Nitrate and nitrite exposure increases anxiety-like behavior and alters brain metabolomic profile in zebrafish

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Abstract

Introduction

Dietary nitrate lowers blood pressure and improves athletic performance in humans, yet data supporting observations that it may increase cerebral blood flow and improve cognitive performance are mixed. Here we tested the hypothesis that nitrate and nitrite treatment would improve indicators of learning and cognitive performance in a zebrafish (*Danio rerio*) model. We also explored the extent to which nitrate and nitrite treatment affected the brain metabolome in order to understand how nitrate and nitrite supplementation may affect indices of cognitive function.

Methods

Fish were exposed to sodium nitrate (606.9 mg/L), sodium nitrite (19.5 mg/L), or control water for 2-4 weeks and free swim, startle response, innate predator avoidance, social cohesion, and shuttle box assays were performed.

Results

Nitrate and nitrite treatment did not change fish weight, length, predator avoidance, or distance and velocity traveled in an unstressed environment. Nitrate- and nitrite-treated fish initially experienced more negative reinforcement and increased time to decision in the shuttle box assay, which is consistent with a decrease in associative learning or executive function however, over multiple trials, all treatment groups demonstrated behaviors associated with learning. Nitrate and nitrite treatment significantly increased anxiety-like behavior but did not alter epinephrine, norepinephrine or dopamine levels. Targeted LC-MS/MS analysis revealed no significant increase

in brain nitrate or nitrite concentrations with treatment. An untargeted metabolomics analysis found 47 metabolites whose abundance was significantly altered in the brain with nitrate and nitrite treatment including an 18-19% reduction in the neurotransmitter γ-aminobutyric acid (GABA), and 17-22% reduction in its precursor, glutamine, which may contribute to the increased anxiety-like behavior.

Conclusion

Nitrate and nitrite treatment did not adversely affect multiple parameters of zebrafish health but was associated with mild anxiety-like behavior, changes in the brain metabolome, and caused a short-term decrease in executive function or associative learning.

Introduction

Nitrate (NO_3^-), a component of leafy green and root vegetables, including beetroot juice (BRJ) and many green leafy vegetables, has blood pressuring-lowering and ergogenic effects in humans[1]. Nitrate supplementation (either as BRJ or sodium nitrate) has also demonstrated benefits pertaining to cardiovascular health[2], such as reducing blood pressure, enhancing blood flow, and elevating the driving pressure of O_2 in the microcirculation to areas of hypoxia or exercising tissue[3,4]. These findings are important to cardiovascular medicine and exercise physiology. Indeed, multiple studies support nitrate supplementation as an effective method to improve exercise performance[5,6]. Additionally, it has been reported that dietary nitrate can modulate cerebral blood-flow (CBF), decrease reaction time in neuropsychological tests, improve cognitive performance and suggest one possible mechanism by which vegetable consumption may have beneficial effects on brain function in humans[7,8]. In contrast, other recent studies have found no significant effect of nitrate or nitrite supplementation on cognitive function and this highlights the need for additional studies to clarify the effect of nitrate and nitrite treatment on cognitive function (reviewed in[9,10]).

Nitric oxide (NO) is a gaseous, free radical signaling molecule produced via enzymatic and non-enzymatic pathways. The enzymatic pathways for NO synthesis are produced by three distinct families of nitric oxide synthase (NOS) enzymes in mammals that use L-arginine and numerous co-factors as substrates[11]. NO conveys essential signaling in the cardiovascular, central nervous, and immune systems[12]. NO, through formation of S-nitrosothiols and nitration of alkenes or other nitrated species, is also considered to have hormone-like properties that take part in different metabolic/endocrine disorders such as diabetes and dysglycemia, thyroid disorders, hypertension, heart failure, and obesity[13]. Furthermore, NO plays an important role in regulation of synaptogenesis and neurotransmission in the central and peripheral nervous system[14,15]. NO can

also be produced by a NO synthase-independent method through the nitrate-nitrite-nitric oxide pathway. Nitrate present in foods or water is reduced endogenously by lingual nitrate reductases in mammals to nitrite (NO_2^-) and, in the stomach, to nitric oxide (NO) before distribution via blood to tissues[16,17]. Several endogenous enzymes, proteins, and chemical species can reduce nitrite to NO including deoxygenated hemoglobin, xanthine oxidoreductase, deoxymyoglobin, mitochondrial enzymes, ascorbic acid, etc.[18] In spite of the vast amounts of research on NO production, NO-related signaling mechanisms, and the effects of nitrate supplementation on the cardiovascular system; there is still a gap in knowledge regarding whether dietary nitrate supplementation affects the brain metabolome, learning, and other brain functions.

In order to determine the physiological and cognitive effects derived from nitrate and nitrite exposure, we carried out a study with the aquatic model organism *Danio Rerio* (zebrafish). Zebrafish was chosen because it is a complex vertebrate organism that was originally established as a prime model for developmental studies and, is increasingly used for behavioral neuroscience research in part because of standardized and high throughput behavioral performance assays[19–23]. Importantly, as in humans, the nitrate-nitrite-nitric oxide pathway and NOS enzymes play important roles in regulating NO levels, along with cardiac and blood vessel development in zebrafish[24]. In addition, high genetic homology exists between zebrafish and humans for genes associated with disease[25,26]. Furthermore, we established that nitrate treatment in zebrafish improves the oxygen cost of exercise[27] as had been observed in humans. While conducting these experiments we also sought to test the hypothesis that nitrate and nitrite treatment would improve indicators of learning and cognitive performance. We also investigated the effects of nitrate and nitrite treatment on zebrafish behavior and the brain metabolome with the aim of elucidating mechanisms that may contribute to the potential improvement of cognitive performance. To this

144 end, adult zebrafish were exposed to sodium nitrate, sodium nitrite, or control water and tested for
145 changes in learning, memory, and behavior. Furthermore, we utilized targeted and untargeted
146 metabolomics analysis to examine the extent to which treatment resulted in changed nitrate or
147 nitrite concentrations in the brain and altered the brain metabolome.

148

149 # Materials and methods

150 **Fish Husbandry**

151 Wild type zebrafish (5D) were raised and maintained at the Sinnhuber Aquatic Research
152 Laboratory (SARL) at Oregon State University on standard lab diet (Gemma Micro. Skretting,
153 Tooele, France) in accordance with protocols approved by the Oregon State University
154 Institutional Animal Care and Use Committee (IACUC). Adult fish 9-16 months of age were
155 maintained at six fish per tank (3 male and 3 female) in 4-liter of aerated water in metal tanks. Fish
156 water was made with reverse-osmosis water supplemented with Instant Ocean® (Spectrum Brands
157 Blacksburg, VA) at 1.4 g of salt/gallon of water and conductivity between 500-600 µS.
158 Experiments contained three treatment groups which were treated for up to 31 days as 1) no
159 treatment (control fish); 2) sodium nitrate-exposed fish (606.9 mg $NaNO_3$ / L water); and 3)
160 sodium nitrite-exposed fish (19.5 mg $NaNO_2$ / L of water). The nitrate dose was chosen because it
161 increased blood nitrate and nitrite levels, improved exercise performance, and was non-toxic in
162 zebrafish[27,28]. The nitrite dose was chosen because it increased blood nitrite levels but was not
163 associated with adverse effects at pathology with the exception of some mild irritation of gill
164 epithelium[27,29]. For labeling experiments, a subset of fish was switched to water containing >99%
165 stable isotopes of $Na^{15}NO_3$, or 100% $Na^{15}NO_2$ (Cambridge Isotope Laboratories, Tewksbury, MA)

166 at day 28 for 3 days of treatment prior to collection. Nitrate and nitrite were dissolved in freshly
167 prepared fish water and, unless otherwise indicated, chemicals were purchased from Sigma-
168 Aldrich (St. Louis, MO). The fish water and treatment exposure were replaced every 36 hours
169 throughout the duration of the experiment to maintain low ammonia levels and consistent
170 treatments; pH was held at 6.8-7, total ammonia levels to 0-2.0 ppm, and temperature at 27-29 °C.
171 Fish were fed a standard lab diet (Gemma Micro. Skretting, Westbrook, ME) at a volume of ~3%
172 body weight/day. For sample collections fish were euthanized with an overdose of the anesthesia
173 drug, tricaine mesylate, and all efforts were made to minimize suffering. Fish were then dried,
174 weighed, measured for standard length, and brains were collected and snap frozen in liquid
175 nitrogen. Samples were stored in -80°C until used for analysis.
176
177 **Nitrate and nitrite quantification in water**
178 Water was collected during the first week of the experiment and saved directly after a water change
179 (designated as fresh), or 36 h post water change (designated as used). For nitrate measurements,
180 fish water was snap frozen directly. For nitrite measurements, 1 mL fish water was mixed with
181 250 µL of a stop solution (containing potassium ferricyanide, N-ethylmaleimide, NP-40) as
182 previously published[30]. Nitrate and nitrite concentrations were determined by ozone
183 chemiluminescence as previously described on a Sievers Nitric Oxide Analyzer (NOA; Zysense,
184 Frederick, CO)[29,31]. Water was collected on the second day of the experiment but was also
185 confirmed to have similar values in an independent water collection 24 days into the experiment
186 (data not shown).
187
188 **Behavioral Assays**

Swimming behavior, startle response, innate predator avoidance, and social cohesion was tested in individual fish between 14-17 days of treatment, using a zebrafish visual imaging system (zVIS) as previously described[32,33]. Briefly, in the free swim assay fish were placed in a tank with 1.7L of water and the data from the first minute was ignored. The location of the fish was then analyzed by region of tank (top, middle, bottom) for the following 7 minutes (stressed, novel tank environment during minutes 1-8), and then during the last 7 minutes of the assay (minutes 11-18) speed and distance fish traveled was measured (unstressed environment). Habituation to an audio startle stimulus was tested in an array of 8 tanks (12cm × 12cm) filled with 750 mL of fish water[32]. Taps were generated by an electric solenoid below each tank. Following a 10-minute acclimation period, a total of five taps were delivered, with 20s following each tap, and the distance moved between taps was quantified. Predator response and social cohesion assays were completed in a tank with single side view of a LCD video projection. Movement and position were recorded during a one-minute acclimation period where there was no stimulus on the screen. Movement was also recorded directly following the acclimation period where one-minute videos were shown of either shoaling zebrafish (social stimulus) or a predator fish attacking its prey (predator stimulus). For data analysis, the tank was subdivided into three zones in relation to the video projection (close, middle, and far) and the time spent in each zone was calculated.

