Sternberg task, Visit was a significant predictor of accuracy [F(5,206.40)=5.67, p<0.001], scanning rate [F(5,219.80)=7.69, p<0.001], and extrapolated RT [F(5,219.23)=3.79, p=0.003]. Again, significant differences were apparent between Visit 2 and Visit 7 for all DVs. Specifically, Sternberg accuracy decreased between Visit 2 (M 90.68, SE 0.82) and Visit 7 (M 88.36, SE 0.83) [p<0.001], although significant decreases were not evident between consecutive intermediate visits. Sternberg scanning rate improved between the Visit 2 (M 33.25, SE 2.05) and Visit 7 (M 23.80, SE 2.08) [p<0.001], however significant gains were only evident between consecutive Visits 2 & 3 [p=0.003]. Sternberg RT improved between Visit 2 (M 574.93, SE 17.30) and Visit 7 (M 541.39, SE 17.39) [p=0.026], although significant improvements were not evident between consecutive, intermediate visits.

Finally, for the Stroop task, Visit predicted interference effect [F(5,219.28)=4.24, p=0.001] and a trend was evident for incongruent RT [F(5,218.94)=1.97, p=0.083]. Pairwise comparisons revealed a decrease in interference effect between Visit 2 (M 60.34, SE 4.68) and Visit 7 (M 42.03, SE 4.73) [p=0.007], however significant improvements were not evident between consecutive, intermediate visits. Marginally significant improvements in incongruent RT were observed between Visit 2 (M 716.61, SE 13.54) and Visit 7 (M 694.93, SE 13.60) [p=0.055] but again significant improvements were not evident between consecutive, intermediate visits.

Order effects, therefore, remained apparent for the majority of cognitive tasks. However, although significant differences were evident between measurements recorded at the beginning and end of the experiment, there were few significant differences between consecutive, intermediate test visits. This suggests that the methodological measures identified in Chapter 3 were moderately successful at minimising order effects. Following the counterbalancing of the order in which all doses were presented to participants, pairwise comparisons revealed no significant baseline differences for any of the cognitive tasks, under any of the individual dose conditions [p>0.1].

4.4.1.2 Immediate & delayed recall

Tabulated means for the immediate and delayed recall tasks can be found in Table 4.2.

For the immediate recall task, Session was a significant predictor of score F(1,264.00)=30.95, p<0.001] and interference errors F(1,264.01)=11.24, p=0.001]. Pairwise comparisons revealed a significant decrease in score between baseline and 1 hour [p<0.001], accompanied by an increase in interference errors [p=0.001]. Similarly, for the delayed recall task, Session was a significant predictor of score F(1,264.00)=100.36, p<0.001] and interference errors F(1,263.06)=58.79, p<0.001]. Again pairwise comparisons revealed a significant decrease in score between baseline and 1 hour [p<0.001], accompanied by an increase in interference errors [p<0.001].

Table 4.2 Episodic memory data for Experiment 2 (n=45)

Recall variable	Dose	Test session: Baseline Mean	Baseline SD	1 hr Mean	1 hr SD
IR score (correct/15)	0mg	9.31	2.70	8.16	2.72
	129mg	9.02	2.97	8.48	3.41
	258mg	9.28	2.69	7.86	2.90
	310mg	9.02	2.72	8.29	3.25
	517mg	8.82	2.60	8.34	3.07
	724mg	9.12	2.32	8.63	2.92
IR interference errors	0mg	0.02	0.15	0.04	0.21
	129mg	0.00	0.00	0.07	0.33
	258mg	0.00	0.00	0.05	0.21
	310mg	0.00	0.00	0.09	0.36
	517mg	0.00	0.00	0.05	0.21
	724mg	0.00	0.00	0.07	0.26
DR score (correct/15)	0mg	7.38	2.76	5.62	3.26
	129mg	7.34	3.37	6.00	3.65
	258mg	7.56	3.45	5.40	3.30
	310mg	6.87	2.87	5.40	3.34
	517mg	6.86	2.83	6.00	3.72
	724mg	7.07	3.01	5.67	3.16
DR interference errors	0mg	0.02	0.15	0.60	1.07
	129mg	0.00	0.00	0.53	1.32
	258mg	0.02	0.15	0.37	0.87
	310mg	0.00	0.00	0.44	0.92
	517mg	0.00	0.00	0.27	0.66
	724mg	0.00	0.00	0.70	1.26

Neither Dose, nor Session x Dose interaction were found to be significant factors in predicting any of the recall DVs. However, more detailed interpretation of pairwise comparisons for the interaction revealed a significant decline in immediate recall score between baseline and 1 hour for the control [p=0.001], 258mg [p<0.001] and 310mg [p=0.037] doses, but not for the 129mg [p=0.124], 517mg [p=0.178], and 724mg [p=0.173] doses. This interaction is shown in Figure 4.1. Immediate recall interference errors increased between baseline and 1 hour for the 310mg dose only [0.031], although a non-significant trend was observed for the 724mg dose [p=0.098]. For delayed recall score and delayed recall interference, pairwise comparisons for the interaction simply revealed a significant decrease in performance over time regardless of dose [p<0.1].

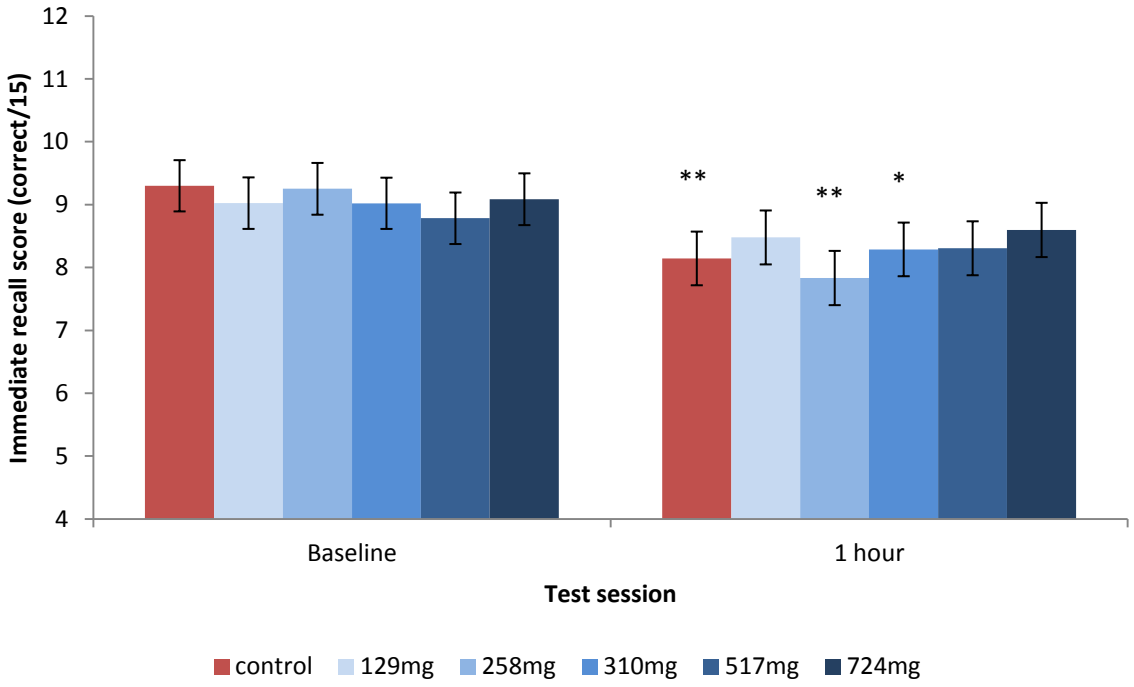


Figure 4.1 The interaction between session and dose for immediate recall score

Values are estimated marginal means. Error bars represent standard error of the mean. Difference from baseline is indicated above the column, ** (p<0.01), * (p<0.05), † (p<0.1).

4.4.1.3 Serial 3s & 7s

Tabulated means for the serial 3s & 7s tasks can be found in Table 4.3 and Table 4.4, respectively.

Table 4.3 Serial 3s data for Experiment 2 (n=45)

		Test session			
Serial 3s	Dose	Baseline		1 hr	
variable		Mean	SD	Mean	SD
Score (correct in 2 mins)	0mg	47.20	19.93	49.29	23.07
	129mg	47.45	20.54	49.93	19.63
	258mg	46.79	16.91	49.60	19.07
	310mg	49.13	22.02	51.27	23.97
	517mg	47.32	17.93	48.98	20.75
	724mg	46.58	18.29	50.12	19.15
Errors (incorrect in 2 mins)	0mg	2.09	1.71	2.44	1.84
	129mg	1.82	2.22	2.02	2.18
	258mg	1.93	2.44	2.07	1.88
	310mg	1.96	1.94	2.50	2.10
	517mg	2.14	2.58	2.16	2.43
	724mg	1.62	1.89	1.95	1.80
RT (ms)	0mg	2464.88	848.23	2330.63	779.69
	129mg	2489.71	867.89	2323.42	748.16
	258mg	2402.68	702.03	2292.59	715.97
	310mg	2443.99	929.38	2277.70	851.01
	517mg	2406.99	691.37	2316.97	715.66
	724mg	2390.97	694.71	2252.78	666.62

Table 4.4 Serial 7s data for Experiment 2 (n=45)

Serial 7s variable	Dose	Test session: Baseline Mean	Baseline SD	1 hr Mean	1 hr SD
Score (correct in 2 mins)	0mg	25.62	11.64	26.24	12.71
	129mg	25.61	12.77	25.65	11.01
	258mg	24.91	10.89	25.56	12.06
	310mg	24.82	13.11	25.20	10.97
	517mg	25.50	11.67	27.23	12.95
	724mg	24.30	11.91	27.14	12.55
Errors (incorrect in 2 mins)	0mg	2.13	1.82	2.02	1.63
	129mg	1.89	1.69	1.86	1.64
	258mg	1.95	1.94	2.56	1.68
	310mg	2.27	1.71	2.23	1.51
	517mg	1.98	1.77	2.20	1.72
	724mg	2.05	1.83	2.37	2.32
RT (ms)	0mg	4641.95	1595.72	4583.39	1770.62
	129mg	4660.56	1652.82	4437.11	1511.59
	258mg	4514.64	1445.50	4527.53	1624.32
	310mg	4828.93	2035.04	4480.39	1561.91
	517mg	4713.68	1558.86	4325.14	1552.65
	724mg	4791.82	1787.96	4427.62	1941.27

For the serial subtraction tasks, Session was a significant predictor of 3s score [$F(1,263.94)=22.98$, $p<0.001$], 3s RT [$F(1,261.91)=63.60$, $p<0.001$], 7s score [$F(1,261.55)=16.52$, $p<0.001$], and 7s RT [$F(1,237.18)=14.59$, $p<0.001$]. A non-significant trend was observed for serial 3s errors [$F(1,254.27)=3.37$, $p=0.067$]. All scores and RTs significantly improved between baseline and 1 hour [$p<0.001$]. Serial 3s errors increased between baseline and 1 hour [$p=0.067$]. Neither Dose, nor Session x Dose interaction were found to be significant factors in predicting any of the serial subtraction variables. For serial 3s score and RT, pairwise comparisons for the interaction simply revealed a significant improvement in performance over time regardless of dose [$p<0.1$]. The only exception was for 3s score following the 517mg dose where no significant change was observed

[p=0.194]. No significant changes were observed for 3s errors. However, significant improvements in serial 7s score and RT were apparent between baseline and 1 hour following the two highest blueberry doses (517mg & 724mg) only [p<0.05] with a non-significant trend for 7s RT following the 310mg dose [p=0.091]. No significant improvements were observed following the control or any of the lower doses [p>0.10]. These interactions are shown in Figure 4.2 and Figure 4.3. For serial 7s errors there was a non-significant trend towards increased errors following the 258mg dose only [p=0.065].

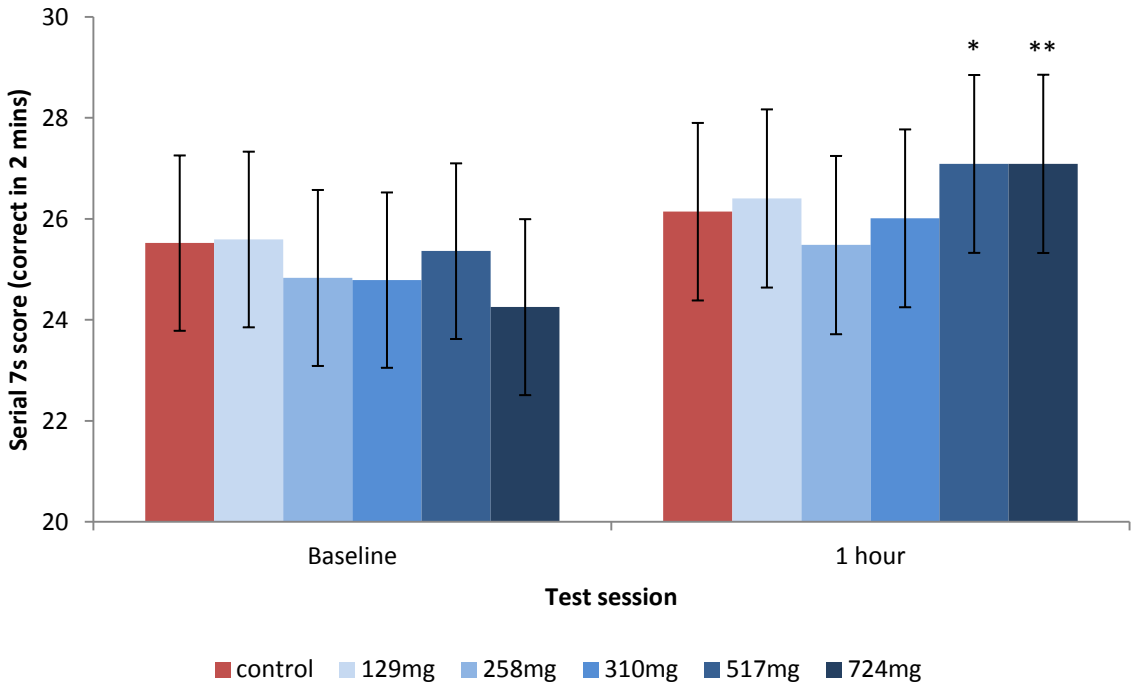


Figure 4.2 The interaction between test session and dose for serial 7s scores

Values are estimated marginal means. Error bars represent standard error of the mean. Difference from baseline is indicated above the column, ** (p<0.01), * (p<0.05), † (p<0.1).

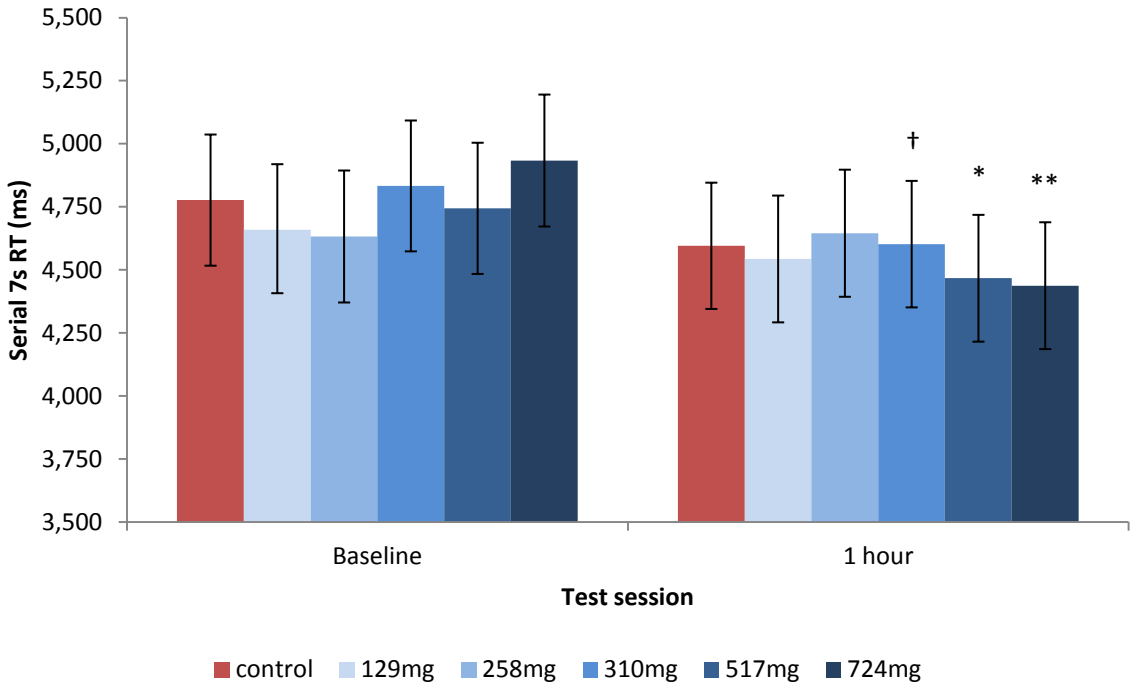


Figure 4.3 The interaction between test session and dose for serial 7s RT

Values are estimated marginal means. Error bars represent standard error of the mean. Difference from baseline is indicated above the column, ** ($p<0.01$), * ($p<0.05$), † ($p<0.1$).

4.4.1.4 Sternberg task

Tabulated means for the Sternberg task can be found in Table 4.5.

For the Sternberg task, Session was a significant predictor of extrapolated RT only $F(1,264.00)=17.37$, $p<0.001$], with faster RTs observed at 1 hour compared with baseline. Neither Dose, nor Session x Dose interaction were predictive of any of the Sternberg variables. Pairwise comparisons for the interaction revealed a significant decrease in extrapolated RT for the control [$p=0.035$], 258mg [$p=0.051$], 310mg [$p=0.010$], and 724mg [$p=0.013$] doses, but not for the 129mg or 517mg doses [$p>0.1$].

Table 4.5 Sternberg data for Experiment 2 (n=45)

Sternberg variable	Dose	Test session: Baseline Mean	Baseline SD	1 hr Mean	1 hr SD
Accuracy (correct/96)	0mg	89.31	7.32	90.37	5.13
	129mg	89.72	5.01	89.02	5.12
	258mg	89.36	6.34	90.15	4.27
	310mg	89.50	5.59	89.42	5.11
	517mg	90.63	4.96	90.40	3.04
	724mg	90.05	4.83	90.12	5.04
Scanning rate (ms/item)	0mg	27.26	20.89	24.83	18.10
	129mg	27.11	13.91	25.48	14.62
	258mg	29.44	16.53	28.80	17.73
	310mg	23.41	14.17	24.50	16.08
	517mg	28.96	15.52	25.22	18.99
	724mg	28.16	14.40	26.90	17.55
Extrapolated RT (ms)	0mg	574.13	140.45	550.17	129.78
	129mg	551.15	126.87	542.37	119.34
	258mg	558.30	107.50	535.69	114.17
	310mg	576.62	134.04	547.38	133.67
	517mg	562.18	127.80	558.98	122.43
	724mg	564.06	118.56	535.20	116.71

4.4.1.5 Stroop task

Tabulated means for the Stroop task can be found in Table 4.6.

Table 4.6 Stroop data for Experiment 2 (n=45)

Stroop variable	Dose	Test session: Baseline Mean	Baseline SD	1 hr Mean	1 hr SD
Accuracy (correct/96)	0mg	91.75	2.89	91.91	3.06
	129mg	91.82	2.90	91.77	3.20
	258mg	91.39	3.71	91.84	2.75
	310mg	91.57	2.85	90.96	3.54
	517mg	91.89	2.91	91.63	3.53
	724mg	92.07	2.98	92.02	2.64
Incongruent RT (ms)	0mg	719.67	97.68	701.96	95.28
	129mg	705.95	98.63	695.75	94.23
	258mg	708.38	95.58	692.47	93.00
	310mg	719.17	102.27	702.26	103.12
	517mg	708.43	86.58	692.83	86.27
	724mg	691.89	88.15	685.34	89.56
Congruent RT (ms)	0mg	667.61	100.22	648.51	105.60
	129mg	651.65	94.87	646.12	93.67
	258mg	662.90	100.78	647.49	94.29
	310mg	667.15	103.65	649.02	100.77
	517mg	646.58	85.72	635.79	88.16
	724mg	645.51	78.03	631.19	83.92
Interference effect (ms)	0mg	52.06	35.22	53.45	31.60
	129mg	54.31	37.69	49.63	35.58
	258mg	45.49	38.71	44.98	39.33
	310mg	52.02	42.48	53.25	43.10
	517mg	61.85	44.64	52.98	39.55
	724mg	46.37	29.94	51.45	30.27

Session was a significant predictor of both congruent RT [$F(1,264.00)=21.99$, $p<0.001$] and incongruent RT [$F(1,264.00)=19.54$, $p<0.001$], but not of accuracy or interference effect [$p>0.1$]. RTs were faster at 1 hour compared with baseline [$p<0.001$]. Dose was a marginally significant predictor

of congruent RT [F(5,218.71)=2.12, p=0.064]; RTs for the 724mg condition were faster than for all other conditions, however statistical significance was not maintained following pairwise comparison. No other significant Dose or Dose x Session interactions were evident from the model. However, pairwise comparisons for the interaction revealed significant improvements in congruent RT between baseline and 1 hour following the control [p=0.008], 258mg [p=0.038], 310mg [p=0.012], and 724mg [p=0.052] doses, but not following the 129mg or 517mg doses [p>0.1]. Significant improvements in incongruent RT were observed between baseline and 1 hour for the control [p=0.020], 258mg [p=0.041], 310mg [0.026], and 517mg [p=0.043] doses, but not for the 129mg or 724mg doses [p>0.1].

4.4.2 Mood analysis

Tabulated means for the self-reported mood measures can be found in Table 4.7

Visit was found to be a significant predictor of mental fatigue only [F(5,219.63)=3.40, p=0.006]. Pairwise comparisons revealed a significant decrease in ratings of mental fatigue between Visit 2 (M 5.12, SE 0.26) and Visit 7 (M 4.20, SE 0.26) [p=0.023]. Significant decreases between consecutive, intermediate visits were observed between Visits 2 & 3 only [p=0.006]. These findings suggest that participants found the tasks particularly mentally challenging at the beginning of the experiment, but quickly became more comfortable with the level of cognitive testing. No similar order effects were apparent for measures of affect [p>0.1]. Pairwise comparisons indicated no significant baseline differences for any of the counterbalanced dose conditions, for any of the mood measures [p>0.1].

Session was found to be a significant predictor of mental fatigue [F(1,264.00)=10.51, p=0.001] and negative affect [F(1,259.58)=4.55, p=0.034], and approached significance for positive affect [F(1,264.00)=3.37, p=0.067]. Mental fatigue decreased between baseline and 1 hour, positive affect increased, and negative affect decreased. These Session effects are most likely attributable to the consumption of a sugary drink, irrespective of dose condition.

Table 4.7 Mood data for Experiment 2 (n=45)

Mood variable	Dose	Test session: Baseline Mean	Baseline SD	1 hr Mean	1 hr SD
Mental fatigue (rating/9)	0mg	4.53	2.14	4.09	2.00
	129mg	4.32	1.96	4.41	1.98
	258mg	4.84	2.00	4.21	2.17
	310mg	4.91	2.11	4.18	1.72
	517mg	4.48	2.10	3.89	1.85
	724mg	4.42	2.33	4.28	2.35
Positive affect (score/50)	0mg	21.29	8.70	21.38	8.86
	129mg	21.68	7.91	22.57	8.21
	258mg	20.33	7.25	22.00	7.76
	310mg	20.76	7.44	22.16	7.91
	517mg	21.20	8.14	22.30	7.83
	724mg	22.26	7.74	21.72	8.73
Negative affect (score/50)	0mg	13.42	4.14	13.11	4.25
	129mg	13.00	3.59	13.05	3.95
	258mg	13.17	3.51	12.79	4.28
	310mg	13.27	4.30	12.16	2.96
	517mg	12.95	3.46	13.00	3.95
	724mg	12.98	3.69	12.57	3.12

Dose and Session x Dose interaction were not significant for any of the mood measures [$p>0.1$]. However, more detailed interpretation of pairwise comparisons for the Session x Dose interaction revealed a significant reduction in mental fatigue between baseline and 1 hour following the 258mg [$p=0.045$] and 310mg [$p=0.017$] doses, and non-significant trend following the 517mg dose [$p=0.056$]. No significant changes were observed for the control, 129mg or 724 mg conditions [$p>0.1$]. A significant decrease in negative affect was observed following the 310mg dose only [$p=0.013$]. These interactions are shown in Figure 4.4 and Figure 4.5.

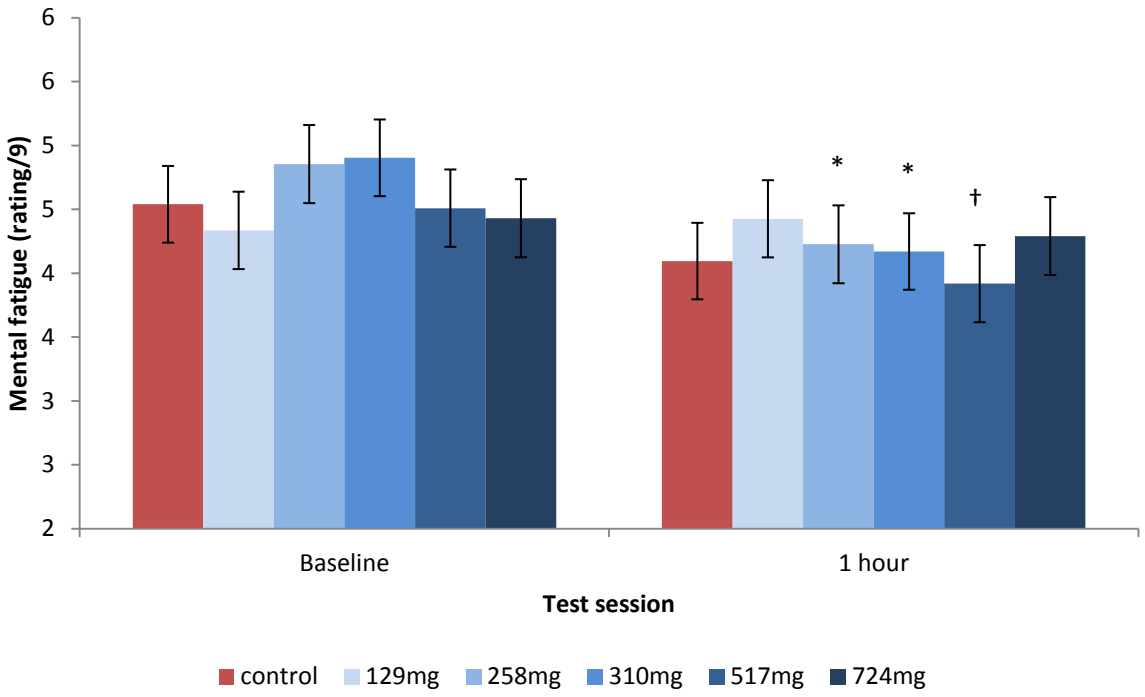


Figure 4.4 The interaction between session and dose for ratings of mental fatigue

Values are estimated marginal means. Error bars represent standard error of the mean. Difference from baseline is indicated above the column, ** ($p<0.01$), * ($p<0.05$), † ($p<0.1$).

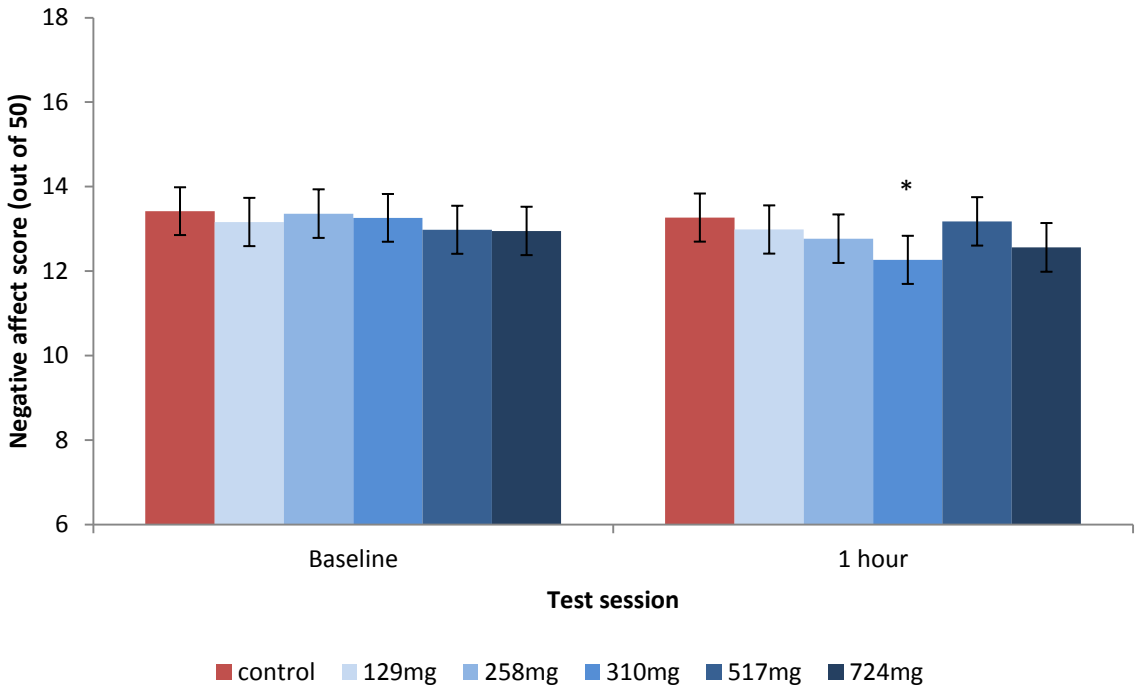


Figure 4.5 The interaction between session and dose for ratings of negative affect

Values are estimated marginal means. Error bars represent standard error of the mean. Difference from baseline is indicated above the column, ** ($p<0.01$), * ($p<0.05$), † ($p<0.1$).

4.4.3 Blood pressure analysis

Tabulated means for all blood pressure data can be found in Table 4.8.

Unexpectedly, Visit was found to be a significant factor in predicting both systolic (SBP) [$F(5,219.10)=2.80$, $p=0.018$] and diastolic (DBP) blood pressure [$F(5,217.05)=3.90$, $p=0.002$]. Pairwise comparisons revealed a significant increase in SBP between Visit 3 (M 102.79, SE 1.59) and Visit 7 (M 105.15, SE 1.59) [$p=0.028$]. Similarly, DBP increased significantly at Visit 7 (M 69.80, SE 0.82) when compared with both Visit 2 (M 67.98, SE 0.81) [$p=0.043$] and Visit 3 (M 67.67, SE 0.81) [$p=0.007$]. This gradual increase in blood pressure observed over the duration of the experiment remains unexplained. No similar changes in heart rate were evident. However, the impact of this order effect was minimal due to the counterbalancing of the doses; no baseline differences in blood pressure or heart rate were evident between any of the dose conditions [$p>0.1$].

