





## Working memory and hippocampal expression of BDNF, ARC, and P-STAT3 in rats: effects of diet and exercise

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### ABSTRACT

**Objectives:** Mounting evidence suggests diet and exercise influence learning and memory (LM). We compared a high-fat, high-sucrose Western diet (WD) to a plant-based, amylose/amylopectin blend, lower-fat diet known as the Daniel Fast (DF) in rats with and without regular aerobic exercise on a task of spatial working memory (WM).

**Methods:** Rats were randomly assigned to the WD or DF at 6 weeks of age. Exercised rats (WD-E, DF-E) ran on a treadmill 3 times/week for 30 min while the sedentary rats did not (WD-S, DF-S). Rats adhered to these assignments for 12 weeks, inclusive of *ad libitum* food intake, after which mild food restriction was implemented to encourage responding during WM testing. For nine months, WM performance was assessed once daily, six days per week, after which hippocampal sections were collected for subsequent analysis of brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeletal protein (ARC), and signal transducer and activator of transcription 3 (P-STAT3, Tyr705).

**Results:** DF-E rats exhibited the best DSA performance. Surprisingly, the WD-S group outperformed the WD-E group, but had significantly lower BDNF and ARC relative to the DF-S group, with a similar trend from the WD-E group. P-STAT3 expression was also significantly elevated in the WD-S group compared to both the DF-S and WD-E groups.

**Discussion:** These results support previous research demonstrating negative effects of the WD on spatial LM, demonstrate the plant-based DF regimen combined with chronic aerobic exercise produces measurable WM and neuroprotective benefits, and suggest the need to carefully design exercise prescriptions to avoid over-stressing individuals making concurrent dietary changes.

### KEYWORDS

Nutrition; high-fat diet; aerobic exercise; cognition; spatial working memory; delayed spatial alternation; learning ; neuroprotection

## Introduction

With the increased prevalence of diet-induced obesity, more attention has been paid to the health risks associated with consuming a diet high in saturated fat and refined sugars. For example, the Western Diet (WD) has been linked to a number of chronic disorders including diabetes, cardiovascular disease, and chronic kidney disease [1–3]. The cognitive impact of consuming a WD, as well as engaging in a sedentary lifestyle, are also being more thoroughly investigated.

The hippocampus is one of the most important brain structures for spatial learning and memory (LM) [4,5]. Consumption of a WD in rats has been shown to result in deleterious structural and organizational changes to the hippocampus such as alterations to volume, vascularization, and local immune cell (e.g. microglial) function [6–8] that appear to contribute to deficits in spatial reference memory (i.e. long-term memory) [9–13] as

well as spatial working memory (WM) [14–20]. Exercise appears to be neuroprotective as both voluntary (e.g. running wheel) and moderate, forced aerobic exercise (e.g. treadmill running) have been shown to increase neuronal density [21–24] and dendritic branching [25,26], promote hippocampal angiogenesis [27], and improve spatial reference and WM [21–24,28–34].

To elucidate a mechanism for the effects of diet and aerobic exercise on spatial memory, many have focused on biochemical markers like brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated protein (ARC). BDNF is a neurotrophin involved in regulating dendritic and synaptic plasticity in the hippocampus [35], while ARC is an immediate-early gene found only in neurons [36] that is critically important in regulating cytoskeletal proteins involved in structural synaptic modifications [37–39]. Together, these proteins are involved in the rearrangement of

cytoskeleton architecture which allows for increased signal conduction through the hippocampus (i.e. long-term potentiation) [40–42]. Consumption of a high-fat diet like the WD has previously been shown to reduce levels of BDNF [14,43,44] and ARC [45,46] in the hippocampus. As more and more studies suggest an impairment in WM and learning with the consumption of a WD, researchers have also shifted focus to investigate potential neuroprotective effects of aerobic exercise. Numerous studies have indicated BDNF levels increase with aerobic exercise in humans [47,48]. Rodent research has reliably demonstrated BDNF levels increase in the hippocampus with aerobic exercise [22,28,29,49] and that inhibiting BDNF during said exercise mitigated the exercise-induced spatial memory benefit previously seen [50]. Interestingly, aerobic exercise has been shown to attenuate the WD-induced reduction in BDNF levels [44] and similar protective effects of exercise were observed on ARC expression in mice exposed to chronic restraint stress [51].

In contrast, research on the cognitive effects of plant-based diets is scarce. The Daniel Fast (DF) is the biblically-inspired, stringent plant-based diet inclusive of *ad libitum* intake of fruits, vegetables, whole grains, nuts, seeds, and oils, while prohibiting the consumption of animal products, processed foods, white flour products, preservatives, additives, sweeteners, caffeine, and alcohol. To our knowledge, no animal or human studies exist that have tested the effects of a plant-based diet on spatial memory, although there are some reports describing the cognitive benefits associated with consuming individual components of plant-diets such as omega-3 fatty acids [52–55] and dietary fiber [56]. Research on the role of neuroinflammation and oxidative stress on exercise- and diet-induced neurogenesis has only just begun [57,58], but there is good evidence to suggest that microglia/macrophages can produce pro-inflammatory cytokines that promote neurogenesis [59,60]. STAT3 is a highly expressed transcription factor in neural tissue which translocates to the nucleus upon phosphorylation (P-STAT3) and targets numerous inflammation mediating genes, particularly during cell proliferation, differentiation, or apoptosis [61–63]. Not surprisingly, neuroinflammation has a negative effect on spatial WM performance [64,65].

This project was done to compare the impact of consuming a diet formulated to resemble a typical Western diet, or another resembling the Daniel Fast, on spatial WM and the expression of BDNF, ARC, and P-STAT3. In addition, the effect of chronic, aerobic exercise on the dependent measures was also explored by dividing each dietary group into sedentary (S) and

exercised (E) cohorts. Based on previously published reports, it was hypothesized that relative to rats consuming the DF, rats consuming the WD rats would exhibit impaired spatial WM performance, decreased hippocampal BDNF and ARC expression, and increased hippocampal P-STAT3 expression. In addition, exercise was expected to ameliorate the negative outcomes associated with consuming the WD.