Custom-built shuttle boxes were used to test learning with a modified protocol as previously described[32,34]. The programmed protocol of this active avoidance conditioning test was designed to condition the zebrafish to leave the compartment with blue light ("reject side") and swim to the dark side ("accept side", also referred to as the correct side). There were a total of 30 trials; each trial consisted of giving the zebrafish 8 seconds to "seek" a dark side of the tank after the blue light came on to avoid a moderate shock. If the fish did not move to the correct side, the

16 second (s) shock period was initiated. A moderate pulse of 5 V was delivered at 1 s intervals, for a duration of 500 ms. Fish were removed from the assay when they did not swim to the correct side during 8 consecutive trials and these fish were counted as repeatedly failed. The statistical method remained as previously described[34], with the data fit using linear regression models to calculate the initial performance of the fish (intercept) and the rate of learning (slopes) for each recorded parameter including the period of time to decision and time shocked[34].

Epinephrine, Norepinephrine, and Dopamine Quantification

Stress hormones epinephrine, norepinephrine, and their precursor dopamine were measured in fish (n=12) using the 3-CAT ELISA (Rocky Mountain Diagnostics, Inc., Colorado Springs, CO) per manufacturer's recommendations. Snap frozen whole zebrafish were ground in liquid nitrogen with mortar and pestle. To normalize variations in fish weight, the resulting whole fish powder was mixed with a buffer at a ratio of 100 mg fish powder to 500 µL HCL buffer solution, containing EDTA and sodium metabisulfite. Samples were centrifuged for 20 minutes at 10,000 × g at 4°C and supernatants collected. A standard curve was generated for each compound concentrations of 0.5, 1.5, 5, 20, and 80 ng/mL, for epinephrine and dopamine, and 0.2, 0.6, 2.0, 8.0, and 32.0 ng/mL for norepinephrine. Samples were diluted 1:1 to be in the range of the standard curve. A Spectramax® M2 plate reader (Molecular Devices, Sunnyvale, CA) was used to measure concentration at 450 nm.

Extraction of zebrafish brains for analysis

Twelve brains per treatment group were snap frozen using liquid nitrogen after four weeks of treatment and two brains were pooled together to compose each sample. Each sample was added

into 2 mL pre-filled tubes containing 300 mg of RNAse and DNAse free zirconium oxide beads (0.5 mm diameter, ceria stabilized, Next Advance, Averill Park, NY). A mixture of 80:20 methanol: water at -80 °C was used as the extraction solvent as previously described[35]. Brains were homogenized with a bullet blender (Precellys® 24-bead-based homogenizer for 2 minutes at 1350 rpm). Extracts were incubated at -20°C for 1 hour and then centrifuged at 13,000 rpm (Eppendorf, Hauppauge, NY) and 4°C for 10 min. The supernatant was split into three 1.5 mL Eppendorf tubes: 100 µL was aliquoted for nitrate and nitrite isotope targeted analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS); 200 µL was aliquoted for untargeted metabolomics analysis, and the remainder (variable volume) was reserved and stored at -80°C.

LC-MS/MS targeted nitrate and nitrite analysis

In order to quantify nitrate and nitrite uptake into the brain, we used a previously described LC-MS/MS approach[36]. Assessing the percent enrichment ($^{15}N/(^{15}N+^{14}N)$ x 100%) allows us to determine the proportion of the nitrate and nitrite that was derived from exogenous sources (stable isotope treatment in water) versus endogenous source (nitrate oxidized from NO produced by NOS enzymes). This method utilizes 2,3-diaminonaphthalene (DAN) derivatization, which reacts with nitrite under acidic conditions to produce 2,3-naphthotriazole (NAT). The production NAT was measured with the previously described method[36] with minor modifications. Briefly, NAT was chromatographically separated on an InfinityLab Poroshell 120 HPH-C18 column (2.7 µm, 2.1 × 50 mm, Agilent, Santa Clara, CA), in a run time of 10 minutes, and detected using a multiple reaction monitoring (MRM) method on an ABSciex 3200 QTRAP mass spectrometer operated in

positive ionization mode. Mass spectrometry allows for the quantification of ^{14}N-NAT (*m/z* 170.1) and ^{15}N-NAT (*m/z* 171.1). The percent enrichment (%) was calculated as: [^{15}N/(^{15}N+^{14}N) × 100].

Un-targeted metabolomics LC-MS/MS

Aliquoted extracts were sonicated for 5 minutes and clarified by centrifugation at 13,000 rpm for 10 minutes. The supernatant was transferred to glass mass spectrometry vials and LC-MS/MS-based metabolomics was performed as previously described[27,37]. Briefly, ultra-high-pressure liquid chromatography (UPLC) was performed on a Shimadzu Nexera system (Shimadzu, Columbia, MD) coupled to a quadrupole time-of-flight mass spectrometer (AB SCIEX TripleTOF 5600). Chromatographic separations were conducted on an Inertsil Phenyl-3 column (4.6 × 150 mm, GL Sciences, Torrance, CA). Elution was achieved using a binary gradient employing as solvent A water, and solvent B methanol, both containing 0.1% formic acid (v/v), as described previously[37]. LC-MS/MS conditions were adapted from Kirkwood et al. (2012)[37] with some modifications. The gradient started with 5% B and was held for 1 min at 5% B, followed by a 11-min linear gradient from 5% to 30 % B. The gradient was increased linearly to 100% B at 23 min, held for 5 min at 100% B and, finally, stepped back to 5% B to equilibrate the column. The flow rate was 0.4 mL/min. The auto-sampler temperature was held at 10°C, the column oven temperature at 50°C, and the injection volume was 5 µL. Time-of-flight (TOF) mass spectrometry (MS) was operated with an acquisition time of 0.25 s and a scan range of 70–1200 Da. Tandem mass spectrometry (MS/MS) acquisition was performed with collision energy set at 35 V and collision energy spread of 15 V. Each MS/MS scan had an accumulation time of 0.17 s and a range of 50–1250 Da using information-dependent MS/MS acquisition (IDA). Ion source gas 1 and 2 and curtain gas (all nitrogen) were set at 50, 40, and 25, respectively. The source temperature was set at 500°C and

the ion spray voltage at 4.5 kV in positive ion mode. The mass calibration was automatically performed every 6 injections using an APCI positive/negative calibration solution (AB SCIEX) via a calibration delivery system (CDS). A separate quality control (QC) pool sample was prepared by combining 5 µL of each sample. Quality control was assured by: (i) randomization of the sequence, (ii) injection of QC pool samples at the beginning and the end of the sequence and between each 10 actual samples, (iii) procedure blank analysis.

Untargeted metabolomics data processing

Raw data was imported into PeakView™ with XIC Manager 1.2.0 (ABSciex, Framingham, MA) for peak picking, retention time correction, and peak alignment. Metabolite identities were assigned as previously described by matching with an in-house library consisting of IROA standards (IROA Technology, Bolton, MA) and other commercially available standards (650 total)[27]. The peak list was exported to MultiQuant 3.0.2 to integrate chromatograms to obtain peak area values for all of the assigned metabolites.

Statistical Analysis

To determine significant differences between three treatment group data were analyzed using a one-way ANOVA with Tukey post hoc test (P-value < 0.05, statistically significant) with GraphPad Prism 4 software (La Jolla, CA). Significant differences were calculated with two-way ANOVA and Tukey post hoc test or a repeated measures two-way ANOVA and Tukey post-hoc test (P–value < 0.05, statistically significant) when both treatment and another condition, like water condition, zone of tank, or a behavioral stimulus, was present[38]. For the shuttle box assay a linear regression was fitted to the data for each treatment to generate initial time and rate of

303 learning graphs, while a separate analysis of variance (AOV) followed by a Tukey's statistical
304 difference was used to calculate statistical significance amongst the groups[34]. For metabolomics
305 data, annotated metabolites were used to conduct multivariate statistical analysis. Pathway analysis
306 and partial least squares-discriminant analysis (PLS-DA), were generated with MetaboAnalyst
307 4.0[39]. The significance of individual metabolites between the treatment groups was assessed with
308 a one-way ANOVA followed by Fisher's post-hoc analysis and Holm FDR-correction, with a *P*-
309 value of < 0.05 and a *q*-value <0.1 indicating significance. If needed, data were logarithmically
310 transformed to correct for unequal variance or non-normal distribution. No outliers were excluded
311 from the statistical analyses. Figures were generated with Prism 8 (GraphPad Software, San Diego,
312 CA), PowerPoint 2016 (Microsoft, Redmond, WA), and MetaboAnalyst 4.0[39].
313

314 Results

315 **Effect of nitrate and nitrite treatment on health parameters and learning**

316 Treatment increased nitrate or nitrite levels in the fresh and used fish water (Fig. 1A and B).
317 Furthermore, both nitrate and nitrite concentrations in control water were maintained at low levels
318 throughout the treatment period (Fig. 1A and B). Several parameters of fish health, including fish
319 length and weight, were not significantly changed with nitrate or nitrite treatment (Fig. 1C and D).
320 Likewise, no significant differences were found between treatment groups for the distance and
321 velocity fish traveled in an unstressed environment (Fig. 1E and F, $P = 0.2089$ and 0.2088,
322 respectively). A startle response assay showed that both nitrate- and nitrite-treated fish became
323 habituated to the vibration, similar to control fish, but nitrate-treated fish traveled a small but
324 significant less distance (10%) following the startle (Fig. 1G).
325

In order to address if nitrate and nitrite treatments altered learning, fish were tested in a learning and memory assay using custom-built shuttle box, where over 30 consecutive trials they learned to avoid an adverse event (mild shock) by moving when a light came on (Fig. 2A). As seen from the linear regression calculated from the data, both nitrate and nitrite treated fish initially took longer to make a decision and were shocked longer (Fig. 2B). Over subsequent trials, more nitrate- and nitrite-treated fish (5-7% of the population tested) had to be removed from the assay because they repeatedly failed to learn (Fig. 2C). However, data from all trial periods show that both nitrate and nitrite treated fish were able to learn and had improved decision time and time shocked, as reflected in their rate of learning (Fig. 2D). It should be noted that the rate of learning (a negative slope) has a larger negative value with nitrate and nitrite treatment, relative to control because these fish had greater potential to improve based on their behavior at the beginning of the assay (Fig. 2B and D). When all fish that were tested are considered, nitrate and nitrite treatment was associated with a significant higher percentage of fish that failed to make a decision and were shocked (Fig. 2E). When the population is filtered to include only fish that could learn (i.e., completed the assay), the nitrite-treated fish were no longer significantly impaired but significant deficits were still present in nitrate-treated fish for time shocked and time to decision (Fig. 2E).