Table 4.8 Blood pressure data for Experiment 2 (n=45)

		Test session			
Blood pressure	Dose	Baseline		1 hr	
Variable		Mean	SD	Mean	SD
Systolic BP (mmHg)	0mg	102.62	11.35	105.73	12.16
	129mg	101.18	9.79	104.20	11.58
	258mg	102.65	10.77	105.93	11.14
	310mg	101.69	10.24	105.51	11.63
	517mg	101.89	11.80	105.34	11.93
	724mg	101.16	10.60	104.79	10.86
Diastolic BP (mmHg)	0mg	67.93	5.41	69.70	5.22
	129mg	67.34	5.22	69.57	6.91
	258mg	66.62	4.83	69.14	5.18
	310mg	67.47	6.32	69.93	5.80
	517mg	66.59	5.22	69.09	5.23
	724mg	67.77	6.16	69.62	5.14
Heart rate (bpm)	0mg	72.00	7.20	68.73	8.30
	129mg	71.91	9.60	70.45	8.64
	258mg	72.26	10.24	69.63	8.86
	310mg	71.04	8.84	69.58	9.60
	517mg	72.59	9.92	71.98	9.88
	724mg	72.70	11.27	71.28	8.92

Session was observed to be a significant predictor of SBP [$F(1,264.00)=153.18$, $p<0.001$], DBP [$F(1,259.02)=107.86$, $p<0.001$] and heart rate [$F(1,263.40)=28.60$, $p<0.001$]. Both SBP and DBP increased significantly after consuming the intervention drinks, whereas heart rate decreased. Although Dose and Session x Dose interaction were not significant for any of the physiological measures [$p>0.1$]; more detailed investigation of pairwise comparisons for the interaction revealed that significant postprandial reductions in heart rate were observed following the control and lower doses, however these were attenuated in the higher dose conditions. Specifically, a significant

difference between baseline and 1 hour was observed following the control [p<0.001] and 258mg dose [p=0.002] but not following the 517mg dose [p=0.465]. Non-significant trends were observed following the 129mg, 310mg, and 724mg doses, [p=0.084], [p=0.078], and [p=0.096] respectively. At the 1 hour test session heart rate was significantly lower in the control condition compared with the 517mg condition [p=0.021], indeed this was the only between-condition effect observed during this experiment. All other reported effects were within-condition only. This dose dependent interaction is shown in Figure 4.6.

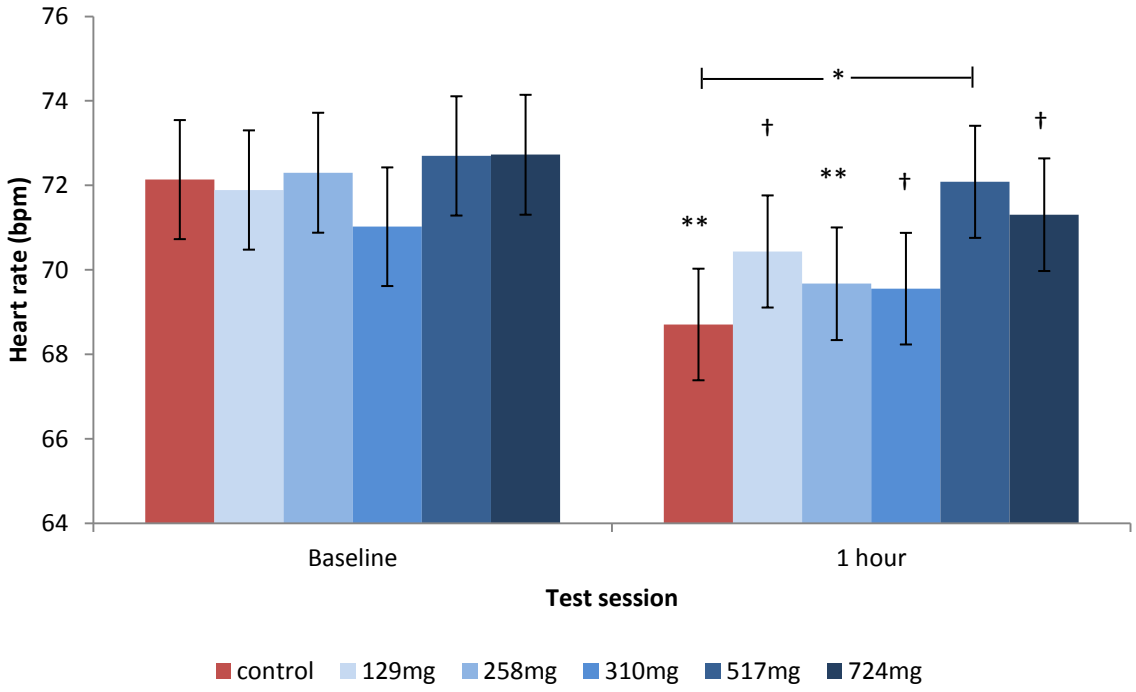


Figure 4.6 The interaction between test session and dose for measurements of heart rate

Values are estimated marginal means. Error bars represent standard error of the mean. Difference from baseline is indicated above the column, difference between conditions is indicated by a horizontal bar, ** (p<0.01), * (p<0.05), † (p<0.1).

4.4.4 Palatability analysis

LMM analysis of the palatability data revealed a significant effect of dose for 'bland' [F(5,44.52)=2.82, p=0.027], 'tasty'[F(5,44.54)=10.07, p<0.001] , 'pleasant' [F(5,44.36)=10.26, p=<0.001], 'sour' [F(5,44.21)=3.97, p=0.005], 'satisfying' [F(5,44.29)=5.10, p=0.001], 'how much more could you consume?' [F(5,43.24)=6.82, p<0.001] , and 'how easy was it to consume?' [F(5,43.75)=10.61, p<0.001] rating dimensions, but not for 'sweet' ratings [F(5,44.23)=1.57, p=0.190]. The effect of dose on each rating dimension is shown in Figure 4.7. The 310mg dose was rated most 'tasty'; ratings were significantly higher than the control [p=0.006], 517mg [p=0.073] and

724mg [p<0.001] doses. The 310mg dose was also rated most 'pleasant' and most 'satisfying'; significantly more so than the 724mg dose, [p<0.001] and [p=0.001] respectively. The 724mg dose was rated more 'sour' than the control [p=0.082], 129mg [p=0.001], 258mg [p=0.011], and 310mg [p=0.009] doses. The 724mg was also rated the least 'easy' to drink and was similarly rated worst in terms of how much more participants felt they could consume. The control drink was rated most 'bland'; approaching significance when compared with the 129mg [p=0.060] and 517mg [p=0.059] doses. Therefore, although the intervention drinks appeared well-matched for sweetness, noticeable differences were evident for all other taste dimensions.

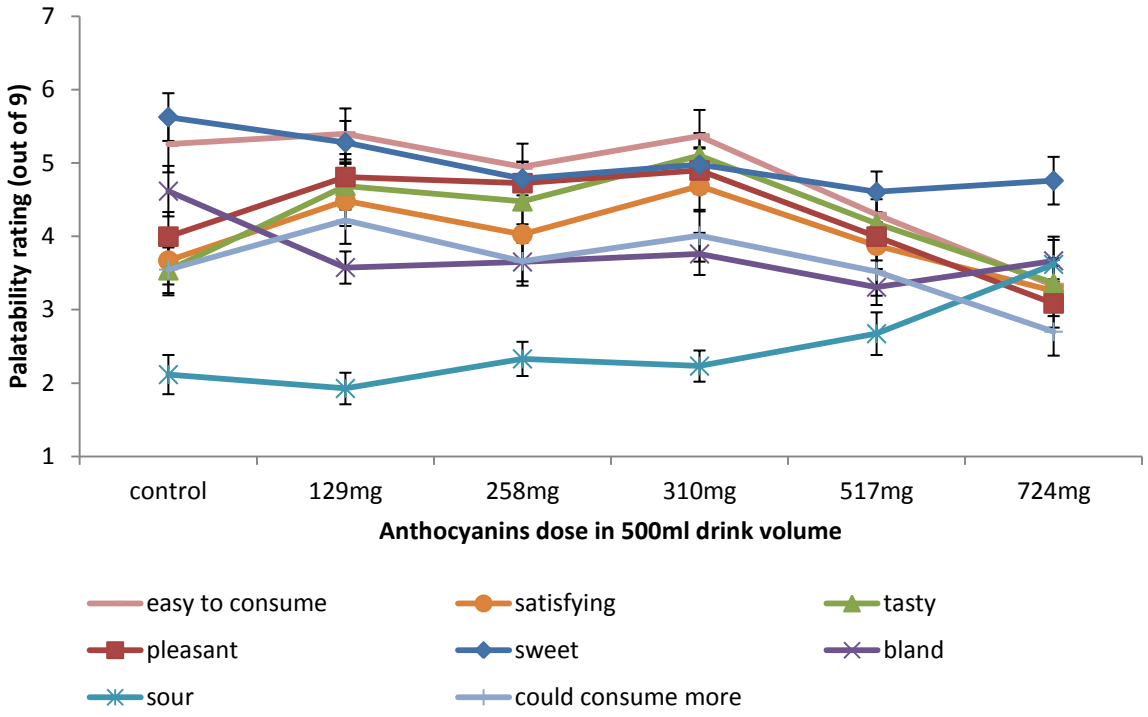


Figure 4.7 Palatability ratings for all intervention doses

Values are estimated marginal means. Error bars represent standard error of the mean.

4.4.5 Cohen's d effect sizes

Cohen's d effect sizes for all significant interactions (identified by pairwise comparisons) are shown in Table 4.9. Effect sizes were calculated from estimated marginal means and standard error values using the method described in Chapter 2. For cognition, mood and heart rate effects, difference from baseline following blueberry intervention was compared with difference from baseline following the control. Positive values indicate performance improvements compared to the control. Negative values indicate detriments to performance.

Table 4.9 Cohen's d effect sizes for Experiment 2

Variable	Dose	Cohen's d
Cognition:		
Immediate recall score	129mg	(**)0.297
	517mg	(**)0.291
	724mg	(**)0.285
Immediate recall interference errors	310mg	*-0.247
	724mg	†-0.174
Serial 3s score	517mg	(†)-0.082
	724mg	**0.14
Serial 7s score	517mg	*0.213
	724mg	**0.423
Serial 7s RT	310mg	†0.055
	517mg	*0.106
	724mg	**0.344
Serial 7s errors	258mg	†-0.325
Sternberg RT	129mg	(*)-0.201
	517mg	(*)-0.275
Stroop congruent RT	129mg	(**)-0.284
	517mg	(**)-0.174
Stroop incongruent RT	129mg	(*)-0.149
	724mg	(*)-0.220
Mood:		
Mental fatigue	258mg	*0.090
	310mg	*0.143
	517mg	†0.072
Negative affect	310mg	*0.318
Physiology:		
Heart rate	517mg	(**)0.506

Table 4.9 continued

Variable	Dose	Cohen's d
	724mg	(**)0.359

Cohen's d values represent small (d=0.2), medium (d=0.5), and large (d=0.8) effect sizes, respectively. Positive values indicate performance improvements, and negative values indicate detriments to performance, compared to the control. Significance of the underlying pairwise comparison with baseline is indicated, **(p<0.01), *(p<0.05), †(p<0.1). The use of brackets, (**), (*), (†) indicates an effect derived through a change from baseline for the control condition, attenuated for the blueberry condition.

4.5 Discussion

The lack of a LMM Session x Dose interaction for any of the DVs suggests that, in this experiment, anthocyanin-rich blueberry had no effect on the cognition, mood or blood pressure of a healthy young adult population, at any dose, when compared with a matched control condition. However, when employing statistically less conservative pairwise comparisons, as recommended by Huck (2015), significant dose-dependent effects on episodic memory, working memory, mood, and heart rate became evident. These pairwise effects were corrected for multiple comparisons, and so have been reported despite the non-significance of the LMM interaction. Possible reasons for this lack of a significant LMM interaction are considered later in this section.

Where consistent effects have been observed for specific doses, across two or more DVs for the same cognitive task, and in line with previous literature findings, it seems likely that the results are not simply a result of type I error. In particular, consumption of the highest doses (517mg & 724mg) appeared to convey small to medium benefits for the maintenance of constant heart rate and immediate recall memory score, and improvements in serial 7s performance for both score and reaction time. The 310mg dose elicited small to medium benefits in mental fatigue and mood. However the lower (129mg & 258mg) doses showed no consistent domain specific effects.

Immediate recall and serial 7s benefits were evident following the 517mg and 724mg blueberry doses, findings which are consistent with previous literature following supplementation with varying flavonoid-rich food sources and in varying populations e.g. (Dodd, 2012; Kennedy et al., 2002; Massee et al., 2015; Whyte & Williams, 2015). Indeed the serial subtraction benefits were successfully replicated later in this thesis (Chapter 6). However it is unclear how these observations relate to the underlying FMD response previously published for the same doses (Rodriguez-Mateos et al., 2013). The FMD data predicted the greatest benefit to cognition following the 310mg dose. Therefore, alternative mechanisms of action may be relevant here. As reported in the literature

review, a difference in postprandial blood glucose response has previously been observed concurrently with cognitive benefits when an anthocyanin-rich berry intervention was compared with a sugar-matched control (Watson et al., 2015). Therefore, a similar mechanism may be responsible for cognitive improvements here. The link showing that glucose influences cognitive performance is well supported. For example, a study by Benton, Owens, & Parker, (1994) investigating the effects of glucose on cognition in healthy young adults found that immediate and delayed recall memory performance was correlated with individual changes in blood sugar levels at the time of testing. The authors suggest that it is the ability to process a glucose load that influences cognition. The same effect has been demonstrated in older adults (Craft, Zallen, & Baker, 1992). Similar studies have revealed a significant effect of glucose on serial 7s subtraction tasks (Kennedy & Scholey, 2000; Scholey et al., 2001). It has been theorised that increased glucose supply to the brain is a physiological response to a cognitively demanding task, but that individual differences in heart rate and glucose response may influence cognitive outcome (Kennedy & Scholey, 2000). Blood glucose may therefore have influenced memory and serial 7s performance in the current study through a mechanism of altered glucose regulation in the blueberry conditions but not the control condition. Possible mechanisms of action are considered in Chapters 5 and 6.

The lack of a beneficial effect for the Sternberg and Stroop tasks suggests that these tasks were not sensitive to blueberry intervention. The 129mg & 517mg doses were observed to elicit a moderate negative effect on performance during each of these tasks when compared to the control. However, it seems likely that these effects may be attributable to type 1 error as the remaining doses, of both intermediate and higher anthocyanin strength, showed no similar negative outcome. In addition, the previous literature has shown performance on these tasks to be improved by similar nutritional interventions e.g. flavonoid-rich ginkgo biloba (Subhan & Hindmarch, 1984). In the previous chapter, the piloting of these tasks revealed that they were rated amongst the least difficult, and so it may be that a more demanding cognitive load is needed before blueberry benefits become apparent in healthy young adults. This theory is consistent with the serial subtraction task data, where moderate cognitive benefits were observed for the serial 7s task but effect size was far smaller for the less demanding serial 3s. The theory is also compatible with the glucose mechanism proposed above, as increased availability of glucose may only be beneficial during particularly cognitively demanding tasks. As there was a great deal of overlap between the neural correlates for all of the cognitive tasks (Chapter 2), it appears likely that it is cognitive load, particularly working memory demand, that is responsible for these differences in cognitive outcome. Additionally, the computerised serial subtraction tasks used here had a high psychomotor processing component. The extra physical costs of these tasks may therefore contribute to extra demand for glucose.

The observed negative affect and mental fatigue benefits observed following the 310mg dose were consistent with both the FMD data and the previous literature e.g. (Khalid et al., 2017; Scholey et al., 2010, 2012). However, palatability data for the intervention drinks revealed that this dose was rated more pleasant, tasty, and satisfying than all other doses and therefore these mood effects may simply have been due to the influence of positive taste dimensions rather than the anthocyanin-rich blueberry content of the intervention. However, it may also be the case that these were genuine blueberry effects on mood that were also present following the 517mg and 724mg doses but, for these higher dose conditions, subjective ratings were negatively influenced by the poor taste of the intervention drink. Palatability has previously been shown to impact mood (Benton, 2002), in particular negative affect (Macht & Mueller, 2007). In future work it is therefore important to pay greater attention to palatability differences between intervention conditions.

The physiological measure of blood pressure was included in the study as an indirect measure of vasoreactivity, with a lowering of blood pressure expected following anthocyanin-rich berry supplementation (Keane, George, et al., 2016; Keane, Haskell-Ramsay, et al., 2016). However, in this experiment, no significant differences were observed between the control and blueberry doses for measures of systolic or diastolic blood pressure. A significant main effect of Session revealed an increase in blood pressure (SBP & DBP) between pre- and post- intervention time points, irrespective of intervention condition. This is likely attributable to circadian changes in blood pressure approaching a mid-morning peak (Millar-Craig, Bishop, & Raftery, 1978). Blood pressure measurements appeared not to be sensitive to any additional blueberry induced vasodilatory response. However, heart rate results were more interesting. Although the LMM interaction was not statistically significant, pairwise comparisons revealed that consumption of the highest doses (517mg & 724mg) conveyed small to medium protective benefits for the maintenance of a constant heart rate. Conversely, a marked decrease in heart rate was observed following the control and 258mg doses. As with the cognitive observations, glucose effects may similarly be responsible for the observed heart rate effects. Heart rate is known to be highly correlated with blood glucose e.g. (Ostrander & Weinstein, 1964; Valensi et al., 2011), and so the attenuation of postprandial decline in heart rate following these doses is suggestive of a dose-dependent glucoregulatory effect. This observation may point to an interaction between anthocyanins and sugars in the intervention drinks, influencing the postprandial glucose profile. In vitro and in vivo studies have linked polyphenols such as anthocyanins with the modulation of glucose digestion, absorption, and distribution to insulin-sensitive tissue (Cazarolli et al., 2008). Effects on postprandial glucose response have also been previously observed in human studies following anthocyanin-rich berry intervention (Törrönen et al.,

2010; Watson et al., 2015; Wilson et al., 2008). Blood glucose measurements were not recorded during this experiment, but clearly warrant further investigation.

Despite the small to moderate effects on cognition, mood, and physiology described above, LMM analysis found no significant interactions between dose and session for any of the measured DVs. Huck (2015) suggests that having similar means across multiple conditions will negatively impact the statistical outcome of an F test interaction. In addition, a number of potential methodological factors were identified which may further account for the LMM null effects. It was inferred that FMD and CBF timings were likely to be synonymous (Teragawa et al., 2005; Gonzales et al., 2010). In addition, both Francis et al. (2006) and Dodd (2012) observed increased CBF at similar time points for cocoa and blueberry polyphenols respectively. But it is possible that that peak CBF did not occur at the time of testing or that the dose-dependent vasodilatory response was somehow altered. For example, extracellular glucose has been demonstrated in vitro to facilitate vasodilation through a mechanism of insulin-induced endothelial nitric oxide production (Taubert, Rosenkranz, Berkels, Roesen, & Schömig, 2004). While not evident in the published findings for the control drink used in the Rodriguez-Mateos et al. (2013) study, the control was only matched to the second highest dose and contained 28 g total sugars compared to the 40g used in this experiment. Taubert et al. (2004) demonstrated the mechanism to be concentration-dependent, so enhanced nitric oxide production induced by sugar ingestion may have confounded the outcome of the current study by eliciting a vasodilatory response following the control condition, thus reducing the expected difference between the control and blueberry conditions. A further study (Kawano et al., 1999) revealed that hyperglycaemia suppressed endothelium dependant vasodilation, therefore the vasodilatory response in the blueberry conditions may have been attenuated; though this was not evident in the Rodriguez-Mateos et al. (2013) study where the FMD data for the highest dose showed a clear vascular response. A repeat FMD study would be necessary to confirm whether the timings of the cognitive study still coincided with peak vasodilation. Regrettably, this was beyond the timescale and resources of the current thesis. However, it is also unlikely that such an affect would reduce the statistical power of the LMM analysis but not the pairwise comparisons.

A more plausible explanation for the lack of LMM interaction despite significant pairwise comparisons is that, despite methodological efforts to keep them to a minimum, the order effects resulting from the length of study were of greater magnitude than the expected blueberry effects. As a result of the sizeable order effects, the necessary counterbalancing of doses over the six week period may have increased within-dose variance sufficiently to impact the statistical power of the LMM analysis. Additionally, main effects of session revealed order effects of greater magnitude than

those observed during the piloting of the tasks. In Chapter 3 participants received no intervention drink between the baseline and test sessions. This suggests that the intervention drink itself may have been an additional confound. The control and blueberry drinks were matched to the highest dose in terms of sugars (21g fructose and 19g glucose) and vitamin C (9.5mg) content. As previously discussed, glucose is the main fuel for brain function and is highly correlated with cognitive performance, so the high sugar content of the drinks may have boosted cognitive performance to a ceiling following both the control and blueberry doses. There was no evidence that participants were operating at ceiling for any of the cognitive tasks deployed, however it is possible that following the sugary intervention drink participants may have achieved a cognitive ceiling in terms of their individual potential for improvement, particularly on the tasks where no significant pairwise comparisons were observed.

In conclusion, only limited dose-dependent cognition and mood effects were apparent. Small mood benefits following the 310mg dose, while consistent with the literature, may have been confounded by the poorly matched flavour of the intervention drink. It is possible that subjective mood may have been positively influenced by the taste of the intervention rather than the blueberry content. Conversely, blueberry induced mood effects following the higher doses may have been negated by their poor taste. The pattern of results for cognition appeared inconsistent with the previous FMD data. The two highest doses (517mg & 724mg) conveyed the strongest cognitive benefits rather than the expected 310mg dose. However, heart rate data following the same two highest doses suggested that blood glucose effects may, at least in part, have been responsible for these findings. From these results it was considered necessary to investigate the postprandial glycaemic response for the control and blueberry drinks used in the study. A more detailed literature review of flavonoid effects on postprandial glucose regulation is reported in the next chapter along with the findings of the glycaemic response experiment.

Chapter 5
Experiment 3: A study of glycaemic effects following acute anthocyanin-rich blueberry supplementation in healthy young adults; implications for cognition and type 2 diabetes

An abridged version of this chapter has been published as Bell, L., Lamport, D. J., Butler, L. T., & Williams, C. M. (2017). A study of glycaemic effects following acute anthocyanin-rich blueberry supplementation in healthy young adults. *Food & Function, 8*, 3104-3110.

5.1 Introduction

In the previous chapter, protection against postprandial heart rate decline was observed in conjunction with benefits to episodic memory, working memory and mood, particularly following the higher doses of anthocyanin-rich blueberry. Specifically, pairwise comparisons revealed that consumption of the highest doses (517mg & 724mg) conveyed benefits for the maintenance of a constant heart rate and maintenance of immediate recall memory score. Additionally, the same two doses elicited improvements to serial 7s performance for measures of both score and reaction time. The 310mg dose was observed to benefit mental fatigue and mood. However the lower (129mg & 258mg) doses showed no consistent cognitive effects. Irrespective of dose, blueberry intervention failed to impact Stroop or Sternberg task performance. It was posited that the high sugar content of the intervention drinks may have influenced the experimental outcome, through a dose-dependent modulation of postprandial glucoregulation. Therefore, it was deemed important to investigate the postprandial glucose response for the doses of blueberry administered during the previous experiment.

Flavonoid-rich foods have been previously observed to influence blood glucose and glucose homeostasis. For example, key mechanisms have been identified in vitro including inhibition of carbohydrate digestion and glucose absorption, facilitation of insulin synthesis and secretion, and facilitation of glucose uptake by cells (Cazarolli et al., 2008; Hanhineva et al., 2010; Norberto et al., 2013; Sancho & Pastore, 2012; Williamson, 2013; Zia Ul Haq, Riaz, & Saad, 2016). In vivo animal models have demonstrated that these mechanisms can impact upon the immediate postprandial period in addition to longer term glucoregulatory health (Hanamura, Mayama, Aoki, Hirayama, &

Shimizu, 2006). Epidemiological data support these findings (Jennings, Welch, Spector, Macgregor, & Cassidy, 2014; Wedick et al., 2012); in particular, this epidemiological evidence suggests that higher intake of the flavonoid subclass anthocyanins is associated with lower incidence of type 2 diabetes and increased insulin sensitivity. However few research trials have been conducted in humans that investigate modulation of the glycaemic response following anthocyanin-rich foods (Burton-Freeman, 2010), or have considered how these effects may impact on cognitive function.

Research to date has produced mixed findings. Attenuated and/or delayed postprandial blood glucose concentrations have been observed in healthy adults in a number of studies. Following a 480ml serving of cranberry juice, Wilson et al., (2008) observed attenuated and delayed postprandial plasma glucose concentrations when compared with a sugar matched control. 187 participants took part in this between-subjects study investigating six different control and active conditions. The outcome reported here was for the normal calorie juice. Anthocyanins levels were reportedly quantified however were not documented in the paper. The authors attributed the findings to the proanthocyanidin content of the juice rather than the anthocyanins that were also present. Other studies have similarly linked proanthocyanidins (oligomers of the subgroup of flavonoids known as flavanols) with altered glycaemic response e.g. (Sulaiman, 2014). Torronen et al (2010) investigated the postprandial glucose response, in 12 healthy adults, following sucrose consumption (35g) either with or without a mixed berry puree (bilberry, blackcurrant, cranberry & strawberry) containing an estimated 300mg anthocyanins. This crossover study found that the mixed berry condition was associated with a delayed and attenuated glucose peak relative to the sugar-matched control. Additionally, after 150 minutes blood glucose levels remained significantly elevated in the mixed berry condition relative to the control condition. Nyambe-Silavwe & Williamson (2016) observed significant lowering of peak postprandial glucose and insulin, in a dose-dependent manner, when white bread was consumed with a flavonoid-rich intervention combining green tea and a mixture of freeze-dried fruits. This was a crossover intervention study with 16 participants. Incremental area-under-the-curve (iAUC) values for glucose were reduced following both high and low flavonoid doses, when compared with sugar-matched controls. Insulin iAUC was reduced for the high dose only. The exact flavonoid content of the intervention, again, was not stated. Based on tabulated values supplied for the individual fruits, the total flavonoid content of the high dose was estimated to be 690mg, of which an estimated 250mg were anthocyanins.

In a study of eight middle aged males (Kay & Holub, 2002), significant postprandial glucose effects were observed following a wild blueberry intervention consumed with a high fat, high carbohydrate fast food meal that included potatoes and white bread. Glucose levels were observed to remain

significantly elevated at 3 & 4 hours postprandially when compared with a blueberry-free control condition. Interestingly though, the glucose effect was attributed to the fructose content of the blueberry rather than the 1160mg anthocyanins present in the intervention; the rationale being that fructose consumption has been associated with a delayed appearance of glucose in blood serum following the conversion of fructose to glucose in the liver. The control condition in the study had not been matched for fructose content, making it impossible to differentiate anthocyanins and fructose effects in this case. The above studies were all conducted in healthy adults, however effects have also been observed in clinical populations. For example, in 10 glucose intolerant subjects (J. Hidalgo et al., 2014), attenuation of peak glucose was observed in the immediate 60 minute postprandial period following boiled rice when consumed with a maqui berry extract containing 20mg anthocyanins.

Despite these positive findings, other similar studies have failed to replicate anthocyanin-rich intervention effects on glycaemic response. For example, in eight healthy adults, Cao, Russell, Lischner, & Prior, (1998) observed no significant effect of a 240g fresh strawberry intervention on postprandial glucose, when consumed with a breakfast meal. Vinson, Bose, Proch, Al Kharrat, & Samman, (2008) investigated the effect of cranberry juice on postprandial glucose response in 10 healthy adults following consumption of high fructose corn syrup. The intervention containing 24mg anthocyanins was observed to blunt the initial glucose peak and reduce AUC; however statistical significance was not achieved. The authors suggest that the true peak for the control condition may have been missed due to individual differences in the response profiles for different subjects that failed to coincide with the timing of blood samples. Clegg, Pratt, Meade, & Henry, (2011) investigated the postprandial glucose response in 12 healthy subjects following a starchy carbohydrate intervention of pancakes fortified with 100g blueberries or 100g raspberries. However, no differences in iAUC were observed for either of the berry conditions when compared to pancakes alone. Again, the anthocyanins content of the berry interventions were not quantified. In other studies, even though glucose effects were not observed, insulin effects were evident. For example, in 24 overweight adults, freeze-dried strawberry powder containing 39mg anthocyanins taken with a high carbohydrate meal, significantly attenuated postprandial plasma insulin concentrations when compared with a control beverage containing no strawberry (Edirisinghe et al., 2011). Alqurashi, Galante, Rowland, Spencer, & Commane, (2016) investigated postprandial glucose and insulin response in 23 mildly obese subjects following an acai fruit and banana smoothie consumed with a high fat breakfast. The intervention contained 493mg anthocyanins. No significant differences in postprandial blood glucose were observed compared with a control condition, although mean insulin peak was found to be greater in the acai condition. But in this study, blood sampling only

occurred at hourly intervals, so it is possible that glucose differences in the immediate 60 minute postprandial period may have been missed. Most recently, the postprandial glucose and insulin effects of freeze-dried strawberry powder were measured in 21 obese adults with insulin resistance (Park et al., 2016). Four doses containing 0mg, 42mg, 88mg, & 155mg anthocyanins, matched for energy and fibre, were administered in drink form with a high fat, high carbohydrate meal. Although the glucose response following the highest dose appeared to show an attenuated peak, and extended availability of glucose at later postprandial time points, measurements were not statistically different from the control. However, postprandial insulin concentrations were significantly lower than control between baseline and 2 hours following the same dose. Interestingly, a significant inverse correlation was observed between plasma concentrations of anthocyanin metabolites and both glucose and insulin levels, indicative of a possible mechanism of action.

As reviewed above, there were many methodological differences between previous studies, not least the variability in the anthocyanin content of the berry interventions investigated (ranging from 20mg up to 1160mg). Significant methodological limitations were evident in a number of these studies. For example: low anthocyanins dose (Edirisinghe et al., 2011; Vinson et al., 2008), no quantification of anthocyanins dose (Cao et al., 1998; Clegg et al., 2011; Nyambe-Silavwe & Williamson, 2016; Wilson et al., 2008), infrequent blood sampling time points (Alqurashi et al., 2016), and an inadequately matched control condition (Kay & Holub, 2002). Whilst findings are not conclusive, there remains enough supporting evidence to suggest that anthocyanin consumption can modulate the postprandial glycaemic response. The degree of this response and the associated implications for cognitive outcomes in humans remain to be determined. A major shortcoming of the current literature is the absence of clearly defined flavonoid concentrations and subclasses. Therefore, not surprisingly, knowledge of the specific impact of anthocyanins on blood glucose remains limited (Zia Ul Haq et al., 2016). In light of this, the aim of the current study was to determine postprandial blood glucose profiles following known doses of anthocyanin-rich blueberry in healthy adults. Two blueberry doses, containing 310mg & 724mg anthocyanins, were selected from the previous experimental chapter. These doses represented the upper and lower limits of the range for which changes in heart rate, cognition and mood were observed. Blueberry doses were investigated with and without added sugar in order to better understand the impact of sugar-matching on postprandial glucose response. Therefore blueberry effects were compared with both sugar-matched and no-added-sugar controls. The aims of the study were twofold. First, the aim was to determine likely blood glucose status of participants at the time of cognitive testing in the previous thesis chapter. Second, the study aimed to determine whether anthocyanin-rich blueberry evoked a dose-dependent effect on postprandial glucose response that might provide a plausible

mechanism of action for the cognitive benefits observed during Experiment 2, and in the previous cognitive literature.

