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Cognitive Effects Following Acute Wild Blueberry Supplementation on 7 – 10 Year Old Children.

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Introduction

It is widely accepted that diet has an influence on the cognitive capabilities and development of children [1]. To date, research has focused primarily on maternal diet during pregnancy, the chronic effects of micronutrients and polyunsaturated fatty acids during childhood, and the acute effects of eating occasions (such as breakfast) and different carbohydrate load [2-4]. Few intervention studies have investigated the acute and chronic effects of specific micro- or macronutrients on cognitive performance in school aged children. However, where this research has been done, benefits have been found on non-verbal intelligence resulting from multi-vitamin supplementation [5, 6] and for sustained attention following acute carbohydrate intervention [7, 8] though evidence of beneficial effects following polyunsaturated fatty acids intervention remains equivocal [9]. To our knowledge, however, no acute fully controlled, double blinded research on the effects of flavonoids, found naturally in foods such as fruit, vegetables, teas and fruit juices, on cognitive behaviour of children has been carried out.

The health benefits of flavonoids, such as improvements in coronary and vascular function, are well documented [10]. There is also a growing body of research from preclinical and adult human trials indicating that both chronic and acute interventions with flavonoids can lead to cognitive improvements in animals and humans [11- 14] with berries (the main source of anthocyanins, a particular class of flavonoids, in the human diet) being known to protect against neuronal stress [15] and positively mediate signalling pathways in the brain [16]. Indeed, pre-clinical work has found that 7-12 weeks of supplementation with blueberry anthocyanins produces significant improvements in rodent visuo-spatial memory [17, 18]. Similarly, following one-off interventions in adults, improvements have been reported following acute cocoa flavonol interventions on memory related areas such as spatial working memory [19] and attention-related executive function tasks such as the serial 3s and RIVP [20]. Chronic supplementation has also shown improvements in visuo-spatial memory following supplementation with pinus radiata extract of proanthocyanins for 5 weeks or 3 months [21, 22] and immediate verbal memory following 12 week supplementation with blueberry and grape anthocyanins [23, 24]. The mechanisms by which flavonoids exert these actions on cognitive performance are still being elaborated, including evidence which suggests that they may increase cerebral blood flow (CBF) [25, 26, 27] as well as modulate the activation status of neuronal receptors, signalling proteins and gene expression [17, 18, 27, 28]

As discussed above there has been little research investigating flavonoid related cognitive interventions with children. Currently, only two short reports have been published. Firstly, a study by Calderón-Garcidueñas and colleagues showed that supplementing children (mean age 10.55) with a 680 mg dose of cocoa flavanols for a period of between 9 –

24 days produced marginally significant improved performance on letter and object span tests for 15 of the 18 participants. Importantly however, this study did not control for duration of intervention or amount of sugar consumed with each drink [29]. More latterly from our own laboratory, in a within-subjects pilot study testing children aged between 8 -10 years [30], we found improvements in delayed memory performance following acute intervention with a 200g fresh high-bush blueberry drink containing 143mg anthocyanins. Though, on this occasion, we did not find improved performance on executive function tasks, the positive memory findings give a solid basis upon which to expect further cognitive benefits to become evident in a more comprehensive time course and dose response study.

Seven- to ten-year-olds were chosen for the present study because, coinciding with a spurt in frontal lobe growth, children of this age have sufficient cognitive ability to competently perform the type of executive function and memory tasks which have shown improvement in adult studies [31, 32, 33]. With regards to the tasks, inhibition tasks such as the Go-NoGo have proved sensitive during adult flavonoid interventions [19, 20] and a study by Hilman et al. [34] has shown an interference task, the Modified Flanker Task (MFT), to be sensitive to acute exercise (which induces increases in Brain Derived Neurotrophic Factor (BDNF) and CBF in a similar way to acute flavonoid interventions) in 9.5 ± 5 years children**.** Furthermore, the MFT and Go-NoGo tasks are known to activate the same brain areas in children (dorsolateral prefrontal cortex, anterior cingulate cortex) [27] which have been found to show increased activation in adult research following a flavonoid intervention [35, 36]. Given that flavonoid intervention has a positive effect on BDNF levels, and also that studies have shown learning and memory effects following flavonoid intervention [25, 26, 30], a modified version of the Rey's Auditory Verbal Learning Task (AVLT) was developed. This task examines performance in both learning and memory recall and has also proven to be sensitive to levels of attention in children aged 8 – 12 [37]. Finally, in order to ascertain whether flavonoids have an effect on processing speed, a levels-of-processing Picture Matching Task (PMT) [38] was introduced.

Lamport *et al*. [11] note that, though positive cognitive effects are often found, an association between polyphenol (and therefore flavonoid) dose, duration of intervention, and cognitive performance has yet to be established. Given this lack of consistency in the data obtained to date, future work should aim to establish dose, duration and performance effects of flavonoid supplementation on cognition. Such work is of relevance in children and adolescents because the positive cognitive effects, if translated to this age group, would be beneficial in an educational setting. Here, therefore, we describe a dose and time course study examining the acute effects of blueberries (spp. vaccinium angustifolium; rich in anthocyanins) on children aged 7 – 10 years completing a range of cognitive tasks.

Method

This study was reviewed by the University of Reading Research Ethics Committee and was given a favourable ethical opinion for conduct.

Participants

An *a priori* power analysis (using G Power 3.1.9.2) based on the significant findings from our previous work (Whyte & Williams 2015) revealed that 23 participants would be required to achieve a power of 0.8. In order to achieve counter-balancing of the 3 interventions, 24 participants (14 female) aged 7-10 years old of varying ethnicity were recruited from two local schools located in ABC1 areas of the UK. Written consent was obtained from parents or legal guardians in advance of the child's participation. On initial recruitment, parents or legal guardians confirmed that the children spoke English as a first language, had not been diagnosed with ADHD or dyslexia, and had no known fruit or fruit juice intolerance. Participants completed the children's version of Ravens Coloured Progressive Matrices (RCPM) as a measure of fluid intelligence, and 'word definitions' and 'verbal similarities' from the British Ability Scales II (BAS II) as measures of crystallised word understanding. Parents or guardians completed the ADHD rating scale IV and the Edinburgh handedness inventory on behalf of the participants. No participants were extreme outliers on any these measures. Three participants were excluded because they failed to consume at least one of the treatments. Thus, all analyses were completed on data from 21 participants. A post hoc power analysis was therefore conducted calculating power values for all significant treatment related results reported below. This revealed an average post hoc power of 0.68.