Effect of nitrate and nitrite treatment on behavior and catecholamine levels

The effect of nitrate and nitrite exposure on predator avoidance and social cohesion was tested. Unexpectedly, nitrate-treated fish spent a statistically significant more time close to the monitor during the acclimation period (72%), while the nitrite-treated fish spent 37% more time close to the monitor (Fig. 3A). The social video stimulus did not significantly alter fish behavior in any treatment group as compared to the acclimation period (Fig. 3A). Nitrate and nitrite treated fish

moved away from the monitor when a predator video was shown, as seen by the significant decrease in time spent in the area close to the monitor (Fig. 3A and Supplemental Figure 1). Fish behavior was also tested in the free swim assay where fish were placed in a novel tank. As expected, control fish spent similar amounts of time at all three depths of the tank, balancing safety from predation and opportunity to find food (Fig. 3B). In contrast, there was a significant difference between the time the nitrate- and nitrite-treated fish spent between the bottom and top zones (Fig. 3B). Nitrate- and nitrite-treated fish spent 22-35% more time in the bottom zone, as compared to control fish. The increase in bottom-dwelling is consistent with anxiety-like behavior. Since anxiety can be associated with stress, we measured the levels of some stress hormones and found neither nitrate, nor nitrite treatment significantly increased epinephrine or norepinephrine levels (Fig. 3C). It appeared that nitrite-treated fish experienced lower concentrations of these hormones yet high variability between fish led to no significant differences being detected. Nitrate or nitrite treatment also did not significantly change dopamine concentrations which is the precursor for epinephrine and norepinephrine (Fig. 3C).

Nitrate and nitrite uptake into the brain

For the last three days of the experiment, a subset of fish was treated with ^{15}N-nitrate or ^{15}N-nitrite in order to study the uptake of nitrate and nitrite into brain tissue. The resulting percent enrichment results show the proportion of nitrate and nitrite derived from exogenous sources (the treatment in water) versus endogenous sources such as oxidation of NO from NOS-mediated production. We observed a low uptake of nitrate (14%) and almost no uptake of nitrite (0.1%) in the brain, which can be seen by comparing the fish that received labeled nitrate or nitrite as compared to the respective unlabeled nitrate or nitrite treatment conditions. (Fig. 4A and B). Furthermore, no

372 significant changes in nitrate or nitrite concentrations were detected in the brain of animals treated
373 with nitrate or nitrite (Fig. 4A and B) when compared with the control group. Taken together, these
374 results suggest that the behavioral changes observed with nitrate and nitrite exposure are likely
375 due to indirect effects of treatment on brain metabolism, rather than a direct effect via influx of the
376 nitrate or nitrite into the brain.
377
378 **Metabolomics Results**
379 One hundred twenty-four (124) metabolites were annotated using our in-house library (SI Table
380 S1). Of these metabolites, 47 were significantly changed among at least one treatment group, as
381 compared to the others and FDR-corrected *P*-values (*q*-values) for all significantly changed
382 metabolites, between all treatment groups, are listed in SI Table S2. For example, deoxyadenosine
383 diphosphate (dADP) was significantly up-regulated (q = 0.018) in fish exposed to nitrate and
384 nitrite, and desmosterol, the immediate precursor of cholesterol in the Bloch pathway of
385 cholesterol biosynthesis, was significantly down-regulated (q = 0.018) in fish exposed to nitrate
386 and nitrite.
387 Partial least squares discriminant analysis (PLS-DA) demonstrates spatial clustering and
388 separation between treatment groups when considering all the annotated compounds (Fig. 5A).
389 There are two importance measures in PLS-DA: one is variable importance in projection (VIP)
390 and the other is the weighted sum of absolute regression coefficients. The VIP graph of the most
391 relevant 30 features (when considering the three treatments) is shown in Fig. 5B. The colored
392 boxes on the right indicate the relative concentrations of the corresponding metabolite in each
393 group under study. Among the positively correlated metabolites with the highest VIP scores

394 associated with the PLS-DA were dADP, desmosterol, linoleic acid, suberic acid, oleic acid and
395 guanine.

396 Notably, nitrate or nitrite treatment resulted in significant differences among multiple
397 metabolites involved in purine metabolism like hypoxanthine, xanthine, inosine, guanine,
398 guanosine, deoxyadenosine diphosphate (dADP) and cyclic adenosine monophosphate (cAMP).
399 Interestingly we also observed a significant decline in nicotinamide adenine dinucleotide
400 phosphate (NADP) and NAD. We observed a significant depletion in the annotated fatty acids
401 (linoleic acid (LA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA)) with nitrate and
402 nitrite treatments. Remarkably, LA was depleted by 50% by nitrate treatment and by ~90% in the
403 nitrite treatment when considering normalized peak intensity values. Similarly, ARA was depleted
404 by 80 and 60% in the nitrate and nitrite treatments, respectively. We also observed lower
405 abundances for some of the annotated TCA (tricarboxylic acid cycle) cycle intermediates (i.e.
406 malate and succinic acid). A depletion in amino acids threonine, N-acetyl-L-methionine (NAM),
407 and phosphoserine, was observed with nitrate and nitrite treatment. Notably, we observed that
408 nitrate and nitrite treatment had an effect on γ-aminobutyric acid (GABA, Fig. 6A), the chief
409 inhibitory neurotransmitter in the developmentally mature central nervous system and its
410 precursor, glutamine (Fig. 6B)[40]. Nitrate treatment caused a significant 22% reduction in the
411 abundance of glutamine and 19% reduction in GABA in zebrafish brains. Nitrite treatment also
412 caused a significant 17% reduction in the abundance of glutamine and 18% reduction of GABA in
413 zebrafish brains. Interestingly, no significant differences in the abundance of the excitatory
414 neurotransmitter glutamate were found with nitrate or nitrite treatments, thus GABA abundance
415 changed in congruence with glutamine, but not glutamate levels.
416

Discussion

Here we disproved the hypothesis that nitrate, and nitrite treatment would improve indicators of learning and cognitive performance in a zebrafish model. While nitrate and nitrite treatment did not adversely affect multiple parameters of health, these treatments were associated with mild anxiety-like behavior and an initial deficit in learning, which was consistent with either decreased executive function or associative learning. While we have previously shown the nitrate and nitrite doses used here increased blood and whole body nitrate and nitrite levels, the treatments were not associated with a significant increase in the concentration of nitrate or nitrate in the brain, and only a minor, or almost no uptake of these chemicals into the brain. Nevertheless, some brain metabolites including GABA and glutamine were significantly decreased by nitrate and nitrite treatment suggesting that the changes in behavior and learning may be due to indirect effects of nitrate and nitrite treatment on the nervous system.

The anxiety-like behavior we observed with nitrate or nitrite treatment was mild compared to other anxiogenic and anxiolytic substances tested in adult zebrafish[41]. For example, ethanol exposure caused concentration- and time-dependent effects on brain ethanol levels and modulated locomotor-, aggression-, anxiety-, and fear-like behaviors in zebrafish[42,43]. Likewise, cannabinoids exposure triggered hypolocomotion, and deficits in spatial memory performance and fear learning[44–47]. Nicotine, morphine, and psychedelic drug exposure, or withdrawal, have numerous and expected anxiolytic and anxiogenic effects in zebrafish[21]. The nitrate- or nitrite-induced anxiety was more similar in scale to zebrafish that were not allowed to exercise[48].

The zebrafish in this study exhibited increased anxiety, as evidenced by staying near the bottom of the novel tank. Nitrate and nitrite treatment also changed the behavior of fish during the

acclimation period of the predator and social stimulus assay. We also observed an initial delay in zebrafish decision making following a light stimulus and increased time being shocked initially in the shuttle box task which could represent an initial deficit in associative learning and/or executive function (e.g., decision making). This is inconsistent with literature that showed nitrate, given as BRJ supplement, improved reaction time and cognitive performance[7,49–51]. A plausible mechanism underlying nitrate-induced cognitive improvements is increased vasodilation, yielding improved CBF[7,9,52,53]. This is exemplified by a study in older adults where two days of consuming a high nitrate diet increased regional cerebral perfusion in frontal lobe white matter, particularly between the dorsolateral prefrontal cortex and anterior cingulate cortex[52]. These brain regions participate in executive function, which may have been affected by nitrate and nitrite treatment in our study. In contrast with our results, multiple studies show no significant association with foods containing nitrate and changes in cognitive function or mood[54–60]. Possible factors contributing to conflicting cognitive responses reports and our own study include different routes of treatment (continual in water vs episodic in meals), different nitrate doses and lengths of treatment, food matrix effects, age and health status of participants, or unidentified species-specific effects.

NO's myriad roles in the brain include acting as a anterograde neurotransmitter, a retrograde neurotransmitter, regulator of presynaptic plasticity in gabaergic and glutamatergic neurons, and effecting dendritic spine growth (reviewed in[61]). NO is directly involved in learning and memory, and NO modulators are being explored for the treatment of anxiety[62]. Manipulation of brain NO levels in rodents decreased anxiety when specific doses of L-arginine (NO precursor), L-NAME (NOS inhibitor), or sodium nitroprusside (NO donor) were given, but a high dose of L-NAME decreased locomotor activity, similar to our result[63,64]. Consistent with our increased anxiety-like behavior, studies in mice showed anxiogenic effects of sildenafil (NO donor), or the

462 combined treatment of sildenafil and ascorbic acid[65–67]. As manipulation of NO levels in the brain
463 can yield both anxiolytic or anxiogenic effects, it is possible that the changes in behavior we
464 observed may be attributable to high NO levels, but we have not assessed surrogate markers of
465 this phenomenon, such as nitrated tyrosine levels in brain tissue[62]. However, our results showing
466 low uptake of nitrate and nitrite treatments into the brain, and no significant changes in brain nitrate
467 and nitrite concentrations indicate it is important to consider other indirect mechanisms and the
468 metabolomics dataset can help inform this.