## Method

### Subjects

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were also aligned with those outlined in The Public Health Service Policy on Humane Care and Use of Laboratory Animals [66] and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research [67]. Twenty-eight, male Long-Evans rats were single-housed in a standard shoebox cage on a 12-h light–dark cycle (lights on 0400 h). The dietary and aerobic exercise intervention began at six weeks of age, when rats received either the Daniel Fast (DF;  $n = 14$ ) or the Western Diet (WD;  $n = 14$ ). Half of the rats in each dietary condition were assigned to the exercise condition (E) with the remaining half assigned to the sedentary condition (S). As such, the four treatment conditions ( $n = 7/\text{group}$ ) were DF-E, DF-S, WD-E, and WD-S. Mild food restriction (90% of each rat's free-feeding weight) began 12 weeks after the start of the dietary intervention at the onset of spatial WM testing and remained in place until the end of the experiment (a total of 9 months of observation). The mild food restriction was done to ensure adequate motivation to respond for food rewards. Rats were always able to obtain water *ad libitum*.

### Dietary conditions

The DF (Research Diets; New Brunswick, NJ) was a specially formulated plant-based rat pellet designed to model the popular DF consumed by humans. The DF consisted of 15% protein, 59% carbohydrate, and 25% fat. The source of protein was soy, the main source of carbohydrate was a 70/30 amylose amylopectin blend, and the primary source of fat was a mixture of flaxseed and safflower oil. The DF contained 7.4% saturated fat, 46.3% monounsaturated fat, and 46.2% polyunsaturated fat. The omega 6–3 fatty acid ratio for the DF diet was 0.5, and it also contained a significant amount of Vitamin C, as well as cellulose and inulin. While a standard chow diet is viewed as a 'healthy' alternative to a WD,

the typical chow diet does not contain nearly the amount of dietary fiber as compared to the DF, in addition to being devoid of flaxseed oil as a source of dietary fat. We believed the inclusion of the fiber content and type, in addition to the flaxseed oil, was important relative to our outcome measures.

The WD (Research Diets) was also a specially formulated animal-based pellet designed to model the typical WD. The WD caloric breakdown was 17% protein, 43% carbohydrate, and 40% fat. The main source of protein was casein, the main source of carbohydrates was sucrose, and the primary source of fat was milk fat. This diet contained 62.4% saturated fat, 30.7% monounsaturated fat, and 6.9% polyunsaturated fat. The omega 6–3 fatty acid ratio for this diet was 3.61, and it also contained high levels of cholesterol. There was no dietary source of inulin or Vitamin C present in the WD. A complete breakdown of dietary compositions of both the DF and WD has been previously published [68].

In both conditions, the rats were weighed seven days a week to maintain them at approximately 90% of their target weight. In particular, the sedentary rats received 20 grams of food if more than 10 grams under their target weight, 15 g of food if 5–10 g under their target weight, 10 g of food per day if they were within 5 grams of their target weight, 5 grams of food if 5–10 grams over their target, or 2.5 g of food if more than 10 grams over their target weight. The guidelines were similar for rats in the exercise condition, except that the exercise rats received an additional 2.5 g more food daily than the sedentary rats in order to offset the additional energy requirements of the exercise protocol. All rats were fed their daily food ration after they completed cognitive testing.

### **Aerobic exercise conditions**

Rats in the exercise conditions ran on a motorized treadmill for 30 min on Mondays, Wednesdays, and Fridays for the duration of the experiment. The protocol for weekly exercise included 35 min at 25 m/min with no incline. It should be noted that no testing of  $VO_{2max}$  was conducted in this study. Therefore, we are uncertain as to the exact percentage of  $VO_{2max}$  the animals were training at. That said, considering multiple other literature sources of treadmill running in rodents, and understanding the degree of variation that can exist between animals, the workload used in the present study represented approximately 50–60%  $VO_{2max}$ . During the 3x weekly exercise sessions, the rats were carefully monitored. If the animal remained on the electrified deck for a period of 30 s, the animal was deemed to be exhausted and removed from the treadmill for that day. No rat was

removed from the treadmill more than three times over the 9-months. Sedentary rats were transported to the testing room and placed on the treadmill but not exercised.

### **Apparatus**

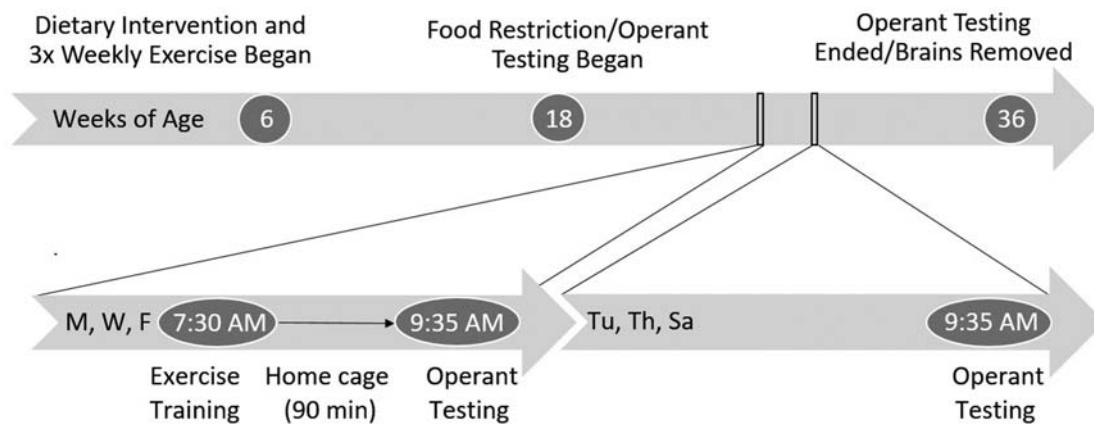
Behavioral testing was conducted in 10 automated operant testing chambers (Med Associates; St. Albans, VT). These chambers were both sound attenuated and well-ventilated with a fan. On one wall of the chamber, was a food magazine positioned in the middle with two retractable response levers symmetrically aligned on each side of the magazine. The rats were rewarded with a small grain-based dustless precision pellet (F0165, Bioserv; Flemington, NJ) and the amount of reward pellets was limited in order to better control nutrient intake. Each response lever was 7 cm from the floor and 5.7 cm from the midline of the wall with a cue light positioned directly above. A house light was situated on the wall opposite of the levers. All operant programs were controlled via a PC equipped with Med-PC V software (Med Associates).