5.2 Methods

Methodological detail common to all experiments in this thesis can be found in Chapter 2. Methodology specific to this intervention study is reported here.

5.2.1 Participants

Seventeen young adults, 4 male, with a mean age of 24.12 years (SD 4.85) and a mean BMI of 23.82 kg/m^2 (SD 3.56) were recruited for the study. The participants were of similar demographic to those who took part in the previous experiment. Full participant details are reported in Chapter 2

5.2.2 Design

The study was a double-blind, five-condition, counterbalanced, crossover design comparing doses of anthocyanin-rich blueberry powder containing 0mg, 310mg and 724mg anthocyanins in both sugar-matched and no-added-sugar conditions (see Table 1). All testing took place in the morning. Participants attended a total of five regularly spaced visits. The minimum number of days between visits was 4, (M 7.69, SD 2.80). Blood glucose measurements were recorded at regular intervals (specified below) for 2 ½ hours post intervention. Equipment details and measuring procedure are reported in Chapter 2.

Table 5.1 Composition of each experimental condition in Experiment 3

Condition	Anthocyanins (mg)	Vitamin C (mg)	Fructose (g)	Glucose (g)
No-added-sugar control	0	9.5	0	0
Sugar-matched control	0	9.5	21	19
No-added-sugar low dose	310	9.5	9	8
Sugar-matched low dose	310	9.5	21	19
Sugar-matched high dose	724	9.5	21	19

All conditions were prepared by dissolving the required quantities of freeze dried blueberry powder, sugars and ascorbic acid in 500ml water. Sugar-matched conditions were matched to the naturally occurring sugar content of the highest blueberry dose.

5.2.3 Procedure

Participants were required to follow the low polyphenol diet (Chapter 2) for 24 hours prior to each visit and were asked to fast for 2 hours immediately prior to attending the laboratory. On arrival,

baseline blood glucose was recorded. Participants then consumed one of five test condition drinks. Drink order was counterbalanced. Participants were asked to consume the drink as quickly as possible within a maximum 10 minute period (Alqurashi et al., 2016; Rodriguez-Mateos et al., 2013). Blood glucose measurements were recorded at pre-consumption, 15, 30, 45, 60, 90, 120 and 150 minutes postprandially. Equipment and procedural details for the sampling of blood glucose can be found in Chapter 2.

5.3 Data analysis

Data were analysed with a linear mixed model (LMM), using an unstructured covariance matrix for repeated blood glucose measurements. Time, Dose and Time x Dose interaction were included as fixed factors in the model. Subjects were included as random effects and BMI was included as a covariate (Model 3.1). Pairwise comparisons with Bonferroni corrections were applied to investigate any significant effects. The analysis aim was to determine whether blueberry dose had a mediating effect on postprandial glucose response.

iAUC was subsequently calculated, from the glucose response curve for each dose, using the trapezoid rule (Le Floch, Escuyer, Baudin, Baudon, & Perlemuter, 1990). Incremental analysis, determined by excluding the area below baseline, has been shown to be a more representative measure of rapid postprandial changes in glycaemic status than total AUC analysis which is strongly correlated with basal blood glucose level (Le Floch et al., 1990). Between-subjects differences in baseline glucose were taken into account during the calculation. LMM analysis was used to determine the effect of dose on iAUC, again using an unstructured covariance matrix for repeat doses. Dose was included as a fixed factor in the model, and BMI was included as a covariate (Model 3.2). The aim of this analysis was to determine whether blueberry dose had a mediating effect on postprandial iAUC.

5.4 Results

Raw glucose results can be found in the supplementary data accompanying this thesis. All LMM results are presented in Appendix L. For clarity, only statistically significant effects are reported in full within the text below.

5.4.1 Blood Glucose Effects

Mean blood glucose concentrations are summarised in Figure 5.1. All factors were statistically significant: Time [$F(7,84.00)=45.17$, $p<0.001$], Dose [$F(4,59.06)=15.12$, $p<0.001$], Time x Dose [$F(28,84.00)=4.53$, $p<0.001$]. BMI was a significant covariate [$F(1,15.99)=5.64$, $p=0.030$] with higher

BMI predicting higher glucose levels (beta=0.047). Pairwise comparisons for the three sugar-matched conditions revealed that the highest anthocyanin dose (724mg) extended the glycaemic response such that blood glucose remained significantly elevated at 120 minutes compared with baseline levels [p=0.008]. Blood glucose remained significantly elevated compared with baseline at 90 minutes [p=0.016] following the sugar-matched low dose (310mg), and at 45 minutes [p<0.001] following the sugar-matched control. As observed in Figure 5.1, the sugar-matched blueberry doses produced non-significant reductions in mean peak compared with the sugar-matched control condition at postprandial time points of 15 minutes (p>0.999) and 30 minutes (p>0.999). The no-added-sugar control condition profile confirmed that there was no significant blood glucose effect (hyper- or hypoglycaemic) associated with simply consuming a 500ml sugar-free beverage.

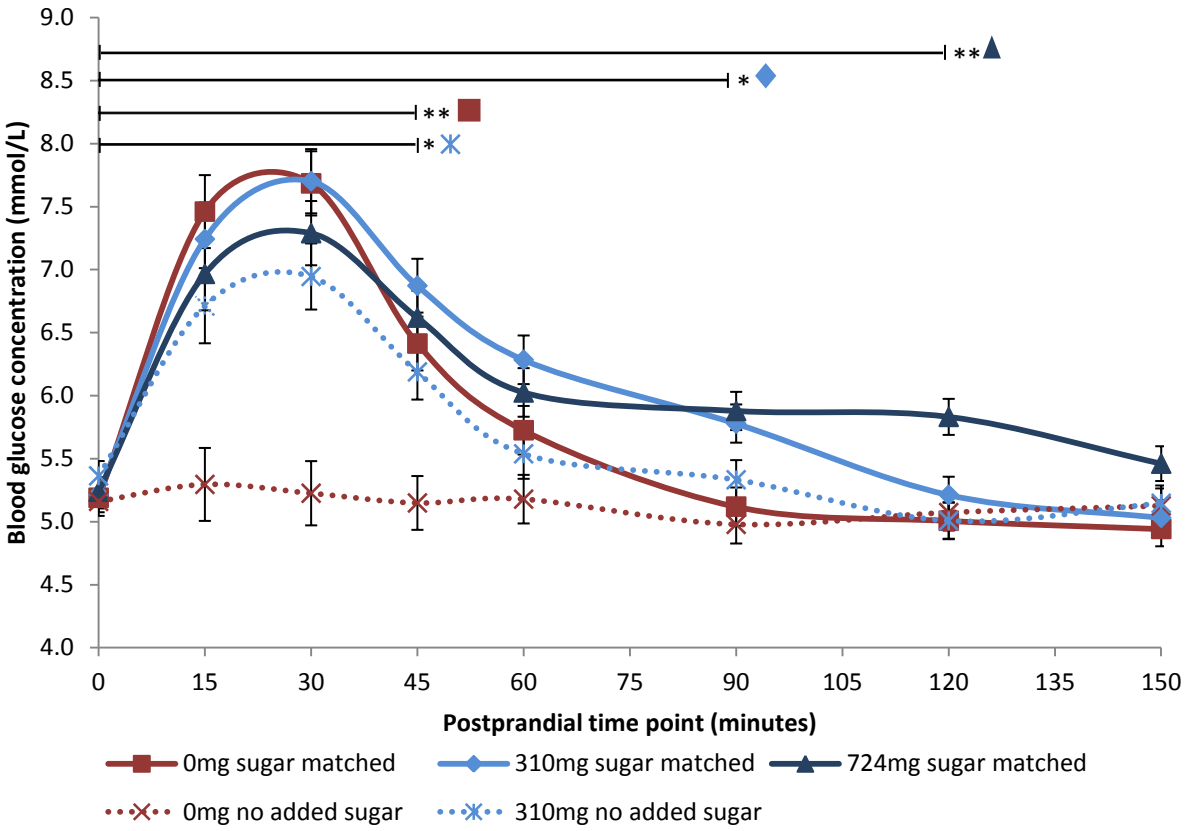


Figure 5.1 Mean blood glucose concentration recorded at baseline and following each dose at fixed postprandial time points

All sugar-matched doses contained the same quantity of sugars naturally present in the highest dose (40g total sugars). The 310mg no-added-sugar dose contained 17g total sugars. Values are estimated marginal means adjusted for BMI. Error bars represent standard error of the mean. Horizontal bars indicate the final time point

at which blood glucose remained significantly elevated compared with baseline for each dose, **(p<0.01),*(p<0.05).

5.4.2 iAUC

Mean iAUC values are summarised in Table 5.2. Dose was observed to be a significant predictor of iAUC [F(4,17.12)=14.02, p<0.001]. BMI did not reach significance as a covariate [F(1,17.88)=0.07, p=0.795]. Post hoc comparisons revealed no significant difference in iAUC between any of the three sugar-matched conditions [p>0.1]. Other comparisons are summarised in Table 5.2.

Table 5.2 Mean iAUC values for each intervention condition in Experiment 3 (n=17)

Condition	iAUC (mmol.min/l)	
	Mean	SE
No-added-sugar control (0mg)	34.38[a]	8.66
No-added-sugar low dose (310mg)	78.47[ab]	15.73
Sugar-matched control (0mg)	119.00[b]	17.38
Sugar-matched low dose (310mg)	140.03[b]	16.73
Sugar-matched high dose (724mg)	138.14[b]	16.34

Values are estimated marginal means adjusted for BMI. Means not sharing a common superscript are significantly different from each other (p<0.05).

5.5 Discussion

The results show that anthocyanin-rich blueberry significantly extended the postprandial glycaemic response compared to the equivalent sugar dose in the absence of blueberry. Indeed, blood glucose levels remained significantly elevated above baseline for 2 hours following a blueberry dose containing 724mg anthocyanins, and for 1.5 hours following a lower 310mg dose. These post-peak elevations were in the range of 0.5-1.5mmol/l above fasting baseline which is well within the healthy postprandial blood glucose range (American Diabetes Association, 2014) and is metabolically beneficial through the avoidance of reactive hypoglycaemic episodes (Willett, Manson, & Liu, 2002) where glucose level falls close to, or below, fasting levels.

In addition, the results demonstrate that at the time of cognitive testing used in Chapter 4 (60 minutes postprandially), glucose levels were already in decline, but still remained significantly elevated following the 724mg and 310mg sugar-matched blueberry doses (and by inference the

517mg dose). Following the sugar-matched control dose, while glucose levels appeared to remain elevated at 60 minutes (Figure 5.1), the difference from baseline was not found to be statistically significant at this time point. Indeed, it is possible that the limited cognitive effects observed in Chapter 4 may, at least in part, have been due to differences in blood glucose at the time of testing. The cognitive benefits were of small effect size when compared with the control, which might be explained by marginally elevated blood glucose levels in the control condition at the time of cognitive testing. The potential impact of improved blood glucose regulation on cognition is discussed in more detail later in this chapter.

The findings of the current study are consistent with previous human research (Törrönen et al., 2010; Wilson et al., 2008) where extension of the postprandial glycaemic response was observed for mixed berry and cranberry interventions respectively. Here we have demonstrated an effect for blueberries, and have shown that the effect is not due to the presence of fructose as previously reported (Kay & Holub, 2002), but is likely related to anthocyanin content. However unlike many previous studies (Hidalgo et al., 2014; Nyambe-Silavwe & Williamson, 2016; Törrönen et al., 2010; Wilson et al., 2008), we did not observe a statistically significant attenuation of peak glucose during the immediate postprandial period of 15-30 minutes, despite the apparent differences in peak height shown in Figure 5.1. A possible explanation is that the intervention drinks were consumed after a 2 hour fast period in the absence of any starchy food. Previous studies included additional food items, therefore attenuation of peak glucose may be more noticeable in the presence of a high sucrose (Törrönen et al., 2010) or more complex carbohydrate load (Hidalgo et al., 2014; Nyambe-Silavwe & Williamson, 2016).

The findings may, in part, also be due to the mediating effect of BMI on postprandial glucose response, as reflected by the significance of BMI as a covariate in this analysis. Postprandial glucose response has been shown to be associated with BMI (Carroll, Kaiser, Franks, Deere, & Caffrey, 2007; Hopper, Koch, & Koch, 2013) and the issue of individual variability in glucose response has been previously highlighted (Carroll et al., 2007; Vinson et al., 2008). It is of particular interest here that BMI predicted blood glucose even in a group of young adults with a healthy BMI range, an effect not apparent in the current literature. Future studies should consider whether the effects of anthocyanin-rich foods on postprandial metabolism are mediated by BMI; it is possible that overweight adults with high levels of subcutaneous fat may show a greater reduction in peak glucose following an anthocyanin-rich intervention relative to healthy normal weight adults; however this hypothesis could not be tested in our small sample of healthy young adults. Additionally, individual differences in the time taken to consume the drink may also have influenced the outcome. Ten

minutes were allowed, but some participants consumed the drinks considerably faster than this (range 0.5-10 minutes). Peak glucose may have occurred prior to the onset of postprandial testing, or between the earlier testing time points for some of the participants, particularly following rapid absorption of glucose in the sugar-matched control which contained free sugars not bound with other nutrients. Thus, mean glucose peak may not have accurately reflected the true value for the control condition. Future studies should impose tighter control over the time taken to consume an intervention, and consider the use of continuous glucose monitoring to identify true peaks.

Another important consideration here is that the drinks were not matched for fibre content. Fibre is known to influence postprandial glucose response. For example, guar gum and sugar beet fibre (both non-starch polysaccharides) have been observed to attenuate peak postprandial blood glucose levels and reduce overall glucose AUC (Morgan, Tredger, Wright, & Marks, 1990). However, the maintenance of raised glucose over a longer postprandial period, as observed in the current study, has not been previously associated with a high fibre intervention. It has been posited that it is predominantly soluble fibre that attenuates postprandial glucose through viscosity effects (Törrönen et al., 2010). As berries are typically low in soluble fibre and no significant attenuation of peak glucose was observed, the extended postprandial response following blueberry supplementation observed here is unlikely due to fibre confounds. Indeed, insulin regulatory effects of anthocyanin-rich berries have previously been observed where control and experimental conditions were matched for fibre (although apparent glucose effects were not found to be statistically significant) (Park et al., 2016). As an alternative mechanism of action, anthocyanins have been observed to inhibit intestinal alpha-amylase and alpha-glucosidase activity (Hanamura et al., 2006; McDougall et al., 2005; Nyambe-Silavwe & Williamson, 2016; Tadera, Minami, Takamatsu, & Matsuoka, 2006), thereby slowing the rate of carbohydrate digestion. Furthermore, anthocyanins and other berry polyphenols have also been associated with inhibition of glucose transport from the intestine to blood plasma (Hanamura et al., 2006). Specific mechanisms include delayed intestinal absorption of glucose through inhibition of the sodium glucose co-transporter SGLT1 (Hidalgo et al., 2014; Johnston, Sharp, Clifford, & Morgan, 2005), and the glucose transporter GLUT2 (Kwon et al., 2007; Song et al., 2002). Therefore, any combination of these mechanisms is likely to underlie the immediate postprandial effects observed here and these potential mechanisms of action warrant further investigation in future studies.

There are a number of important implications for this work. Numerous cognitive benefits of flavonoid-rich foods, including foods rich in anthocyanins, have been previously documented including reduced risk of neurodegenerative disease, and short term improvements to cognition

during the immediate postprandial period. It is possible that previously observed cognitive effects, following acute intervention with anthocyanin-rich foods, are mediated by variations in blood glucose levels rather than, or in addition to, other hypothesised mechanisms of actions associated with flavonoid intake, such as increased cerebral blood flow (Rendeiro, Rhodes, & Spencer, 2015). Increased glucose availability in the late postprandial period following ingestion of anthocyanin-rich foods may convey a cognitive advantage, given that low circulating glucose levels are often correlated with cognitive deficits. Indeed, it has been demonstrated that foods which elicit a favourable glycaemic response are beneficial for cognition (Lamport, Lawton, Mansfield, Moulin, & Dye, 2014), and such effects may even extend beyond the first postprandial response period, continuing to influence cognition after subsequent food intake (Lamport, Hoyle, Lawton, Mansfield, & Dye, 2011). Therefore, it is possible that circulating anthocyanin metabolites may also induce 'second meal' effects (Lamport et al., 2011), which is an important consideration for flavonoid intervention studies where a standardised meal is provided prior to extended cognitive testing time points. Further work is needed to confirm whether effects on glycaemic response, such as the effects following anthocyanin-rich blueberry observed here, correlate with changes in cognitive performance. However, it is possible that cognitive benefits may be limited in a metabolically healthy population such as the young adults investigated here.

The postprandial response to ingested carbohydrate is also recognised as a marker of metabolic health. For example, postprandial hyperglycaemia is observed in type 2 diabetes mellitus and has been shown to be a risk factor for cardiovascular disease (Bonora & Muggeo, 2001). Cognitive deficits including neurodegenerative disease are also associated with type 2 diabetes (Chung et al., 2015; Lamport, Lawton, Mansfield, & Dye, 2009) and hypertension (Muela et al., 2017). Therefore, interventions which moderate postprandial glucose by limiting periods of hyper- or hypoglycaemia are desirable. Indeed, although not directly relevant to the aims of this thesis, the present data may also have implications for type 2 diabetes where regulation of postprandial glucose response is important in the prevention and treatment of the disease, and in the reduction of associated risk factors such as cardiovascular disease (Bonora & Muggeo, 2001). Glucoregulatory effects similar to the current study have been reported following the consumption of low glycaemic index foods, which are reported to reduce incidence of hypoglycaemia and type 2 diabetes (Willett et al., 2002). It should be noted, however, that no hypoglycaemic episodes were observed following the control intervention in the current study, suggesting that these benefits may only be relevant in subjects with pre- or type 2 diabetes, rather than in healthy young adults. Nevertheless, anthocyanin-rich blueberries have been positively associated with multiple health outcomes including enhanced cognition (Bell et al., 2015; Lamport et al., 2012), decreased blood pressure (Basu et al., 2010), and

improved vasoreactivity (Rodriguez-Mateos et al., 2013), all of which are compromised in type 2 diabetes (Chung et al., 2015; Lamport et al., 2009). Furthermore, anthocyanins, and particularly blueberry consumption, are significantly associated with lower type 2 diabetes mellitus risk (Jennings et al., 2014; Wedick et al., 2012). In addition to the regulatory mechanisms described previously, anthocyanins have also been reported to further inhibit alpha-glucosidase activity in synergy with acarbose (Akkarachiyasit, Charoenlertkul, Yibchok-Anun, & Adisakwattana, 2010), a common anti-diabetic drug. Other reviewed blueberry effects on metabolic syndrome include evidence of a lowering of leptin concentrations thereby decreasing the tendency towards obesity, improved pancreatic beta cell function facilitating insulin secretion (Martineau et al., 2006), and subsequent improved insulin sensitivity (Norberto et al., 2013). Other flavonoid subclasses have also been observed to regulate tissue and cell specific isoforms of glucose transporters (GLUTs1-5) (Cazarolli et al., 2008), the rate determining step for glucose uptake by all cells including transport of glucose across the blood-brain barrier (Vannucci, Maher, & Simpson, 1997). Additional evidence also suggests that berry anthocyanins may inhibit glycative activity, where glucose interacts with proteins and lipids in both exogenous food sources and endogenous body tissue (Thangthaeng, Poulose, Miller, & Shukitt-Hale, 2016). The reactive products of such activity (AGEs) have been linked with both hyperglycaemia and cognitive decline. Cognitive outcomes have not been assessed in the current study, however these mechanisms would support enhancement of cognition in type 2 diabetes following anthocyanin-rich intervention. The combined evidence suggests there is clear potential for a diet-based blueberry intervention to benefit the risk factors and co-morbidities associated with type 2 diabetes.

In conclusion, blood glucose levels were found to be significantly elevated at the time of cognitive testing utilised in Chapter 4 following the sugar-matched blueberry doses, but not following the sugar-matched control. It is unclear at this stage whether the sugar levels in the intervention drinks confounded the previous experiment, or provided an additional mechanism of action for observed cognitive benefits. In the current glycaemic experiment, anthocyanin-rich blueberry also influenced the postprandial glucose response such that blood glucose availability was extended, in a dose-dependent manner, for up to 2 hours postprandially. The potential impact of this experimental outcome has been discussed with reference to cognition and metabolic health. In relation to the current thesis, it was decided to design a follow-up experiment, incorporating blood glucose measurements and cognitive testing at baseline, 1 hour and 2 hour postprandial time points. The aim was to determine whether cognitive benefits following blueberry intervention were directly related to blood glucose levels at the time of cognitive testing and whether extended availability of

blood glucose conveyed cognitive benefits at a slightly later time point. The outcome of this follow-up study is reported in the next chapter.

Chapter 6

Experiment 4: A dose-response study of cognitive and blood glucose effects following acute anthocyanin-rich blueberry supplementation in healthy young adults

6.1 Introduction

As with Experiment 2 (Chapter 4), this study aimed to determine a dose-response for the cognitive effects of blueberry flavonoids, using doses matched to those used by Rodriguez-Mateos et al. (2013). This time cognitive testing was performed pre-, 1 hour, and 2 hours post-intervention in order to monitor changes in cognition at time points with and without expected differences in blood glucose concentration, as identified in the previous chapter. Blood glucose measurements were included in the current study in order to confirm glucose status at the time of cognitive testing. The cognitive tasks previously used in Experiment 2 were identified as sensitive to flavonoid intervention in the literature review, but for this experiment the task battery was modified to place greater emphasis on mood, working memory and attention. There were a number of reasons for this; newly published research showed marked effects in these domains following berry intervention (Khalid et al., 2017; Watson et al., 2015); as a possible mechanism, glucose has previously been linked with improved attention and working memory (Benton, Brett, & Brain, 1987; Benton et al., 1994; Kennedy & Scholey, 2000; Scholey et al., 2001); a shortened cognitive test battery was needed to maintain two distinct post-intervention time points. In particular, the shortened battery ruled out the testing of episodic memory. The tasks were all investigated for practice effects prior to use, as reported in Chapter 3. Again, the methodology used here reflects the findings of that study.

6.2 Methods

6.2.1 Participants

The participants were 41 healthy adults, aged 18-36 years (M 23.54, SD 5.14 years), 11 male, BMI (M 23.44, SD 4.99). The participants were of similar demographic to those who took part in all previous experiments. Full participant details are reported in Chapter 2.

6.2.2 Design

A double-blind crossover intervention study design was used. Independent variables were blueberry dose (4 levels; 3 doses and a matched control) and time (3 levels; pre-, 1 hour, and 2 hours post-intervention). Additional postprandial time points of 15 minutes and 30 minutes were included for blood glucose measurement only. Participants attended a total of 5 regularly spaced visits. The minimum number of days between visits was 3 (M 6.97, SD 4.27). Again, in order to minimise the impact of practice on cognitive task performance, the first visit was treated as a familiarisation visit. Cognitive and blood glucose data collected during this visit were not included in subsequent analyses. Participants were not made aware of this and followed the same procedure as for all other visits, with the exception that participants were permitted as much practice as they required in order to feel comfortable with the cognitive tasks. The different blueberry doses were administered in drink form. The drink consumed at the familiarisation visit was identical to the control. At subsequent visits, the control and all blueberry doses were administered in counterbalanced order, determined using Williams matrices (Williams, 1949).

6.2.3 Procedure

At each visit participants arrived 2 hours fasted having previously followed a 24 hour low polyphenol diet (Chapter 2). They completed a pre-intervention battery of cognitive tasks and blood glucose measurements. Participants then consumed a blueberry or control drink. In order to minimise drink consumption rate variability, participants were required to finish the drink within a 5 minute time period, after which they were asked to remain in the lab waiting room. As in Experiment 2, participants completed a 24 hour retrospective food diary and a post-drink palatability questionnaire (Chapter 2). Further blood glucose measurements were recorded 15 minutes and 30 minutes after consuming the intervention. Participants resumed testing, repeating the cognitive task battery and blood glucose measurements at the post-intervention time points of 1 and 2 hours. A return appointment was then booked for the following week.

6.2.4 Blueberry intervention drinks

The blueberry doses used in the drinks were again aligned with those used in a previous vascular study (Rodriguez-Mateos et al., 2013), however in this case two of the previous doses (129mg and 258mg) were omitted. Thus, three blueberry doses, containing 310mg, 517mg & 724mg anthocyanins, were selected as these doses represented the range for which heart rate, cognition and mood effects were observed in Experiment 2. Additionally, as described in Chapter 2, a new batch of freeze-dried blueberry powder was used in this experiment. The anthocyanin content of

this new batch was proportionally greater than the powder used in previous experiments. Indeed, by matching the anthocyanin content of the doses between experiments, the overall polyphenol content of each dose was greatly reduced in the current experiment when compared with Experiments 2 & 3. Therefore, the total polyphenol content of the lower, 129mg & 258mg, doses fell below that observed to elicit a vasodilatory response in the vascular study (Rodriguez-Mateos et al., 2013), giving further justification for the omission of these doses. Similarly, the total sugar content of the highest dose was reduced compared with Experiments 2 & 3. All doses were sugar-matched to this new value. The compositional analyses of all intervention drinks are shown in Table 6.1. The drinks were again prepared immediately prior to consumption by adding the appropriate amounts of freeze-dried blueberry powder, sugars and vitamin C to water. However, this time a lower volume of 300ml water was used. Lower amounts of powder were needed to achieve the doses and so the corresponding amount of water was reduced to maintain taste and consistency of the drinks. As a result the drinks were also easier to consume within the reduced time limit.

Table 6.1. Composition of blueberry and control doses in Experiment 4

		Freeze-dried wild blueberry powder (g)		
Composition	Control	16.3	27.2	38.1
Anthocyanins (mg)	0	310	517	724
Procyanidins (mg)	0	Not quantified	Not quantified	Not quantified
Total polyphenols (mg)	0	473	789	1105
Vitamin C (mg)	128	128	128	128
Fructose (g)	13.7	13.7	13.7	13.7
Glucose (g)	13.0	13.0	13.0	13.0

6.2.5 Cognitive & subjective measures

Full details of the selected cognitive tasks are given in Chapter 2. The order of the tasks was as follows: serial 3s & 7s subtraction tasks, RVIP, and digit vigilance, presented in between-subjects counterbalanced order; PANAS Now mood questionnaire.

6.2.6 Physiological measures

Blood glucose was measured at baseline and at 15, 30, 60 and 120 minutes. At each time point that coincided with a cognitive test session (baseline, 1 hour, 2 hours), measurements were recorded

prior to cognitive testing. Blood sampling equipment details and measuring procedure are reported in Chapter 2.

6.3 Data analysis

Data collected during Visit 1 were discarded prior to analysis and any outliers were removed according to criteria previously described in Chapter 2.

Blood glucose data were analysed by LMM using an unstructured covariance matrix to model successive repeat measurements. Visit, Time, Dose, and Time x Dose interaction were included as fixed factors in the model. Subjects were included as random effects. BMI was included as a covariate (Model 4.1). Where the covariate was found to be a significant predictor of performance, the analysis was performed with the further addition of covariate interactions (Model 4.2).The analysis aim was to determine whether blueberry dose had a mediating effect on postprandial glycaemic response. Pairwise comparisons were used to investigate significant effects and all interactions. A Bonferroni correction was applied to all multiple comparisons. iAUC values were calculated for each dose using the trapezoid rule. iAUC data were analysed by LMM, again using an unstructured covariance matrix. Visit and Dose were included as fixed factors in the model and BMI was included as a covariate (Model 4.3). The analysis aim was to determine whether blueberry dose had a mediating effect on the total amount of glucose absorbed into the blood stream post ingestion. Pairwise comparisons with Bonferroni correction were again used to investigate any significant effects and all interactions.

All cognitive and mood data were similarly analysed by LMM, also using an unstructured covariance matrix to model successive repeat measurements. Visit, Session, Dose, and Session x Dose interaction were included as fixed factors in the model. Subjects were included as random effects. The analysis aim was to determine whether blueberry dose was a significant predictor of cognitive or mood performance. In order to determine whether blood glucose concentration was also a significant predictor of cognition or mood, the analysis was performed both without (Model 4.4) and with the addition of this repeated covariate (Model 4.5). Where the covariate was found to be a significant predictor of performance, the analysis was performed with the further addition of covariate interactions (Model 4.6). Pairwise comparisons with Bonferroni correction were used to investigate any significant effects and all interactions for each of the models.

Palatability ratings for the intervention drinks were analysed by LMM using an unstructured covariance matrix to model successive ratings. Dose was the only fixed factor in the model (Model

4.7). The analysis aim was to determine whether the intervention drinks were adequately matched for flavour across a number of different taste dimensions.

6.4 Results

Raw data for all measured variables can be found in the supplementary data accompanying this thesis. Tabulated LMM results can be found in Appendix L. For clarity, only significant F-statistics are reported in full in the text.

6.4.1 Glucose analysis

LMM analysis revealed that Time [$F(4,164.00)=215.62$, $p<0.001$], Dose [$F(3,90.27)=4.81$, $p=0.004$], and Time x Dose interaction [$F(12,164.00)=3.36$, $p<0.001$] were all statistically significant factors in the prediction of blood glucose concentration. Visit was not observed to be a significant factor [$F(3,120.95)=2.03$, $p=0.113$], indicating that order effects were not present in the data. BMI was a significant covariate [$F(1,39.01)=9.96$, $p=0.003$]; higher BMI was predictive of higher glucose [beta=0.043]. When added to the model, no covariate interactions were observed to be significant. The interaction between blueberry dose and postprandial glucose response is shown in Figure 6.1. For all doses, blood glucose levels were significantly elevated compared to baseline at 15minutes [$p<0.001$] and 30minutes [$p<0.001$], but were no longer elevated at 1 hour [$p>0.1$]. At 2 hours, glucose levels were significantly reduced for all doses compared with baseline values [$p<0.001$]. Significant differences in peak glucose concentration were observed between 15 minutes and 30 minutes postprandially: at 15 minutes the 724mg dose was significantly lower than both the 310mg [$p=0.011$] and 0mg [$p<0.001$] doses, and the 517mg dose was significantly lower than the 0mg dose [$p=0.009$]; at 30 minutes the 724mg dose was significantly lower than both the 517mg [$p=0.048$] and 0mg [$p=0.001$] doses. No significant differences were found between doses at baseline, or at 1 and 2 hours postprandially, despite apparent differences at 1 hour seen in Figure 6.1 .