469 Reduction of brain GABA and glutamine levels we observed with nitrate and nitrite
470 treatment, be it through increased NO in the brain or indirect mechanisms, is noteworthy because
471 perturbations in the GABAergic system have been associated with anxiety and depression and thus
472 may be an important player in the behavioral changes we observed[68–70]. Glutamine is a substrate
473 for GABA production and serves as an important energy source for the nervous system. We also
474 observed changes in brain purine-related metabolites, which is consistent with the known
475 relationship between exogenous and endogenous pathways that generate NO[18,24,27]. The nitrate-
476 and nitrite-induced reductions in fatty acids, neurotransmitters, signaling molecules, tricarboxylic
477 acid cycle intermediates, and amino acids are also of interest and warrant future investigation. A
478 significant limitation of this study is the use of whole zebrafish brains to derive metabolomics data,
479 limiting our ability to draw inferences to specific functional structures within the brain, like the
480 zebrafish equivalent of the prefrontal cortex[71]. Interestingly, a study in older adults using a more
481 focused technique measured brain *N*-acetyl aspartate, creatine, choline, or myo-inositol levels and
482 found no change with 3 day BRJ supplement[57].

483 It is also possible that the changes in zebrafish behavior we observed were because nitrate
484 and nitrite treatment caused a headache or migraine[72]. Headaches are a predominate side effect

485 from therapeutic use of organic nitrates, which are prodrugs for NO, cause vasodilation of blood
486 vessels in the brain, and "immediate" mild-to-medium severity headaches or "delayed" migraines
487 which involves cGMP or NO dependent S-nitrosylation-mediated changes in ion channel
488 function[73]. Nitroglycerin has been used to model migraines in multiple species including fish[74,75].
489 Also, headache is the most common side effect in patients taking sildenafil, which promotes blood
490 flow to organs like the brain, through cGMP. Furthermore, consumption of high nitrite foods was
491 associated with headaches in some people[76]. Migraines have also been correlated in humans with
492 oral microbiomes that increased abundances of nitrate, nitrite, and NO reductase genes supporting
493 that nitrite and NO could promote migraines[77]. Given these various findings, it is possible in
494 zebrafish that nitrate- or nitrite-induced production of NO in blood vessels stimulated vasodilation
495 and caused a headache or migraine. While it is beyond the scope of this study to assess this
496 possibility, future cognitive studies with nitrate, nitrite or BRJ treatment in the clinic should make
497 note of the incidence of headaches and migraines.

498 It is also important to note that a body of literature shows that nitrate pollution in aquatic
499 ecosystems can have adverse effects for a variety of species (reviewed in[78]). Likewise, human
500 consumption of nitrate- and nitrite-contaminated water or excessive intake from vegetables may
501 also cause adverse effects[79]. An endocrine disrupting role of nitrate and nitrite has been observed
502 in various species, and the possible pathways of altering steroidogenesis have been proposed[80].
503 Both glutamate and GABA are involved in pituitary hormone release in fish. There is also good
504 evidence for the involvement of GABA in luteinizing hormone release in fish[81]. Other studies have
505 indicated that high nitrate and nitrite exposure from drinking water and diet may exert adverse
506 effects on the development of the human nervous system[82,83]. Nitrate and nitrite can also perturb
507 the activity of dopaminergic (DA) neurons by acting through estrogen receptor (ER) in early

development of zebrafish[84] at concentrations around the safety limit for drinking water recommended by the Environmental Protection Agency (EPA) and the World Health Organization (WHO) (10 mg/L NO_3-N and 1 mg/L NO_2-N, respectively)[85]. While many of these studies were conducted during embryonic development, and are different from own limited adult exposure, they highlight that nitrate and nitrite can have significant effects on the central nervous system.

As with all studies conducted in model organisms there are some specific contextual factors that make comparison to humans difficult. While zebrafish are used to model complex brain disorders, including anxiety, limitations exist because we must infer pain, discomfort or other behaviors through observation[21 22,41,72]. Another unique aspect of zebrafish exposure is ammonia in water. To address this potentially toxic metabolite, we regularly measured ammonia and found no effect of nitrate or nitrite treatment on water ammonia levels. Due to the large number of animals needed to conduct the study, we were limited in the number of doses we could test and thus focused on a nitrate dose and exposure duration associated with improvements in exercise performance[27]. More and larger studies are needed to delineate the potential benefits and risks associated with nitrate and/or nitrite treatment on CBF, mood, and cognitive function, particularly in populations of people with differing ages and underlying health status. Importantly, a study in humans is underway to look at the effect of increasing doses of nitrate on cognition-related outcomes[86]. We also cannot differentiate between the direct effects of nitrate or nitrite in the fish, or indirect effects that could be generated by increased NO availability. Nevertheless, we show that nitrate and nitrite treatment in a zebrafish model did not adversely affect multiple parameters of health but was associated with mild anxiety-like behavior, changes in brain metabolome, and an initial decrease in executive function or associative learning.

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Figure captions

Fig 1. Nitrate and nitrite treatment did not adversely affect multiple parameters of health but nitrate treatment significantly decreased movement following a startle. Adult zebrafish were treated with control water, sodium nitrate, or sodium nitrite and (A) nitrate and (B) nitrite concentrations were measured in newly treated fish water (fresh) and water at the end of 42-hour use (used) (n = 7-10). Fish (C) length and (D) weight was measured after 28-31 days of treatment (n = 18-33). At 14 – 17 days of treatment the (E) distance and (F) velocity zebrafish traveled was quantified in the voluntary swimming assay (n = 39-42) or (G) the response to five sequential acoustic startles (as taps against the fish tank) was quantified (n = 84-90). (A-G) Bars represent the mean ± SEM.

Fig 2. Nitrate and nitrite treatment were associated with an initial decline in learning but fish learned over repeated tests. Adult zebrafish were treated with control water, sodium nitrate, or sodium nitrite for 14-17 days when learning and memory were tested in the shuttle box assay (n = 72-109). (A) Time-to-decision and time shocked was recorded for each fish and trial (dots) and linear regression of the data were calculated (lines). As calculated from the linear regression the bars indicate (B) initial periods of time fish spent for the indicated measure and (D) rates of learning as quantified by the slope from the linear regression. (C) Bars indicate the percentage of fish that were removed from the assay because they did not swim to the correct side during eight consecutive trials. (E) Statistical summary of shuttle box results as calculated by an analysis of variance (AOV) followed by a Tukey's post-test where "All" indicates data from all fish analyzed, while "Completed trials" excludes data from fish that repeatedly failed.

Fig 3. Nitrate and nitrite treatment increased anxiety-like behavior in zebrafish. Adult zebrafish were treated with control water, sodium nitrate, or sodium nitrite for 14-17 days. Fish movement was recorded and (A) the time fish spent in the zone closest to a monitor during an acclimation (no stimulus), or in the presence of a stimulus of a video of shoaling fish (social), or a predator (n = 42-84) was recorded. (B) Likewise, movement in a novel tank was recorded and the percent of time spent in the bottom, middle and top zones of tank are indicated (n = 83-89). (C) Concentrations of hormones were measured in whole fish by ELISA (n = 12). (A-G) Bars represent the mean ± SEM.

Fig 4. Little uptake of nitrate or nitrite from treatments was found in the brain. The concentration of (A) nitrate or (B) nitrite was measured using targeted LC-MS/MS in control animals or animals treated with (A) unlabeled and ^{15}N-labeled nitrate, or (B) unlabeled and ^{15}N-labeled nitrite. (A-B) Zebrafish brains were collected on day 31 and percent enrichment (in boxes), indicates the relative amount of nitrate or nitrite in the brain that was derived from the treatment. Bars represent the mean concentration ± SEM (n = 6).

Fig 5. Nitrate and nitrite treatment significantly altered the abundance of some brain metabolites. Adult zebrafish were treated with control water, sodium nitrate, or sodium nitrite for 31 days and brain metabolites were measured using untargeted LC-MS/MS. (A) Partial least squares discriminant analysis (PLS-DA) scored plot demonstrates spatial clustering and separation between treatment groups when considering all the annotated compounds. (B) PLS-DA variable importance in projection (VIP) graph of the most relevant 30 features (when considering the three treatments). Colored boxes at right indicate the mean relative concentrations of the corresponding metabolite in each treatment group under study. Red color indicates higher abundance, while green color indicates lower abundance. The PLS-DA model display 95% confidence region.

598 Abbreviations: dADP (deoxyadenosine diphosphate), NAM (N-acetyl-L-methionine), 3-
599 Hydroxybenzo (3-Hydroxybenzoic acid), 1-Aminocyclopr (1-Aminocyclopropane-1-
600 carboxylate), 3PG (3-Phosphoglyceric acid), NADP (Nicotinamide adenine dinucleotide
601 phosphate), ARA (Arachidonic acid), NAD (Nicotinamide adenine dinucleotide), EPA
602 (Eicosapentanoic acid), 12-Hydroxydode (12-Hydroxydodecanoic acid), MMA (Methylmalonic
603 acid), 2-Hydroxybutyrate (2-Hydroxybutyric acid).

604 **Fig 6. Nitrate and nitrite treatment decrease gamma-aminobutyric acid (GABA) and**
605 **glutamine levels in zebrafish brain.** Adult zebrafish were treated with control water, sodium
606 nitrate, or sodium nitrite for 31 days. Abundance of (A) GABA and (B) glutamine was measured
607 in brain tissue by LC-MS/MS. Bars represent the mean peak area ± SEM (n = 6).

608

609 Supplemental Figure Captions

610 **Supplemental Fig 1. Nitrate and nitrite treated fish avoided predators.** Adult zebrafish were
611 treated with control water, sodium nitrate, or sodium nitrite for 14-17 days. Fish movement was
612 recorded and (A) the time fish spent in the zone closest to a monitor during an acclimation (no
613 stimulus), or in the presence of a predator (n = 84-42) was recorded. Bars represent the mean ±
614 SEM.