### **Procedure**

The experimental timeline is presented in Figure 1. Operant testing occurred six days per week (Monday–Saturday) at the same time of day every day, and at least 90 min after exercise training which only occurred on Monday, Wednesday, and Friday. Each operant chamber was tested prior to each session to ensure proper functioning. Prior to spatial WM testing, rats were first trained to press the lever using previously published autoshaping and fixed-ratio training procedures (2–3 sessions/task) [69,70]. Any lever press resulted in a 45 mg reward pellet being dispensed into the food magazine. [The reward pellets were manufactured by Bio-Serv and consisted of a caloric breakdown of 64.4% carbohydrate, 25.4% protein, and 10.2% fat. The carbohydrates were sucrose, dextrose, and fructose. The protein came from both casein and whey. The fat breakdown was 46.4% polyunsaturated, 30.2% monounsaturated, and 23.4% saturated.] A maximum of 100 pellets were allowed during autoshaping and fixed-ratio training. Once rats were reliably pressing both levers (~5–7 sessions), they moved on to alternation training.

### **Cued alternation**

The Cued Alternation (CA) program followed Fixed-Ratio training and included performance-based advancement. During CA both levers were extended, but only one of the cue lights was illuminated. The



**Figure 1.** *Top:* full experimental timeline. The diet and aerobic exercise intervention began when the rats were 6 weeks of age and continued for the duration of the experiment. Food restriction and operant testing began when the rats were approximately 18 weeks old. *Bottom:* representative daily schedule. On Mondays (M), Wednesdays (W), and Fridays (F) the exercised rats ran on the treadmill for 35 min at least 90 min before operant testing began. (Sedentary rats were taken to the room and placed on the treadmill but not exercised). On Tuesdays (Tu), Thursdays (Th), and Saturdays (Sa), operant testing was conducted at the same time of day as on exercise days, but rats did not receive exercise. training first.

animal had to press the lever beneath the illuminated cue light to receive a reward pellet. If the incorrect lever was pressed, no reinforcer was delivered. Once a lever was pressed, the opposite cue light from the lever just pressed was illuminated on the next trial. Each CA session lasted until 200 trials occurred. In order to progress to the next testing phase, each animal had to successfully achieve 80% accuracy over three consecutive days. All rats achieved this criterion in 4–7 sessions.

#### Non-cued alternation

The Non-cued alternation (NCA) phase was an extension of the previous CA protocol with the exception that illumination of the cue light associated with the correct lever was discontinued. As during CA, to receive a reinforcer for any given lever press, it had to occur on the lever opposite the one pressed on the previous trial. Each NCA session ended after 200 trials occurred. Progression from NCA to the next testing phase occurred after 10 days of testing regardless of performance. If the animal alternated their response to the lever opposite the previous trial, it earned a food reinforcer. For NCA, the primary dependent measure was percent correct.

#### Delayed spatial alternation

The Delayed Spatial Alternation (DSA) phase was the final testing phase and it lasted approximately 4.5 months. For DSA, both levers were extended but both cue lights remained extinguished. As with NCA, the rats were required to alternate levers to earn a reinforcer. However, after each reward was presented, both

levers were retracted and remained so for either 0, 10, 20, or 40 s before being extended again. The retraction time between trials was randomly determined via an algorithm in the DSA program such that there were 50 trials at each delay. If the animal alternated their response to the lever opposite the previous trial, it earned a food reinforcer. Each DSA session ended after 200 trials occurred. For DSA, the primary dependent measure was the number of reinforcers earned.

*Win-stay and lose-stay errors.* Within the DSA task, if the animal failed to earn a reinforcer it was because the animal made one of two different types of errors. The first error was called a win-stay error. This occurred if the animal first correctly alternated levers (i.e. a 'win' where it alternated from the right lever to the left or the left lever to the right and received a food pellet), but then incorrectly made the next lever press on the same lever as the previous trial (i.e. a 'stay' – no food reward presented). The second error was called a lose-stay error. This occurred if the animal incorrectly failed to alternate levers (i.e. a 'lose' where it responded two times on the same side with no food reward) followed by a third response in a row on the very same side (i.e. another 'stay' with no food reward). Thus, a lose-stay error represented at least three consecutive responses on the same response lever.

#### Tissue extraction and western blotting

The day after DSA testing ended (when rats were approximately 9 months old), they were euthanized via CO<sub>2</sub> inhalation and decapitated. The hippocampus was rapidly dissected on ice, and then immediately



snap-frozen in liquid nitrogen after which it was stored at  $-70^{\circ}\text{C}$ . The hippocampal samples were homogenized in a high-speed tissue grinder in T-PER Reagent (Thermo Scientific, Rockford, IL) + Protease Inhibitor (PI) (Roche, Nutley, NJ). Protein concentration was determined by Coomassie Plus (Thermo Scientific, Rockford, IL) Bradford analysis. Samples were prepared with Lane Marker Reducing Sample Buffer 5X (Thermo Fisher, Waltham, MA) and denatured at  $95^{\circ}\text{C}$  for 5 min. The samples were loaded onto 4–20% Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Protein Gels (Biorad, Hercules, CA) at 40  $\mu\text{g}$  of protein per well and run via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 135 V for 55 min. Following the SDS-PAGE, the proteins were transferred to a PVDF Membrane at 0.25 Amps for 1.5 h. Membranes were blocked with 5% bovine serum albumin (BSA) solution dissolved in Tris-Buffered Saline with 0.1% tween 20 (TBST) for 1 h. Membranes were then incubated in primary antibodies for BDNF (Abcam, Cambridge, UK), ARC (Abcam, Cambridge, UK), Phospho-STAT3 (Tyr705; Cell Signaling Technology, Danvers, MA), STAT3 (Cell Signaling Technology, Danvers, MA), and GAPDH (Santa Cruz, Dallas, TX) overnight, diluted according to the manufacturer in 5% BSA solution. Blots were then washed with TBST before being incubated in appropriate secondary antibody, diluted 1:5000 with 5% BSA in TBST, for 2 h. The blots were then washed with TBST before the protein bands were visualized with a chemiluminescent agent and imaged using a Fotodyne<sup>®</sup> 60-7020 (FOTODYNE Incorporated, Hartland, WI) bench-top image and gel documentation system. All bands were quantified via densitometry (AlphaEaseFC<sup>™</sup>, San Leandro, CA, USA) and mean values were analyzed. Densitometry means for BDNF and ARC in each sample were normalized to corresponding GAPDH values while Phospho-STAT3 values were normalized to STAT3 values.