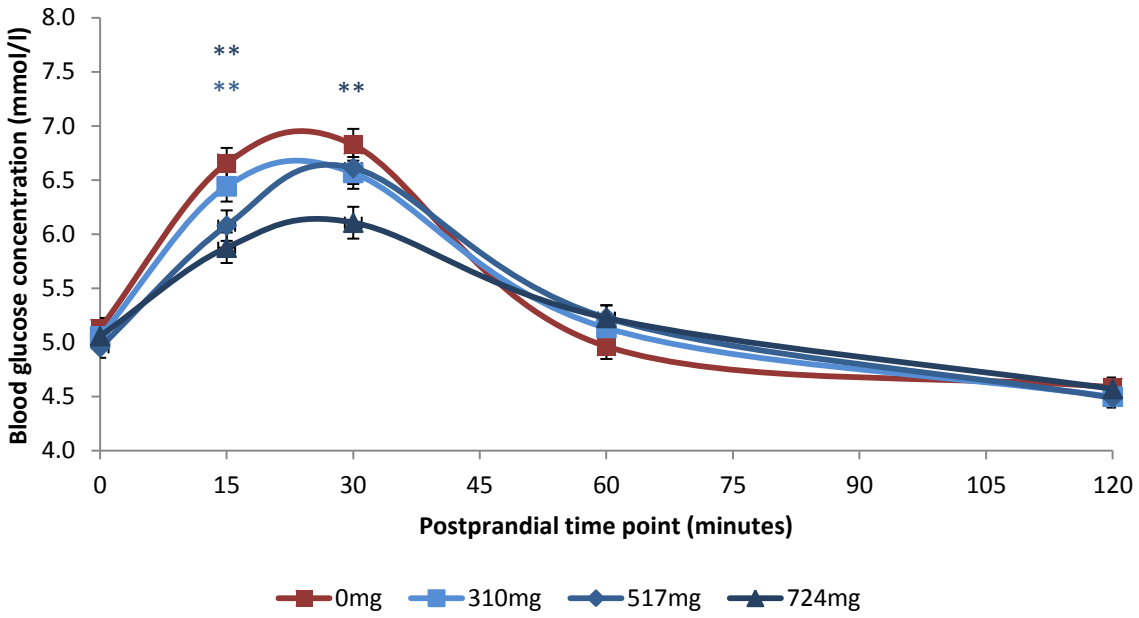


Figure 6.1 The interaction between blueberry dose and postprandial blood glucose concentration

All doses were sugar-matched to contain the same quantity of sugars naturally present in the highest dose (26.7g total sugars). Values are estimated marginal means adjusted for BMI. Error bars represent standard error of the mean. Coloured asterisks indicate a significant difference between the corresponding blueberry dose and the 0mg control condition at that time point, ** ($p<0.01$).

Analysis of iAUC data revealed that neither Visit [$F(3,40.03)=0.09$, $p=0.965$] nor Dose [$F(3,92.64)=2.15$, $p=0.100$] significantly predicted iAUC. BMI was not a significant covariate [$F(1,39.86)=0.33$, $p=0.569$]. Therefore iAUC did not significantly differ between conditions.

Table 6.2 Mean iAUC values for each intervention condition in Experiment 4 (n=41)

Anthocyanins dose	iAUC (mmol.min/l)	
	Mean	SE
0mg	63.98	6.97
310mg	68.17	7.01
517mg	70.26	6.98
724mg	55.43	6.99

Values are estimated marginal means adjusted for BMI

6.4.2 Cognitive analysis

Tabulated means for cognitive data can be found under each individual task subheading. LMM results for the analyses of all cognitive variables are reported in full in Appendix M. In the text significant F-statistics are reported in full. Non-significant effects are reported as p values only.

6.4.2.1 Order effects and baseline differences

The factor Visit was included in the LMM model for each cognitive dependent variable in order to determine the nature of any residual order effects. For the digit vigilance task, Visit was a significant predictor of both score [F(3,113.74)=3.42, p=0.020] and commission errors [F(3,121.23)=2.83, p=0.041] , but not RT [p=0.082]. Pairwise comparisons revealed a significant decrease in digit vigilance score between Visits 2 & 5 [p=0.025]. A corresponding trend for increased commission errors was evident over the same period [p=0.094]. For the RVIP task, Visit was a significant predictor of RT only [F(3,122.19)=2.86, p=0.040]. Pairwise comparisons revealed a significant slowing of RT between Visits 3 & 5 [p=0.045]. No other significant order effects were evident for this task. Visit was a significant predictor of 3s score [F(3,120.61)=97.76, p<0.001], 3s RT [F(3,116.29)=31.53, p<0.001], 7s score [F(3,118.96)=35.01, p<0.001], and 7s RT [F(3,114.31)=24.56, p<0.001]. Post hoc comparisons revealed a significant increase in both 3s and 7s scores between all consecutive visits [p<0.05]. Significant decreases for 3s RT occurred up to Visit 3 [p<0.05], however for 7s RT significant improvements continued between all visits [p<0.05] apart from between Visits 3 & 4 where apparent improvements were non-significant [p=0.281]. Therefore significant order effects were evident for both serial 3s and 7s tasks. In general, order effects therefore remained apparent despite methodological measures employed to keep them to a minimum. However, following the successful counterbalancing of the order in which all doses were presented to participants, no significant baseline differences were evident for any of the cognitive tasks, under any of the individual dose conditions [p>0.1].

6.4.2.2 Digit vigilance

Tabulated means for the digit vigilance task can be found in Table 6.3.

Table 6.3 Digit vigilance data for Experiment 4 (n=41)

Digit vigilance variable	Dose	Test session: Baseline Mean	Baseline SD	1 hr Mean	1 hr SD	2 hr Mean	2 hr SD
Score (correct/45)	0mg	42.85	2.89	42.75	2.36	42.31	2.92
	310mg	42.41	2.87	42.20	3.16	43.05	2.16
	517mg	42.66	3.41	42.83	2.65	42.74	2.62
	724mg	42.80	2.76	42.41	2.95	42.15	3.43
Commission errors	0mg	1.53	1.58	1.63	1.80	1.93	1.80
	310mg	1.56	1.19	1.95	1.92	1.45	1.57
	517mg	1.76	1.76	1.40	1.32	1.83	1.89
	724mg	1.56	1.45	2.07	1.89	2.08	1.61
RT (ms)	0mg	442.05	40.82	448.92	45.89	451.65	48.35
	310mg	446.50	50.20	449.34	44.21	448.66	47.40
	517mg	436.76	38.72	449.19	46.51	446.44	41.90
	724mg	440.31	48.30	449.64	51.73	451.08	48.91

For the digit vigilance task, Session was a significant predictor of RT [$F(2,163.68)=7.59$, $p=0.001$], but not of score or errors [$p>0.1$]. RTs slowed significantly between baseline and 1 hour [$p=0.002$]. Neither Dose, nor Session x Dose interaction was found to be a significant factor in predicting any of the digit vigilance variables. However, more detailed interpretation of pairwise comparisons for the interaction revealed a significant slowing of RT between baseline & 1 hour [$p=0.016$] and a trend towards a slowing of RT between baseline & 2 hours [$p=0.085$] following the 517mg dose, however no corresponding changes in score or errors were observed. When added to the model as covariate, glucose concentration was not found to be a significant factor [$p>0.1$].

6.4.2.3 RVIP

Tabulated means for the RVIP task can be found in Table 6.4.

Table 6.4 RVIP data for Experiment 4 (n=41)

RVIP variable	Dose	Test session					
		Baseline		1 hr		2 hr	
		Mean	SD	Mean	SD	Mean	SD
Score (correct/40)	0mg	23.20	8.89	25.00	9.44	23.93	9.43
	310mg	22.93	8.68	23.66	9.72	23.44	9.59
	517mg	23.63	9.36	25.02	9.51	23.98	10.16
	724mg	23.88	9.67	23.85	9.32	23.08	9.58
Commission errors	0mg	12.07	13.17	11.20	12.47	11.35	13.68
	310mg	11.39	12.15	12.46	13.40	12.41	14.00
	517mg	12.76	14.26	11.54	14.02	12.17	14.67
	724mg	12.56	14.23	11.51	14.73	11.13	13.07
RT (ms)	0mg	388.39	39.45	394.62	48.28	384.37	46.21
	310mg	398.26	47.75	391.45	45.06	392.79	43.00
	517mg	395.01	47.16	386.69	40.57	391.24	44.84
	724mg	387.76	48.96	392.33	46.48	388.51	38.74

For the RVIP task, Session was a significant predictor of score [$F(2,163.61)=4.54$, $p=0.012$], but not of RT or errors [$p>0.1$]. Scores significantly increased between baseline and 1 hour [$p=0.038$]. Neither Dose, nor Session x Dose interaction were found to be significant factors in predicting any of the RVIP variables, however pairwise comparisons for the interaction revealed a trend towards increased score between baseline & 1 hour following the control intervention [$p=0.063$]. No corresponding changes in RT or error rate were observed. When added to the model as covariate, glucose concentration was not found to be a significant factor [$p>0.1$].

6.4.2.4 Serial 3s & 7s

Tabulated means for the serial 3s & 7s tasks can be found in Table 6.5 & Table 6.6, respectively.

Table 6.5 Serial 3s data for Experiment 4 (n=41)

Serial 3s variable	Dose	Test session					
		Baseline		1 hr		2 hr	
		Mean	SD	Mean	SD	Mean	SD
Score (correct in 2 mins)	0mg	53.08	19.33	55.93	19.73	55.47	18.43
	310mg	50.79	19.50	53.00	19.64	53.05	18.89
	517mg	51.73	20.84	55.63	20.88	57.28	20.76
	724mg	52.53	18.32	55.60	19.41	56.13	16.15
Errors (incorrect in 2 mins)	0mg	1.15	1.49	1.63	1.64	1.92	1.92
	310mg	1.44	2.01	1.82	1.76	1.88	2.05
	517mg	1.39	1.36	1.90	2.01	1.51	1.80
	724mg	1.63	1.71	1.71	1.68	1.62	1.95
RT (ms)	0mg	2283.43	957.26	2170.61	1017.07	2085.89	869.50
	310mg	2428.45	1171.80	2258.22	1102.35	2249.03	1029.78
	517mg	2361.19	1005.97	2125.26	874.77	2128.29	863.73
	724mg	2315.17	1094.88	2025.87	862.48	2139.95	873.66

Table 6.6 Serial 7s data for Experiment 4 (n=41)

Serial 7s variable	Dose	Test session					
		Baseline		1 hr		2 hr	
		Mean	SD	Mean	SD	Mean	SD
Score (correct in 2 mins)	0mg	26.18	8.64	26.18	10.85	28.92	13.94
	310mg	25.30	11.24	26.93	11.05	26.82	12.96
	517mg	26.30	13.26	28.33	14.89	27.95	10.57
	724mg	26.23	13.27	26.77	11.14	27.31	11.21
Errors (incorrect in 2 mins)	0mg	1.68	1.68	2.32	1.89	1.56	1.74
	310mg	1.88	1.93	2.05	2.28	2.46	2.05
	517mg	1.98	2.04	2.27	1.95	2.39	2.38
	724mg	1.93	1.57	1.95	2.05	2.25	2.15
RT (ms)	0mg	4332.43	1907.26	3953.11	1626.98	4257.97	1993.81
	310mg	4513.37	1906.53	4471.30	2036.34	4380.48	2058.33
	517mg	4302.26	1619.31	4156.56	1729.86	4070.61	1740.44
	724mg	4403.49	1772.28	4105.09	1636.97	4420.54	2112.45

For the serial subtraction tasks, Session was a significant predictor of 3s score [$F(2,158.32)=18.62$, $p<0.001$], 3s RT [$F(2,156.34)=31.24$, $p<0.001$], 7s score [$F(2,156.55)=11.62$, $p<0.001$], and 7s RT [$F(2,145.93)=10.99$, $p<0.001$]. All scores and RTs significantly improved between baseline and 1 hour [$p<0.001$] but no further improvements were observed between 1hour and 2 hours [$p>0.1$]. Error scores remained stable between all visits and sessions. Neither Dose, nor Session x Dose interaction were found to be significant factors in predicting any of the serial subtraction variables. However, pairwise comparisons for the interaction revealed significant improvements in serial 3s score and RT between baseline & 1 hour, and between baseline & 2 hours following the control, 517mg and 724mg conditions [$p<0.05$], but not the 310mg condition [$p>0.10$]. For the control condition, these improvements were accompanied by a trend towards an increase in error rate [$p=0.052$] that was not observed for any of the blueberry doses, shown in Figure 6.2.

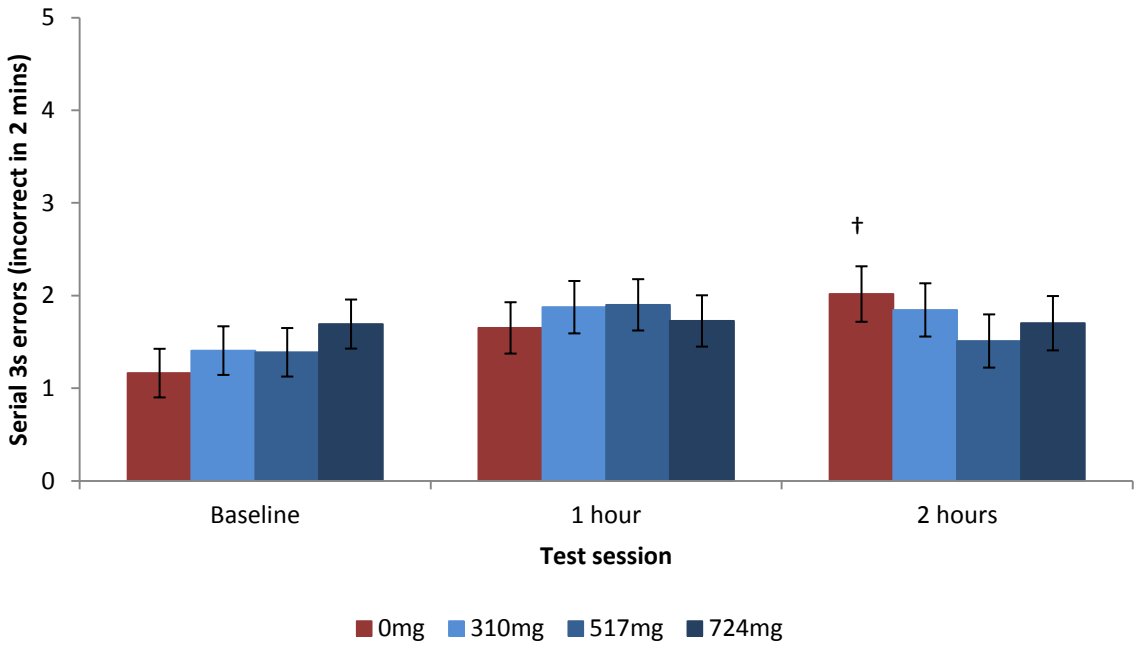


Figure 6.2 The interaction between test session and dose for serial 3s errors

Values are estimated marginal means. Error bars represent standard error of the mean. A significant difference from baseline is indicated above the column, † ($p<0.10$).

Pairwise comparisons for the serial 7s interaction revealed significant improvements in score and RT between baseline & 1 hour, and between baseline & 2 hours following the two highest blueberry doses (517mg & 724mg) only [$p<0.05$]. No significant improvements were observed following the control or 310mg dose [$p>0.10$]. These interactions are shown in Figure 6.3 & Figure 6.4.

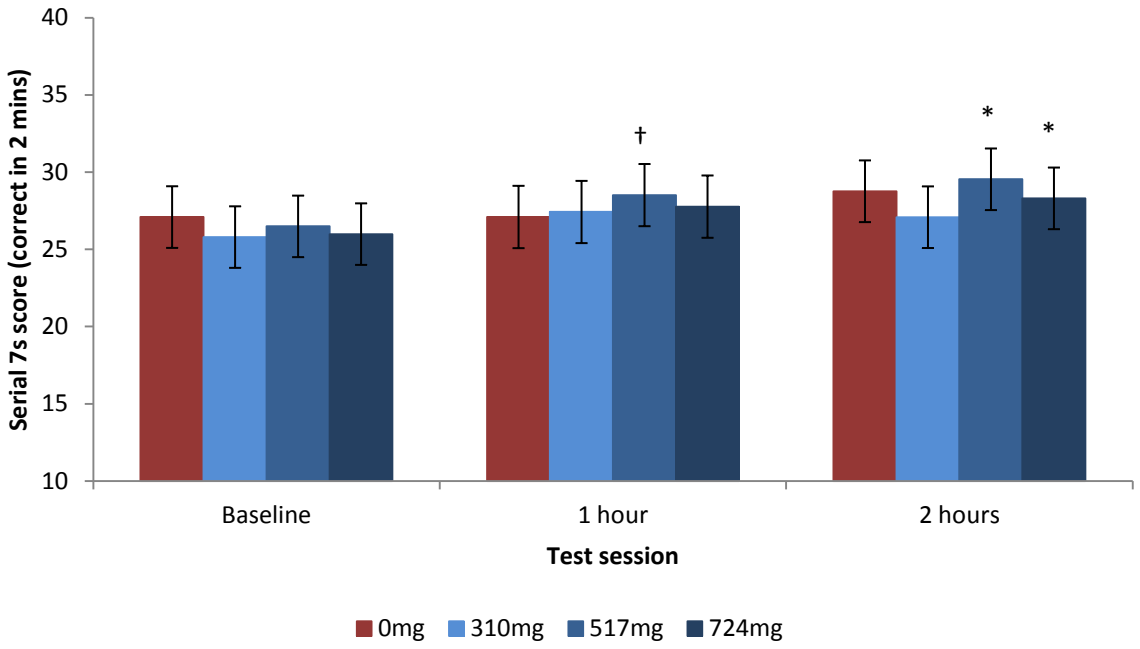


Figure 6.3 The interaction between test session and dose for serial 7s score

Values are estimated marginal means. Error bars represent standard error of the mean. A significant difference from baseline is indicated above the column, *($p<0.05$), †($p<0.10$).

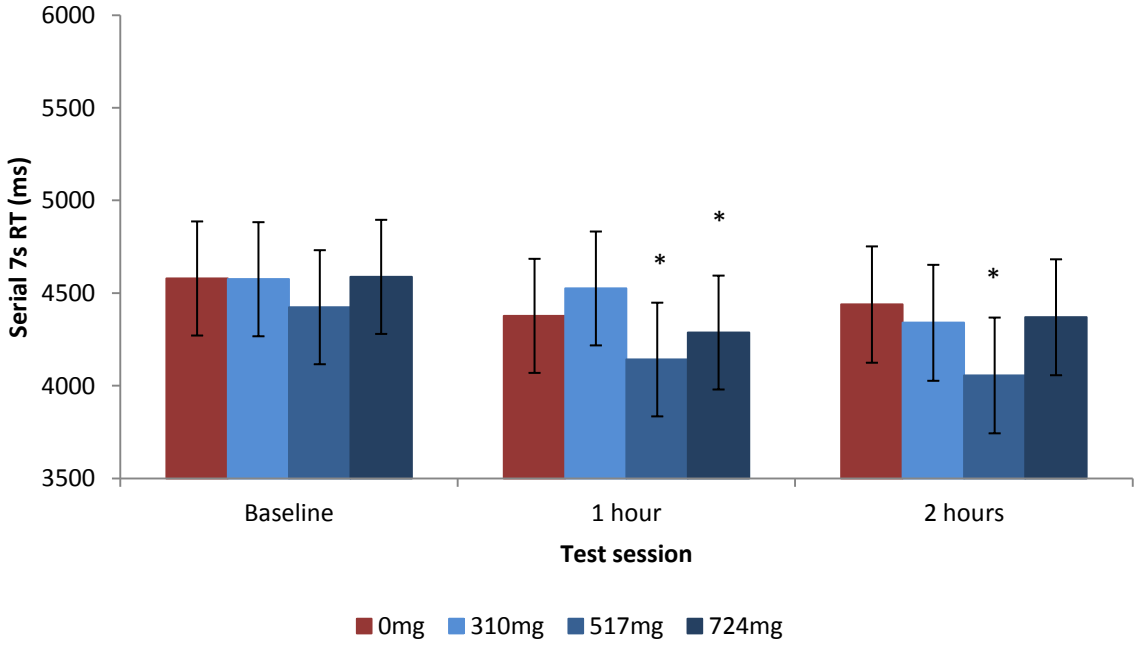


Figure 6.4 The interaction between test session and dose for serial 7s RT

Values are estimated marginal means. Error bars represent standard error of the mean. A significant difference from baseline is indicated above the column, *($p<0.05$).

When added to the model as covariate, glucose concentration was found to be a significant predictor of serial 3s score only [F(1,345.39)=6.81, p=0.009]; higher glucose levels were predictive of better cognitive performance [beta=1.406]. In order to further investigate the relationship between glucose and serial 3s task performance, covariate interactions with the fixed factors Session & Dose were added to the LMM model. Glucose x Session [F(2,208.96)=5.16, p=0.006], and Glucose x Dose [F(3,308.62)=4.81, p=0.003] were found to be statistically significant, however Glucose x Session x Dose was not [F(6,241.69)=1.69, p=0.125]. For the Glucose x Session interaction, beta coefficients were: baseline [beta=1.748]; 1 hour [beta=1.831]; 2 hours [beta=2.841]. Therefore, at later time points a greater proportional difference in serial 3s performance was observed for each unit difference in blood glucose i.e. blood glucose level showed greatest impact on cognitive performance at 2 hours postprandially. For the Glucose x Dose interaction, beta coefficients were: 0mg [beta=2.841]; 310mg [beta=0.308]; 517mg [beta=6.497]; 724mg [beta=5.063]. Therefore, differences in blood glucose showed the greatest impact on cognitive performance following the two highest blueberry doses.

6.4.3 Mood analysis

Tabulated means for all mood data can be found in Table 6.7. LMM results for the analyses of positive affect, negative affect and mental fatigue can be found in Appendix L. For clarity, only significant F-statistics are reported in full in the text.

Table 6.7 Mood data for Experiment 4 (n=41)

Mood measure	Dose	Test session					
		Baseline		1 hr		2 hr	
		Mean	SD	Mean	SD	Mean	SD
Mental fatigue (rating/9)	0mg	4.17	2.10	3.83	2.17	4.00	2.22
	310mg	4.54	2.27	4.34	2.27	4.27	2.11
	517mg	4.34	2.15	3.88	1.82	3.59	1.72
	724mg	4.56	2.26	4.49	2.05	4.05	1.96
Positive affect (score/50)	0mg	23.90	8.52	21.93	7.47	23.67	8.13
	310mg	21.95	8.01	21.51	8.03	23.20	8.35
	517mg	22.78	7.44	23.71	7.77	24.76	9.19
	724mg	22.41	8.39	22.41	7.49	23.73	7.84
Negative affect (score/50)	0mg	12.49	2.70	11.90	3.13	11.97	2.99
	310mg	12.63	4.04	12.51	3.49	12.32	2.71
	517mg	12.76	3.71	12.27	3.32	12.00	2.74
	724mg	13.54	4.15	12.37	3.48	11.98	2.58

For all subjective mood measures Visit was not a significant factor [$p>0.1$], indicating no order effects for these measures. Subsequently, pairwise comparisons revealed there were no significant baseline differences for any of the counterbalanced dose conditions [$p>0.1$]. Dose and Session x Dose interaction were not significant for any of the mood measures [$p>0.1$]. Session, however, was observed to be a significant predictor of positive affect [$F(2,161.97)=4.99$, $p=0.008$], negative affect [$F(2,161.93)=4.63$, $p=0.011$], and mental fatigue [$F(2,163.32)=3.45$, $p=0.034$]. Pairwise comparisons revealed: a significant increase in positive affect between the 1 & 2 hour test sessions [$p=0.007$]; a significant reduction in negative affect between baseline & 1 hour [$p=0.038$], and baseline & 2 hours [$p=0.009$]; and a significant reduction in mental fatigue between baseline & 2 hours [$p=0.028$]. These Session effects are most likely attributable to the consumption of an energy-containing drink, irrespective of dose condition.

More detailed interpretation of pairwise comparisons for the Session x Dose interaction revealed a trend towards decreased positive affect between baseline & 1hour following the control condition

[p=0.091]; no similar trends were observed following any of the blueberry conditions. A trend towards reduced mental fatigue between baseline & 2 hours was observed following the 517mg dose [p=0.076]. The highest (724mg) dose elicited significant reductions in negative affect between baseline & 1hr [p=0.041], and between baseline & 2hrs [p=0.010], as shown in Figure 6.5. When added to the model as a repeated covariate, blood glucose concentration approached significance as a predictor of mental fatigue [F(1,364.76)=3.12, p=0.078]; higher glucose levels were predictive of lower levels of mental fatigue [beta=-0.227]. Following their addition to the model, no covariate interactions were observed to be significant.

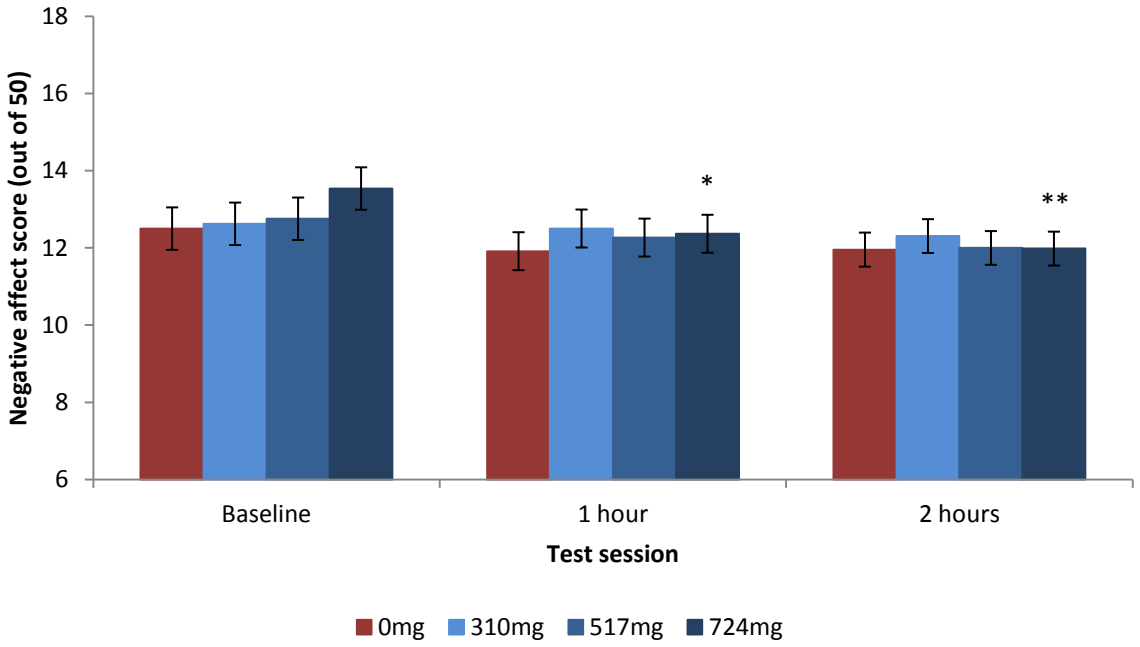


Figure 6.5 The interaction between session and dose for negative affect

Values are estimated marginal means. Error bars represent standard error of the mean. A significant difference from baseline is indicated above the column, **(p<0.05), *(p<0.05).

6.4.4 Cohen's d effect sizes

Cohen's d effect sizes for all significant interactions (identified by pairwise comparisons) are shown in Table 6.8. Effect sizes were calculated from estimated marginal means and standard error values using the method described in Chapter 2. For cognition and mood effects, difference from baseline following blueberry intervention was compared with difference from baseline following the control. Glucose effect sizes indicate the difference compared to control at the stated postprandial time point.

Table 6.8 Cohen's d effect sizes for Experiment 4

Variable	Dose	Time point	Cohen's d
Physiology:			
Blood glucose	517mg	15 mins	**0.647
	724mg	15 mins	**0.876
		30 mins	**0.789
Cognition:			
Digit vigilance RT	517mg	1h	*-0.200
		2h	†-0.023
RVIP score	310mg	1h	(†)-0.219
	517mg	1h	(†)-0.085
	724mg	1h	(†)-0.373
Serial 3s score	310mg	1h	(*)-0.119
		2h	(*)-0.178
	517mg	1h	**0.143
		2h	**0.258
	724mg	1h	*0.031
		2h	*0.021
Serial 3s errors	310mg	2h	(†)0.187
	517mg	2h	(†)0.330
	724mg	2h	(†)0.378
Serial 3s RT	310mg	1h	(*)-0.295
		2h	(*)-0.434
	517mg	1h	**0.167
		2h	**0.633
	724mg	1h	*0.062
		2h	**0.362
Serial 7s score	517mg	1h	†0.362
		2h	*0.248
	724mg	2h	*0.116
Serial 7s RT	517mg	1h	*0.116

Table 6.8 continued

Variable	Dose	Time point	Cohen's d
		2h	*0.297
	724mg	1h	*0.142
Mood:			
Mental fatigue	517mg	2h	†0.241
Positive affect	310mg	1h	(†)0.294
	517mg	1h	(†)0.507
	724mg	1h	(†)0.345
Negative affect	724mg	1 h	*0.197
		2h	**0.305

Cohen's d values represent small (d=0.2), medium (d=0.5), and large (d=0.8) effect sizes, respectively. Positive values indicate performance improvements, and negative values indicate detriments to performance, compared to the control. Significance of the underlying pairwise comparison is indicated, **(p<0.01), *(p<0.05), †(p<0.1). The use of brackets, (*), (†), indicates a change from baseline for the control condition, which was attenuated for the blueberry condition.

6.4.5 Palatability analysis

LMM analysis of the palatability data revealed a significant effect of dose for 'sweet' [F(3,40.81)=15.76, p<0.001], 'bland'[F(3,40.94)=6.79, p=0.001], and 'sour' [F(3,40.63)=8.82, p<0.001]rating dimensions, but nor for 'tasty'[p=0.114], 'pleasant'[p=0.112], 'satisfying'[p=0.382], 'how much more could you consume?'[p=0.429], and 'how easy was it to consume?' [p=0.714] ratings. The effect of dose on each rating dimension is shown in Figure 6.6. The 0mg control drink was rated as tasting significantly sweeter than all of the blueberry doses [p<0.001]. The 517mg and 724mg doses were rated significantly more sour than the control [<0.001], and were each rated marginally more sour than the 310mg dose, [p=0.093] and [p=0.058], respectively. However, although there were noticeable differences between interventions for specific taste dimensions (sweet, bland, sour), they appeared well-matched in terms of overall taste, pleasantness and satisfaction ratings.