615

616 Supplemental Table Captions

617 **Supplemental Table S1. Metabolites annotated using our in-house library.**
618 **Supplemental Table S2. Significantly changed metabolites, between all treatment groups.**

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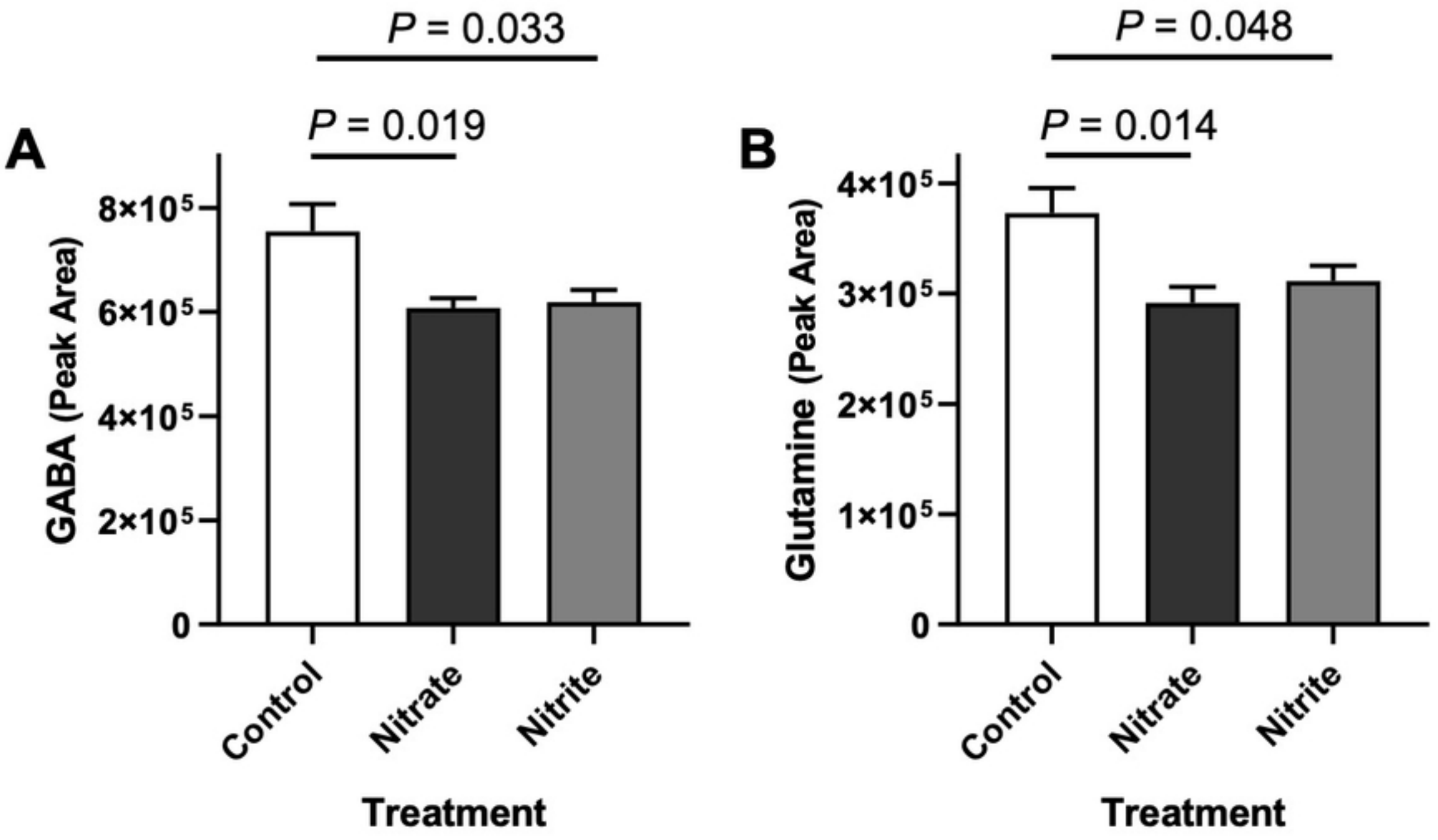
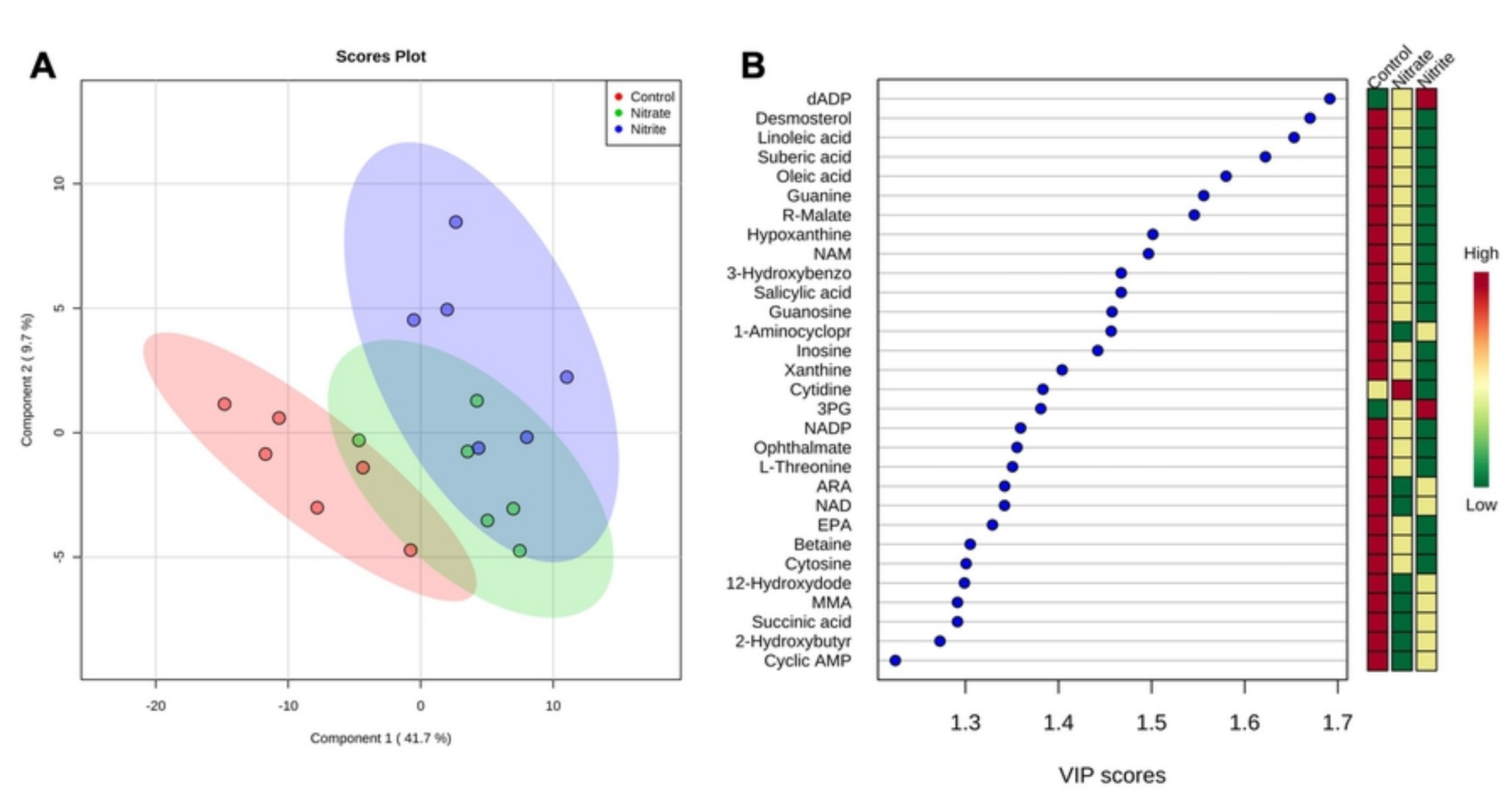
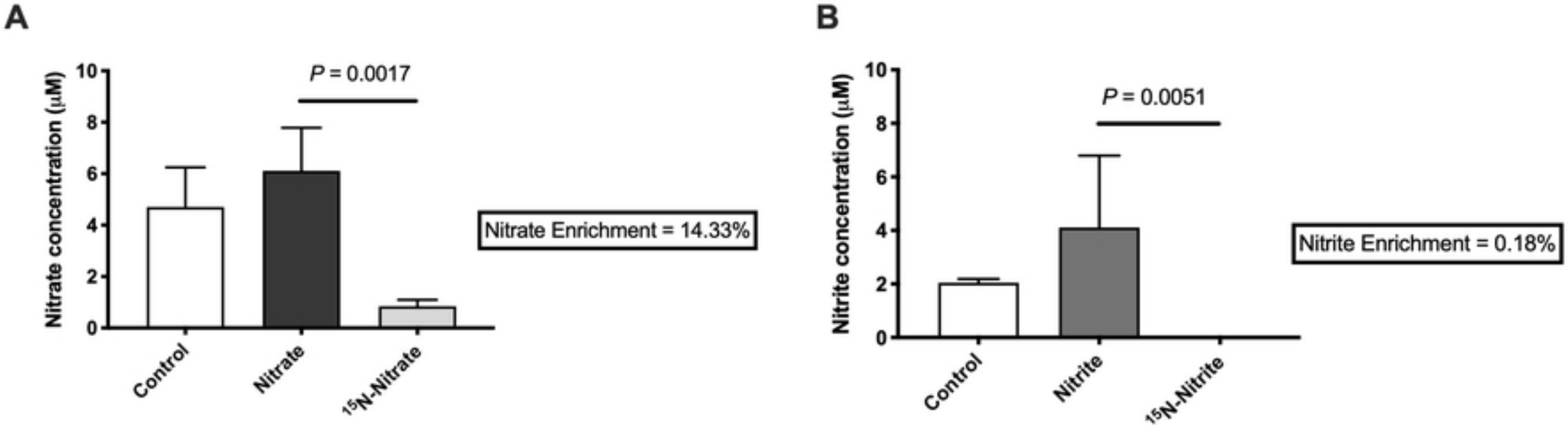
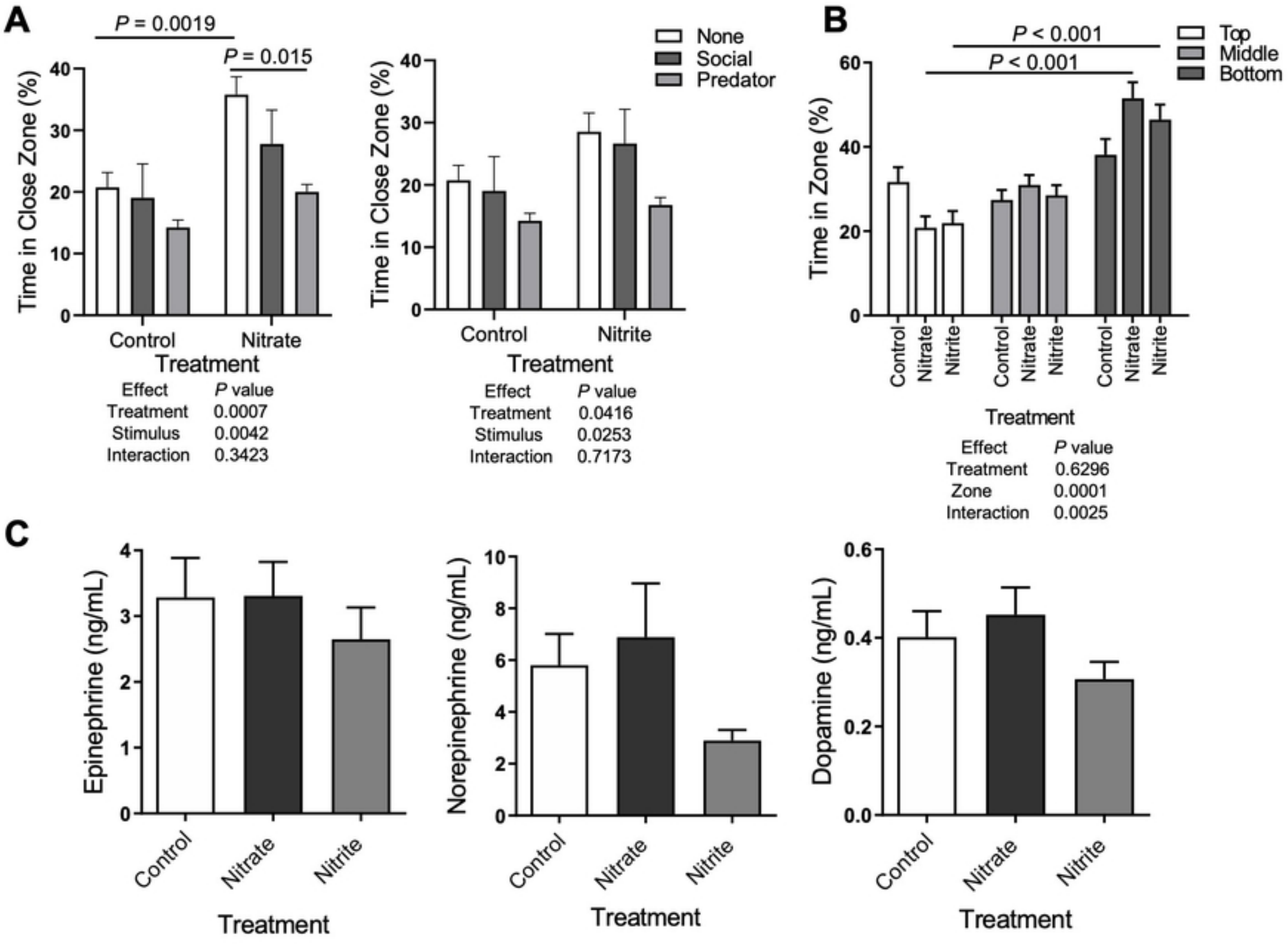
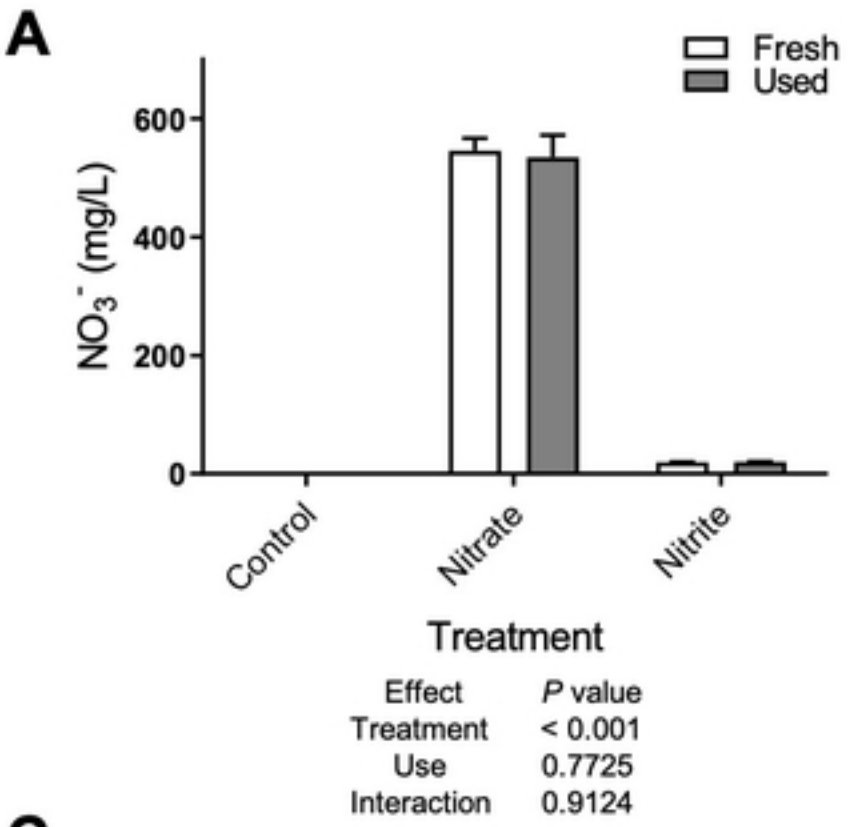