### Design and analyses

As all rats were required to achieve criterion performance on autoshaping, fixed-ratio training, and CA, so no analyses were conducted. For NCA, the percent correct was averaged across the 10 days of testing and analyzed via a 2 (diet)  $\times$  2 (exercise) between-subjects ANOVA. The DSA analysis also included the between-subjects factors of diet and exercise. However, the repeated measures factors of delay and 5-day block (i.e. the 125 daily sessions were averaged into 25, 5-day blocks) were also included in analysis of the DSA data. Post hoc analyses were conducted as appropriate on the DSA data to determine the nature of significant

diet- and exercise-related interactions. The number of win-stay and lose-stay errors during DSA were also analyzed using two separate diet  $\times$  exercise  $\times$  5-day block mixed ANOVAs with corresponding post hoc analyses as appropriate.

For the western blot analysis, three separate independent samples *a priori* *t*-tests were conducted. The first analysis evaluated the impact of diet alone by comparing the DF-S group to the WD-S group, while the remaining analyses evaluated the impact of exercise by comparing the E group to the S group separately for each diet. Associated measures of effect size are also reported to ensure significant differences are accompanied with a medium to large effect size and not due to the increased risk of Type I error associated with multiple comparisons.

## Results

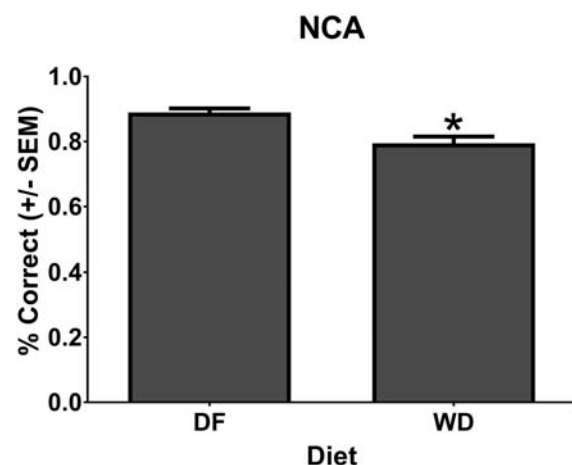
### Non-cued alternation

Analysis of the percent correct during NCA revealed only a significant main effect diet [ $F(1, 24) = 15.514$ ,  $p = .001$ ]. As seen in Figure 2, DF rats performed better than the WD rats.

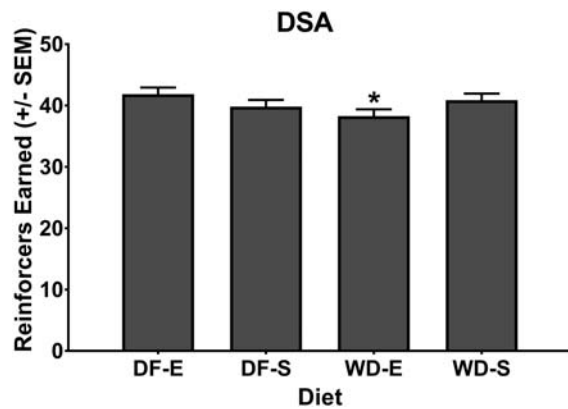
### Delayed spatial alternation

#### Reinforcers earned

The diet  $\times$  exercise  $\times$  delay  $\times$  5-day block mixed ANOVA of reinforcers earned during DSA revealed significant diet  $\times$  exercise [ $F(1, 24) = 4.447$ ,  $p = .046$ ], diet  $\times$  exercise  $\times$  block [ $F(24, 576) = 2.565$ ,  $p < .001$ ], diet  $\times$  exercise  $\times$  delay [ $F(3, 72) = 3.357$ ,  $p = .023$ ], and diet  $\times$