Demographic details of the participants are shown in Table 1.

Table 1. Mean (SD) values for demographic data

Variable	All Participants	Females	Males
N	21	12	9
Age	8.7 (0.67)	8.5 (0.66)	8.9 (0.67)
Ravens	27. 7 (3.25)	27.8 (3.25)	27. 6 (3.43)
Word Definitions	111.3 (17.4)	109 (19.4)	114.3 (14.8)
Verbal Similarities	100.5 (15.6)	102 (16.5)	99.2 (15.2)
ADHD	9 (8.62)	5.7 (6.61)	13.3 (9.37)
Fruit + Veg Portions	4.9 (2.32)	5.5 (2.16)	4.1 (2.38)

Treatments

On each test day, participants were administered a drink containing either 15g or 30g freeze dried wild blueberries (WBB), or vehicle-only treatment. Each participant completed three treatment days with a seven day wash-out between treatments. Our 30g WBB (equivalent to ~240g fresh wild blueberries; 108 kcal) treatment contained 253mg anthocyanins, while our 15g (equivalent to ~120g fresh wild blueberries) WBB treatment contained 127mg anthocyanin. Both the vehicle and 15g WBB treatments had fructose, sucrose and vitamin C added in order to match levels of these nutrients with the 30g WBB treatment**.** Prior to each test day, a confederate placed all powders into an opaque drinking cup with a small opening for a straw, all cups were then labelled with the participant number. Half an hour before intervention all powders were mixed with 30ml a low energy fruit squash (Rocks brand, UK: 8.4 kcal) a low polyphenol drink (13.2mg in total) and 170ml of water giving a total of ~220ml in liquid to consume. This was added by the experimenter to the cup through the opening using a funnel and then shaken to fully mix the contents. Treatments were consumed through a black straw by the participant. Both experimenter and participant therefore remained blind to the treatment on each test day. Treatments were administered in a fully counterbalanced order across participants.

Cognitive Tests

E-Prime V2 (Psychology Software Tools, Inc.) running on a 15" Toshiba Satellite laptop was used to display the stimuli and record participant responses. To present the audio stimuli during the AVLT task, and also to control for external noise, participants wore enclosed headphones throughout all tasks.

1) **Auditory-Verbal Learning Task (AVLT).** This task examined performance in learning, memory recall and recognition. The AVLT consisted of five consecutive free recalls (Recalls 1 to 5) of the same 15 nouns (List A) presented auditorily at a rate of 1 word per second. A further list of fifteen nouns (List B) was then presented as an interference list and recalled once only (Recall B). There was then a further free recall of List A (Recall 6) followed by a fifteen minute delay and then a final free recall of List A (Recall 7). Finally, participants were shown a list of 50 nouns, containing all the words from List A and List B plus an additional 20 filler words, and asked to circle only the words from list A. Different lists were created for each test session with all words having AOA ratings of less than 400 (equivalent to age 7 and below) and being matched (all ps≥ .49) for concreteness and familiarity (see Online Resource 1).

   For each test session we calculated the following outcomes as specified in Lezak, Howieson and Loring [39]: Immediate Word Span (Recall 1) – showing immediate free recall ability; Number of Words Learned (Recall 5 minus Recall 1) – showing learning over the session; Final Acquisition level (Recall 5) – showing the total number of words

learned; Proactive Interference (PI; Recall B minus Recall 1) – indicating the effect of previously encoded words on the encoding of new words; Retroactive Interference (RI; Recall 5 minus Recall 6) – indicating the effect of encoding new words on previously encoded words and Word Recognition expressed as the number of correctly circled words.

2) **Modified Flanker Task (MFT).** This task examined response interference. Using the method of Hillman *et al*. [34] (2009), arrow symbols "<" and ">" were presented in white against a black background five in a row. The middle arrow was either congruent (*i.e.* <<<<< or >>>>>) or incongruent (*i.e.* <<><< or >><>>) with the pairs of arrows on either side. The stimulus was displayed for 120ms and was followed by a pseudorandom inter-stimulus interval of 1000, 1300 or 1500ms. There were 100 trials with presentation randomised so the arrows appeared with equal probability of congruence and direction. Participants were instructed to press the left and right arrow keys on the keyboard according to the direction of the centre arrow.

   Accuracy and Response Times (RTs; with RTs less than 100ms removed) for both congruent and incongruent trials were measured separately. Additionally, we calculated Interference Effect measures separately for accuracy and RT by subtracting incongruent trial performance from congruent trial performance.

3) **Go-NoGo.** This task examined response inhibition. Participants were shown stimulus slides of either a cartoon mole (Go Target) or a cartoon rabbit (NoGo Non-Target) which were presented for 300ms. This was followed by a fixation slide of an empty mole hole presented for 1300ms. The object of the game was explained as saving the garden by using the right hand to press the space bar in order to "whack" the moles as they popped out of the hole, or to avoid "whacking" the rabbit. There were 100 trials 25 of which were NoGo trials.

   A d-prime measure was calculated by subtracting the false alarm rate z-score from the hit rate z-score (both normalized using the Excel "normsinv" function as specified in Stanislaw & Tordorov, [40]) for each child. False Alarm Rate and RTs (Go trials only) were recorded and a speed/accuracy trade off was calculated by converting the False Alarm rate and RTs into z-scores (normalised to SD of overall False alarm rate and RTs respectively) and then dividing the resulting RT z-scores by the False Alarm z-scores.

4) **Picture Matching Task (PMT).** This task investigated both levels of processing and response interference. Line drawings of a banana, book, jack in the box, and umbrella, shown in either open or shut states, similar to those used by Bisanz *et al*. [38] were created giving 8 pictures in total. As can be seen in Fig.1, combinations of pairs of items were shown to participants which were either Physically Different and Name Different (PDND), Physically Different and Name Same (PDNS), or Physically Same and Name Same (PSNS). Participants performed two versions of the task: in the Physical Match Task, participants responded yes (by pressing a green key) or no (by pressing a red key) to the pictures according to whether or not they were an exact match (i.e. a yes response should be made in the PSNS

condition), whereas in the Name Match Task, participants were instructed to respond yes or no according to whether or not the pictures had the same name (i.e. a yes response should be made for both the PDNS and PSNS conditions). Participants were shown the picture pairs on either side of a fixation cross which remain displayed until the participant responded. Trial intervals were filled with a 200ms fixation cross.