A
P = 0.033
P = 0.019
GABA (Peak Area)
8×10^5
6×10^5
4×10^5
2×10^5
0
Control
Nitrate
Nitrite
Treatment
B
P = 0.048
P = 0.014
Glutamine (Peak Area)
4×10^5
3×10^5
2×10^5
1×10^5
0
Control
Nitrate
Nitrite
Treatment

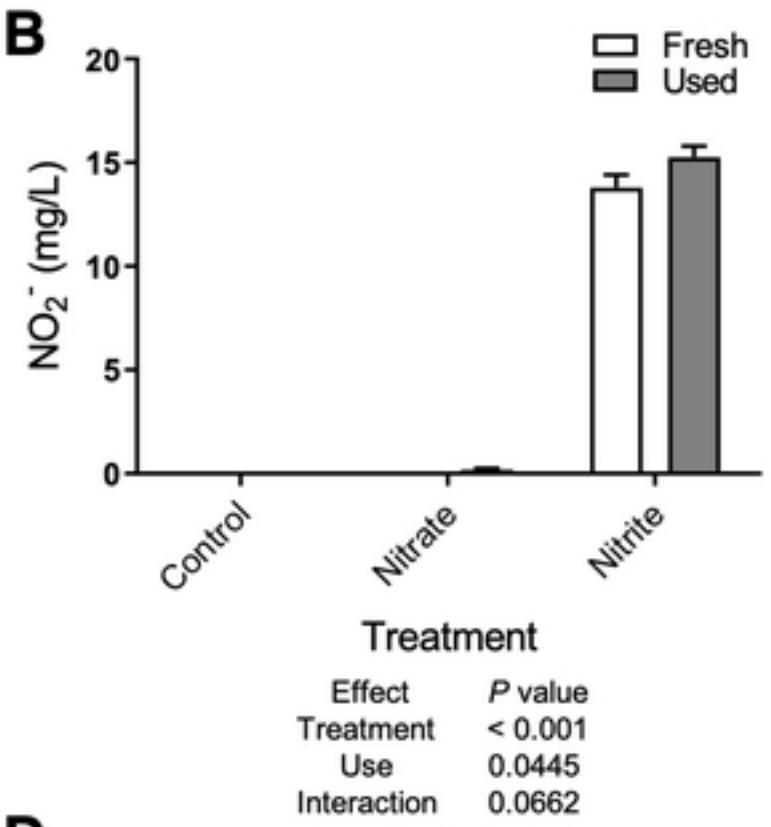

A
Scores Plot
Control
Nitrate
Nitrite
Component 2 (9.7 %)
Component 1 (41.7 %)
B
dADP
Desmosterol
Linoleic acid
Suberic acid
Oleic acid
Guanine
R-Malate
Hypoxanthine
NAM
3-Hydroxybenzo
Salicylic acid
Guanosine
1-Aminocyclopr
Inosine
Xanthine
Cytidine
3PG
NADP
Ophthalmate
L-Threonine
ARA
NAD
EPA
Betaine
Cytosine
12-Hydroxydode
MMA
Succinic acid
2-Hydroxybutyr
Cyclic AMP
Control
Nitrate
Nitrite
High
Low
VIP scores


A
Nitrate concentration (μM)
10
8
6
4
2
0
P = 0.0017
Nitrate Enrichment = 14.33%
Control
Nitrate
15N-Nitrate
B
Nitrite concentration (μM)
10
8
6
4
2
0
P = 0.0051
Nitrite Enrichment = 0.18%
Control
Nitrite
15N-Nitrite