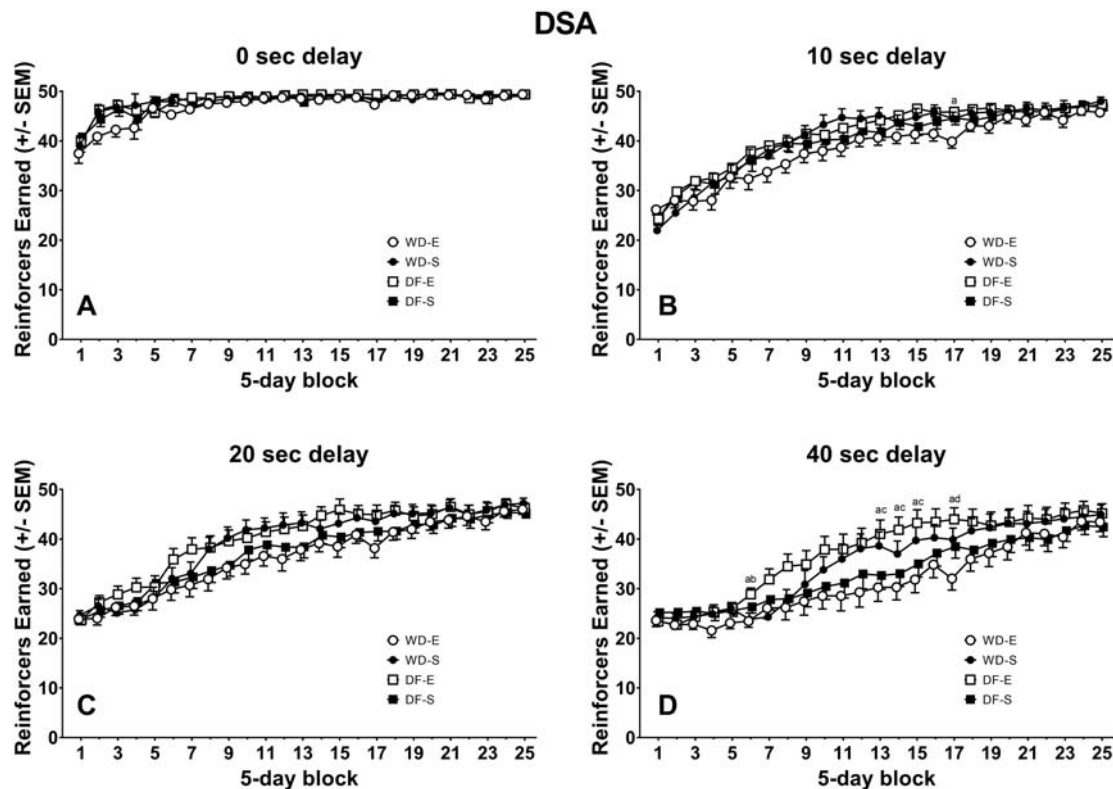
**Figure 2.** Rats who were fed the Daniel Fast (DF;  $n = 14$ ) diet performed significantly better on the Non-cued Alternation (NCA) task than rats that were fed the Western Diet (WD;  $n = 14$ ).  $*p = .001$ .



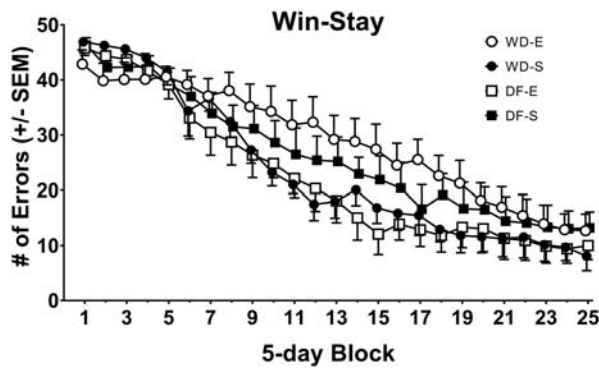
**Figure 3.** Analysis of the number of reinforcers earned revealed a significant diet  $\times$  exercise interaction ( $p = .046$ ). Rats who were fed the DF diet who exercised (DF-E;  $n = 7$ ) outperformed their sedentary counterparts (DF-S;  $n = 7$ ) while Western diet rats who were sedentary (WD-S;  $n = 7$ ) received a higher average number of reinforcers than Western Diet rats that exercised regularly (WD-E;  $n = 7$ ).  $*p = .030$  from DF-E group.

exercise  $\times$  delay  $\times$  block [ $F(72, 1728) = 2.551, p < .001$ ] interactions. Figure 3 presents the number of reinforcers earned across the four treatment conditions (i.e.

DF-E, DF-S, WD-E, and WD-S) averaged across delay (max = 50) and testing block. Exercise appeared to benefit rats in the DF group as they received more reinforcers than their sedentary counterparts. However, this effect was reversed in the rats fed the WD. Post hoc LSD analyses, however, revealed a significant difference only between the DF-E and WD-E groups ( $p = .030$ ). Figure 4 presents the number of reinforcers earned across the four treatment conditions as a function of both delay and 5-day testing block. As expected, the number of reinforcers earned dropped for all groups as the implemented delay got longer (panels A–D). With an increasing delay, the degree of decline was somewhat different for the different treatment groups and was most evident in the intermediate testing blocks. Specifically, after initial acquisition (i.e. beginning around block 8), rats in all of the treatment groups appear to be earning all 50 possible reinforcers at the 0-s delay (Figure 4(A)). However, as the delay got longer (Figure 4(B–D)), the DF-E and WD-S groups appear to be earning more reinforcers than the other two groups during intermediate testing blocks. Post hoc simple effects analyses on data generated following the 10-s delay showed



**Figure 4.** Analysis of the number of reinforcers earned revealed a significant diet  $\times$  exercise  $\times$  delay  $\times$  5-day testing block interaction ( $p < .001$ ). The most profound differences among treatment groups were observed during intermediate trials during the 40-s delay. <sup>a</sup>Significant difference between Daniel Fast exercise (DF-E;  $n = 7$ ) and Western Diet exercise (WD-E;  $n = 7$ ) group,  $p \leq .01$ ; <sup>b</sup>significant difference between DF-E and Western Diet sedentary group (WD-S;  $n = 7$ ) group,  $p = .009$ ; <sup>c</sup>significant difference between DF-E and Daniel Fast sedentary group (DF-S;  $n = 7$ ) group,  $p < .05$ ; <sup>d</sup>significant difference between WD-E and WD-S group,  $p = .025$ .



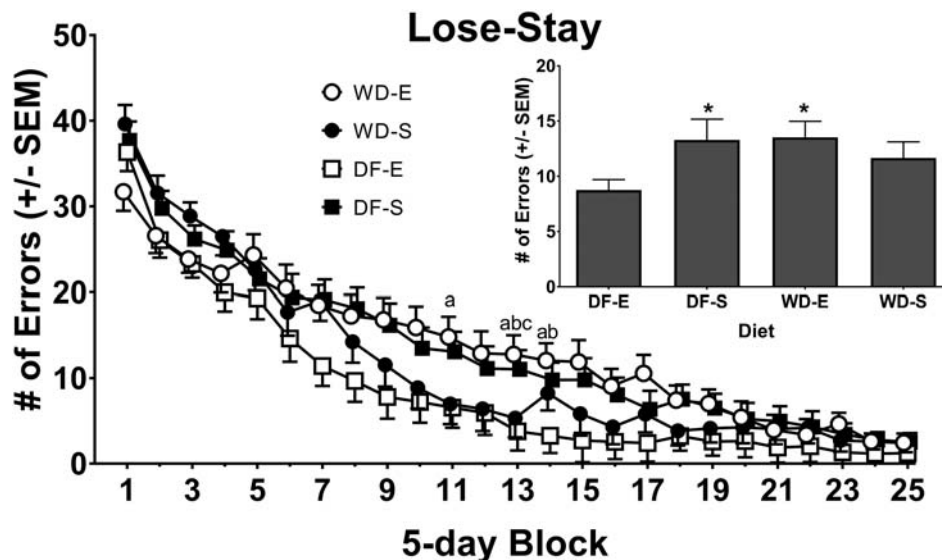
**Figure 5.** Analysis of the number of win-stay errors revealed a significant diet  $\times$  exercise  $\times$  5-day testing block interaction ( $p < .001$ ). Post hoc simple effects analysis did not reveal any significant treatment effects within any of the 5-day testing blocks. DF = Daniel Fast, WD = Western Diet, E = exercise, S = sedentary ( $n = 7$ /treatment group).