Median reaction time (with RTs less than 100ms removed) and accuracy was measured for all PDND, PDNS, and PSNS trials.

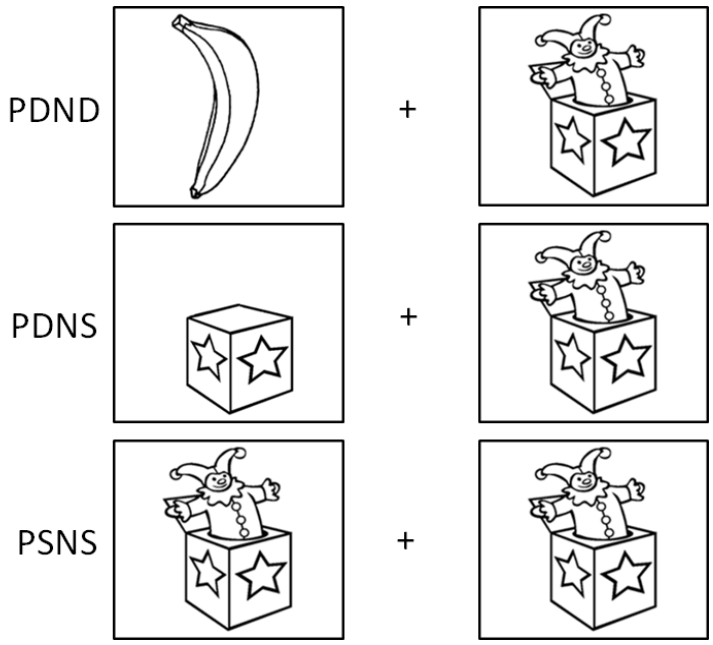

Fig.1 – Example of pairs of stimuli as used in the PMT. Physically Different/Name Different (PDND) should always receive a "no" in both the physical match task and name match task. Physically Different/Name Same (PDNS) should receive a "no" response in the Physical match task and a "yes" response in the Name match task. Physically Same/Name Same (PSNS) should always receive a "yes" response in both the Physical match task and the Name match task.

Procedure

Pre-test session: On the evening before the first test session, a pre-test session took place at the participants' home address where the RCPM, Word Definitions and Verbal Similarities from the BAS II, the ADHD rating scale IV and the Edinburgh Handedness Inventory were administered. Participants also completed 11 practice trials of the Go-NoGo, 12 of the MFT and 16 trials each of the physical and named conditions of the PMT in order to become accustomed to these tasks prior to the test days. Where participants showed less than 50% accuracy on a task further trials were completed until the participant achieved at least 50% accuracy. All participants achieved >50% accuracy for the first practice of the Go-NoGo (mean = 75%) and PMT task (physical match task mean = 85.4%; name match task mean = 88.5%) conditions. The MFT was found to be more demanding with two children needing two attempts, eight children needing three attempts, one child needing six attempts and one needing nine attempts before achieving >50% accuracy. Parents or guardians of the

participants were provided with a list of foods to avoid in order to ensure a low flavonoid diet was consumed on the evening before each test session.

Treatment days: On each test day, a low-flavonoid breakfast, snacks and lunch were provided. The breakfast consisted of 30g cornflakes with 125ml of semi-skimmed milk and 5g of sugar for breakfast (254 kcal in total). The mid morning snack was a small (125g) banana (~80 kcal) and lunch consisted of a 25g bag of salted potato crisps and a plain sandwich containing 2 slices of white sliced bread, 10g of butter and either 26g ham, 20g chicken or 22.5g cheese as a filling (matched for energy content – 404 kcal in total). Participants drank only water during the test day and were allowed to consume as much as they wished. Participants were tested in quiet unoccupied rooms in the two schools from which the participants were drawn. On each treatment day, following breakfast, an initial baseline test session took place at 0830hrs (test session 1). The treatment was then consumed at 0900hrs with the participants being given 10 minutes to consume the drink in its entirety. Three further test sessions took place (±10mins) at 1015hrs (test session 2), 1200hrs (test session 3), and 1515hrs (test session 4). The snack and lunch were consumed immediately after the second and third test sessions respectively. During each test session participants completed the tasks in the order, AVLT Recalls 1-6, MFT, Go-NoGo, PMT, AVLT Recall 7 and Recognition (see Fig. 2).

Following the final test session, participants were debriefed regarding the purpose of the study and informed of the contents of the drinks. All children received a £10 book token though this payment was not disclosed until completion of the study.

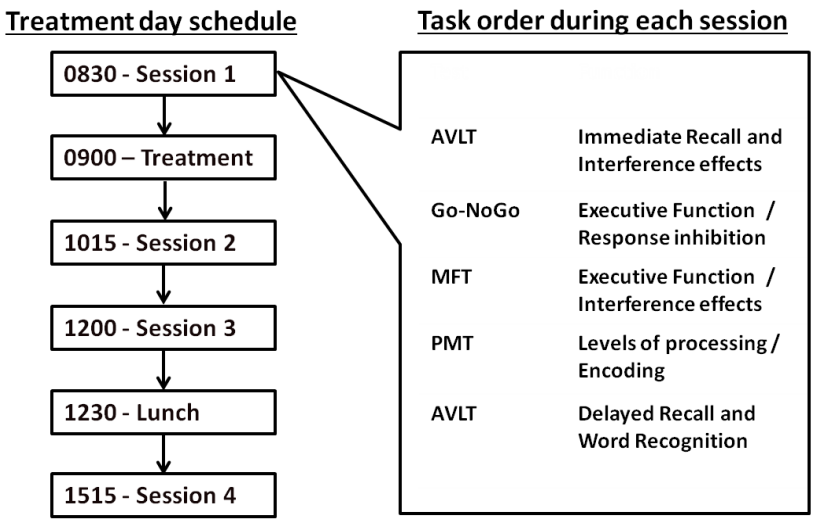

Fig. 2. Schedule of sessions, intervention and task order for each test day.