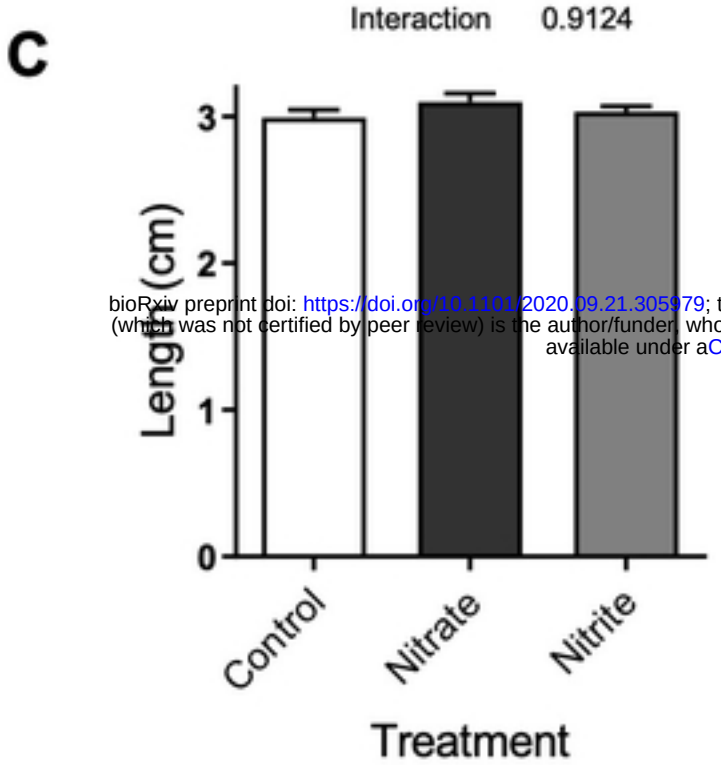

A
P = 0.0019
P = 0.015
Time in Close Zone (%)
Control
Nitrate
Treatment
Effect P value
Treatment 0.0007
Stimulus 0.0042
Interaction 0.3423
None
Social
Predator
Time in Close Zone (%)
Control
Nitrite
Treatment
Effect P value
Treatment 0.0416
Stimulus 0.0253
Interaction 0.7173
B
P < 0.001
P < 0.001
Top
Middle
Bottom
Time in Zone (%)
Control
Nitrate
Nitrite
Treatment
Effect P value
Treatment 0.6296
Zone 0.0001
Interaction 0.0025
C
Epinephrine (ng/mL)
Control
Nitrate
Nitrite
Treatment
Norepinephrine (ng/mL)
Control
Nitrate
Nitrite
Treatment
Dopamine (ng/mL)
Control
Nitrate
Nitrite
Treatment

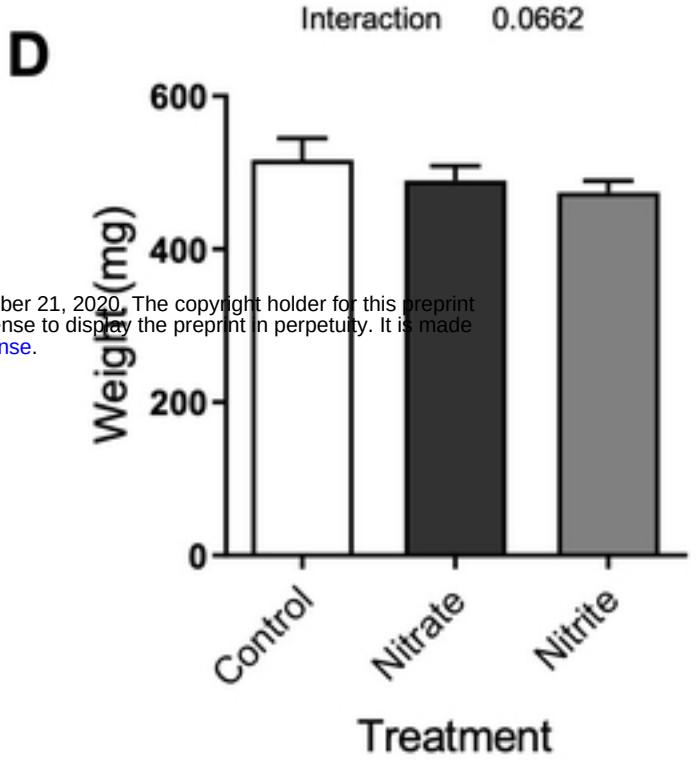


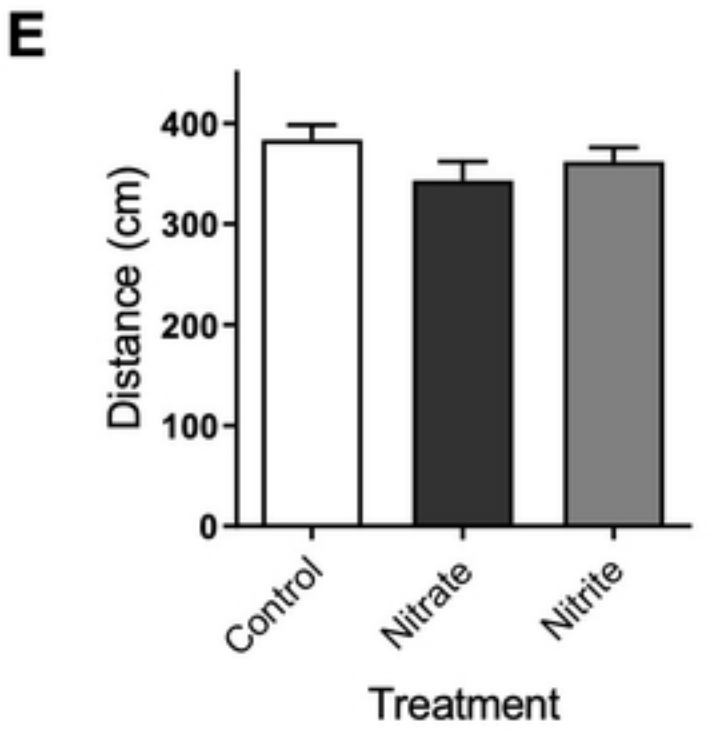
Effect	*P* value
Treatment	< 0.001
Use	0.7725
Interaction	0.9124

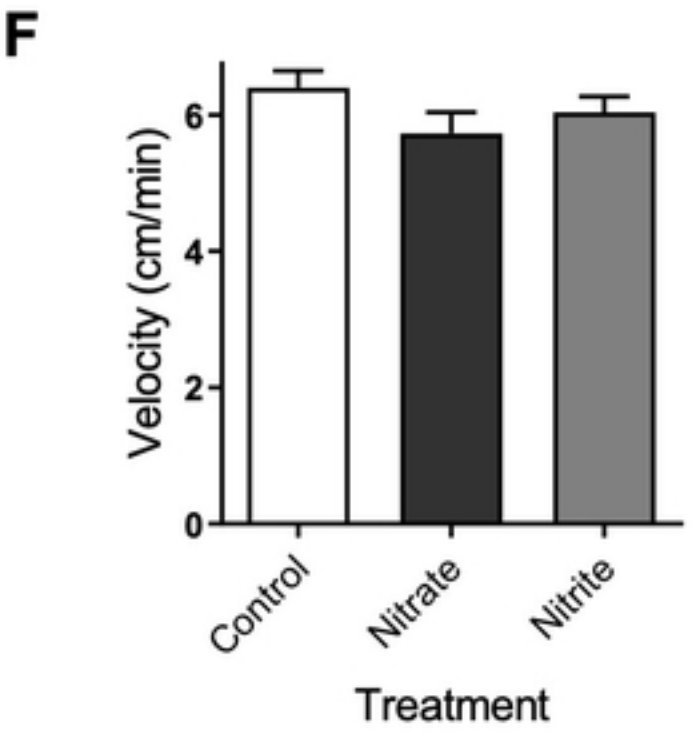


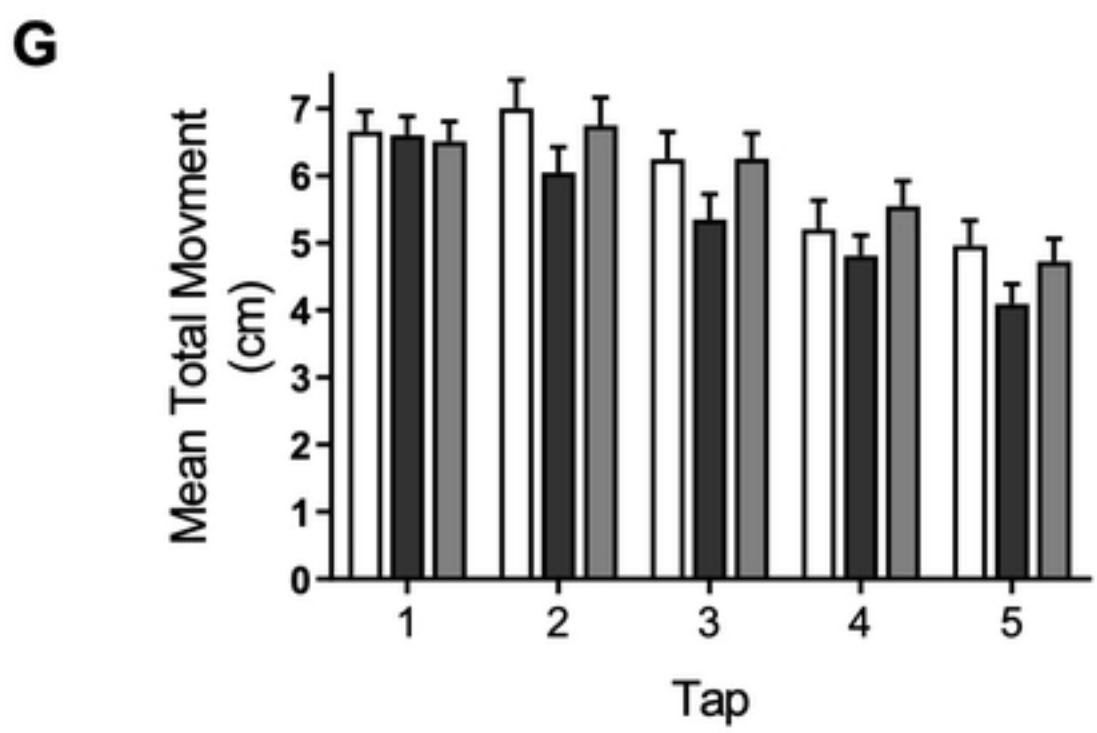
Effect	*P* value
Treatment	< 0.001
Use	0.0445
Interaction	0.0662