one significant treatment effect during block 17 ( $p = .021$ ) wherein the DF-E group earned more reinforcers than the WD-E group. However, this effect seems to be driven by the rather low performance of the WD-E group during that testing block (Figure 4(B)). Looking at the data generated during the 20-s delay, treatment effects appear more prevalent especially during intermediate testing blocks; however, post hoc analysis did not reveal any significant treatment effects in any of the 5-day testing blocks (Figure 4(C)). During the 40-s delay, the effect of treatment in the intermediate testing blocks appeared to be larger and post hoc analyses

revealed a significant effect of treatment during blocks 6 ( $p = .020$ ; DF-E significantly different from WD-E and WD-S), blocks 13–15 ( $p = .041$ ,  $0.023$ , and  $0.028$  respectively; DF-E significantly different from DF-S and WD-E in all cases), and block 17 ( $p = .011$ ; DF-E and WD-S significantly different from WD-E; Figure 4 (D)).

#### Win-stay/lose-stay errors

The mixed ANOVA on number of win-stay errors made (Figure 5) revealed only a significant diet  $\times$  exercise  $\times$  5-day block interaction [ $F(24, 576) = 3.597$ ,  $p < .001$ ]. However, post hoc simple effects analysis did not reveal any significant treatment effects within any of the 5-day testing blocks. The mixed ANOVA on number of lose-stay errors made also revealed a significant diet  $\times$  exercise  $\times$  5-day block interaction [ $F(24, 576) = 3.641$ ,  $p < .001$ ] as well as a diet  $\times$  exercise interaction [ $F(1, 24) = 4.758$ ,  $p = .039$ ]. As can be seen in Figure 6, post hoc analyses revealed a significant effect of treatment during blocks 11 ( $p = .041$ ; DF-E significantly different from WD-E), 13 ( $p = .024$ ; DF-E significantly different from DF-S and WD-E and WD-S significantly different from WD-E), and 14 ( $p = .035$ ; DF-E significantly different from DF-S and WD-E). Across all testing blocks, the DF-E group made fewer lose-stay errors than the DF-S ( $p = .039$ ) and the WD-E ( $p = .031$ ) groups (Figure 6 inset).