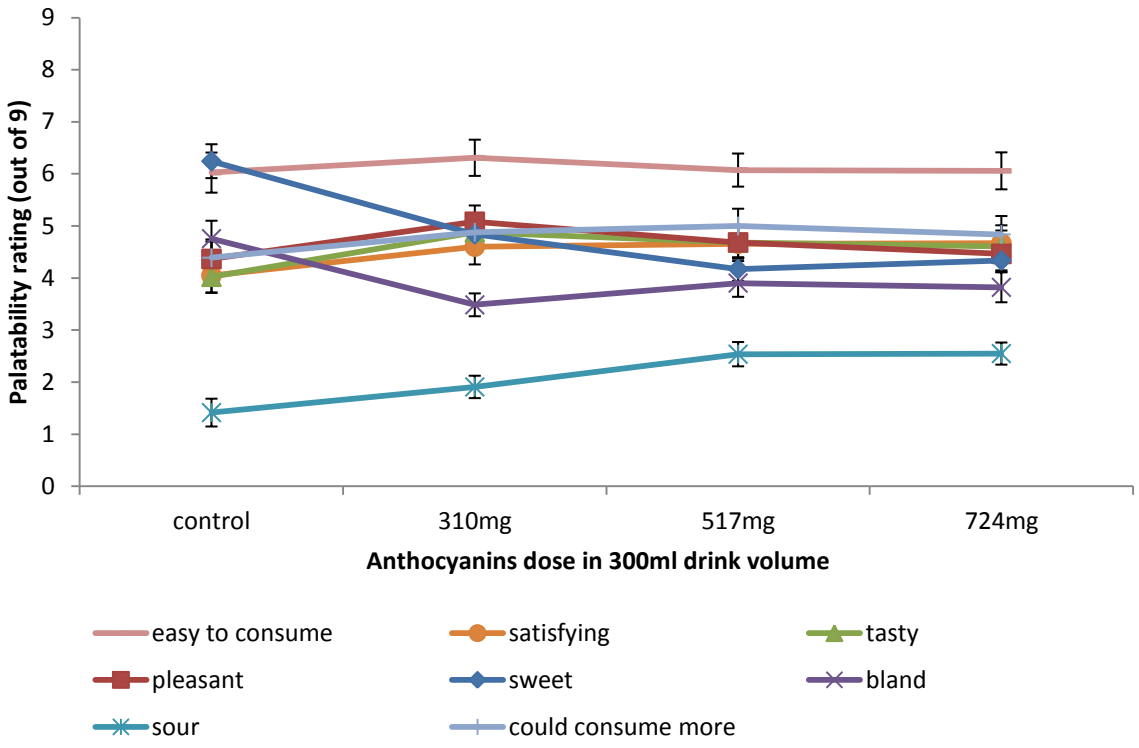


Figure 6.6 Palatability ratings for all intervention doses in Experiment 4

Values are estimated marginal means. Error bars represent standard error of the mean.

6.5 Discussion

LMM analysis revealed a significant dose-dependent effect of anthocyanin-rich blueberry on postprandial glucose response. Specifically, the 724mg and 517mg doses, but not the 310mg dose, significantly attenuated peak postprandial glucose compared to the control condition. Observed effect sizes were large following the 724mg dose and moderate following the 517mg dose. However, unlike my previous results in Experiment 3 (Chapter 5), no extended elevation of blood glucose was observed following any of the blueberry doses.

The lack of a LMM Dose x Session interaction for any of the cognitive or mood DVs suggests that, in this experiment, blueberry anthocyanins had no effect on mood or cognition at any dose when compared with the matched control condition. However, when employing statistically less conservative pairwise comparisons, as recommended by Huck (2015), significant effects on mood and working memory became evident. In particular, the 724mg dose elicited a significant reduction in negative affect between baseline and 1 & 2hrs, while positive affect decreased at 1 hour following the control but not following any of the blueberry doses. Improvements in serial 3s score & RT, at 1 & 2 hour time points, were evident following the two highest (517mg & 724mg) blueberry doses but

not the lowest 310mg dose. In this case, similar score & RT improvements were also observed following the matched control, although here the improvements were accompanied by an increase in errors that approached significance at 2 hours. No such increased error rate was observed for any of the blueberry conditions. Improvements in serial 7s score & RT were evident at 1 & 2 hours following the 516mg & 724mg doses only.

No cognitive benefits were observed for the digit vigilance or RVIP tasks suggesting that they were not sensitive to flavonoid-rich intervention in this study. During the piloting of the tasks (Chapter 3), the digit vigilance task was rated the easiest of all the tasks in the cognitive battery so it may be that a more demanding cognitive load is needed before blueberry effects become apparent. Indeed, a similar observation has been made in a study of executive function in children following blueberry intervention (Whyte, Shafer, & Williams, 2017). However, the RVIP task was rated at a similar level of difficulty as the serial 7s task. It is unclear why intervention with anthocyanin-rich blueberry should elicit improvement in serial 7s but not RVIP, as the tasks were well matched for cognitive load, with some overlap in cognitive domain. The RVIP task has previously shown sensitivity to flavonoid-rich intervention in young adult populations (Scholey et al., 2010; Watson et al., 2015). While Scholey et al. (2010) investigated flavanol-rich cocoa, Watson et al. (2015) observed maintenance of RVIP accuracy compared to control following a freeze-dried blackcurrant extract rich in anthocyanins. However in the same study, a juiced extract from a different cultivar did not elicit the same effect. Conversely, significant improvements in digit vigilance RT were observed following the juice but not the freeze-dried extract. Therefore it may be that different profiles of anthocyanins or other polyphenols also present, or indeed other components of the food matrix, may impact very specific cognitive domains via different mechanisms of action. For example, in the same experiment, the juice elicited altered postprandial glucose response and inhibited monoamine oxidase, whereas the freeze-dried extract showed neither effect. Due to cost implications, the exact polyphenol and anthocyanin profiles for the freeze-dried powder used here were not determined, but such analyses should be considered in future work.

The improvements to working memory following the highest doses of anthocyanin-rich blueberry are similar to the effects observed in Experiment 2 (Chapter 4); the observed small to moderate effect sizes compare favourably between the two thesis chapters and are in agreement with literature values. Similar effects on serial subtraction performance have been observed following flavonoid-rich intervention e.g. (Kennedy et al., 2002; Massee et al., 2015; Scholey et al., 2010) and are reported in Chapter 1. No significant differences in blood glucose were evident at the time of cognitive testing in the current study, suggesting that elevated glucose could not be a cause of the

observed cognitive effects. However, the two highest doses were observed to significantly attenuate the peak postprandial glucose response. Therefore, changes to blood glucose regulation may still, at least in part, underlie these working memory effects through other related mechanisms of action, for example through insulin effects. The co-regulation of insulin and glucose is particularly important when considering the impact of carbohydrate on cognitive function as it is insulin that facilitates the uptake of glucose to the brain e.g.(Gibson, 2007; Kullmann et al., 2016). Indeed, acute increases in insulin have been observed to improve cognition and vasoreactivity (Novak et al., 2014). Insulin was not measured for this thesis but should be included in future work. As discussed in the previous chapter, it has been demonstrated that foods which elicit a favourable postprandial glycaemic response are beneficial for cognition (Lamport, Lawton, et al., 2014), even at later time points where no blood glucose differences are apparent (Benton & Nabb, 2003; Lamport et al., 2011). Therefore the overall glucose (or insulin) profile may be important, not just the plasma glucose level at the time of cognitive testing. Indeed, at the 2 hour time point, where mean blood glucose fell below baseline for all dose conditions, effect sizes for improvements in serial 3s RT following the 517mg and 724mg doses were at their greatest. Further, irrespective of dose condition or time point, blood glucose concentration was observed to be a significant predictor of serial 3s score and approached significance as a predictor of mental fatigue in the current experiment. As marginal decreases in mental fatigue were also observed following the 517mg dose, there is some suggestion that increases in task performance may be facilitated by immediate availability of plasma glucose, perhaps affording protection against mental fatigue. Such a mechanism may be particularly relevant in cognitively demanding working memory tasks that are combined with a rapid speed of processing component, such as in the serial subtraction tasks used here. Indeed similar observations have been made in the literature (Massee et al., 2015; Scholey et al., 2010).

When considering the original hypothesis that cognitive benefits following blueberry intervention might be related to CBF increases, the results of this experiment echo my observations in Experiment 2 (Chapter 4) and do not appear compatible with the findings of Rodriguez-Mateos et al. (2013), where peak vasodilatory effects occurred following the 310mg anthocyanins dose. Therefore the outcome of this study casts further doubt on whether increased CBF following flavonoid-rich intervention may be the main mechanism for acute cognitive enhancement. In light of the discussed evidence relating to anthocyanin-facilitated improvements to blood glucose regulation (Chapter 5), it seems more likely that cognitive effects may arise from a synergy between increased CBF and improved glucoregulation. A well-powered intervention study similar to the one performed here, manipulating both flavonoid dose and glucose dose, and combining postprandial cognition and

blood glucose testing with FMD or fMRI analysis in a subset of participants, may help to confirm this hypothesis.

The observed mood effects, again identified through pairwise comparisons, compare favourably with the literature. For example, Khalid et al. (2017) observed improvements in positive affect, in young adults, 2 hours after a consumption of a blueberry dose containing 253mg anthocyanins. Increases in ratings of calmness have also been reported following anthocyanin-rich grape intervention (Haskell-Ramsay et al., 2017). Studies investigating interventions rich in other flavonoid subgroups have also shown similar effects on self-reported ratings of mood and mental fatigue e.g. (Boolani et al., 2017; Massee et al., 2015; Scholey et al., 2012). In the current study, a marginal reduction in positive affect, at 1 hour following the control condition, was attenuated following all three blueberry doses. The 517mg dose reduced mental fatigue, and interestingly, the highest (724mg) dose also significantly reduced negative affect; an effect not observed by Khalid et al. (2017). While a similar reduction in negative affect, following the 310mg dose in Chapter 4, may have been confounded by differences in the pleasantness of the doses, in this experiment no such differences were evident. Therefore it is unlikely that the observed effects on positive, or negative, affect here are simply due to the consumption of a pleasant beverage.

Positive and negative affect, as determined by PANAS, are distinct facets of mood. Low positive affect is an indicator of depression, whereas high negative affect is an indicator of anxiety (D. Watson et al., 1988), therefore different flavonoid mechanisms may be responsible for observed changes in either scale. Here, it has been posited that acute cognitive improvements following flavonoid-rich intervention are likely due to an interaction between improvements in blood glucose regulation and CBF. However, the underlying mechanisms for acute mood improvements may be more complex. Khalid et al. (2017) posit that glucose consumption may enhance uptake of tryptophan, a precursor of serotonin, to the brain. This insulin driven mechanism of action requires competing large neutral amino acids to be selectively transported into peripheral tissue, increasing the ratio of circulating tryptophan available for transport across the blood brain barrier. However this has been reported not to occur if glucose is consumed with even small levels of plant or animal protein, which are naturally low in tryptophan compared with other amino acids and thereby oppose any increase in the ratio of circulating tryptophan (Benton & Donohoe, 1999; Benton & Nabb, 2003; Gibson & Green, 2002). Therefore this mechanism is unlikely to be responsible for the blueberry influence on negative affect observed here. Increased availability of glucose has also been associated with a reduction in self-reported tension (Benton & Owens, 1993). The mechanism remains unclear but is again suggestive of an impact on negative affect. Falling glucose levels during

cognitively demanding tasks have been associated with increases in mental fatigue (Owens, Parker, & Benton, 1997), therefore flavonoid induced improvements in glucoregulation may afford protection against mental fatigue, thereby attenuating a dip in mood. Blood glucose effects are likely, then, to contribute to flavonoid induced mood effects. But other flavonoid related mood mechanisms have been observed. For example, anxiolytic effects have been observed via flavonoid interaction with GABA receptors, leading to the upregulation of the inhibitory neurotransmitter GABA (Hanrahan, Chebib, & Johnston, 2011; Wasowski & Marder, 2012). Anthocyanins have also been observed to inhibit monoamine oxidase (MAO) (Watson et al., 2015), resulting in the upregulation of monoamine type neurotransmitters such as serotonin and dopamine. These mechanisms were not tested in the current study, but may have contributed to the observed mood effects, and therefore warrant further investigation.

When considering the varying physiological, cognitive and mood effects observed in this experiment following different blueberry doses, it should be noted that the doses administered were from a batch of blueberry powder different from that used previously, both in earlier chapters of this thesis and in the Rodriguez-Mateos et al (2013) study. The lowest blueberry dose containing 310mg anthocyanins elicited no glucose, mood or cognitive effects in the current study. Although the dose contained a level of anthocyanins comparable to previous research, which has shown significant cognition or mood effects (Khalid et al., 2017; Whyte & Williams, 2015; Whyte et al., 2016), the total polyphenol content of this dose (473mg total polyphenols) was lower than that found in the equivalent dose (766mg polyphenols) for which a peak vasodilatory response had been previously observed (Rodriguez-Mateos et al., 2013). It is possible that anthocyanins may not be effective in isolation; other polyphenols also present may be a contributory factor in improvements to vasodilation, cognition and mood. However, the 310mg dose did not elicit any cognitive effects in Experiment 2 (Chapter 4) either, when the previous batch of powder was used. Negative affect was reduced following this dose in Chapter 4, but this outcome may have been influenced by taste differences rather than flavonoid effects. Therefore, irrespective of total polyphenol content, the 310mg dose appeared relatively ineffective in the current research. Studies using extracts of anthocyanins may be more helpful in isolating their specific benefits, in the absence of other polyphenol confounds.

In the previous investigation of glycaemic response (Chapter 5), extended availability of blood glucose was observed up to 2 hours postprandially following the 724mg dose and up to 90 minutes following the 310mg dose. In that experiment the sugar matched doses and control all contained 40g sugar; substantially higher than the 26.7g in the current experiment. Indeed, an additional

difference between this study and the previous literature is the considerably lower availability of sugars or more complex forms of carbohydrate. In similar flavonoid-rich intervention studies reviewed in Chapter 1 (Dodd, 2012; Field, Williams, & Butler, 2011; Haskell-Ramsay et al., 2017; Khalid et al., 2017; Lamport et al., 2016; Scholey et al., 2010; Watson et al., 2015), this value ranged from 19.9g-55g, and in all cases further forms of carbohydrate were introduced through the provision of standardised breakfasts and lunches or the addition of cordials to improve intervention taste. Therefore the overall carbohydrate consumption in these studies was much greater than in the current study. Given the known effects of flavonoid-rich foods on postprandial blood glucose response, it is possible that in all of these studies blood glucose levels remained elevated for longer periods in the flavonoid-rich conditions compared with the control conditions, due to the abundant availability of glucose. Indeed this was evident in the Watson et al (2015) study, where blood glucose remained significantly elevated up to 2.5 hours after a blackcurrant juice extract. In Experiment 2 (Chapter 4) cognitive testing did not take place at the later time point when differential glucose concentrations were subsequently observed (Chapter 5). However, it is possible that cognitive effects at later time points may be more likely to occur when flavonoid-rich foods are consumed with higher amounts of glucose, due to increased availability combined with facilitated uptake of glucose. None of the other studies reviewed in Chapter 1 recorded blood glucose during cognitive test sessions so this cannot be confirmed as a potential mechanism of action, but nevertheless, warrants further investigation.

When interpreting the cognitive data presented here, it is important to recognise that the dependent variables for each cognitive task (score, errors, RT) are intrinsically related. A limitation of the type of analysis performed here is that each dependent variable is analysed separately. Therefore greater weight should be given to the findings that extend across two or more of these variables. Where only one variable is affected, there may be a greater likelihood of type 1 error. Some of the isolated cognitive effects observed here, such as the worsening performance for RVIP and digit vigilance, may indeed be false positives and should be interpreted with caution. Nevertheless, the effects on working memory appear consistent across scores, errors and RTs, and are in line with previous research. Future research should seek to develop ways of combining related dependent variables in their analyses. Currently available methods such as hits minus false alarms, summation of z scores, or inverse efficiency scores (expressing accuracy as a function of RT) often suffer from a lack of statistical power due to increased variance in the combined data (Bruyer & Brysbaert, 2011). Such methods were, therefore, considered inappropriate for the current work in light of the already considerable variance within the data that resulted from the within-subjects design incorporating multiple conditions and time points.

In conclusion, only limited dose-dependent cognition and mood effects were apparent, as identified through Bonferroni corrected pairwise comparisons. With respect to the research questions, the highest blueberry dose (containing 724mg anthocyanins) has shown a small modulating effect on mood and cognition, and the second highest dose (517mg) has shown effects on cognition, whereas the lowest dose (310mg) has not impacted either domain. This is clearly suggestive of a dose dependent effect that may be related to the concurrently observed effects on blood glucose regulation, and may also relate to the previously observed effects on vasodilation (Rodriguez-Mateos et al., 2013). Indeed a synergy of the two effects offers a plausible mechanism for improved cognition as discussed in Chapter 5. Mood effects may be less straightforward. As discussed above, other potential mechanisms of action such as monoamine oxidase inhibition and GABA upregulation have not been investigated here but may be responsible for observed mood effects. Finally, although dose-dependent effects were evident, it remains unclear whether the anthocyanin dose or the total polyphenol dose was the determining factor for the observed effects on either cognition or mood.

Chapter 7
Final Discussion

7.1 Summary of findings

Following recent interest in the maintenance of health and wellbeing through dietary intervention, the acute cognitive and mood benefits of wild blueberries were investigated in a young adult population. The main thesis aims were to determine whether cognition and mood effects following wild blueberry supplementation were dose dependent, and whether modulation of blood glucose regulation was a likely mechanism of action. The cognitive, mood, blood pressure, and blood glucose effects of various wild blueberry doses, with known postprandial metabolite and FMD response profiles, were investigated across three separate experiments, each using a crossover design. Key findings included dose-dependent cognitive benefits to episodic memory, working memory, and mood. In addition, dose-dependent effects on postprandial blood glucose regulation were observed, providing evidence for a possible mechanism of action. Indeed, in the case of working memory, blood glucose at the time of cognitive testing was found to be a significant predictor of performance. The outcome of each experiment is discussed in more detail below.

7.1.1 Experiment 1

All cognitive tasks were first piloted in Experiment 1 in order to determine appropriate design strategies for minimizing cognitive practice effects. Methodological recommendations following the outcome of Experiment 1 were that participants should attend a familiarization visit on a separate occasion prior to data collection, and that well-correlated alternate task forms should be used at each test point, with familiarization trials also included immediately before each period of data collection. Visit order was also included as a factor in the subsequent analysis of all cognitive data in order to statistically model any residual practice effects, thereby accounting for some of the variance in the data that may otherwise have been attributed to intervention effects.

7.1.2 Experiment 2

Following on from Experiment 1, an investigation into the effects of wild blueberry on cognitive function was performed using the previously piloted cognitive tasks. In Experiment 2, a double-blind, crossover intervention study compared the effects of five separate wild blueberry doses (containing 129mg, 258mg, 310mg, 517mg, and 724mg anthocyanins) with a sugar-matched control. Measures included cognition, mood, blood pressure and heart rate. Testing was performed at baseline, and 1

hour postprandially. No significant blood pressure effects were observed for any of the doses when compared with the control. However, consumption of the highest doses (517mg & 724mg) appeared to convey small to medium protective benefits for the maintenance of a constant heart rate. Following the control and 258mg blueberry dose heart rate was observed to drop significantly. Heart rate is strongly correlated with blood glucose and so the attenuation of postprandial decline in heart rate following these doses is suggestive of a dose-dependent glucoregulatory effect. The same doses similarly showed maintenance of immediate recall memory score, and improvements in serial 7s performance for both score and reaction time. The 310mg dose elicited small to medium benefits in mental fatigue and mood, although these findings may have been confounded by taste differences between the intervention doses. The lower (129mg & 258mg) doses showed no consistent domain specific effects for cognition or mood.

7.1.3 Experiment 3

Based on the physiological and cognitive observations from Experiment 2, and the probable link between postprandial heart rate and blood glucose availability, it was deemed necessary to investigate the underlying glucose response for the doses that elicited heart rate, cognition and mood effects. Therefore in Experiment 3, the impact of anthocyanin-rich wild blueberry on postprandial glucose response was investigated for two of the doses used in Experiment 2. Blueberry doses containing 310mg and 724mg anthocyanins were administered to participants, in both sugar-matched and no-added-sugar conditions. Plasma glucose was determined by a capillary sampling method at baseline and at regular intervals up to 2.5 hours postprandially. The results demonstrated that wild blueberry significantly extended the postprandial glycaemic response compared with the equivalent sugar dose in the absence of blueberry. Indeed, blood glucose levels remained significantly elevated above baseline for 2 hours following a blueberry dose containing 724mg anthocyanins, and for 1.5 hours following a lower 310mg dose. These post-peak elevations were in the range of 0.5-1.5mmol/l above fasting baseline which is well within the healthy postprandial blood glucose range (American Diabetes Association, 2014) and is metabolically beneficial through the avoidance of reactive hypoglycaemic episodes (Willett et al., 2002) where glucose level falls close to or below fasting levels. Furthermore, blueberry was observed to reduce peak postprandial glucose levels, although statistical significance was not achieved. The results were suggestive of a possible glucoregulatory mechanism of action for the observed heart rate, cognition, and mood effects in Experiment 2, through extended availability of blood glucose in the blueberry conditions. Indeed, at the time of cognitive testing in the previous experiment (1 hour postprandially), blood glucose remained significantly elevated above baseline following the 310mg and 724mg sugar-

matched doses, but not following the sugar–matched control. However, apparent blood glucose differences between each of these three matched conditions did not reach statistical significance at the 1 hour time point. This may be due to a lack of statistical power as this was a small study, but also suggests that anthocyanin-rich blueberries may not facilitate improvements to cognition solely through availability of glucose, but also through other related mechanisms such as insulin regulation leading to increased efficiency of glucose uptake. Clear differences in glucose availability were evident at later time points providing a rationale for the investigation of cognitive effects beyond 1 hour.

7.1.4 Experiment 4

As Experiment 3 revealed extended availability of blood glucose following anthocyanin-rich blueberry, a repeat of the cognitive investigation performed in Experiment 2 was carried out, but with the addition of a later testing time point at 2 hours postprandially. A modified cognitive task battery was also administered to investigate whether glucose differences at this later time point would elicit effects not previously measured by the cognitive battery from Experiment 2. Specifically, a greater emphasis was placed on attention as both glucose and flavonoid interventions have been independently reported to impact attention. Therefore in Experiment 4, a double-blind, crossover intervention study compared the effects of three separate wild blueberry doses (containing 310mg, 517mg, and 724mg anthocyanins) with a sugar-matched control. Measures included cognition, mood, and postprandial glucose response. Testing was performed at baseline, and 1 and 2 hours postprandially. The wild blueberry doses containing 517mg and 724mg anthocyanins elicited significant improvements in working memory at 1 and 2 hours, as measured by the serial subtraction task. Following the 724mg dose, participants also reported a significant reduction in negative affect. Both the 517mg and 724mg doses significantly attenuated the peak postprandial glucose response, when compared to the control, at a time point of 15 minutes. However, blood glucose levels following wild blueberry intervention were not found to be significantly different from those observed following the control at either time of cognition and mood testing. The 310mg dose produced no significant cognitive, mood or glucose effects. Interestingly, this dose was previously observed to elicit the greatest vasodilatory response when measured for the same range of wild blueberry doses. The findings suggest that benefits to cognition and mood following wild blueberry supplementation may be related to concurrent alterations in blood glucose regulation. Indeed, irrespective of experimental condition, blood glucose level was observed to be a significant predictor of serial subtraction performance. A similar observation was made by Kennedy & Scholey (2000) during a glucose intervention, where serial 7s performance correlated with blood glucose level.

7.2 General discussion

In this section the cognitive and physiological effects of anthocyanin-rich blueberries reported above are compared with the literature. Potential mechanisms of action are discussed in relation to the thesis hypotheses.

7.2.1 Cognitive effects

The episodic memory effects observed in Experiment 2 are consistent with the findings of Whyte et al. (2016), where increased immediate recall was observed at 1.25 hours postprandially in children. Here the same effect has been demonstrated in young adults. The effect size appears somewhat diminished in the young adults (d=0.29) compared with the children (d=0.80). Indeed, in the children immediate recall improved between baseline and test following blueberry intervention, whereas in the young adults blueberry merely attenuated a decline in performance. The differences in effect may reflect developmental differences in the capacity for improvement. Interestingly, in the child study it was the higher of two blueberry doses (253mg anthocyanins) that elicited a significant effect. In young adults, effects were similarly observed following the higher blueberry doses (517mg and 724mg), although the 129mg dose did also elicit an immediate recall effect. In general however, there is some evidence for dose-dependency with higher doses being more effective. Differences in the effective dose sizes between the two studies may simply reflect body mass differences between the different age groups. Indeed, for an average child weighing 27.5kg (based on NHS growth curve data), the effective dose in Whyte et al. (2016) was calculated to be 9.2mg/kg body weight. For a 60kg young adult this equates to an anthocyanin dose of 552mg, which is comparable to the 517mg and 724mg doses that were observed to elicit significant effects in Experiment 2.

The working memory effects observed in Experiments 2 and 4 are consistent with the findings of Scholey et al. (2010), Massee et al. (2015), and Kennedy et al. (2002), who observed serial 3s and serial 7s improvements following acute doses of cocoa and ginkgo in young adults. However this is the first study to demonstrate a similar effect following anthocyanin-rich blueberry. Again, the effect sizes are smaller in this thesis (d=0.02-0.42) compared with the previous work (d=0.47-0.56). As with cocoa, significant effects following blueberry were evident at 1 - 2 hours postprandially, whereas ginkgo effects occurred at a later time point of 6 hours, reflecting differences in the absorption and metabolism rates of the different flavonoid subclasses present. A notable difference between the previous and current work is that effect sizes for blueberry were clearly dose dependent; in Experiment 2 effect sizes were largest following the 724mg dose and no significant effects were observed for doses of 310mg or lower. In Experiment 4 the largest effect sizes were observed

following the 517mg dose, but were still evident following the 724mg dose. Again, no significant performance improvements were evident following the 310mg dose. However, fewer doses were investigated in the previous literature and the flavonoid profiles of cocoa and ginkgo are very different to blueberry, making direct dose comparisons difficult.

Previous literature on acute mood changes following flavonoid-rich intervention is limited. However the effects on positive and negative affect observed in Experiments 2 and 4 are broadly in agreement with recent published data. Khalid et al. (2017) observed significant improvements in positive affect following a single blueberry dose containing 253mg anthocyanins in both children and young adults, and Boolani et al. (2017) observed a reduction in anxiety in young adults following cocoa. The outcomes of these two studies suggest that flavonoid-rich interventions can impact different facets of mood; depression or anxiety. In the current thesis the findings of Experiment 2 revealed a reduction in negative affect following the 310mg dose only. This observation may have been confounded by significant taste differences between doses, but in Experiment 4, where taste differences were minimal, maintenance of positive affect was observed for all blueberry conditions when compared with a marginally significant drop in the control condition, and a significant reduction in negative affect was observed following the 517mg and 724mg doses. The findings suggest that, at varying doses, anthocyanin-rich blueberries may exert effects on both anxiety and depression scales of mood.

7.2.2 Physiological effects

Experiment 2 failed to replicate the reductions in blood pressure observed following acute supplementation with cherry juice (Keane et al 2016). Therefore, it may be that different flavonoid-rich fruits elicit different cardiovascular effects due to differences in their flavonoid profile. Differences in flavonoid composition are considered in the limitations section below. However despite the lack of a blood pressure effect, constant heart rate was maintained following the 517mg blueberry dose, despite a significant drop following the control. To a lesser degree this decline in heart rate was also attenuated following the 129mg, 310mg and 724mg blueberry doses. This effect was unexpected as similar findings have not been previously reported in the literature, but is suggestive of a glucoregulatory effect as heart rate is correlated with glucose availability (Valensi et al., 2011). Indeed, when postprandial glucose response was subsequently investigated in Experiments 3 and 4, significant differences in glucose profile were observed between the control and blueberry conditions. Similar glucoregulatory effects have been previously observed for a number of different anthocyanin-rich berry interventions e.g. (Törrönen et al., 2010; Watson et al., 2015; Wilson et al., 2008). Heart rate and blood glucose were not recorded concurrently during any

of the experiments carried out during this thesis. On reflection it may have been helpful to do so in Experiment 4. Nevertheless, evidence of the interaction between glucose, heart rate and cognition has previously been reported following direct supplementation with glucose (Kennedy & Scholey, 2000).

7.2.3 Mechanisms of action

Combined, the cognitive findings of each of the blueberry intervention experiments reported in this thesis support the hypothesis that wild blueberry effects on cognition are dose-dependent, with doses containing 517mg and 724mg anthocyanins eliciting the greatest cognitive effects. However, contrary to expectation, the most effective doses were higher than the 310mg dose previously observed to elicit the greatest vasodilatory response by Rodriguez-Mateos et al. (2013). No vasodilatory measures were recorded during any of the current experiments; vasodilatory response was inferred from the previous research, using the same source of blueberry powder in participants of a similar age. Nevertheless, the results suggest that other mechanism(s) may play a role in improvements to cognition. Indeed, it was hypothesised that dose-dependent blood glucose effects would likely contribute to the cognitive outcome of the study through a mechanism of increased availability of glucose to the brain. Few cognitive studies have measured blood glucose to date, however Watson et al. (2015) demonstrated enhanced cognition and concurrently elevated blood glucose following an anthocyanin-rich berry intervention. As reported in Chapter 5 there are a number of mechanisms whereby flavonoid interventions, and in particular those rich in anthocyanins, have been shown to impact glucoregulation, supporting the idea that flavonoid intervention may improve availability or efficiency of glucose uptake by the brain during cognitive exertion. This hypothesis was supported in Experiment 4, where cognitive performance and blood glucose were measured concurrently, and the 517mg and 724mg doses elicited significant modulation of the postprandial blood glucose profile and significant improvements to working memory and mood, whereas the 310mg dose did neither. In the same experiment, blood glucose level was also observed to be a significant predictor of working memory performance irrespective of dose condition, providing further evidence that cognitive outcome is dependent on the availability of glucose. Improved glucoregulation did not appear to facilitate global increases in cognition as there were no significant effects observed for the RVIP or digit vigilance tasks. Observed effects were clearly domain specific, relating to episodic memory, working memory, and mood. The availability of extracellular glucose and improved glucoregulation have been associated with improved episodic memory and working memory (for a comprehensive review see Smith, Riby, Eekelen, & Foster, 2011). There is some evidence that hippocampus function may be selectively enhanced by glucose,

possibly due to the increased number of insulin receptors in this area of the brain (Smith et al., 2011). There is also some evidence that glucose facilitates the synthesis of acetylcholine and influences the action of other neurotransmitters including dopamine and serotonin (Benton & Nabb, 2003; Benton et al., 1994). These neurotransmitter effects have the potential to facilitate global cognition and mood improvements such as the serial subtraction and PANAS effects observed in this thesis. However, it remains unclear why such mechanisms should not also impact attention. No consistent attention effects were observed on any of the tasks deployed in this work, although other authors have reported such actions e.g. (Haskell-Ramsay et al., 2017; Kennedy et al., 2000; Scholey et al., 2010; Watson et al., 2015). A possible explanation may be that the task versions used here were not cognitively demanding enough; the most robust effects of glucose have been observed during divided attention or at times of high cognitive demand (Benton & Nabb, 2003; Scholey et al., 2001; Smith et al., 2011). Although improved glucoregulation appears a viable mechanism of action for the cognition and mood effects observed throughout this thesis, it is possible that such a glucoregulatory mechanism of action may occur in synergy with increased cerebral blood flow. Increased CBF is a much posited mechanism for cognitive improvements following flavonoid-rich interventions. However, it should be noted that neither CBF nor other potential mechanisms of action, such as MAO inhibition or BDNF synthesis, have been directly investigated here, and require further study.