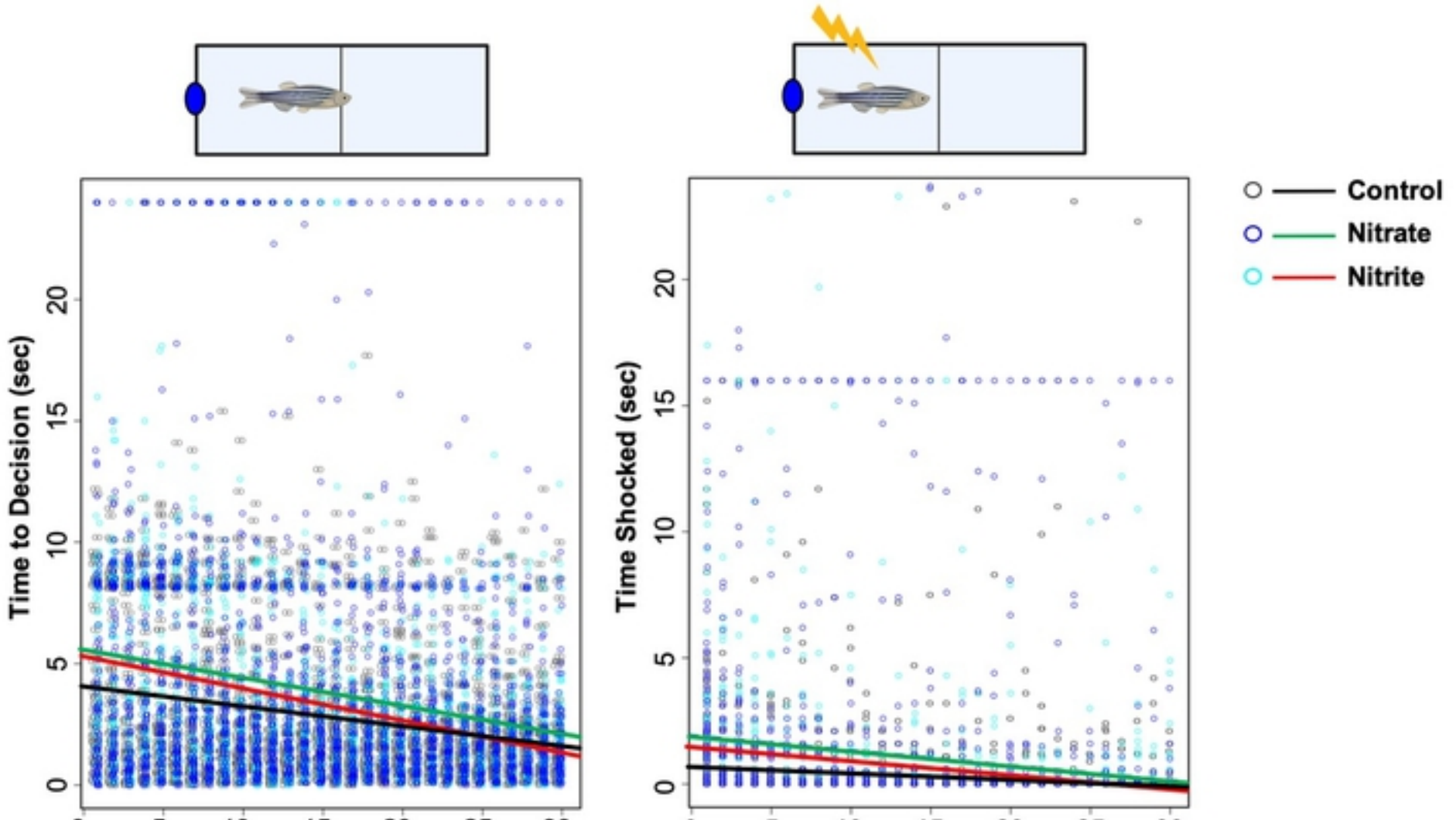




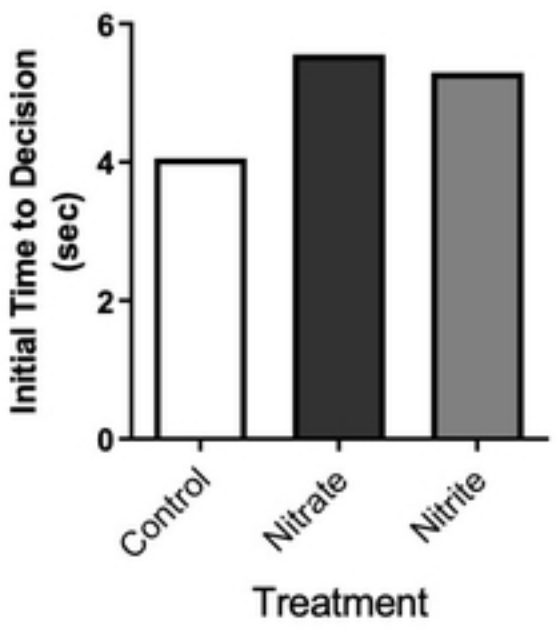


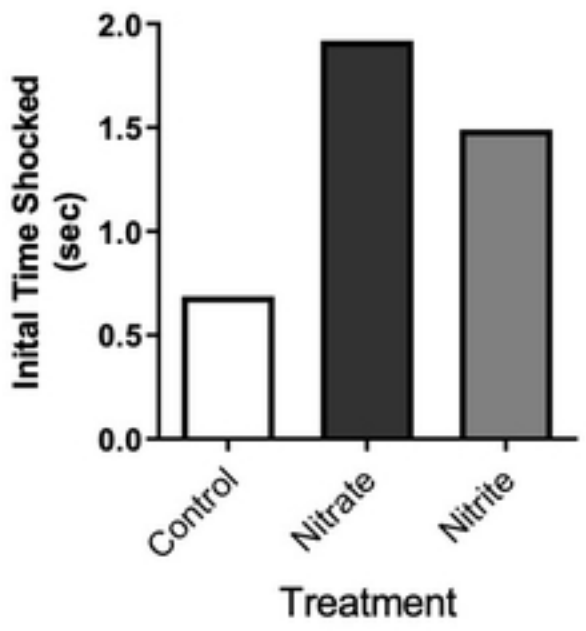
Effect	*P* value
Tap	< 0.0001
Nitrate	0.0101
Nitrite	0.6446

A

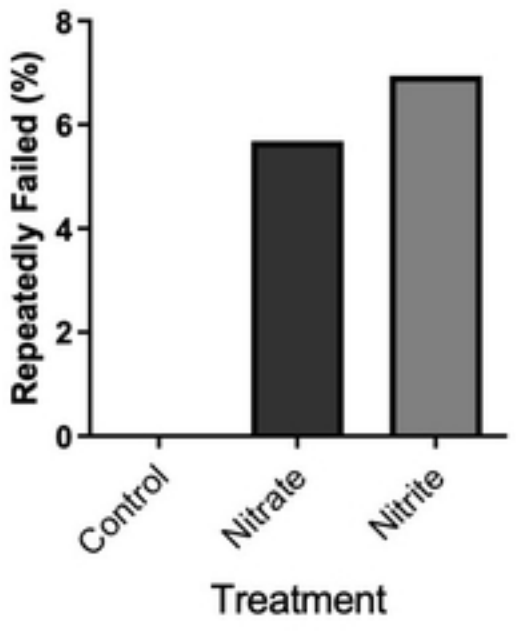


B

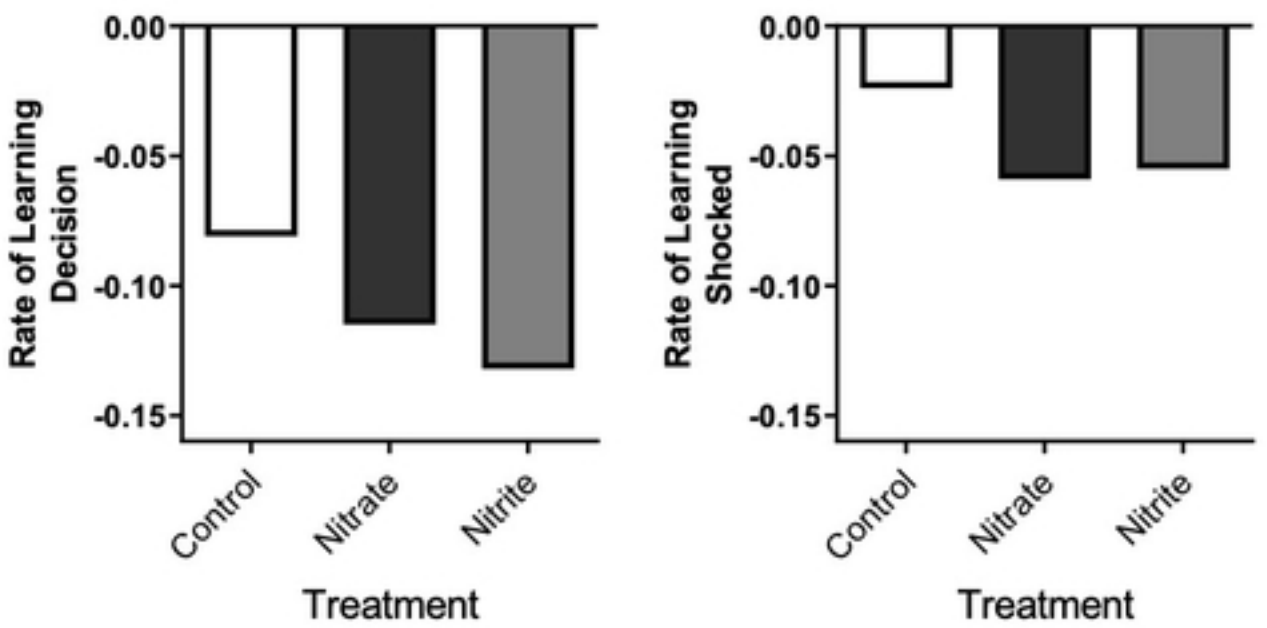




C



D



E

Time to Decision

		All						Completed Trials		
Treatment	**n**	**% Learned**	**% Failed**	**Intercept**	**Slope**	**p value**	**sig**	**% Learned**	**p value**	**sig**
Control	109	51.376	48.624	4.057	-0.081	NA	NA	51.376	NA	NA
Nitrate	88	40.909	59.091	5.556	-0.115	0	Yes	43.373	0	Yes
Nitrite	72	50	50	5.298	-0.132	0	Yes	53.731	0.5804	No

Time Shocked

		All						Completed Trials		
Treatment	**n**	**% Learned**	**% Failed**	**Intercept**	**Slope**	**p value**	**sig**	**% Learned**	**p value**	**sig**
Control	109	61.468	36.697	0.687	-0.024	NA	NA	61.468	NA	NA
Nitrate	88	38.636	56.818	1.919	-0.059	0	Yes	40.964	0	Yes
Nitrite	72	41.667	58.333	1.491	-0.055	0	Yes	44.776	0.9960	No