Analysis

To confirm that performance was broadly consistent with previous studies, an initial non change from baseline analysis was conducted where appropriate using raw behavioural data. Additionally, we compared the pre-intervention baseline data for each condition on each test day to ensure there were no significant differences in baseline performance. For all test conditions, comparisons between baseline performance on each test day were non-significant (all *ps* > .082). All post-intervention measures for AVLT and Go-NoGo and MFT interference effect were then analysed as change from baseline using 3 x 3 (Treatment x Session) repeated measures ANOVAs. In order to further explore dose dependency, linear and quadratic contrasts were performed for treatment in the above ANOVAs. Dependent variables (DV) for the MFT were analysed separately as change from baseline using 3 x 2 x 3 (Treatment x Congruency x Session) repeated measures ANOVAs. Dependent variables for the PMT name-match and physical-match tasks were analysed separately as change from baseline using 3 x 3 x 3 (Treatment x Picture Type x Session). Again, linear and quadratic contrasts were performed for treatment effects in the above ANOVAs. Bonferroni-adjustment for multiple comparisons was used for all post hoc analyses. Finally, to analyse the composite effects of anthocyanin dose on overall cognitive performance across the battery, all change from baseline measures for all participants at each time point were analysed by a Page's test [38] for monotonic ordered treatment effects.

SPSS v 19-21 was used to carry out the analyses.

Results

Auditory-Verbal Learning Task.

As shown in Fig. 3, during each test session, participants recalled a greater number of words from the primary word list on each subsequent attempt of the first five recalls (shown as Rec1 – 5) indicating a significant learning effect, $F(2,80) = 107.3$, $p < .001$, $\eta_p^2 = .843$. Additionally, as we would expect, there was also evidence of cumulative, between list, Proactive Interference (PI) over the course of each test day, with participants, irrespective of treatment, showing a decrease in the average number of words recalled in each subsequent test session, $F(3,60) = 14.8$, $p < .001$, $\eta_p^2 = .426$.

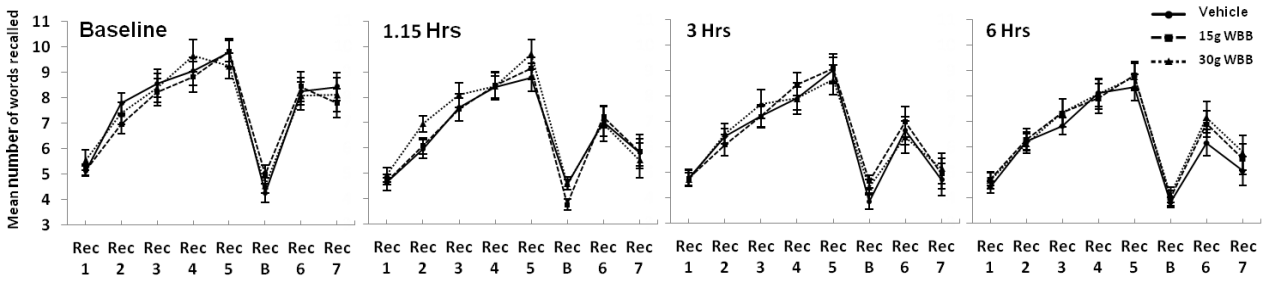

Fig. 3. Word recall performance on the AVLT for the baseline session and three post intervention sessions at 1.15, 3 and 6hrs. Maximum score for each recall is 15. Mean immediate and delayed recall (± standard error of the mean) performance for the AVLT is shown for each recall attempt by vehicle, 15g and 30g WBB. Performance shows the expected learning and within session proactive interference effects (as shown in the poorer performance for recall 6) and between session proactive interference effects (as shown in a general decline in words recalled on each subsequent session).

As can be seen in Fig. 4a, participants showed a reduction in final acquisition for all time points throughout the day with the exception of 1.5hrs following intervention where the 30g WBB intervention showed an increase from baseline of 0.57 words. A Drink X Session ANOVA suggested a weak trend for session, $F(2,40) = 2.56$, $p = .09$, $\eta_p^2 = .113$, along with a significant simple main effect of treatment also being evident 1.15 hrs following intervention, $F(2,40) = 4.13$, $p = .023$, $\eta_p^2 = .171$. A significant positive linear trend was also found for treatment at 1.15 hrs where, as can be seen from figure 4a, the vehicle shows the greatest negative change (mean = -.95) and 30g WBB the greatest positive change (mean = .57), $F(1,20) = 8.76$, $p = .008$, $\eta_p^2 = .305$. Post hoc analysis of this main effect suggests that the improvement in the 30g WBB condition contrasted significantly with the vehicle which showed a decrease in recall of 0.95 words at this time point, $p = .023$, following Bonferroni correction for 3 comparisons. However, no significant difference from vehicle was found for the 15g WBB treatment which showed a decrease in recall of 0.6 words. The effect seen at 1.15hrs was not maintained over the remaining test sessions.

For all treatments there was a significant decrease in participants' Delayed Recall (Rec7 in Fig. 3) at 1.15, 3 and 6 hrs after the baseline session, $F(3,60) = 23.5$, $p < .001$, $\eta_p^2 = .541$. However, for the 15g WBB treatment this decrease was smaller in magnitude than both the vehicle and 30g WBB treatments, indeed our change from baseline analysis (Fig. 4b) shows a trend towards significantly different performance between our vehicle and 15g WBB treatments indicating less negative effect on Delayed Recall, $F(1,20) = 3.46$, $p = .078$, $\eta_p^2 = .147$. No significant linear or quadratic trend effects were found for the change from baseline analysis on this measure.

No significant effects were found for the PI and RI measures.