7.3 Limitations of the research

7.3.1 Experimental confounds

7.3.1.1 Practice effects

Methodology was specifically tailored to minimise cognitive practice effects after an investigation of the size and distribution of these effects was carried out in Experiment 1. However previous research suggests that it is impossible to completely eradicate practice effects and so a low level of confound is still likely to have remained during the two blueberry intervention studies. Indeed practice related improvements were evident in all experiments, as identified by the significance of visit number as a predictor of cognitive performance for the majority of tasks. Residual practice effects are likely to have increased the variance in each counterbalanced dose condition, thus reducing the statistical power of each experiment to detect small changes in cognition and mood. Therefore, residual practice effects are likely to have impacted the statistical significance of the session x dose interaction term when employing conservative LMM analyses of cognition and mood in Experiments 2 and 4. The problem was overcome by employing Bonferroni corrected pairwise

comparisons as a less conservative alternative to the F test interaction (Huck, 2015). Previously published crossover studies with multiple conditions appear to have adopted a similar analysis strategy e.g. Kennedy et al. (2000) and Kennedy et al. (2002). Replication is important when judging the validity and reliability of a statistical approach (Keppel, 1991). Working memory and mood effects evident in Experiment 2 were replicated in Experiment 4, further demonstrating the suitability of this statistical strategy.

7.3.1.2 Habitual diet

The interfering effects of habitual diet were addressed by asking participants to follow a low polyphenol diet for 24 hours before participation, to eat the same breakfast on each visit date, and fast for 2 hours immediately prior to baseline testing. Compliance was recorded using self-report. The presence of a few minor misdemeanours in these reports suggests that participants were honest in their recording of dietary intake. No attempt was made to quantify differences in the intake of any prohibited items between participants or between visits; rather any deviance from the diet was immediately discussed with the participant to ensure that they would not repeat the mistake, and therefore further minimise any interference from polyphenol consumption other than the test intervention. Tighter controls on diet could have been implemented. For example a standardised evening meal could have been provided for all participants on the day before testing, along with a standardised breakfast across all participants (rather than within-participants) on the morning of testing. This may have further reduced any dietary confound. Standardisation of food intake may therefore have helped to reduce variance in the data. However, if such measures are required to detect the acute cognitive effects of flavonoids, then observed effects may arguably have little real world relevance. As such, there is a counter argument for conducting studies with no dietary restrictions.

7.3.1.3 Taste matching of doses

An additional confound was that the intervention drinks were not always well matched in terms of taste. Variations in pleasantness may have influenced mood ratings in Experiment 2 in particular, as palatability has previously been observed to influence negative affect (Benton, 2002; Macht & Mueller, 2007). Drink ratings were more similar in Experiment 4 following the use of a different batch of wild blueberry powder, but further improvements could be made, for example through the addition of flavourings and thickeners to better match both taste and texture of all interventions. There were also notable hydration differences between the two cognitive studies. In Experiment 2, drinks were mixed using 500ml water, whereas in Experiment 4 this volume was reduced to 300ml in

order to make the drinks easier to consume within a shorter time period. Hydration has been previously shown to influence cognition and mood (Masento, Golightly, Field, Butler, & van Reekum, 2014) and so minor differences between the study outcomes could, at least in part, be due to hydration differences. However, the task batteries for the two studies were different making direct comparison difficult.

7.3.1.4 Presence of other polyphenols

In this thesis, wild blueberry doses were quantified in terms of their anthocyanin content. Anthocyanins form the largest group of flavonoids, and indeed polyphenols, in wild blueberries. It was considered appropriate to quantify the doses in this way as anthocyanins consumption has specifically been linked to vasodilatory (Bell & Gochenaur, 2006) and glucoregulatory (Cazarolli et al., 2008; Hanhineva et al., 2010; Sancho & Pastore, 2012) mechanisms of action in vitro, and has been linked with improved cognition in vivo (Rendeiro et al., 2013). Epidemiological studies have also linked anthocyanins to reduced incidence of type 2 diabetes (Jennings et al., 2014; Wedick et al., 2012). The Oxygen Radical Absorbance Capacity (ORAC) of blueberry also correlates strongly with anthocyanins (Howard, Clark, & Brownmiller, 2003; Lohachoompol et al., 2004; Zheng & Wang, 2003). However, it should be noted that other polyphenols are also present in moderate quantities and so the effects observed in this thesis cannot solely be attributed to anthocyanins and should be considered as blueberry effects. Human supplementation with pure extracts would be needed to confirm if acute benefits to cognition and mood were attributable to anthocyanins in isolation. However, pure extracts are costly and arguably unnecessary given that blueberries provide a relatively cheap and convenient way to consume beneficial quantities of anthocyanins and other polyphenols within a normal healthy diet.

7.3.1.5 The role of gut microbiota in the biotransformation & metabolism of polyphenols

As described in Chapter 1, notable individual differences have been observed in the absorption profiles of a range of flavonoids and their associated metabolites (Bresciani et al., 2017; Mennen et al., 2008; Rodriguez-Mateos et al., 2015); not only in the types and quantities of metabolites present, but also in the timings of their appearance (Bresciani et al., 2017). Recent research has attributed many of these individual differences to gut microbiota; specific bacteria have been identified that act on different flavonoids (Braune & Blaut, 2016). Individuals have varying polyphenol metabolising phenotypes, largely dependent on their gut microbiota profile (Tomás-Barberán et al., 2016). The effectiveness of a flavonoid intervention is therefore dependent on the phenotype of the subject; polyphenol biotransformation and metabolism by microbiota in the gut is

pivotal to the subsequent biological activity of an intervention. If the relevant bacteria are not present then the intervention may show little or no effect at postprandial time points where the ingested intervention has reached the large intestine. Interestingly, a flavonoid-rich diet can have a positive impact on the profile of gut microbiota (Cardona et al., 2013; Hidalgo et al., 2012; Valdés et al., 2015), and so subjects who regularly consume flavonoids are likely to produce greater quantities of biologically active metabolites, and may therefore experience greater cognitive health benefits in the immediate postprandial period following their consumption, although this has not yet been investigated. Therefore it is important to identify the gut microbiota phenotype of participants. Small effect sizes may simply be due to the diluting effect of participants with a mismatched phenotype, for whom little or no flavonoid effects are likely to be observed. The young adult student participants in this research reportedly consumed around 4-5 daily portions of fruit and vegetables, suggesting a relatively healthy lifestyle. However, this was a self-report measure and appears very close to the '5-a-day' Government target, so reporting may have been influenced by demand characteristics. If participants routinely consumed below this level, then this may explain the small cognitive and mood effect sizes observed here. All of the experiments in this thesis used a within-subjects design in order to minimise the impact of individual differences such as gut microbiota, but phenotypes were not determined. In future research, studies may benefit from the assessment of gut micriobiota phenotype so that its impact can be systematically investigated.

7.3.2 Methodology

7.3.2.1 Population

The data collected throughout this thesis relate to healthy young adults. The data cannot therefore be generalised to older or younger populations, or those with clinical conditions. In order to fully understand the acute effects of anthocyanin-rich blueberries it is recommended to extend this work to other age ranges and clinical populations who may further benefit from acute blueberry supplementation.

7.3.2.2 Physiological measures

Physiological measures that were recorded during this research were limited to blood pressure and blood glucose. The absorption, metabolism, and vasodilatory response profiles following varying doses of wild blueberry were not measured directly but were inferred from previous research conducted by Rodriguez-Mateos et al. (2013); therefore the study would have been strengthened by the inclusion of these measures. Similarly, cerebral blood flow was not measured; again, peripheral and cerebral vasodilation was assumed from the published observations of Rodriguez-Mateos et al.

(2015) and Dodd (2012). A methodological strength of Experiments 2 and 3 was that the intervention drinks were prepared using the same batch of freeze-dried blueberry powder as used by Rodriguez-Mateos and colleagues, so metabolism and vasodilation profiles were likely to be similar. However in Experiment 4 a new batch, still from the same cultivar, was used. Therefore, it is unknown whether the same underlying response occurred in this final experiment. It should also be noted that individual differences, for example in gut microbiota, may have altered absorption, metabolism and vasodilatory response profiles or effect sizes throughout this research, when compared with previous work. Any differences may potentially have reduced the statistical power of this series of experiments to observe cognitive changes resulting from direct or indirect metabolite effects.

With respect to the measurement of glucose regulation, only blood glucose values were recorded. However, blueberry-induced changes to the corresponding insulin response may also have influenced the availability of glucose in these experiments, for example through facilitated uptake of glucose by cells. Indeed, as described in Chapter 5, insulin effects have previously been observed following anthocyanins-rich berry interventions (Alqurashi et al., 2016; Edirisinghe et al., 2011; Nyambe-Silavwe & Williamson, 2016). Future flavonoid research should consider the interaction between postprandial glucose and insulin response, and how this relationship may influence cognitive outcome.

7.3.2.3 Cognitive measures

Although a range of cognition, mood and memory tasks were deployed in Experiments 2 and 4, it should be noted that significant outcomes relate only to performance on those specific tasks. It is not valid to generalise these effects to other related tasks or domains.

7.3.2.4 Compositional differences between blueberry powder batches

Two different batches of freeze dried blueberry powder were used during the course of this research. The total anthocyanin content was matched between studies; however there remained considerable differences in the total polyphenol content. There were also considerable differences in the vitamin C content of the two batches. It is unclear whether vitamin C differences arose from actual differences between the batches or differences in the analytical techniques used for each assay. The analysis of the second batch (used in Experiment 4) was carried out using a reducing agent to convert dehydroascorbic acid (DHA), the oxidised form of ascorbic acid, into ascorbic acid prior to determination. This method is commonly used for the determination of vitamin C in fruits and vegetables (Campos, Ribeiro, Della Lucia, Pinheiro-Sant'Ana, & Stringheta, 2009) as DHA is

known to be a biologically available and active form of vitamin C (Wilson, 2002). However the same method was not used in the analysis of the first batch (used in Experiments 2 & 3). This may have resulted in inconsistencies in the vitamin C matching of blueberry and control drinks. Regrettably, fibre was not quantified for the first batch of blueberry powder making direct comparison between batches impossible. It is unlikely that the total fibre content differed greatly between the two batches; however after anthocyanin-matching between experiments, differences in the total fibre content of the intervention drinks may have been introduced.

Anthocyanin profiling was not performed for either batch of blueberry powder used in this thesis. Anthocyanins are a group of compounds with similar structural properties. There are reportedly more than 600 unmodified anthocyanidin and modified anthocyanin compounds (Babu, Liu, & Gilbert, 2013). It is possible that these compounds may exert different physiological and cognitive effects. For example, in vitro glucose stimulation of insulin secretion was reported to be augmented by pelargonidin-3-galctoside, cyanidin -3-glucoside, delphinidin-3-glucoside, and marginally by delphinidin, whereas cyanidin, pelargonidin, malvidin and petunidin showed no effect (Jayaprakasam, Vareed, Olson, & Nair, 2005). Additionally, as described in Chapter 1, Watson et al. (2015) observed different cognitive and physiological outcomes following two blackcurrant cultivars with different polyphenol and anthocyanin profiles. Therefore anthocyanin profiles for a particular intervention may be important in determining cognitive and/or physiological outcomes.

Observed differences in the outcomes of Experiment 2 and Experiment 4 in this thesis reflect differences in the study design, such as changes to the task battery and the addition of an extra testing time point. For the cognition and mood tasks in common between the two experiments, the outcome was very similar. Blood glucose response profiles were quite different between Experiment 3 and Experiment 4, but as identical cultivars were used for all experiments it is likely that these differences were due to variation in sugar content rather than anthocyanin profile. Nevertheless, polyphenol, vitamin C and fibre differences between the experiments, as described above, may have contributed to the outcome. With hindsight, it may have been methodologically preferable to match the sugar, fibre and vitamin C content across studies in order to better control for these differences. Future work should seek to better define the full compositional profile, including anthocyanin subclasses present in any intervention, and control for as many compositional elements as possible.

7.4 Future work

Now that this work has identified that higher doses of anthocyanin-rich blueberry (517mg & 724mg) appear the most effective doses in terms of mood and working memory outcomes, the natural

progression would be an acute intervention trial focussing on a single similar dose, with the aim of obtaining sufficient statistical power to confirm a significant dose x session interaction when comparing the blueberry dose with a well-matched control (e.g. matched for flavour, colour, viscosity, sugars, protein, vitamin C and fibre) at pre- and post-intervention time points. It would also be desirable to concurrently measure postprandial blood glucose and insulin, and to profile participants' gut microbiota. Measurement of vasodilatory response should also be considered, but may require testing of a subgroup of participants as there are inherent methodological difficulties in recording cognition and vasodilation simultaneously. A series of such studies might further investigate the impact of age, gender, health status, cognitive domain, or cognitive load with respect to acute wild blueberry intervention. Well-powered studies, such as those described, may provide a fuller understanding of the acute cognitive benefits of wild blueberry and their likely mechanisms of action.

The observed effects on blood glucose regulation strongly suggest that wild blueberry may have a clinical application in the prevention or treatment of metabolic disorders such as type 2 diabetes. As discussed in Chapter 5, the postprandial response to ingested carbohydrate is recognised as a marker of metabolic health. Interventions such as wild blueberry which modulate postprandial glucose by limiting periods of hyper- or hypoglycaemia are desirable. In type 2 diabetes, regulation of postprandial glucose response is important not only in the prevention and treatment of the disease, but also in the reduction of associated risk factors such as cardiovascular disease (Bonora & Muggeo, 2001). Cognition, blood pressure and vasoreactivity are all reportedly compromised in type 2 diabetes (Chung et al., 2015; Lamport et al., 2009) and, as reviewed in Chapter 1, these areas have all shown improvement following acute anthocyanins-rich interventions. Furthermore, habitual diets rich in anthocyanins, and particularly blueberry consumption, are significantly associated with lower type 2 diabetes mellitus risk (Jennings et al., 2014; Wedick et al., 2012). The combined evidence suggests there is clear potential for a diet-based blueberry intervention to benefit the risk factors and co-morbidities associated with type 2 diabetes. This should be further investigated through intervention trials on a clinical population of type 2 diabetes patients, or those with pre-diabetes.

7.5 Final conclusions

With some statistical caveats, this programme of research is the first to demonstrate a dose-dependent effect of anthocyanin-rich wild blueberries on episodic memory, working memory and mood. In particular effects appear strongest for the maintenance of immediate recall on a single-trial word list learning task, the attenuation of negative affect using a self-report questionnaire, and the

improvement of working memory on a serial subtraction task combining a high cognitive load with a psychomotor component. However, in order to increase the statistical power, allowing future studies to detect these small cognitive changes following flavonoid-rich intervention, additional controls for the reduction of variance in collected data may be needed. In particular, research is recommended into the mediating effect of gut microbiota on the effectiveness of acute wild blueberry supplementation.

This research is also the first to indentify a dose-dependent glucoregulatory effect of wild blueberries that may provide a plausible mechanism of action for observed cognitive and mood benefits, through extended glucose availability and/or facilitated uptake of glucose to the brain. Therefore, future research should consider the potential application of wild blueberry as a treatment or preventative intervention for metabolic disorders such as type 2 diabetes, where both cognition and glucoregulation are typically impaired.

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Appendices

Appendix A Ethical approval for Experiments 1-4

A.1 SREC Ethics committee approval for Experiment 1.1

From: Philip T. Smith [p.t.smith@reading.ac.uk]

Sent: 19 March 2014 12:28

To: claire.williams@reading.ac.uk

Cc: l.a.forrest@reading.ac.uk

Subject: Ethics 2014/033/CW

Dear Claire

Ethics proposal: Examination of the occurrence of practice effects in the Sternberg task and other cognitive tasks commonly used in crossover dietary intervention studies.

I have been asked to look at this proposal. This project is in line with University of Reading ethics guidelines, and may proceed.

Regards

A.2 SREC Ethics committee approval for Experiment 1.2

From: **Philip T. Smith** <p.t.smith@reading.ac.uk>

Date: Thu, Dec 3, 2015 at 10:41 AM

Subject: Ethics 2014/033/CW

To: Lynne Bell <l.bell@pgr.reading.ac.uk>, Claire Michelle Williams <claire.williams@reading.ac.uk>

Cc: PCLS Ethics <pclsethics@reading.ac.uk>

Dear Claire and Lynne

Thank you for the request to revise this project. This project is in line with University of Reading ethics guidelines, and may proceed.

There is just one detail: it is not clear to me whether Emma and Nasser are undergraduates, and whether they will conduct any of the testing without any more senior member of the School being present. If so, they need to sign the consent form, beneath each participant's signature. But I can rely on you to make these adjustments, if they are necessary: I don't need to see the application again.

Regards

Philip

A.3 UREC Ethics committee approval for Experiment 2

Coordinator for Quality Assurance in Research
Dr Mike Proven, BSc(Hons), PhD
Office of the University Secretary
Whiteknights House
Whiteknights, PO Box 217
Reading RG6 6AH
phone +44 (0)118 378 7119
fax +44 (0)118 378 8979
email m.j.proven@reading.ac.uk

Dr Claire Williams
School of Psychology and Clinical Language Sciences
University of Reading
RG6 6AL

10 July 2014

Dear Claire

UREC 14/28: A dose-response study of the acute effects of blueberry juice on cognitive performance in healthy young adults. Provisional opinion

Thank you for your revised application (your email, including attachment, dated 10 July 2014 refers) addressing the minor issues raised by the UREC Sub-committee at its June meeting. On the basis of these revisions, I can confirm that the Chair is pleased to confirm a favourable ethical opinion.

Please note that the Committee will monitor the progress of projects to which it has given favourable ethical opinion approximately one year after such agreement, and then on a regular basis until its completion.

Please also find attached Safety Note 59: Incident Reporting in Human Interventional Studies at the University of Reading, to be followed should there be an incident arising from the conduct of this research.

The University Board for Research and Innovation has also asked that recipients of favourable ethical opinions from UREC be reminded of the provisions of the University Code of Good Practice in Research. A copy is attached and further information may be obtained here: http://www.reading.ac.uk/internal/res/QualityAssuranceInResearch/reas-RSqar.aspx.

Yours sincerely

Dr M J Proven
Coordinator for Quality Assurance in Research (UREC Secretary)
cc: Dr John Wright (Chair); Dr Laurie Butler, Head of School

A.4 SREC Ethics committee approval for Experiment 3

2015-053-CW - An investigation of the glycaemic response following blueberry drink ingestion in healthy young adults: a dose response study – Claire Williams, Laurie Butler, Daniel Lamport, & Lynne Bell

11th May 2015

In my opinion, this study meets the requirements for ethical approval, and I am happy for it to proceed

John Harris

A.5 SREC Ethics committee approval for Experiment 4

From: **PCLS Ethics** <pclsethics@reading.ac.uk>

Date: Mon, Jan 25, 2016 at 12:45 PM

Subject: FW: 2014-047-CW - A dose-response study of the acute effects of blueberry juice on cognitive performance in healthy young adults

To: "claire.williams@reading.ac.uk" <claire.williams@reading.ac.uk>, Lynne Bell <l.bell@pgr.reading.ac.uk>

From: Anastasia Christakou [mailto:a.christakou@reading.ac.uk]

Sent: 25 January 2016 12:43

To: PCLS Ethics

Subject: Re: 2014-047-CW - A dose-response study of the acute effects of blueberry juice on cognitive performance in healthy young adults

These amendments have SREC approval.

Anastasia

[sent from a mobile device]

Appendix B Intervention study eligibility criteria

Age 18-40 years

Non-smoker

Not pregnant

Able to consume the beverages

No significant vision, hearing or language difficulties

Should not suffer from any of the following diseases:

- Major mental illness
- Liver disease
- Diabetes mellitus (Type 1 and 2)
- Heart disease
- Renal or gastrointestinal disorders

Should not be taking blood pressure lowering or anticoagulant medication

Should not be consuming more than the Government recommended units of alcohol per week

Should not be vigorous exercisers (restricted to < 4 hours per week for the duration of the study)

Should not be taking nutritional supplements (for the duration of the study)

Should not be taking recreational drugs (either illegal or legal for the duration of the study)

Appendix C Health screening questionnaire

****** Please answer the following questions as accurately as possible. All information provided will remain confidential ******

Demographic Information:

Age _____

Gender _____

Questions about health and lifestyle: *(Circle the relevant answer)*

Do you smoke? Yes / No

If 'Yes', please state how many per day _____.

Do you drink alcohol? Yes/No

If 'Yes', approximately how many units per week? _____

Do you drink tea? Yes / No

If 'Yes', approximately how many cups of tea per day? _____

Do you drink coffee? Yes / No

If 'Yes', approximately how many cups of coffee per day? _____

Are you currently on a weight-reducing or other special diet? Yes/No

If 'Yes', please give details.

_____

_____

_____

Do you exercise regularly? Yes/No

If 'Yes', please give an estimate of the number of hours you spend exercising per week.

_____

Do you have allergies to any foods? In particular, any fruits or cocoa, nut, milk or gluten based products? Yes/No

If 'Yes', please list foods you have an allergy to below:

_____

_____

_____

Are you pregnant? Yes/No/Not applicable

Please indicate if you had or have any if the following conditions: (*place a tick next to any relevant conditions*)

[ ] Diabetes – if yes, please circle: Type I / Type II

[ ] Heart disease – if yes, have you had open heart/bypass surgery? Yes / No

[ ] High blood pressure – if yes, are you currently taking medication to control your blood pressure? Yes / No

Did you start taking medication immediately after diagnosis? Yes / No

How long have you been taking medication for?_____

[ ] Nervous system disease / Degenerative disorder (e.g. Multiple Sclerosis)

[ ] Stroke or Transient Ischaemic Attack

[ ] Chronic kidney disease/impaired kidney function

[ ] Chronic liver disease/impaired liver function

[ ] Chronic thyroid disease/impaired thyroid function

[ ] Mental or emotional problems for which you were admitted to hospital

[ ] Renal or Gastrointestinal disorders – if yes, please give details

_____

[ ] Any other serious illnesses – if yes, please give details

_____

Are you currently, or have you been within the last month, on any of the following medication?

[ ] Warfarin

[ ] Heparin

[ ] Rivaroxaban

[ ] Dabigatran

[ ] Apixaban

[ ] Aspirin

[ ] Plavix (Clopidogrel)

[ ] Persantin

[ ] Other anticoagulant medication- if yes, please give details

_____

[ ] Any other medication – if yes, please give details

_____

Appendix D Information sheets

D.1 Participant information sheet for Experiment 1.1

Title of Study: A comparison of practice effects for the Sternberg task and other cognitive tasks commonly used in crossover intervention studies

Information Sheet

Supervisors:	Email:	Phone:
Dr Claire M. Williams	claire.williams@reading.ac.uk	0118 378 7540
Dr Laurie T. Butler	l.t.butler@reading.ac.uk	0 118 378 7543

Experimenter:
Lynne Bell l.bell@pgr.rdg.ac.uk

We would be grateful if you could please assist us by participating in our study exploring practice effects for the Sternberg task. Specifically we are interested to find out whether prolonged practice on this task improves performance and whether or not the level of observed improvement differs from other commonly used cognitive tasks.

Your participation will take approximately 2.5 hours, on each of 3 separate visits, with 1 week between each visit. During each visit you will perform a range of cognitive tasks, measuring episodic memory, working memory and executive function. At each visit the complete set of tasks will be performed twice with a one hour break between each set. During the break you may spend the time as you wish, but you are requested not to consume any food and to only drink water. At your first visit you will also be asked to record what you consumed for your last meal (breakfast for morning participants and lunch for afternoon participants). For your subsequent visits you will be asked to eat the same meal (or as close as possible) before attending.

Your data will be kept confidential and securely stored, with only an anonymous number identifying it. Information linking that number to your name will be stored securely and separately from the data you provide us. All information collected for the project will be destroyed after a period of 5 years from the completion of the project. Taking part in this study is completely voluntary; you may withdraw at any time without having to give any reason. Please feel free to ask any questions that you may have about this study at any point.

This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct

Thank you for your help.

D.2 Participant information sheet for Experiment 1.2

Title of Study: Examination of the occurrence of practice effects in cognitive tasks commonly used in crossover dietary intervention studies

Information Sheet

Supervisors:	Email:	Phone:
Dr Claire M. Williams	claire.williams@reading.ac.uk	0118 378 7540
Dr Daniel J. Lamport	d.j.lamport@reading.ac.uk	0118 378 7937
Dr Laurie T. Butler	l.t.butler@reading.ac.uk	0 118 378 7543
Experimenter:		
Lynne Bell	l.bell@pgr.rdg.ac.uk	0118 378 7928
Emma Coleman	e.coleman2@student.reading.ac.uk	
Nasser Al-Farhan	Nasser.s.al.farhan@gmail.com	

We would be grateful if you could please assist us by participating in our study exploring practice effects in cognitive testing. Specifically we are interested to find out whether prolonged practice on cognitive tasks improves performance and whether or not the level of observed improvement differs between different cognitive domains.

Your participation will take approximately 2.5 hours, on each of 3 separate visits, with 1 week between each visit. During each visit you will perform a range of cognitive tasks. Each task will be performed 3 times on each visit, with a one hour break between each set of tasks. During the break you may spend the time as you wish, but you are requested not to consume any food and to only drink water. At your first visit you will also be asked to record what you consumed for your last meal (breakfast for morning participants and lunch for afternoon participants). For your subsequent visits you will be asked to eat the same meal (or as close as possible) before attending.

Your data will be kept confidential and securely stored, with only an anonymous number identifying it. Information linking that number to your name will be stored securely and separately from the data you provide us. All information collected for the project will be destroyed after a period of 5 years from the completion of the project. Taking part in this study is completely voluntary; you may withdraw at any time without having to give any reason. Please feel free to ask any questions that you may have about this study at any point.

This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct

Thank you for your help.

D.3 Participant information sheet for Experiment 2

Title of study: A dose-response study of the acute effects of blueberry juice on cognitive performance in healthy young adults

Participant Information Sheet

Supervisors:

Name:	E mail:	Telephone:
Dr L.T. Butler	l.t.butler@reading.ac.uk	+44 (0)118 378 7543

School of Psychology and Clinical Language Sciences.

Name:	E mail:	Telephone:
Dr C.M.Williams	claire.williams@reading.ac.uk	+44 (0)118 378 7540

School of Psychology and Clinical Language Sciences.

Experimenter:

Name:	E mail:	Telephone:
Lynne Bell	l.bell@pgr.reading.ac.uk	PCLS Office: +44 (0)118 378 8523

School of Psychology and Clinical Language Sciences.

Introduction

Flavonoids are dietary polyphenols (plant chemicals found in certain foods such as fruit and vegetables) and previous studies have shown both cognitive and cardiovascular benefits of these compounds. This research project is looking at the short-term effects of a range of doses of flavonoid-rich blueberry on measures of cognition, which will be administered in the form of juice drinks. To avoid interference you will be asked to restrict your intake of flavonoid-rich foods prior to participating in the study and to fast for 2 hours prior to each test session. During the study you will undergo a range of cognitive tests. You will also be asked to give permission for blood pressure measurements to be taken. This is in order to determine the effects of flavonoids on vascular reactivity.

Project Description

The following is a brief description of the procedure for this research project. If you have any additional questions or require further explanation, please do not hesitate to ask.

Participation in this research project will require you to be available to attend the Psychology department on seven separate days and times that will be agreed with you in advance. Please let the Experimenter know if you cannot attend for any reason and the appointment can be rearranged.

Screening

Before participating in the study you are asked to fill in a short health questionnaire and return this to the experimenter via email.

All visits - Test sessions

For the 24 hours preceding all visits we ask that you follow a restricted diet, and do not partake in any vigorous physical activities. Please also refrain from eating or drinking for 2 hours immediately prior to each test visit. During the first half of each visit you will have your blood pressure recorded and complete a battery of computerised cognitive tasks. You will then be given a 600ml drink that may have a thick smoothie-like texture and will be asked to complete a food consumption questionnaire. You will then have one hour of free time but we ask that you stay within the Department and do not eat or drink anything else other than water. After the one hour break you will complete a second set of computer based cognitive tasks and further blood pressure measurements will be taken. The whole visit should take no longer than 2 $^{3}/_{4}$ hours. Before leaving we will also confirm your next visit date which will be one week later. The procedure will be exactly the same for all test visits, with the exception that you will be given a different drink.

All of your data throughout the study will be kept confidential by the use of an identification code and therefore your name will not be attached to any of the data collected. Data will be securely stored and retained for 5 years then destroyed. Please note that participation in this study is entirely voluntary and you have the right to withdraw from the study at any time without having to give a reason. The juice drinks are safe for human consumption and no negative side-effects are expected. This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. This research will be carried out by a PhD student from the University of Reading.

Thank you for your help.

D.4 Participant information sheet for Experiment 3

Title of study: An investigation of the glycaemic response following blueberry drink ingestion in healthy young adults: a dose response study

Participant Information Sheet

Supervisors:

Name:	E mail:	Telephone:
Prof C.M.Williams	claire.williams@reading.ac.uk	+44 (0)118 378 7540

School of Psychology and Clinical Language Sciences.

Name:	E mail:	Telephone:
Dr D.J.Lamport	d.j.lamport@reading.ac.uk	+44 (0)118 378 7937

School of Psychology and Clinical Language Sciences.

Name:	E mail:	Telephone:
Prof L.T. Butler	l.t.butler@reading.ac.uk	+44 (0)118 378 7543

School of Psychology and Clinical Language Sciences.