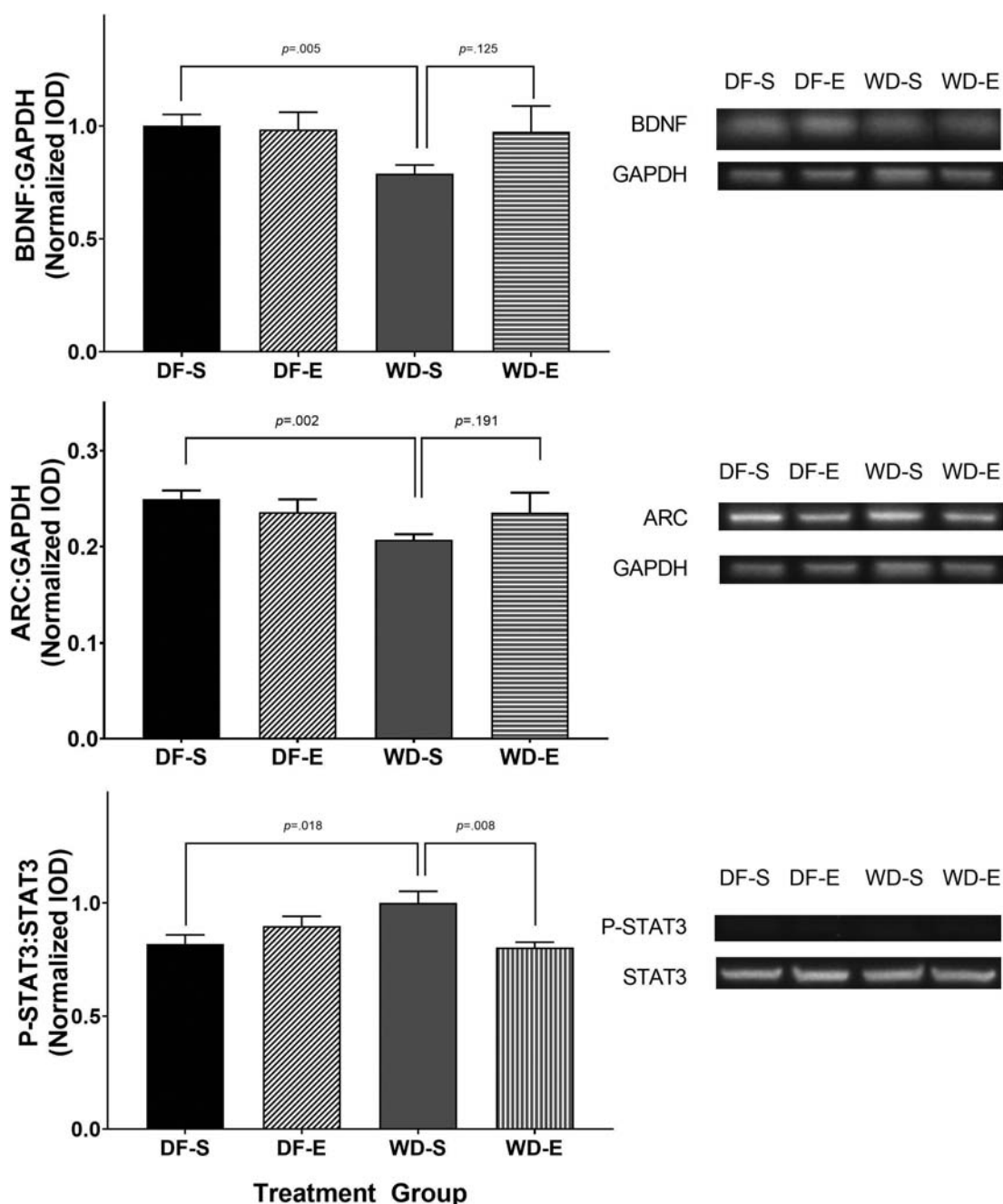


**Figure 6.** Analysis of the number of lose-stay errors revealed a significant diet  $\times$  exercise  $\times$  5-day testing block interaction ( $p < .001$ ). Post hoc simple effects analysis revealed a significant treatment effect during blocks 11, 13, and 14. <sup>a</sup>significant difference between Daniel Fast exercise (DF-E;  $n = 7$ ) and Western Diet exercise (WD-E;  $n = 7$ ) group,  $p \leq .02$ ; <sup>b</sup>significant difference between DF-E and Daniel Fast sedentary group (DF-S;  $n = 7$ ) group,  $p < .005$ ; <sup>c</sup>significant difference between Western Diet sedentary (WD-S;  $n = 7$ ) and WD-E group,  $p = .027$ ; \*significant different from DF-E group,  $p < .05$ .

### Protein analysis

Compared to the DF-S group, the WD-S group had a significant decrease in the expression of BDNF ( $p = .005$ ) and ARC ( $p = .002$ ) and a significant increase in P-STAT3 ( $p = .018$ ; Figure 7). The expression of P-STAT3 was also significantly higher in the WD-S group compared to the

WD-E group ( $p = .008$ ). Visual inspection of the data suggested the expression of BDNF ( $p = .125$ ) and ARC ( $p = .191$ ) were lower in the WD-S than the WD-E group, but neither difference reached the criterion for statistical significance. No significant differences were found when comparing the DF-E to the DF-S groups.



**Figure 7.** Western Blot analysis of hippocampal tissue for (A) BDNF, (B) ARC, and (C) P-STAT3 presented as normalized integrated optical density (IOD). *A priori* t-tests examining the effect of diet (without exercise) compared the DF-S group to the WD-S group and revealed significant decreases in BDNF ( $p = .005$ ) and ARC ( $p = .002$ ) and a significant increase in STAT3 phosphorylation ( $p = .019$ ). Similar *a priori* analyses examining the effect of exercise separately for each diet were also conducted. While it appeared that ARC and BDNF were lower in the WD-E rats relative to the WD-S rats, the difference did not reach the criterion for statistical significance. There was, however, a significant decrease in STAT3 phosphorylation in the WD-E group relative to WD-S ( $p = .009$ ). No significant differences were found when comparing the DF-E to the DF-S group.



## Discussion

Results from this study demonstrate the complexity of the combined influence of diet and chronic, aerobic exercise on spatial WM. In terms of diet composition, rats fed the WD did not perform as well as rats fed the DF during NCA, suggesting the DF rats acquired the alternation task quicker than the WD rats. After progressing to the DSA task, significant effects were not detected for dietary composition alone; however, when exercise status and length of delay were taken into account, results were surprising in that aerobic exercise improved performance of rats fed the DF but impaired performance in those fed the WD. The enhanced spatial WM performance of the DF-E group during the DSA was the most discernable as the delay between trials increased and during intermediate trial blocks. This appeared to be a result of the DF-S and WD-E group making more lose-stay errors during the DSA task compared to the DF-E and WD-S groups, especially following a 40-s delay. Lose-stay errors are reflective of response perseveration, indicative of a possible deficit in cognitive flexibility which is an aspect of executive function modulated by the prefrontal cortex (PFC) [71]. Working memory is also an executive function, and spatial WM tasks (like DSA) are also modulated by the PFC [72–74]. There were no obvious treatment group differences during early trial blocks (i.e. during response acquisition) or during later blocks when all rats were well-trained on the task.

Surprisingly, the WD-S group sometimes outperformed the WD-E group, which deviates from previous research demonstrating treadmill exercise has a beneficial effect on spatial WM performance [24,31]. Even though all rats had at least 90 min between the end of exercise training and the start of operant testing, we considered the possibility that exercise training prior to operant testing may have caused fatigue in the WD-E group, such that they were unable to perform adequately during the spatial WM task. Recall that the DF-E rats also ran on the treadmill prior to operant testing, but it is possible that the overall health benefits conferred on the rats by the DF diet were protective against fatigue. To assess this possibility, we compared the DSA results from the WD-E group on days when the exercise protocol was administered (i.e. Mondays, Wednesdays, and Fridays) to those days when it was not (i.e. Tuesdays, Thursdays, and Saturdays). A paired-samples *t*-test was conducted comparing percent correct on exercise versus non-exercise days in the WD-E group averaged across blocks 6–16. The result was not significant [exercise = 76.95% ( $\pm 3.11$  SEM); non-exercise = 77.52% ( $\pm 3.00$  SEM)] suggesting exercise-induced

fatigue was not responsible for the unexpectedly poor performance in the WD-E group.