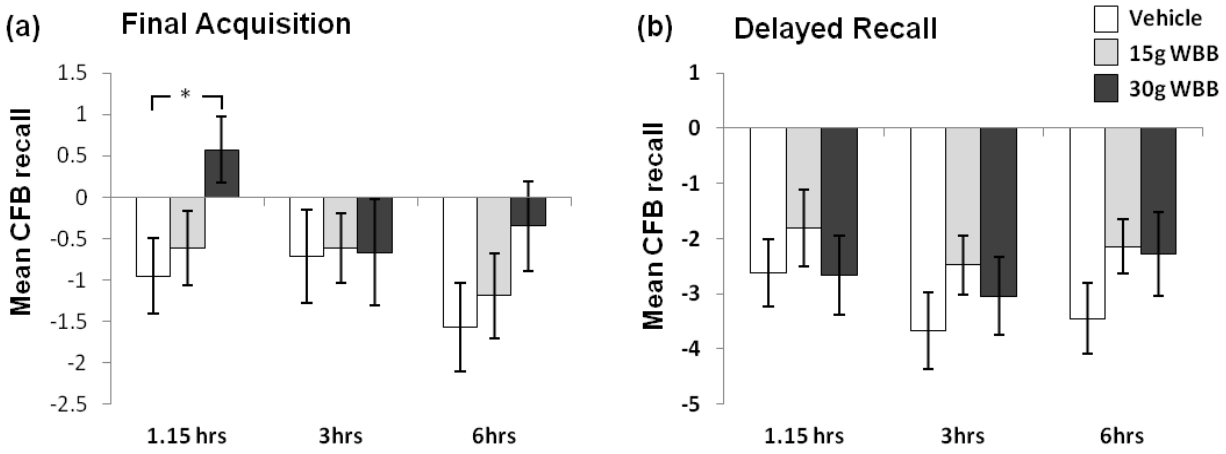

Fig. 4. Mean change from baseline recall (± standard error of the mean) for **a** Final Acquisition (recall 5), and **b** Delayed Recall performance at all time points following vehicle, 15g or 30g WBB intervention. All scores calculated by subtracting number of words recalled at baseline (max 15) from number of words recalled at each subsequent post intervention session. Final Acquisition performance can be seen to significantly improve (* = p<.05 following Bonferroni correction) at 1.15 hours following 15g intervention in comparison to vehicle. There is evidence of a less steep decline in Delayed Recall performance at all time points following 15g or 30g intervention in comparison to vehicle, however, this fails to reach significance.

For all treatments, there was a decrease in Word Recognition at 1.15, 3 and 6hrs following the baseline session, $F(3,60) = 23.3$, $p < .001$, $\eta_p^2 = .538$ (Fig. 5a). There was a significant change from baseline main effect of treatment, $F(2,40) = 3.94$, $p = .027$, $\eta_p^2 = .164$, with the vehicle showing a greater decrease in the number of words recognised in comparison to both the 15g and 30g WBB treatments. A significant positive linear trend was also found for treatment, $F(1,20) = 6.86$, $p = .016$, $\eta_p^2 = .255$. Subsequent analysis found decrease in performance for the vehicle drink was significantly different to the decrease seen for both the 15g WBB, $F(1,20) = 6.57$, $p = .019$, $\eta_p^2 = .247$, and 30g WBB treatments, $F(1,20) = 6.86$, $p = .016$, $\eta_p^2 = .255$. As can be seen in Fig. 5b, the vehicle treatment showed the poorest change from baseline recognition performance 6 hrs post-intervention where a decrease of 3.66 words could be seen. In comparison the 15g WBB treatment showed a decrease of only 1.85 words, $p = .038$ following Bonferroni correction for 3 comparisons, and the 30g treatment showed a decrease of only 1.81 words, however, this failed to reach significance, $p = .131$ following Bonferroni correction for 3 comparisons.

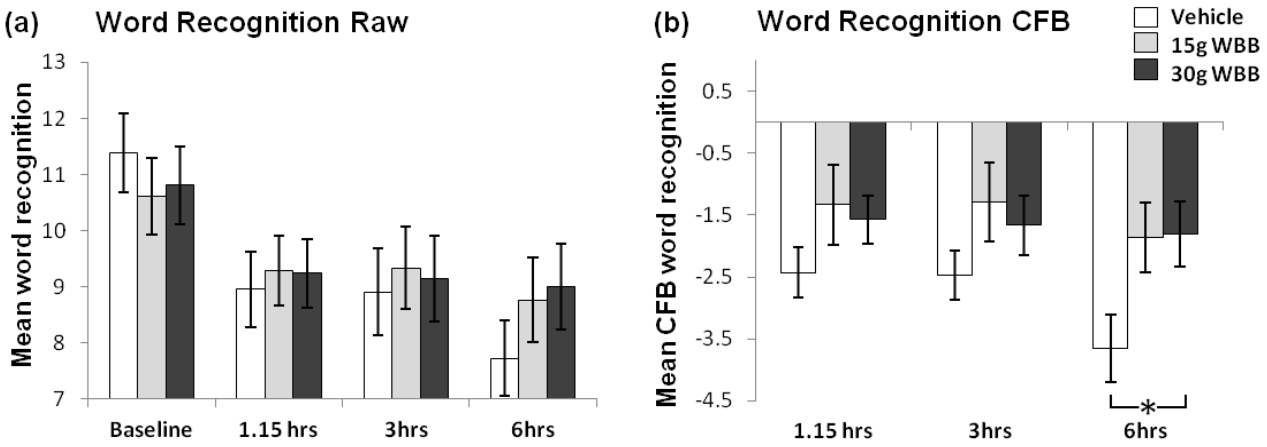

Fig. 5. AVLT word recognition performance. **a** Raw mean word recognition with a maximum score of 15 (± standard error of the mean) showing, for each dose, a decrease in performance for the three post baseline sessions which is particularly marked for the vehicle. **b** Mean change from baseline word recognition (± standard error of the mean) indicating a greater reduction in performance at all time points following vehicle in relation to the 15g and 30g doses which reaches significance at 6hrs following intervention (* = p<.05 following Bonferroni correction). Scores calculated by subtracting number of words recalled at baseline (max 15) from number of words recalled at each subsequent post intervention session.

Modified Flanker Task

As expected, our analyses revealed that response interference was evident with participants showing significantly lower accuracy, $F(1,20) = 1.83$, p <.001, $\eta_p^2 = .522$, and slower response times, $F(1,20) = 22.8$, p <.001, $\eta_p^2 = .532$ for the incongruent trials in relation to the congruent trials. Furthermore, change from baseline analysis for accuracy also found a significant effect of congruence for all treatments, $F(1,20) = 4.59$, $p = .045$, $\eta_p^2 = .522$.