Experimenter:

Name:	E mail:	Telephone:
Lynne Bell	l.bell@pgr.reading.ac.uk	+44 (0)118 378 7924

School of Psychology and Clinical Language Sciences.

Introduction

This research project is looking at the short-term effects of a range of doses of blueberry on blood glucose. Blueberries are a rich source of flavonoids (plant chemicals found in certain foods such as fruit and vegetables) and will be administered in the form of a smoothie-like drink. To avoid interference you will be asked to restrict your intake of flavonoid-rich foods prior to participating in the study and to fast for 2 hours prior to each test session.

Blood glucose measurement

During this study you will be required to give a finger prick sample which is used to measure your blood glucose level. This is a relatively painless procedure which will cause you minimal distress. If you would like to experience giving a finger prick sample before consenting to take part in this research please tell

the experimenter. If during the experiment you decide that you do not want to give a finger prick sample then you can withdraw from the experiment without giving a reason. Unusually low or high glucose levels can be indicative of abnormalities in glucose regulation. We will inform you verbally if we observe unusual glucose levels and in such cases we recommend that you contact your GP and ask for an official glucose assessment. If you prefer we can contact your GP directly and pass on your readings. This finger prick procedure has been has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Procedure

The following is a brief description of the procedure for this research project. If you have any additional questions or require further explanation, please do not hesitate to ask.

Participation in this research project will require you to be available to attend the Psychology department on five separate days and times that will be agreed with you in advance. Please let the Experimenter know if you cannot attend for any reason and the appointment can be rearranged.

Initial screening

A short medical questionnaire has been emailed to you along with this information sheet. Please complete and return by email to the experimenter. You will then be given a date for your first test session. At this session you will be asked to sign a consent form before commencing the study.

Test sessions

For the 24 hours preceding these sessions we ask that you follow a restricted diet (see attached), and do not partake in any vigorous physical activities. Please also refrain from eating or drinking for 2 hours immediately prior to each test session. You may eat a light breakfast (still following the restricted diet) before the 2 hour fasting period. If you choose to do so, you are kindly asked to eat an identical breakfast before all subsequent visits. At the start of each session you will complete a food consumption questionnaire, before having your baseline blood glucose level recorded. You will then be given a 600ml drink that may have a thick smoothie-like texture. The drink must be fully consumed within 10 minutes. After this you will remain in the lab for a period of 2 ½ hours, during which further blood glucose measurements will be recorded at intervals of 15 minutes. The whole visit should take no longer than 3 hours. Before leaving we will also confirm your next session date which will be one week later. The procedure will be exactly the same for all test sessions, with the exception that you will be given a different drink.

Data protection and ethical conduct

All of your data throughout the study will be kept confidential by the use of an identification code and therefore your name will not be attached to any of the data collected. Data will be securely stored and retained for 5 years then destroyed. Please note that participation in this study is entirely voluntary and you have the right to withdraw from the study at any time without having to give a reason. The blueberry drinks are safe for human consumption and no negative side-effects are expected. This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. This research will be carried out by a PhD student from the University of Reading.

Thank you for your help.

D.5 Participant information sheet for Experiment 4

Title of study: A dose-response study of the acute effects of blueberry juice oncognitive performance in healthy young adults

Participant Information Sheet

Supervisors:

Name:	E mail:	Telephone:
Dr D.J.Lamport	d.j.lamport@reading.ac.uk	+44 (0)118 378 7937

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Name:	E mail:	Telephone:
Dr L.T. Butler	l.t.butler@reading.ac.uk	+44 (0)118 378 7543

School of Psychology and Clinical Language Sciences.

Name:	E mail:	Telephone:
Dr C.M.Williams	claire.williams@reading.ac.uk	+44 (0)118 378 7540

School of Psychology and Clinical Language Sciences.

Experimenter:

Name:	E mail:	Telephone:
Lynne Bell	pf008864@pgr.reading.ac.uk	+44 (0)118 378 7928

School of Psychology and Clinical Language Sciences.

Introduction

This research project is looking at the short-term effects of a range of doses of blueberry on measures of cognition. Flavonoids are dietary polyphenols (plant chemicals found in certain foods such as fruit and vegetables) and will be administered in the form of a juice drink. To avoid interference you will be asked to restrict your intake of flavonoid-rich foods prior to participating in the study and to fast for 2 hours prior to each test session. During the study you will undergo a range of cognitive tests. You will also be asked to give permission for blood glucose measurements to be taken. This is in order to determine the effects of flavonoids on blood glucose regulation.

Blood glucose measurement is a relatively painless procedure involving a finger prick which will cause you minimal distress. If you would like to experience giving a finger prick sample before consenting to take part in this research please tell the experimenter. If during the experiment you decide that you do

not want to give a finger prick sample then you can withdraw from the experiment without giving a reason. Unusually low or high glucose levels can be indicative of abnormalities in glucose regulation. We will inform you verbally if we observe unusual glucose levels and in such cases we recommend that you contact your GP and ask for an official glucose assessment. If you prefer we can contact your GP directly and pass on your readings. This finger prick procedure has been has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Project Description

The following is a brief description of the procedure for this research project. If you have any additional questions or require further explanation, please do not hesitate to ask.

Participation in this research project will require you to be available to attend the Psychology department on five separate days and times that will be agreed with you in advance. Please let the Experimenter know if you cannot attend for any reason and the appointment can be rearranged.

Screening

Before participating in the study you are asked to fill in a short health questionnaire and return this to the experimenter via email.

All visits - Test sessions

For the 24 hours preceding these visits we ask that you follow a restricted diet, and do not partake in any vigorous physical activities. Please also refrain from eating or drinking for 2 hours immediately prior to each test session. At the start of each session you will complete a food consumption questionnaire, before having your blood glucose level recorded and completing a short battery of computerised cognitive tasks. You will then be given a 300ml drink that may have a thick smoothie-like texture. After this you will have your blood glucose re-measured at 15 mins and 30 mins followed by a further 30 mins of free time. We ask that you stay within the Department and do not eat or drink anything else other than water. After the break you will complete a second set of computer based cognitive tasks. After a further break of around 45 minutes you will complete the cognitive tasks for a third time. Blood glucose measurements will also be taken each time you resume cognitive testing (5 finger pricks in total per visit). The whole visit should take no longer than $2\ ^{3}/_{4}$ hours. Before leaving we will also confirm your next visit date which will be one week later. The procedure will be exactly the same for all test visits, with the exception that you will be given a different drink.

All of your data throughout the study will be kept confidential by the use of an identification code and therefore your name will not be attached to any of the data collected. Data will be securely stored and retained for 5 years then destroyed. Please note that participation in this study is entirely voluntary and you have the right to withdraw from the study at any time without having to give a reason. The juice drinks are safe for human consumption and no negative side-effects are expected. This application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. This research will be carried out by a PhD student from the University of Reading.

Thank you for your help**.**

Appendix E Consent forms

E.1 Consent form for Experiment 1.1

Title of Study: Examination of the occurrence of practice effects in the Sternberg task and other cognitive tasks commonly used in crossover dietary intervention studies

CONSENT FORM

I, …… agree to participate in the study '**Examination of the occurrence of practice effects in the Sternberg task and other cognitive tasks commonly used in crossover dietary intervention studies**', being conducted by Lynne Bell at The University of Reading. I have seen and read a copy of the Participants Information Sheet and have been given the opportunity to ask questions about the study and these have been answered to my satisfaction. I understand that all personal information will remain confidential to the Investigator and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me. I understand that participation in this study is voluntary and that I can withdraw at any time without having to give an explanation.

I am happy to proceed with my participation.

Signature ----------

Name (in capitals) ----------

Date ----------

E.2 Consent form for Experiment 1.2

Title of Study: Examination of the occurrence of practice effects in cognitive tasks commonly used in crossover dietary intervention studies

CONSENT FORM

I, …… agree to participate in the study '**Examination of the occurrence of practice effects in cognitive tasks commonly used in crossover dietary intervention studies**', being conducted by Lynne Bell, Emma Coleman & Nasser Al-Farhan at The University of Reading. I have seen and read a copy of the Participants Information Sheet and have been given the opportunity to ask questions about the study and these have been answered to my satisfaction. I understand that all personal information will remain confidential to the Investigator and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me. I understand that participation in this study is voluntary and that I can withdraw at any time without having to give an explanation.

I am happy to proceed with my participation.

Signature ----------

Name (in capitals) ----------

Date ----------

Researcher signature ----------

Name (in capitals) ----------

Date ----------

E.3 Consent form for Experiment 2

A dose-response study of the acute effects of blueberry juice on cognitive performance in healthy young adults

Consent Form

When you have read each statement below, please put your initials in the box next to it to show that you understand and agree with the statement

1. I agree to participate in the project 'A dose-response study of the acute effects of blueberry juice on
cognitive performance in healthy young adults', being conducted by the Nutrition and Cognition Research Group at the University of Reading. ☐

2. I have read and been given a copy of the Participant's Information Sheet and this consent form. I have had the opportunity to ask any questions about the project and these have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

3. I am aware that this application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

4. I understand that participation is entirely voluntary and that I have the right to withdraw from the project at any time, without giving a reason, and that this will be without detriment to any care or services I may be receiving or may receive in the future. ☐

5. I understand that all personal information will remain confidential to the Investigator and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me. ☐

6. I am happy to proceed with my participation. ☐

In the presence of:

Name:.....(Volunteer) **Name:**.....(Researcher)

Signed:.....(Volunteer) **Signed:**.....(Researcher)

Date:.....(Volunteer) **Date:**.....(Researcher)

E.4 Consent form for Experiment 3

An investigation of the glycaemic response following blueberry drink ingestion in healthy young adults: a dose response study

Consent Form

When you have read each statement below, please put your initials in the box next to it to show that you understand and agree with the statement

1. I agree to participate in the project 'An investigation of the glycaemic response to varying doses of blueberry in healthy young adults', being conducted by the Nutrition and Cognition Research Group at the University of Reading. ☐

2. I have read the information sheet and I have been told the reasons why a finger prick blood sample is required. I have had the opportunity to ask any questions about the project and these have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

3. I consent to a series of finger prick samples being taken. ☐

4. I authorise the Investigator to consult my General Practitioner if abnormal glucose readings are observed. ☐

5. I am aware that the finger prick procedure has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

6. I understand that participation is entirely voluntary and that I have the right to withdraw from the project at any time, without giving a reason, and that this will be without detriment to any care or services I may be receiving or may receive in the future. ☐

7. I understand that all personal information will remain confidential to the Investigator and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me. ☐

8. I am happy to proceed with my participation. ☐

In the presence of:

Name:.....(Volunteer)

Signed:.....(Volunteer)

Date of birth:.....(Volunteer)

Name and address of GP:.....(Volunteer)

.....

.....

.....

Name:.....(Researcher)

Signed:.....(Researcher)

Date:.....(Researcher)

E.5 Consent form for Experiment 4

A dose-response study of the acute effects of blueberry juice on cognitive performance in healthy young adults

Consent Form

When you have read each statement below, please put your initials in the box next to it to show that you understand and agree with the statement

1. I agree to participate in the project 'A dose-response study of the acute effects of blueberry juice on
cognitive performance in healthy young adults', being conducted by the Nutrition and Cognition Research Group at the University of Reading. ☐

2. I have read the information sheet and I have been told the reasons why a finger prick blood sample is required. I have had the opportunity to ask any questions about the project and these have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation. ☐

3. I consent to a series of finger prick samples being taken ☐

4. I authorise the Investigator to consult my General Practitioner if abnormal glucose readings are observed ☐

5. I am aware that the finger prick procedure has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

6. I am aware that this application has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct. ☐

7. I understand that participation is entirely voluntary and that I have the right to withdraw from the project at any time, without giving a reason, and that this will be without detriment to any care or services I may be receiving or may receive in the future. ☐

8. I understand that all personal information will remain confidential to the Investigator and arrangements for the storage and eventual disposal of any identifiable material have been made clear to me. ☐

9. I am happy to proceed with my participation. ☐

In the presence of:

Name:.....(Volunteer) **Name:**.....(Researcher)

Signed:.....(Volunteer) **Signed:**.....(Researcher)

Date:.....(Volunteer) **Date:**.....(Researcher)

Name and address of GP:.....(Volunteer)

.....

.....

.....

Appendix F Low polyphenol diet

Please **avoid** alcohol or other recreational drugs for **48** hours before **each** visit to the Nutritional Psychology Unit and for the duration of each study day.

Please **avoid** eating foods shown below for **24** hours before **each** visit to the Nutritional Psychology Unit and for the duration of each study day.

- All berries
- Fruit and vegetables (except potatoes)
- Fruit juices
- Jams and preserves
- Red wine
- Fruit teas
- Soy products
- Chocolate/cocoa
- Tea (black, green. earl grey etc)
- Coffee
- All high energy and/or caffeinated drinks, eg: Coca-Cola, Red Bull, Lucozade.
- All dietary supplements
- Alcohol or other recreational drugs (avoid for **48** hours)

Foods you may eat include those shown below.

- Potatoes
- Rice
- Sweetcorn
- Mushrooms
- Carrots
- Bananas
- Pasta
- Meat/fish
- Dairy products

Appendix G 24 hour food record

Participant No: Participant ID: Visit No:

Food intake record

Please write down everything you consumed during the 24 hours prior to your 2 hour fast. Please include details of all meals, snacks and beverages:

Appendix H Blueberry intervention palatability questionnaire

Participant number:_____ Visit:_____ Drink:_____ Date: ____/____/___

Please answer the following questions by circling the appropriate number response on the scale. Please regard the ends of each scale as indicating the most extreme sensation you have ever felt.

1. How **sweet** did you find the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

2. How **bland** did you find the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

3. How **tasty** did you find the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

4. How **pleasant** did you find the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

5. How **sour** did you find the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

6. How **satisfying** did you find the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

7. How **much more** of this drink do you think you could consume?

None at all1 2 3 4 5 6 7 8 9 A lot more

8. How **easy** did you find it to consume the drink?

Not at all 1 2 3 4 5 6 7 8 9 Extremely

9. Any additional comments about the drink:

_____
_____
_____

Appendix I Immediate & delayed recall word lists

1	2	3	4	5	6	7
hose	latch	swamp	inn	cane	dew	scout
tank	fox	fudge	yacht	spike	prune	pit
seed	branch	sail	worm	owl	knight	shell
bone	brick	gun	soil	pearl	drain	blade
card	tool	board	stew	lung	crown	flag
wood	star	pine	film	church	nurse	oil
bin	ball	pot	coin	jar	broom	tray
clock	meat	neck	soap	dog	leaf	brush
salt	spoon	fruit	men	bag	knee	book
prison	metal	penny	doorway	needle	forest	palace
shepherd	armour	woodland	sardine	ferry	dairy	lily
chapel	measles	arrow	knuckle	package	cotton	gravel
jewel	linen	kitten	valley	bullet	iron	gravy
hotel	chicken	rubber	candy	shoulder	teacher	wire
toilet	garden	oven	pocket	picture	paper	building

8	9	10	11	12	13	14
lime	fawn	herd	wick	sword	crane	beast
calf	doll	rake	barn	vest	shrub	crow
oak	duck	rod	dart	cliff	cone	heel
weed	shore	mud	net	deck	pole	shark
van	bush	sweat	thread	frost	stool	chin
blood	land	rug	heart	cloth	beard	throat
school	glove	sock	wheel	stone	corn	sleeve
rain	ring	soup	hall	seat	plate	lamp
face	tree	plant	room	door	box	girl
tack	bowl	sheet	cheek	pipe	pig	shield
marble	beehive	camel	ruby	kennel	cellar	farmyard
rifle	barrel	mansion	slipper	napkin	daisy	laundry
tractor	hammer	soldier	cherry	lemon	salad	movie
office	motor	piano	dentist	pepper	brother	uncle
orange	button	ticket	shower	mirror	bedroom	pillow

Appendix J Motivation & task difficulty questionnaire

Participant No: Participant ID: Visit No: Session No:

<Cognitive task name>

How difficult did you find the <cognitive task name> task?

1 2 3 4 5 6 7 8 9

Not at all Extremely

How motivated were you to do well during the <cognitive task name> task?

1 2 3 4 5 6 7 8 9

Not at all Extremely

Appendix K Fruit & vegetable consumption questionnaire

To answer the next questions it is important that you understand what a portion of fruit is and also what a portion of vegetables is so below is a quick guide:

Quick guide to fruit and vegetable portions

FRUIT	**One portion of fruit is:**
Fresh	***Small-sized fruit*** **- 2 satsumas, 2 kiwi fruits, 14 cherries, 2 handfuls of raspberries or blueberries**
	Medium-sized fruit **- 1 medium fruit, e.g. 1 apple, banana, pear, orange or nectarine**
	Large fruit **- 1 (5cm slice) of melon, 1 large slice of pineapple, 2 (5cm slices) of mango**
Dried	**1 tablespoon of raisins, currants, sultanas, 3 apricots**
Juice	**A glass (150ml) of 100% juice (or smoothie) counts as 1 portion, but *you can only count juice as 1 portion per day, however much you drink***
Tinned	**Roughly the same quantity of fruit that you would eat as a fresh portion: 2 pear or peach halves, 3 heaped tablespoons of fruit salad**
VEGETABLES	**One portions of vegetables is:**
Green	**2 broccoli spears, 8 cauliflower florets**
Cooked	**3 heaped tablespoons of cooked vegetables such as carrots, peas or sweetcorn**
Salad	**3 sticks of celery, 1 medium tomato, 7 cherry tomatoes**
Tinned & frozen	**Roughly the same quantity as you would eat as a fresh portion**
Pulses & beans	**3 heaped tablespoons of baked beans, haricot beans, kidney beans, cannelloni beans, butter beans or chick peas. Remember that beans and pulses do count, but *you can only count beans/pulses as 1 portion per day, however much you eat***

1. **How many portions of fruit, of any sort, do you eat on a typical day? _____ portions**
2. **How many portions of vegetables, excluding potatoes, do you eat on a typical day? _____ portions**

Appendix L LMM results tables

Table L.1 LMM results for Experiment 1.1

Experiment 1.1	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Cognition:						
Immediate recall score (correct/15)	1.1	716.50	Visit	F(2,54.00)	15.91	<0.001
			Session	F(1,69.62)	1.83	0.181
			Visit x Session	F(2,82.13)	2.92	0.059
	1.2	704.10	Visit	F(2,54.20)	16.82	<0.001
			Session	F(1,79.39)	0.02	0.881
			Visit x Session	F(2,81.91)	4.36	0.016
			Motivation	F(1,136.77)	13.56	<0.001
Immediate recall interference errors	1.3	-14.71	Visit	F(2,73.11)	0.07	0.934
			Session	F(1,61.39)	0.38	0.538
			Visit x Session	F(2,71.07)	0.67	0.517
Delayed recall score (correct/15)	1.1	798.11	Visit	F(2,55.09)	2.87	0.065
			Session	F(1,72.68)	19.20	<0.001
			Visit x Session	F(2,67.28)	2.03	0.140
	1.2	791.03	Visit	F(2,53.22)	2.79	0.070
			Session	F(1,71.43)	17.08	<0.001
			Visit x Session	F(2,65.15)	2.13	0.127
			Motivation	F(1,127.22)	7.64	0.007
Delayed recall interference errors	1.3	295.14	Visit	F(2,54.91)	11.31	<.001
			Session	F(1,67.05)	44.29	<.001
			Visit x Session	F(2,54.76)	9.16	<.001
Serial 3s score	1.1	1188.55	Visit	F(5,51.08)	72.00	<0.001

Table L.1 continued

Experiment 1.1	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
(correct in 2 mins)			Session	F(1,74.78)	41.29	<0.001
			Visit x Session	F(2,74.32)	4.39	0.016
	1.2	1186.78	Visit	F(2,49.95)	70.48	<0.001
			Session	F(1,76.87)	42.98	<0.001
			Visit x Session	F(2,76.20)	3.87	0.025
			Motivation	F(1,155.27)	1.92	0.168
Serial 3s RT (ms)	1.1	2716.36	Visit	F(2,53.86)	33.98	<0.001
			Session	F(1,84.73)	16.13	<0.001
			Visit x Session	F(2,47.79)	1.98	0.149
	1.2	2713.70	Visit	F(2,53.89)	35.30	<0.001
			Session	F(1,86.05)	16.59	<0.001
			Visit x Session	F(2,50.31)	1.45	0.243
			Motivation	F(1,118.84)	2.85	0.094
Serial 3s errors (incorrect in 2 mins)	1.1	725.55	Visit	F(2,48.98)	0.11	0.893
			Session	F(1,68.38)	0.67	0.416
			Visit x Session	F(2,80.10)	1.04	0.356
	1.2	724.30	Visit	F(2,50.64)	0.07	0.930
			Session	F(1,66.62)	0.69	0.411
			Visit x Session	F(2,79.75)	0.95	0.391
			Motivation	F(1,40.60)	1.39	0.246
Serial 7s score (correct in 2 mins)	1.1	1078.76	Visit	F(2,48.54)	26.88	<0.001
			Session	F(1,57.88)	9.13	0.004
			Visit x Session	F(2,67.26)	0.37	0.690

Table L.1 continued

Experiment 1.1	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
	1.2	1062.77	Visit	F(2,46.26)	32.86	<0.001
			Session	F(1,69.02)	10.74	0.002
			Visit x Session	F(2,71.52)	0.71	0.496
			Motivation	F(1,135.85)	19.05	<0.001
Serial 7s RT (ms)	1.1	2963.58	Visit	F(2,66.09)	11.37	<0.001
			Session	F(1,67.10)	17.01	<0.001
			Visit x Session	F(2,73.84)	4.39	0.016
	1.2	2963.53	Visit	F(2,66.14)	11.43	<0.001
			Session	F(1,67.33)	17.04	<0.001
			Visit x Session	F(2,73.47)	4.39	0.016
			Motivation	F(1,124.54)	0.05	0.825
Serial 7s errors (incorrect in 2 mins)	1.1	773.58	Visit	F(2,53.59)	0.52	0.595
			Session	F(1,77.42)	0.52	0.474
			Visit x Session	F(2,86.86)	0.51	0.600
	1.2	759.72	Visit	F(2,49.04)	0.88	0.423
			Session	F(1,76.45)	0.36	0.548
			Visit x Session	F(2,87.48)	0.67	0.513
			Motivation	F(1,82.72)	16.49	<0.001
Sternberg accuracy (correct/96)	1.1	963.90	Visit	F(2,89.31)	1.51	0.226
			Session	F(1,95.03)	0.68	0.412
			Visit x Session	F(2,79.32)	0.25	0.777
	1.2	926.88	Visit	F(2,89.83)	1.84	0.165
			Session	F(1,86.21)	0.37	0.542
			Visit x Session	F(2,81.68)	0.33	0.722

Table L.1 continued

Experiment 1.1	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Motivation	F(1,131.06)	44.25	0.000
Sternberg scanning rate (ms/item)	1.1	1466.11	Visit	F(2,60.33)	7.08	0.002
			Session	F(1,58.80)	0.10	0.754
			Visit x Session	F(2,70.08)	0.26	0.774
	1.2	1464.85	Visit	F(2,60.89)	6.87	0.002
			Session	F(1,58.29)	0.07	0.789
			Visit x Session	F(2,71.51)	0.25	0.777
			Motivation	F(1,130.19)	1.37	0.243
Sternberg extrapolated RT (ms)	1.1	2149.69	Visit	F(2,60.81)	2.80	0.069
			Session	F(1,56.18)	8.80	0.004
			Visit x Session	F(2,71.12)	1.13	0.328
	1.2	2146.33	Visit	F(2,60.23)	2.82	0.067
			Session	F(1,55.60)	9.37	0.003
			Visit x Session	F(2,71.45)	1.06	0.351
			Motivation	F(1,128.73)	3.60	0.060
Stroop accuracy (correct/96)	1.1	988.00	Visit	F(2,72.10)	0.03	0.966
			Session	F(1,56.97)	0.04	0.835
			Visit x Session	F(2,58.63)	1.18	0.316
	1.2	982.40	Visit	F(2,66.97)	0.06	0.938
			Session	F(1,55.68)	<0.01	0.949
			Visit x Session	F(2,60.04)	1.20	0.307
			Motivation	F(1,102.34)	6.35	0.013
Stroop incongruent RT (ms)	1.1	1833.20	Visit	F(2,52.04)	12.66	<0.001
			Session	F(1,58.63)	23.75	<0.001

Table L.1 continued

Experiment 1.1	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Visit x Session	F(2,74.99)	8.91	<0.001
	1.2	1830.40	Visit	F(2,52.41)	13.62	<0.001
			Session	F(1,57.19)	24.57	<0.001
			Visit x Session	F(2,74.68)	8.70	<0.001
			Motivation	F(1,140.55)	3.24	0.074
Stroop congruent RT (ms)	1.1	1858.71	Visit	F(2,48.90)	6.17	0.004
			Session	F(1,47.64)	44.81	<0.001
			Visit x Session	F(2,83.96)	3.45	0.036
	1.2	1853.27	Visit	F(2,43.04)	6.65	0.003
			Session	F(1,42.89)	50.08	<0.001
			Visit x Session	F(2,83.05)	3.52	0.034
			Motivation	F(1,140.24)	5.82	0.017
Stroop interference effect (ms)	1.1	1753.17	Visit	F(2,55.68)	0.32	0.729
			Session	F(1,58.75)	5.51	0.022
			Visit x Session	F(2,84.38)	2.29	0.108
	1.2	1750.37	Visit	F(2,55.57)	0.29	0.748
			Session	F(1,61.19)	6.50	0.013
			Visit x Session	F(2,84.16)	2.40	0.097
			Motivation	F(1,145.5)	3.19	0.076
Mood:						
Mental fatigue (rating/9)	1.1	657.59	Visit	F(2,58.82)	5.18	0.008
			Session	F(1,69.09)	7.24	0.009
			Visit x Session	F(2,83.19)	0.52	0.599

Table L.1 continued

Experiment 1.1	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Positive affect (score/50)	1.1	1034.15	Visit	F(2,53.70)	5.18	0.009
			Session	F(1,61.90)	13.24	0.001
			Visit x Session	F(2,71.46)	3.13	0.050
Negative affect (score/50)	1.1	789.83	Visit	F(2,75.48)	0.31	0.737
			Session	F(1,58.12)	2.69	0.107
			Visit x Session	F(2,54.22)	0.40	0.673
Perceived difficulty (rating/9)	1.4	3597.07	Task	F(5,186.29)	20.12	<0.001
			Visit	F(2,595.30)	7.38	<0.001
			Session	F(1,602.72)	<0.01	0.947
			Task x Visit	F(10,595.30)	1.07	0.387
			Task x Session	F(5,602.72)	7.22	<0.001
			Visit x Session	F(2,544.08)	6.54	0.002
			Task x Visit x Session	F(10,544.08)	1.87	0.047
Motivation (rating/9)	1.4	3287.93	Task	F(5,262.17)	10.42	<0.001
			Visit	F(2,537.87)	8.03	<0.001
			Session	F(1,481.32)	15.14	<0.001
			Task x Visit	F(10,537.87)	0.80	0.632
			Task x Session	F(5,481.32)	2.72	0.019
			Visit x Session	F(2,547.79)	0.28	0.759
			Task x Visit x Session	F(10,547.79)	0.61	0.803

Table L.2 LMM results for Experiment 1.2

Experiment 1.2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Cognition:						
Digit vigilance score (correct/45)	1.1	1354.2	Visit	F(2,71.98)	2.31	0.106
			Session	F(2,113.33)	8.35	<0.001
			Visit x Session	F(4,86.69)	0.25	0.908
	1.2	1340.72	Visit	F(2,80.15)	1.87	0.160
			Session	F(2,117.55)	5.87	0.004
			Visit x Session	F(4,85.72)	0.32	0.867
			Motivation	F(1,172.34)	6.54	0.011
Digit vigilance commission errors	1.1	1035.88	Visit	F(2,90.77)	6.64	0.002
			Session	F(2,132.04)	0.52	0.598
			Visit x Session	F(4,101.48)	0.74	0.568
	1.2	1020.55	Visit	F(2,93.92)	4.94	0.009
			Session	F(2,132.14)	0.09	0.917
			Visit x Session	F(4,98.55)	0.58	0.677
			Motivation	F(1,224.24)	10.48	0.001
Digit vigilance RT (ms)	1.1	2636.74	Visit	F(2,63.25)	1.31	0.277
			Session	F(2,125.79)	6.70	0.002
			Visit x Session	F(4,88.65)	0.89	0.475
	1.2	2613.18	Visit	F(2,65.49)	0.78	0.463
			Session	F(2,127.82)	4.21	0.017
			Visit x Session	F(4,88.14)	0.75	0.560
			Motivation	F(1,237.80)	8.14	0.005
RVIP score (correct/40)	1.1	1701.31	Visit	F(2,73.09)	24.45	<0.001
			Session	F(2,138.86}	3.77	0.026

Table L.2 continued

Experiment 1.2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Visit x Session	F(4,98.89)	2.47	0.049
	1.2	1671.44	Visit	F(2,71.74)	26.88	<0.001
			Session	F(2,138.85)	5.51	0.005
			Visit x Session	F(4,100.73)	2.09	0.088
			Motivation	F(1,243.60)	9.49	0.002
RVIP commission errors	1.1	2017.52	Visit	F(2,61.71)	0.47	0.627
			Session	F(2,115.62)	3.13	0.048
			Visit x Session	F(4,90.25)	3.01	0.022
	1.2	1979.62	Visit	F(2,64.14)	0.61	0.548
			Session	F(2,116.65)	3.06	0.050
			Visit x Session	F(4,86.94)	3.22	0.016
			Motivation	F(1,206.91)	2.31	0.130
RVIP RT (ms)	1.1	2763.36	Visit	F(2,82.07)	3.97	0.023
			Session	F(2,133.40)	4.44	0.014
			Visit x Session	F(4,100.73)	0.91	0.464
	1.2	2725.26	Visit	F(2,82.56)	3.59	0.032
			Session	F(2,133.46)	5.38	0.006
			Visit x Session	F(4,97.61)	0.86	0.490
			Motivation	F(1,231.89)	1.68	0.196
Serial 3s score (correct in 2 mins)	1.1	1959.48	Visit	F(2,39.61)	46.21	<0.001
			Session	F(2,104.14)	16.73	<0.001
			Visit x Session	F(4,84.61)	2.81	0.031
	1.2	1943.22	Visit	F(2,35.86)	49.65	<.001