The results cannot be explained by an effect of diet-associated energy restriction on overall food-seeking (i.e. motivation to obtain food). If this were true, one would expect to see a difference between the DF and WD groups across ALL delays (including the 0-s delay) which was not the case. However, it is possible that energy restriction was a mediating factor such that as the task got harder (i.e. with increasing delay), energy restriction differentially affected working memory performance for the two different diets. However, the results of a study by Del Arco et al. [75] suggest this is not the case. Starting at three months of age, rats were fed *ad libitum* (control group), or had 40% caloric restriction (CR) until they were 6, 15, or 24 months of age (i.e. 3, 12, or 21-month intervention, respectively). At 6 and 15 months of age, CR significantly increased BDNF expression compared to rats fed *ad libitum*. In addition, no differences in working memory were observed between the control or any of the CR groups 6 on a water escape, T-maze alternation task which did not rely on food rewards. An accurate interpretation becomes more complicated however, when caloric restriction is combined with exercise. A recent study found that a 12-week intervention consisting of treadmill running (30 min, 5 $\times$ /week) significantly increased BDNF expression in the hippocampus compared to control rats [76]. These results suggest that exercise (like caloric restriction) is protective. However, when hippocampal BDNF expression was measured in rats that received the same exercise regimen along with caloric restriction (i.e. 40% less food), the benefit of exercise on BDNF expression was suppressed [76].

Notably, we did not obtain a biomarker of exercise intensity such as lactate. Given that the rats on the WD were heavier than those on the DF diet, it is possible that WD rats had to expend more energy than those on the DF diet. However, because all of the rats were engaged in relatively moderate-intensity exercise, we do not believe the lactate values would have been more than a few mM in either group making it very difficult to observe group differences. Also, body mass does not always equate to perceived difficulty in running performance, so future research should rely on a better indicator of intensity such as oxygen consumption.

Bloomer and colleagues [68] recently reported circulating inflammatory and oxidative stress markers, as well as post-intervention run-time to exhaustion, following three months of adhering to a WD or DF diet. Post-intervention aerobic exercise testing resulted in the DF-E group significantly outperforming the WD-E

group. While both exercise groups trained under the same conditions (speed, duration, incline), the regular exercise was undertaken by the WD-E group was likely more intense for this group than the DF-E group. Although circulating cytokine values were not significantly different, exercise-induced inflammation can last days after strenuous exercise, which could explain the mean pro-inflammatory IL-1 $\beta$  values that were nearly six times higher in the WD-E groups versus DF-E groups. This elevation in pro-inflammatory markers was also accompanied by rises in the anti-inflammatory marker IL-10. These changes to cytokines followed a similar pattern to the spatial WM performance reported here, such that WD + E experience worsened inflammatory status (i.e. elevated IL-1 $\beta$  and reduced IL-10) than WD alone while DF + E experience improved inflammatory status relative to DF alone. Indeed, the connection between brain inflammation and impaired cognitive performance has been discussed previously [77,78].

In terms of hippocampal inflammatory biochemical markers, p-STAT3:STAT3 expression was significantly greater in the WD-S group compared to the WD-E. Furthermore, p-STAT3:STAT3 expression was also significantly greater in WD-S rats than DF-S. Admittedly, STAT3 activation may not be the best standalone neuroinflammatory marker, as functions vary based on cell-type [79], phosphorylation can be activated by both pro- and anti-inflammatory markers through the Jak-STAT3 pathway [80], and leptin is known to activate STAT3 [81]. With increased body mass, specifically fat mass, plasma leptin is known to increase proportionally as leptin resistance develops over time [82]. It is possible elevated leptin levels in the WD rats caused increased p-STAT3:STAT3 which was more pronounced in the WD-S group. Future research will need to examine this issue as circulating leptin levels were not measured here.

The WD-S group had significantly lower BDNF and ARC relative to the DF-S group, coinciding with their poorer spatial WM performance (Figure 7(A,B)). These findings corroborate others [14,43–46] who have demonstrated that spatial memory impairment following consumption of a high-fat diet is associated with decreases in BDNF and ARC. Interestingly, even though the WD-E group performed worse than the WD-S group during those intermediate sessions, the WD-E groups showed a trend toward greater expression of BDNF and ARC suggesting exercise may still be neuroprotective even if not manifested in actual performance. Notably, the samples were taken after all blocks of DSA testing had been completed and no significant differences were observed in DSA performance during the

later testing blocks. Based on the dynamic nature of neuroplasticity, it is entirely possible that hippocampal BDNF and ARC expression may have been quite different during the intermediate trials had brain tissue been harvested at that time.

When considering our findings, a potential limitation is our omission of a standard chow condition. The adoption of plant-based diets, which are very high in dietary fiber and balanced in terms of omega 3 and 6 fatty acids, has become quite popular in recent years. Many individuals move from a typical American Diet (i.e. Western Diet) to a form of purified vegan diet for purposes of health enhancement. In the present study, we attempted to mimic such a change, using animals in a very controlled environment. While our findings support the potential role of a plant-based diet to favorably impact cognitive performance, what remains unknown is which specific components of the diet are the main drivers behind our findings. Future research into the specific components of the DF are needed to more fully elucidate these answers.

A second limitation is the absence of a detailed physiological profile (e.g. body mass, blood glucose, insulin, etc.) for the various treatment groups. However, previously published reports from our lab provide some information about what differences were likely present. For example, Bloomer et al. [68] reported a significant difference in overall body mass and fat mass (but not lean mass) in rats that received the same diet and exercise interventions as those described in the current study for three months starting at 6 weeks of age. Specifically, the overall body mass of rats in the WD-S group was significantly higher than the WD-E, DF-E, and DF-S groups. For fat mass, both DF groups were lower than both WD groups, and the WD-E group was lower than the WD-S group. Likewise, Smith et al. [83] fed mice the DF and WD for 7 weeks (no exercise intervention) and found the fasting blood glucose and insulin were significantly lower in the DF mice compared to the WD mice. The DF mice also exhibited significantly better glucose tolerance and significantly lower insulin resistance than the WD mice.

In summary, these results support previous research demonstrating the negative effects of the WD on spatial learning and memory. They also demonstrate that the plant-based DF regimen, when combined with exercise, produces measurable spatial WM benefits and other neurobiological benefits that appear to be neuroprotective. On the other hand, exercise in the WD fed rats had a negative effect on spatial WM performance, with a less clear impact on proteins associated with neuroinflammation/neuromodulation. Overall, the results suggest the need to carefully design exercise prescriptions to

avoid over-stressing individuals who may be making concurrent dietary changes.

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No potential conflict of interest was reported by the author(s).

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## Data availability statement

All data will be made available upon request by emailing the corresponding author.

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