Data were then analysed separately for both the congruent and incongruent conditions with only incongruent trials showing a significant effect of session, $F(2,40) = 3.39$, $p = .044$, $\eta_p^2 = .145$. As can be seen in Fig. 6a, there were modest dips in accuracy on incongruent trials at 1.15hrs for all treatments, and, although this was maintained at 3hrs for the vehicle treatment (mean = -0.03) this contrasted with an improvement for the 30g WBB treatment (mean = 0.055). Importantly, incongruent condition performance between all treatments was found to be significant at the 3hr point, $F(2,40) = 4.02$, $p = .026$, $\eta_p^2 = .167$. Furthermore, a significant positive linear trend was also found for treatment in the incongruent conditions, $F(1,20) = 4.45$, $p = .48$, $\eta_p^2 = .182$. As can be seen in Fig. 6a, participants performed least accurately following vehicle and most accurately following 30g WBB at the 3hr point. Post hoc analysis also revealed a significant difference between vehicle and 30g WBB treatments at the 3hr point, $p = .035$ following Bonferroni correction for 3 comparisons. Performance

for the vehicle treatment returned to levels similar to baseline at 6hrs and, though the 30g WBB treatment performance remained elevated, no statistically significant differences were found. No further significant effects were found for this task.

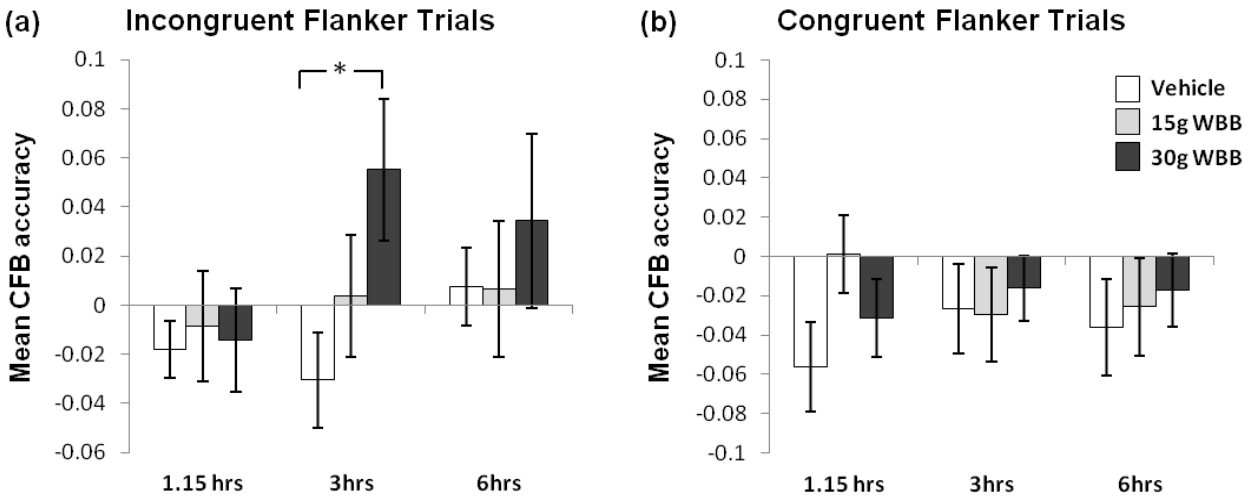

Fig. 6. Change from baseline accuracy for **a** incongruent and **b** congruent flanker task trials (± standard error of the mean) showing improved accuracy performance (* = p<.05 following Bonferroni correction) on incongruent trials at 3 hours following intervention in relation to the control drink. Scores calculated by subtracting average accuracy score (max 1) from accuracy score at each subsequent post intervention session.

Go – NoGo Task

Regardless of treatment, change from baseline performance decreased significantly over each subsequent session for Go trial accuracy, $F(2,34) = 3.73$, p=.034, $\eta_p^2 = .180$, Go trial RT, $F(2,34) = 4.48$, $p = .014$, $\eta_p^2 = .222$, and also showed a similar trend for the d-prime measure, $F(2,34) = 3.04$, $p = .074$, $\eta_p^2 = .142$. Change from baseline comparisons between treatments, however, failed to reach significance for d-prime or Go trial accuracy. For Go trial RT there was a significant effect of treatment at the 1.15hr point, $F(2,34) = 3.59$, $p = .039$, $\eta_p^2 = .174$. As can be seen from Figure 3.8A, the vehicle treatment performance improved by 26.0 ms compared to a modest slowing of 5ms for the 127 mg treatment (p =.078 following Bonferroni correction for 3 comparisons). No significant linear, $F(1,17) = 3.64$, $p = .074$, $\eta_p^2 = .176$, or quadratic, $F(1,17) = 3.55$, $p = .077$, $\eta_p^2 = .173$, RT trends were found for treatment at the 1.15hr point. No significant main effects were found at 3 or 6 hrs. All analyses for the accuracy and response time trade off and false alarm measures failed to reach significance.