Table L.2 continued

Experiment 1.2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Session	F(2,105.74)	22.49	<.001
			Visit x Session	F(4,82.51)	2.87	0.028
			Motivation	F(1,197.14)	17.64	<.001
Serial 3s errors (incorrect in 2 mins)	1.1	1257.89	Visit	F(2,78.14)	2.87	0.063
			Session	F(2,115.35)	0.37	0.691
			Visit x Session	F(4,93.40)	1.45	0.223
	1.2	1255.26	Visit	F(2,77.23)	2.72	0.072
			Session	F(2,117.78)	0.18	0.832
			Visit x Session	F(4,92.95)	1.46	0.222
			Motivation	F(1,158.75)	2.81	0.096
Serial 3s RT (ms)	1.1	4402.4	Visit	F(2,64.08)	31.22	<0.001
			Session	F(2,104.49)	11.44	<0.001
			Visit x Session	F(4,93.00)	1.56	0.192
	1.2	4400.72	Visit	F(2,64.06)	30.25	<.001
			Session	F(2,104.83)	12.12	<.001
			Visit x Session	F(4,93.23)	1.58	0.185
			Motivation	F(1,165.433)	1.82	0.180
Serial 7s score (correct in 2 mins)	1.1	1736.98	Visit	F(2,62.26)	28.94	<0.001
			Session	F(2,123.19)	13.49	<0.001
			Visit x Session	F(4,94.21)	0.84	0.503
	1.2	1715.02	Visit	F(2,63.84)	32.34	<0.001
			Session	F(2,114.35)	16.54	<0.001
			Visit x Session	F(4,92.52)	1.11	0.355
			Motivation	F(1,209.03)	11.28	0.001

Table L.2 continued

Experiment 1.2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Serial 7s errors (incorrect in 2 mins)	1.1	1110.51	Visit	F(2,78.98)	0.61	0.545
			Session	F(2,127.26)	7.94	0.001
			Visit x Session	F(4,94.52)	0.91	0.462
	1.2	1086.54	Visit	F(2,73.78)	1.14	0.325
			Session	F(2,133.11)	8.63	<0.001
			Visit x Session	F(4,98.18)	1.39	0.244
			Motivation	F(1,183.87)	2.72	0.101
Serial 7s RT (ms)	1.1	4745.02	Visit	F(2,79.59)	42.14	<0.001
			Session	F(2,107.78)	25.42	<0.001
			Visit x Session	F(4,89.87)	1.94	0.111
	1.2	4706.44	Visit	F(2,79.61)	44.95	<0.001
			Session	F(2,110.43)	28.16	<0.001
			Visit x Session	F(4,87.32)	2.34	0.061
			Motivation	F(1,219.22)	4.45	0.036
Mood:						
Mental fatigue (rating/9)	1.1	1132.14	Visit	F(2,76.15)	0.18	0.836
			Session	F(2,130.86)	2.00	0.139
			Visit x Session	F(4,94.29)	1.11	0.359
Positive affect (score/50)	1.1	1729.76	Visit	F(2,73.67)	3.48	0.036
			Session	F(2,126.73)	8.93	<0.001
			Visit x Session	F(4,88.15)	0.28	0.888
Negative affect (score/50)	1.1	1309.28	Visit	F(2,71.56)	1.28	0.286
			Session	F(2,136.12)	6.35	0.002
			Visit x Session	F(4,98.66)	0.64	0.635

Table L.2 continued

Experiment 1.2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Perceived difficulty (rating/9)	1.4	3873.52	Task	F(3,204.29)	142.51	<0.001
			Visit	F(2,452.46)	22.98	<0.001
			Session	F(2,586.29)	37.75	<0.001
			Task x Visit	F(6,449.48)	2.72	0.013
			Task x Session	F(6,585.91)	2.83	0.010
			Visit x Session	F(4,447.35)	8.86	<0.001
			Task x Visit x Session	F(447.30)	1.18	0.297

Table L.3 LMM results for Experiments 1.1 & 1.2 combined

Experiments 1.1 & 1.2 combined	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Cognition:						
Serial 3s score (correct in 2 mins)	1.5	3161.68	Time of day	F(1,171.56)	0.02	0.891
			Visit	F(2,95.87)	100.94	<0.001
			Session	F(2,144.33)	35.24	<0.001
			ToD x Visit	F(2,97.24)	2.23	0.113
			ToD x Session	F(2,144.55)	1.07	0.345
			Visit x Session	F(4,106.31)	4.00	0.005
			ToD x Visit x Session	F(4,106.30)	0.91	0.463
	1.6	3141.77	Time of day	F(1,171.33)	<0.01	0.999
			Visit	F(2,95.38)	107.35	<0.001
			Session	F(2,148.61)	41.71	<0.001
			ToD x Visit	F(2,96.33)	2.72	0.071
			ToD x Session	F(2,147.96)	0.75	0.475
			Visit x Session	F(4,107.74)	3.65	0.008
			ToD x Visit x Session	F(4,107.80)	0.70	0.596
			Motivation	F(1,388.76)	21.02	<0.001
Serial 3s errors (incorrect in 2 mins)	1.5	1983.35	Time of day	F(1,69.15)	5.34	0.024
			Visit	F(2,117.59)	2.04	0.135
			Session	F(2,144.40)	0.90	0.410
			ToD x Visit	F(2,119.15)	0.73	0.485
			ToD x Session	F(2,144.70)	0.77	0.467
			Visit x Session	F(4,110.41)	1.64	0.168
			ToD x Visit	F(4,110.55)	1.02	0.401

Table L.3 continued

Experiments 1.1 & 1.2 combined	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			x Session			
	1.6	1978.14	Time of day	F(1,71.49)	6.66	0.012
			Visit	F(2,115.97)	1.79	0.172
			Session	F(2,144.19)	0.62	0.540
			ToD x Visit	F(2,117.31)	0.77	0.465
			ToD x Session	F(2,143.69)	0.96	0.387
			Visit x Session	F(4,110.69)	1.67	0.163
			ToD x Visit x Session	F(4,110.83)	0.96	0.433
			Motivation	F(1,194.51)	5.40	0.021
Serial 3s RT (ms)	1.5	7118.82	Time of day	F(1,146.48)	0.45	0.505
			Visit	F(2,119.15)	63.75	<0.001
			Session	F(2,205.13)	21.52	<0.001
			ToD x Visit	F(2,121.59)	0.15	0.857
			ToD x Session	F(2,204.88)	0.71	0.491
			Visit x Session	F(4,146.74)	1.57	0.184
			ToD x Visit x Session	F(4,147.48)	0.79	0.536
	1.6	7115.79	Time of day	F(1,144.47)	0.41	0.521
			Visit	F(2,118.64)	62.76	<0.001
			Session	F(2,206.34)	22.69	<0.001
			ToD x Visit	F(2,120.63)	0.17	0.844
			ToD x Session	F(2,205.55)	0.54	0.582
			Visit x Session	F(4,145.76)	1.47	0.215

Table L.3 continued

Experiments 1.1 & 1.2 combined	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			ToD x Visit x Session	F(4,147.02)	0.86	0.487
			Motivation	F(1,270.60)	3.16	0.077
Serial 7s score (correct in 2 mins)	1.5	2812.56	Time of day	F(1,151.74)	1.87	0.174
			Visit	F(2,111.21)	52.10	<0.001
			Session	F(2,173.06)	17.45	<0.001
			ToD x Visit	F(2,113.08)	6.44	0.002
			ToD x Session	F(2,173.41)	0.82	0.441
			Visit x Session	F(4,115.67)	0.99	0.414
			ToD x Visit x Session	F(4,115.88)	0.80	0.531
	1.6	2776.99	Time of day	F(1,151.59)	0.87	0.353
			Visit	F(2,110.47)	57.18	<0.001
			Session	F(2,156.24)	22.27	<0.001
			ToD x Visit	F(2,111.67)	5.80	0.004
			ToD x Session	F(2,154.61)	1.20	0.304
			Visit x Session	F(4,116.54)	1.14	0.342
			ToD x Visit x Session	F(4,117.30)	1.21	0.311
			Motivation	F(1,380.09)	27.13	<0.001
Serial 7s errors (incorrect in 2 mins)	1.5	1915.13	Time of day	F(1,81.57)	0.06	0.805
			Visit	F(2,107.19)	1.25	0.289
			Session	F(2,163.85)	4.60	0.011
			ToD x Visit	F(2,109.03)	0.12	0.890
			ToD x Session	F(2,164.00)	0.25	0.782
			Visit x Session	F(4,111.94)	0.19	0.942

Table L.3 continued

Experiments 1.1 & 1.2 combined	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			ToD x Visit x Session	F(4,112.33)	1.16	0.334
	1.6	1889.38	Time of day	F(1,82.59)	0.08	0.780
			Visit	F(2,105.37)	1.91	0.153
			Session	F(2,156.62)	3.85	0.023
			ToD x Visit	F(2,106.15)	0.12	0.887
			ToD x Session	F(2,155.14)	0.20	0.819
			Visit x Session	F(4,107.47)	0.23	0.920
			ToD x Visit x Session	F(4,108.27)	1.21	0.309
			Motivation	F(1,326.80)	11.78	0.001
Serial 7s RT (ms)	1.5	7672.87	Time of day	F(1,161.61)	0.25	0.618
			Visit	F(2,102.79)	42.23	<0.001
			Session	F(2,152.25)	27.73	<0.001
			ToD x Visit	F(2,103.56)	2.53	0.085
			ToD x Session	F(2,152.36)	0.66	0.519
			Visit x Session	F(4,106.37)	2.19	0.075
			ToD x Visit x Session	F(4,106.64)	1.06	0.379
	1.6	7634.93	Time of day	F(1,164.69)	0.58	0.448
			Visit	F(2,100.57)	42.40	<0.001
			Session	F(2,154.15)	29.24	<0.001
			ToD x Visit	F(2,100.65)	2.32	0.103
			ToD x Session	F(2,152.83)	0.62	0.540
			Visit x Session	F(4,107.45)	2.71	0.034

Table L.3 continued

Experiments 1.1 & 1.2 combined	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			ToD x Visit x Session	F(4,108.36)	1.05	0.383
			Motivation	F(1,310.62)	2.20	0.139
Mood:						
Mental fatigue (rating/9)	1.5	2779.06	Time of day	F(1,79.41)	0.91	0.343
			Visit	F(2,115.12)	1.61	0.205
			Session	F(2,158.09)	0.45	0.638
			ToD x Visit	F(2,116.97)	0.66	0.521
			ToD x Session	F(2,158.09)	1.74	0.179
			Visit x Session	F(4,111.79)	0.40	0.808
			ToD x Visit x Session	F(4,112.25)	0.16	0.960
Positive affect (score/50)	1.5	2085.36	Time of day	F(1,129.26)	3.18	0.077
			Visit	F(2,106.24)	6.44	0.002
			Session	F(2,138.03)	15.91	<0.001
			ToD x Visit	F(2,107.87)	4.32	0.016
			ToD x Session	F(2,138.11)	0.38	0.683
			Visit x Session	F(4,96.71)	1.51	0.205
			ToD x Visit x Session	F(4,97.03)	1.42	0.235
Negative affect (score/50)	1.5	1821.22	Time of day	F(1,97.94)	0.01	0.929
			Visit	F(2,118.14)	1.49	0.229
			Session	F(2,168.32)	6.57	0.002
			ToD x Visit	F(2,119.59)	1.93	0.150
			ToD x Session	F(2,168.34)	0.91	0.404
			Visit x Session	F(4,114.36)	1.28	0.283

Table L.3 continued

	LMM fixed effects, interactions & covariates					
Experiments 1.1 & 1.2 combined	Model	Fit (-2LL)	Factor	df	F statistic	p value
			ToD x Visit x Session	F(4,115.34)	0.05	0.995

Table L.4 LMM results for Experiment 2

Experiment 2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Cognition:						
Immediate recall (score/15)	2.1	2213.36	Visit	F(5,219.21)	1.88	0.098
			Session	F(1,264.00)	30.95	<0.001
			Dose	F(5,216.82)	0.36	0.875
			Session x Dose	F(5,264)	1.23	0.293
Immediate recall interference errors	2.2	-680.2	Visit	F(5,264.06)	1.07	0.379
			Session	F(1,264.01)	11.24	0.001
			Dose	F(5,263.30)	0.16	0.978
			Session x Dose	F(5,264.01)	0.33	0.898
Delayed recall (score/15)	2.1	2259.32	Visit	F(5,219.14)	2.98	0.013
			Session	F(1,264.00)	100.36	<0.001
			Dose	F(5,217.31)	0.76	0.583
			Session x Dose	F(5,264.00)	1.40	0.225
Delayed recall interference errors	2.2	199.64	Visit	F(5,264.04)	0.67	0.647
			Session	F(1,263.06)	58.79	<0.001
			Dose	F(5,263.17)	0.96	0.441
			Session x Dose	F(5,263.06)	1.03	0.401
Seral 3s score (correct in 2 mins)	2.1	3648.66	Visit	F(5,219.20)	80.02	<0.001
			Session	F(1,263.94)	22.98	<0.001
			Dose	F(5,218.38)	1.25	0.287
			Session x Dose	F(5,263.94)	0.25	0.941
Serial 3s errors (incorrect in 2 mins)	2.1	2152.71	Visit	F(5,213.88)	0.83	0.531
			Session	F(1,254.27)	3.37	0.067
			Dose	F(5,214.96)	0.61	0.695
			Session x Dose	F(5,254.27)	0.25	0.942
Serial 3s RT (ms)	2.1	7417.28	Visit	F(5,218.15)	109.92	<0.001
			Session	F(1,261.91)	63.60	<0.001
			Dose	F(5,212.89)	1.47	0.201

Table L.4 continued

Experiment 2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Session x Dose	F(5,261.89)	0.57	0.727
Seral 7s score (correct in 2 mins)	2.1	3191.56	Visit	F(5,218.11)	27.62	<0.001
			Session	F(1,261.55)	16.52	<0.001
			Dose	F(5,217.68)	0.57	0.721
			Session x Dose	F(5,261.63)	1.16	0.328
Serial 7s errors (incorrect in 2 mins)	2.1	2025.52	Visit	F(5,221.58)	0.75	0.585
			Session	F(1,263.97)	1.64	0.201
			Dose	F(5,221.65)	0.65	0.661
			Session x Dose	F(5,263.97)	0.67	0.650
Serial 7s RT (ms)	2.1	8481.82	Visit	F(5,192.26)	20.45	<0.001
			Session	F(1,237.18)	14.59	<0.001
			Dose	F(5,193.34)	0.32	0.898
			Session x Dose	F(5,236.58)	1.51	0.189
Sternberg accuracy (correct/96)	2.1	2747.04	Visit	F(5,206.40)	5.67	<0.001
			Session	F(1,245.07)	0.45	0.501
			Dose	F(5,206.75)	1.07	0.376
			Session x Dose	F(5,245.25)	0.43	0.824
Sternberg scanning rate (ms/item)	2.1	4211.07	Visit	F(5,219.80)	7.69	<0.001
			Session	F(1,264.15)	1.77	0.185
			Dose	F(5,219.15)	1.85	0.104
			Session x Dose	F(5,264.13)	0.42	0.833
Sternberg extrapolated RT (ms)	2.1	5991.01	Visit	F(5,219.23)	3.79	0.003
			Session	F(1,264.00)	17.37	<0.001
			Dose	F(5,219.24)	1.49	0.194
			Session x Dose	F(5,264.00)	0.91	0.476
Stroop accuracy (correct/96)	2.1	2444.58	Visit	F(5,218.39)	0.79	0.560
			Session	F(1,260.78)	0.08	0.772
			Dose	F(5,218.22)	1.09	0.367

Table L.4 continued

Experiment 2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Session x Dose	F(5,260.71)	0.72	0.610
Stroop incongruent RT (ms)	2.1	5601.64	Visit	F(5,218.94)	1.97	0.083
			Session	F(1,264.00)	19.54	<0.001
			Dose	F(5,218.95)	1.82	0.109
			Session x Dose	F(5,264.00)	0.33	0.893
Stroop congruent RT (ms)	2.1	5564.72	Visit	F(5,218.91)	0.61	0.691
			Session	F(1,264.00)	21.99	<0.001
			Dose	F(5,218.71)	2.12	0.064
			Session x Dose	F(5,264.00)	0.49	0.785
Stroop interference effect (ms)	2.1	5153.71	Visit	F(5,219.28)	4.24	0.001
			Session	F(1,263.11)	0.11	0.741
			Dose	F(5,219.96)	1.28	0.272
			Session x Dose	F(5,263.10)	0.69	0.630
Mood:						
Mental fatigue (rating/9)	2.1	2098.79	Visit	F(5,219.63)	3.40	0.006
			Session	F(1,264.00)	10.51	0.001
			Dose	F(5,219.58)	0.40	0.845
			Session x Dose	F(5,264.00)	1.08	0.375
Positive affect (score/50)	2.1	3368.94	Visit	F(5,219.44)	1.86	0.102
			Session	F(1,264.00)	3.37	0.067
			Dose	F(5,218.87)	0.41	0.838
			Session x Dose	F(5,264.00)	0.66	0.654
Negative affect (score/50)	2.1	2496.64	Visit	F(5,219.03)	0.39	0.852
			Session	F(1,259.58)	4.55	0.034
			Dose	F(5,219.06)	0.48	0.793
			Session x Dose	F(5,259.65)	1.05	0.389
Physiology:						
Systolic BP (mmHg)	2.1	3122.99	Visit	F(5,219.10)	2.80	0.018
			Session	F(1,264.00)	153.18	<0.001
			Dose	F(5,218.27)	1.46	0.205

Table L.4 continued

Experiment 2	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Session x Dose	F(5,264.00)	0.21	0.957
Diastolic BP (mmHg)	2.1	2843.16	Visit	F(5,217.05)	3.90	0.002
			Session	F(1,259.02)	107.86	<0.001
			Dose	F(5,216.67)	0.71	0.620
			Session x Dose	F(5,259.87)	0.30	0.915
Heart rate (bpm)	2.1	3322.48	Visit	F(5,219.50)	1.62	0.155
			Session	F(1,263.40)	28.60	<0.001
			Dose	F(5,210.58)	1.46	0.204
			Session x Dose	F(5,263.39)	1.45	0.206
Palatability:						
'Sweet' rating (out of 9)	2.3	987.07	Dose	F(5,44.23)	1.57	0.190
'Bland' rating (out of 9)	2.3	978.25	Dose	F(5,44.52)	2.82	0.027
'Tasty' rating (out of 9)	2.3	1049.05	Dose	F(5,44.54)	10.07	<0.001
'Pleasant' rating (out of 9)	2.3	1046.32	Dose	F(5,44.36)	10.26	<0.001
'Sour' rating (out of 9)	2.3	945.97	Dose	F(5,44.21)	3.97	0.005
'Satisfying' rating (out of 9)	2.3	1053.19	Dose	F(5,44.29)	5.10	0.001
'How much more could you consume?' rating (out of 9)	2.3	1051.72	Dose	F(5,43.24)	6.82	<0.001
'How easy was it to consume?' rating (out of 9)	2.3	1100.45	Dose	F(5,43.75)	10.61	<0.001

Table L.5 LMM results for Experiment 3

Experiment 3	LMM fixed effects, interactions & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Physiology:						
Blood glucose (mmol/l)	3.1	1194.95	Time	F(7,84.00)	45.17	<0.001
			Dose	F(4,59.06)	15.12	<0.001
			Time x Dose	F(28,84.00)	4.53	<0.001
			BMI	F(1,15.99)	5.64	0.030
iAUC	3.2	908.44	Dose	F(4,17.12)	14.02	<0.001
			BMI	F(1,17.88)	0.07	0.795

Table L.6 LMM results for Experiment 4

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Glucose:						
Blood glucose (mmol/l)	4.0	1555.11	Visit	F(3,121.85)	2.02	0.115
			Time	F(4,164.00)	215.62	<0.001
			Dose	F(3,91.99)	4.93	0.003
			Time x Dose	F(12,164.00)	3.36	<0.001
	4.1	1546.29	Visit	F(3,120.95)	2.03	0.113
			Time	F(4,164.00)	215.62	<0.001
			Dose	F(3,90.27)	4.81	0.004
			Time x Dose	F(12,164.00)	3.36	<0.001
			BMI	F(1,39.01)	9.96	0.003
	4.2	1535.77	Visit	F(3,121.01)	2.39	0.072
			Time	F(4,164.00)	11.30	<0.001
			Dose	F(3,91.02)	0.23	0.878
			Time x Dose	F(12,164.00)	0.62	0.824
			BMI	F(1,44.38)	8.48	0.006
			Time x BMI	F(4,164.00)	0.84	0.504
			Dose x BMI	F(3,91.12)	0.19	0.906
			Time x Dose x BMI	F(12,164.00)	0.40	0.962
iAUC	4.0	1646.42	Visit	F(3,39.99)	0.09	0.965
			Dose	F(3,91.69)	2.11	0.104
	4.3	1646.1	Visit	F(3,40.03)	0.09	0.965
			Dose	F(3,92.64)	2.15	0.100
			BMI	F(1,39.86)	0.33	0.569
Cognition:						

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
Digit vigilance score (correct/45)	4.4	2078.6	Visit	F(3,113.74)	3.42	.020
			Session	F(2,154.02)	.48	.621
			Dose	F(3,112.93)	.29	.836
			Session x Dose	F(6,154.00)	1.22	.297
	4.5	2078.58	Visit	F(3,113.98)	3.37	.021
			Session	F(2,180.71)	.42	.656
			Dose	F(3,112.81)	.29	.835
			Session x Dose	F(6,153.77)	1.23	.295
			Glucose	F(1,373.34)	.02	.885
Digit vigilance commission errors	4.4	1660.27	Visit	F(3,121.23)	2.83	.041
			Session	F(2,157.54)	0.63	.535
			Dose	F(3,121.48)	1.02	.388
			Session x Dose	F(6,157.40)	1.20	.308
	4.5	1660.02	Visit	F(3,121.28)	2.74	.046
			Session	F(2,179.16)	0.43	.649
			Dose	F(3,121.36)	1.04	.379
			Session x Dose	F(6,156.89)	1.20	.307
			Glucose	F(1,413.42)	0.27	.607
Digit vigilance RT (ms)	4.4	4512.43	Visit	F(3,123.55)	2.29	.082
			Session	F(2,163.68)	7.59	.001
			Dose	F(3,123.06)	0.65	.581
			Session x Dose	F(6,163.67)	0.58	.748
	4.5	4512.42	Visit	F(3,124.03)	2.28	.083
			Session	F(2,190.03)	7.28	.001
			Dose	F(3,123.13)	0.66	.581
			Session x Dose	F(6,164.07)	0.58	.746

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Glucose	F(1,394.38)	0.01	.915
RVIP score (correct/40)	4.4	2828.14	Visit	F(3,123.36)	0.31	.821
			Session	F(2,163.61)	4.54	.012
			Dose	F(122.70)	0.92	.434
			Session x Dose	F(6,163.61)	0.68	.666
	4.5	2826.45	Visit	F(3,123.72)	0.26	.851
			Session	F(2,192.98)	5.40	.005
			Dose	F(3,122.63)	0.90	.442
			Session x Dose	F(6,164.28)	0.63	.705
			Glucose	F(1,342.04)	1.70	.193
RVIP commission errors	4.4	2936.27	Visit	F(3,123.16)	1.55	.204
			Session	F(2,160.78)	1.60	.206
			Dose	F(3,122.98)	0.07	.977
			Session x Dose	F(6,160.77)	1.03	.405
	4.5	2936.15	Visit	F(3,123.69)	1.51	.216
			Session	F(2,187.78)	1.60	.204
			Dose	F(3,123.09)	0.07	.977
			Session x Dose	F(6,161.14)	1.03	.406
			Glucose	F(1,323.17)	0.12	.726
RVIP RT (ms)	4.4	4603.01	Visit	F(3,122.19)	2.86	.040
			Session	F(2,163.51)	.92	.400
			Dose	F(3,123.36)	1.33	.269
			Session x Dose	F(6,163.51)	1.54	.169
	4.5	4602.68	Visit	F(3,122.75)	2.86	.040
			Session	F(2,190.19)	.48	.622

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Dose	F(3,123.32)	1.34	.263
			Session x Dose	F(6,164.04)	1.58	.156
			Glucose	F(1,416.08)	.33	.567
Serial 3s score (correct in 2 mins)	4.4	3212.85	Visit	F(3,120.61)	97.76	<.001
			Session	F(2,158.32)	18.62	<.001
			Dose	F(3,120.71)	1.84	.143
			Session x Dose	F(6,158.30)	0.66	.684
	4.5	3206.36	Visit	F(3,120.59)	96.90	<.001
			Session	F(2,186.33)	21.80	<.001
			Dose	F(3,120.33)	1.87	.138
			Session x Dose	F(6,158.58)	0.66	.684
			Glucose	F(1,345.39)	6.81	.009
	4.6	3174.72	Visit	F(3,117.04)	106.68	<0.001
			Session	F(2,214.29)	3.62	0.028
			Dose	F(3,308.69)	3.83	0.010
			Session x Dose	F(6,237.09)	1.57	0.155
			Glucose	F(1,405.87)	9.68	0.002
			Glucose x Session	F(2,208.96)	5.16	0.006
			Glucose x Dose	F(3,308.62)	4.81	0.003
			Glucose x Session x Dose	F(6,241.69)	1.69	0.125
Serial 3s errors (incorrect in 2 mins)	4.4	1840.93	Visit	F(3,121.95)	2.19	.093
			Session	F(2,161.68)	2.95	.055
			Dose	F(3,117.66)	0.17	.916

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Session x Dose	F(6,161.74)	0.82	.553
	4.5	1840.76	Visit	F(3,122.43)	2.21	.090
			Session	F(2,184.03)	2.83	.061
			Dose	F(3,117.61)	0.18	.911
			Session x Dose	F(6,159.80)	0.83	.552
			Glucose	F(1,397.38)	0.17	.678
Serial 3s RT (ms)	4.4	7006.66	Visit	F(3,116.29)	31.53	<.001
			Session	F(2,156.34)	31.24	<.001
			Dose	F(3,106.34)	1.00	.394
			Session x Dose	F(6,156.23)	1.44	.204
	4.5	7006.44	Visit	F(3,116.39)	31.60	<.001
			Session	F(2,181.67)	28.98	<.001
			Dose	F(3,106.57)	0.99	.399
			Session x Dose	F(6,156.82)	1.41	.212
			Glucose	F(1,305.07)	0.23	.632
Serial 7s score (correct in 2 mins)	4.4	2909.63	Visit	F(3,118.96)	35.01	<.001
			Session	F(2,156.55)	11.62	<.001
			Dose	F(3,118.73)	1.15	.330
			Session x Dose	F(6,156.43)	0.93	.477
	4.5	2907.69	Visit	F(3,117.46)	36.54	<.001
			Session	F(2,185.35)	12.37	<.001
			Dose	F(3,117.11)	1.22	.305
			Session x Dose	F(6,156.74)	0.81	.566
			Glucose	F(1,366.22)	2.01	.157
Serial 7s errors (incorrect in	4.4	1905.87	Visit	F(3,122.12)	1.77	.157

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
2 mins)			Session	F(2,163.04)	2.42	.092
			Dose	F(3,120.87)	0.94	.423
			Session x Dose	F(6,163.09)	1.06	.389
	4.5	1905.66	Visit	F(3,121.53)	1.81	.149
			Session	F(2,185.23)	2.07	.129
			Dose	F(3,119.52)	0.95	.419
			Session x Dose	F(6,163.16)	1.03	.410
			Glucose	F(1,432.31)	0.22	.642
Serial 7s RT (ms)	4.4	7736.26	Visit	F(3,114.31)	24.56	<.001
			Session	F(2,145.93)	10.99	<.001
			Dose	F(3,115.09)	1.71	.168
			Session x Dose	F(6,146.53)	0.89	.502
	4.5		Visit	F(3,114.47)	24.64	<.001
		7736.06	Session	F(2,170.14)	10.93	<.001
			Dose	F(3,115.05)	1.72	.166
			Session x Dose	F(6,148.88)	0.88	.509
			Glucose	F(1,341.38)	0.20	.655
Mood:						
Mental fatigue (rating/9)	4.4	1882.09	Visit	F(3,123.79)	0.34	0.799
			Session	F(2,163.32)	3.45	0.034
			Dose	F(3,123.45)	2.04	0.112
			Session x Dose	F(6,163.30)	0.53	0.788
	4.5	1879.06	Visit	F(3,124.28)	0.32	0.813
			Session	F(2,193.07)	4.77	0.009
			Dose	F(3,123.30)	2.12	0.102
			Session x Dose	F(6,163.37)	0.60	0.728

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Glucose	F(1,364.76)	3.12	0.078
	4.6	1868.98	Visit	F(3,120.40)	0.27	0.844
			Session	F(2,235.88)	1.09	0.338
			Dose	F(3,321.80)	0.67	0.573
			Session x Dose	F(6,239.18)	0.77	0.593
			Glucose	F(1,434.37)	1.19	0.276
			Glucose x Session	F(2,237.88)	1.79	0.169
			Glucose x Dose	F(3,329.16)	0.37	0.772
			Glucose x Session x Dose	F(6,246.05)	0.92	0.482
Positive affect (score/50)	4.4	3042.2	Visit	F(3,121.50)	2.01	0.116
			Session	F(2,161.97)	4.99	0.008
			Dose	F(3,120.70)	1.31	0.275
			Session x Dose	F(6,161.96)	1.07	0.384
	4.5	3027.96	Visit	F(3,122.47)	1.80	0.150
			Session	F(2,189.76)	5.55	0.005
			Dose	F(3,121.48)	1.13	0.338
			Session x Dose	F(6,163.31)	0.95	0.461
			Glucose	F(1,356.86)	1.36	0.244
Negative affect (score/50)	4.4	2227.91	Visit	F(3,120.75)	0.40	0.751
			Session	F(2,161.93)	4.63	0.011
			Dose	F(3,111.87)	0.63	0.594
			Session x Dose	F(6,162.81)	0.63	0.705
	4.5	2227.86	Visit	F(3,121.18)	0.40	0.750

Table L.6 continued

Experiment 4	LMM fixed effects & covariates					
	Model	Fit (-2LL)	Factor	df	F statistic	p value
			Session	F(2,193.08)	4.23	0.016
			Dose	F(3,111.94)	0.63	0.597
			Session x Dose	F(6,161.77)	0.63	0.708
			Glucose	F(1,307.10)	0.04	0.834
Palatability:						
'Sweet' rating (out of 9)	4.7	602.7	Dose	F(3,40.81)	15.76	<0.001
'Bland' rating (out of 9)	4.7	633.65	Dose	F(3,40.94)	6.79	0.001
'Tasty' rating (out of 9)	4.7	645.36	Dose	F(3,40.62)	2.11	0.114
'Pleasant' rating (out of 9)	4.7	662.16	Dose	F(3,40.43)	2.13	0.112
'Sour' rating (out of 9)	4.7	503.48	Dose	F(3,40.63)	8.82	<0.001
'Satisfying' rating (out of 9)	4.7	650.21	Dose	F(3,40.59)	1.05	0.382
'How much more could you consume?' rating (out of 9)	4.7	664.4	Dose	F(3,40.62)	0.94	0.429
'How easy was it to consume?' rating (out of 9)	4.7	682.43	Dose	F(3,40.56)	0.457	0.714