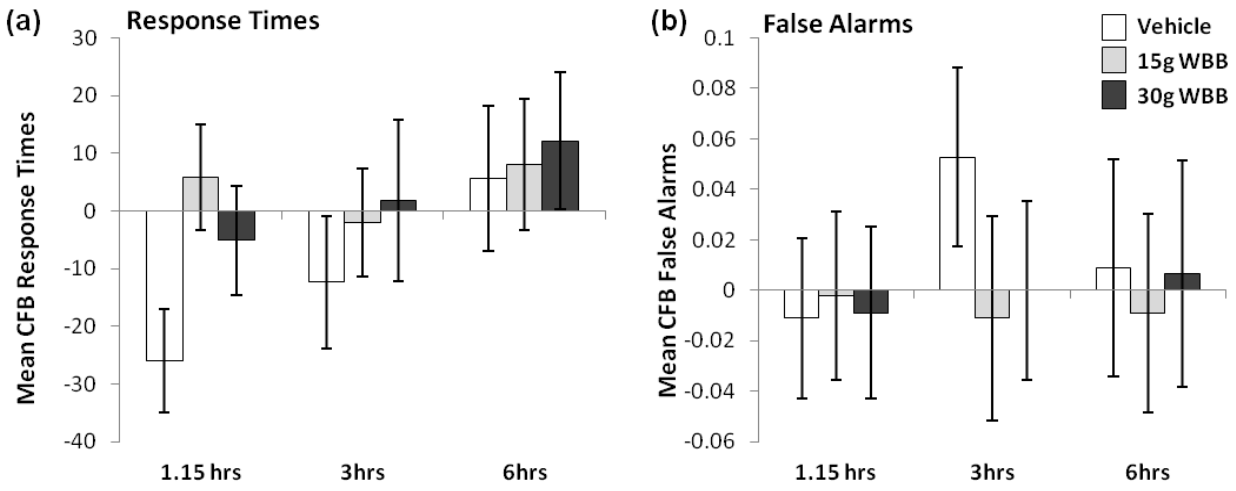

Fig. 7. Change from baseline for **a** Go trial response time (scores calculated by subtracting baseline average response time from average response time at each subsequent post intervention session) shows a trend for faster response times at 1.15hrs for the control intervention in comparison to the 15g WBB dose and **b** number of false alarms (Scores calculated by subtracting baseline average False alarm score (max 1) from average False alarm score at each subsequent post intervention session) shows a non-sig increase in false alarms at 3hrs for the control intervention.

Picture Matching Task

As expected, analysis of the raw data revealed that additional processing time was required for the named picture task with participants showing significantly slower response times in comparison to the physical picture task, $F(1,20) = 40.6$, $p < .001$, $\eta_p^2 = .670$. A main effect of session also revealed faster reaction times as the participants progressed through the four sessions $F(3,60) = 10.6$, $p < .001$, $\eta_p^2 = .347$.

Figures 8a & b show the change from baseline reduction in median reaction time separately for the physical-match and named-match tasks over sessions 2-4. Change from baseline analysis was performed separately on the name-match and physical-match tasks as 3 x 3 x 3 (Treatment x Session x Picture Type) ANOVAs. No significant results were found for the physical-match conditions, however, where the task was cognitively more challenging in the name-match conditions, a significant main effect of picture type was found, $F(2,40) = 4.77$, $p = .014$, $\eta_p^2 = .193$. Though the main effect of treatment was non-significant, $F(1,20) = 2.461$, $p = .098$, $\eta_p^2 = .230$, a significant linear trend was found for this measure $F(1,20) = 5.96$, $p = .024$, $\eta_p^2 = .230$ where, as can be seen from figure 8b, for all time points the greatest improvements were found found for 30g WBB and the smallest for vehicle treatment. Subsequent analysis of the different picture types revealed no significant effects in the PSNS and PDND conditions, however, the PDNS analysis indicated a trend for a main effect of treatment, $F(2,40) = 2.98$, $p = .062$, $\eta_p^2 = .130$. A significant linear trend was also found for treatment on this measure,

$F(1,20) = 6.34$, $p = .020$, $\eta_p^2 = .241$, where it can be seen from Fig. 8c that the greatest improvement in reaction times is found for 30g WBB.

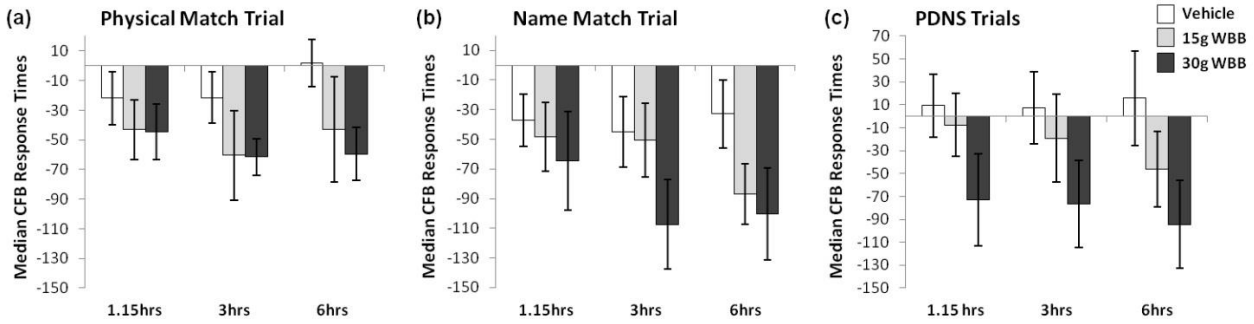

Fig. 8. Change from baseline for median reaction time scores for **a** the Physical-Match and **b** Name-Match Tasks (± standard error of the mean) by treatment and session showing improved change from baseline performance for both the 15g and 30g intervention drinks for all sessions in comparison to vehicle. **c** Change from baseline for median reaction time scores for the PDNS trials of the Name-Match task by treatment and session (± standard error of the mean) showing improved change from baseline performance for the 30g intervention in comparison to the vehicle and 15g treatments. Scores calculated by subtracting baseline median response time from median response time at each subsequent post intervention session.

Analysis of dose effects

As discussed above, there is at present a lack of data concerning dose effects on performance in individual tests (Lamport *et al*., 2012), a critical issue in the exploratory analysis of nutritional interventions on cognition is the extent to which a dose-response effect can be generally observed. In the individual analyses conducted above, although not all tests find specific significant results, there often appears to be a dose response, in which for any given DV, children in the vehicle treatment perform less well than in the 15g WBB treatment who in turn perform less well than in the 30g WBB treatment. This observation is further supported by the significant trends reported above. Indeed, though the omnibus ANOVA analysis may not have revealed a significant effect, as in the case of the picture recognition task, the subsequent linear trend analysis did show a significant effect with vehicle performance showing the smallest decrease in RT and 30g WBB performance showing the greatest. As an unbiased test of this possibility, we conducted a non-parametric test of trend [41] on the change from baseline data for each test, session, and participant. (Data were laid out as three columns, with each line the performance of one child in one test: see Online Resource 2). Importantly, this test revealed a significant monotonic increase in cognitive performance with anthocyanin dose, $L = 11225$, $p = .009$.

Discussion

The general health benefits of berries are well documented with studies providing support for their neuroprotective, antioxidant, anticancer, and anti-inflammatory properties, effects that are ascribed to their high levels of phenolic compounds of which flavonoids are a primary class [51]. A number of animal and adult studies have recently demonstrated positive cognitive effects following acute and chronic [[15, 16, 17, 18, 24,30] blueberry interventions; however of these studies, only one has investigated the effects of berries on children [30]. To our knowledge, therefore, the data presented here is the first fully controlled multi-dose, time course study which demonstrates that acute cognitive benefits can be observed in 7-10-year old-children with an anthocyanin-rich blueberry intervention. The Page's test reveals the consistency and strength of this finding with WBB supplementation leading to significant overall improvements in cognition function, with the best change from baseline performance associated with 30g WBB treatment, intermediate performance with the 15g WBB treatment, and least effective performance with the vehicle treatment. This finding is important because, even when analysed separately, our tasks typically showed a linear relationship between cognitive benefits and increasing WBB dose. The next step in the investigation is clearly to determine more precisely the cognitive locus, and physiological basis, of this effect. Our detailed analyses provide some possible directions for this enquiry.

Analysis of individual cognitive tasks showed that supplementation with anthocyanins produced significant improvements in word acquisition and word recognition, as well as a greater ability to overcome response interference effects as demonstrated in the MFT. These findings give a fuller understanding of the areas where specific benefits in cognition are strongest and may also indicate potential mechanisms driving the effects. Considering the benefits for memory, we have shown that, when compared to the vehicle treatment, participants demonstrated a significant improvement in final acquisition of a repeated list of words following intervention with 30g WBB at 1.15 hrs. This suggests that blueberry intervention had a positive effect on learning at this time point. Additionally, whilst word recognition performance was progressively worse for all doses on each subsequent session throughout the day, this attenuation was significantly less marked for both WBB doses compared with vehicle and suggests a positive effect on secondary (delayed) memory performance. These findings echo our previous research where delayed memory was seen to improve following a fresh high bush blueberry interventions [30] and are also consistent with Krikorian et al (2010) who found improved performance on word list recall following a 12 week intervention on older adults with wild blueberry juice [24].

The delayed word recognition effects observed in this study suggest that 30g WBB and 15g WBB are effective in maintaining delayed memory performance throughout our six hour test period. Previous *in vivo* and *in vitro* research has found that chronic and acute flavonoid interventions positively influence the ERK-CREB-BDNF signalling pathway related to memory formation in both young and aging rats [17, 18, 28, 42, 43]. Furthermore, Dodd [27] found that acute

supplementation with blueberry anthocyanins maintained BDNF plasma levels in adults in contrast to a reduction following vehicle. BDNF is critical for the formation of short and long term memories and learning [44, 45] and there is also evidence to suggest that BDNF contributes most strongly at the point of encoding during recognition tasks [46]. Our findings would seem to be consistent with the maintenance or up-regulation of BDNF levels found following anthocyanin intervention, leading to the facilitation of stronger encoding. Alongside the delayed memory effects seen on the AVLT, children showed greater acquisition of the word lists at 1.15 hours following WBB supplementation which may also be linked to elevated levels of BDNF. One alternative explanation for this improvement is that it was driven by a peak in CBF (known to be between 1-3 hours post-flavonoid intervention in adults [27]) which in turn may have facilitated increased glucose- or oxygen-driven attention at the point of encoding (see [47] for discussion). Furthermore Rodriguez-Mateos *et al*. [48] has shown specific increases in enothelium-dependent vasodilation and availability of anthocyanin metabolites at 1-2 and 6hr but not 3 hours after blueberry anthocyanin intervention in healthy men. As these are the time periods where we have found the most significant improvements in memory, this gives further support to the possibility that improvements may be driven by increased blood flow.

The actions of our WBB were not just restricted to beneficial actions on memory-related processing. Participants also demonstrated improved accuracy on the more cognitively demanding trials of the MFT at 3 hours following intervention with the 30g WBB dose. This indicates a possible beneficial effect of WBB on overcoming the interfering effects of distracting non-target stimuli. No significant effects were found on the cognitively less demanding congruent trials, where it is probable that all children, regardless of treatment, were able to perform at a high level thus giving little room for an improvement to be seen. Similar effects were also noted for the PMT reaction time where no effects were found for the less cognitively demanding physical comparison condition. In the more cognitively-demanding name comparison condition a significant linear trend was evident for RT. Subsequent analysis revealed this was primarily driven by performance on the PDNS trials where participants overcame interference created by presenting items with the same name but shown as different pictures. An emerging pattern from the data for these two tasks would therefore seem to be that improvement is most likely to be found where tasks (or elements of tasks) are sufficiently sensitive to cognitively challenge the child whilst also allowing room for improvement in performance. To our knowledge, this is the first berry related intervention to show an improvement in executive function related performance in children and therefore requires further investigation to confirm and expand these effects with particular reference to the possible sensitivity to cognitive demand.

Although significant attempts were made to control all aspects of this study, a number of potential elements which may have influenced outcomes must be considered. Firstly, the effect of some participants choosing not to consume the

intervention resulted in a reduction in power from the anticipated 0.8 to 0.68. There is therefore an increased probability of type II error in the above analysis. Though the combined Page's test measure goes some way towards demonstrating a global cognitive effect following our treatment, further research is recommended with a larger sample size to confirm and expand on our task specific analysis above. Secondly, though participants were asked for their opinion on drink palatability, no formal investigation was carried out in relation to how participants perceived the drinks to differ in palatability. Previous research has found that palatability can have an effect on mood [49] and it has been proposed that this in turn might enhance cognitive performance [50]. Furthermore consuming palatable food may also lead to an increased glycemic response in comparison to a matched unpalatable control [51] which may in turn have an effect on cognitive performance. It is therefore important that future studies should consider alternative formulations or capsulation in order to improve the palatability and similarity of intervention drinks. A further factor is that testing took place in a school setting in different classrooms, which meant some unavoidable amount of variable noise distraction, particularly during the lunch hour. Though this has the benefit of being a more ecologically valid environment, further research in more a controlled environment, along with blood measures of flavonoid and metabolite content would be useful to confirm and extend our preliminary results. Finally, even though a low flavonoid diet was consumed the day before, and during, each test day, the children who participated in this study reported a relatively high intake of fruit and vegetables in their normal diet which may have reduced the effects of the intervention. It would be interesting to investigate the action of our blueberry intervention in low fruit and vegetable consumers to see if more marked effects on cognitive performance would become evident.

Despite these caveats, we conclude that acute blueberry supplementation has a measurable broad, dose-related, effect on the performance of 7-10 year-old children in a wide variety of cognitive tasks.

Conflict of Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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