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ARTICLE

Drinking a Structured Water Product on Markers of Hydration, Airway Health and Heart Rate Variability in Thoroughbred Racehorses: a Small-scale, Clinical Field Trial

Michael Ivan Lindinger[1*] Foster Northrop[2]

1. The Nutraceutical Alliance, Burlington, ON, Canada
2. Northrop Equine, Louisville, KY, USA

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ABSTRACT

Racehorses in training are in situations of repeated stress that may have effects on hydration and health. It was hypothesized that daily consumption of a structured water (SW) product for 4 weeks will result in improved hydration, improved upper airway health and increased heart rate variability. Two groups of Thoroughbred racehorses matched for physiological, training and racing attributes were studied for 4 weeks. One group (n = 17) received 10 L (about 15%) of their daily water as SW (followed by ad libitum filtered deep well water) and the control group (n = 15) only filtered deep well water. Blood samples and bioelectrical impedance analysis (BIA) measures were obtained at baseline, 2 and 4 weeks. Hydration was assessed using BIA. The upper airway was assessed by nasopharyngeal endoscopy at baseline and weekly within 60 minutes of breezing. On weekly breeze days heart rate was recorded at rest, during exercise and recovery and data were analysed for heart rate variability. Compared to controls, horses drinking SW showed increased hydration improved upper airway health post-breezing and increased heart rate variability. It is concluded that drinking 10 L daily of SW increased hydration and may have conferred some wellness benefits.

1. Introduction

As the most important essential nutrient in most biological systems, water plays central roles in all facets of cellular, tissue, organ and organism function. For more than 50 years we have known that water in biological systems is structured [1-7], and also that structured water (SW) occurs naturally [8,9] or can be man-made [10,11]. However, we still have very little idea what happens to SW when it is taken up into plants or consumed by animals. While the internet is rife with anecdotes, hard science is lacking. The present study describes the effects of a stable, man-made SW in an intact biological system, the Thoroughbred racehorse in training and competition.

Physicists and chemists have been studying SW for nearly 8 decades [1-5]. Liquid water can be structured, defined as an increase in the numbers of water clusters, using variety of methods including magnets, light and other forms of electromagnetic energy [7,12,13]. Magnetized water (water treated by being in the influence of magnets) briefly gains altered water structuring and has received some attention in

**Corresponding Author:*
Michael Ivan Lindinger,
The Nutraceutical Alliance, Canada;
Email: mi.lindinger@gmail.com

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plant and animal agricultural research [14]. While these types of magnetized waters are stable for only a few hours to a few days, the studies universally report benefits to plants and animals unless the intensity of the magnetic field used for treating the water is too high [13,14]. It remains unknown how these types of SW confer their benefits.

Some notable studies reporting effects of SW on cell systems include the following. Culture medium created using SW, compared to control medium, resulted in increased viability of mouse splenocytes, increased viability of RAW 264.7 macrophage cells, and doubling of phagocytic activity [15]. This study also showed up to a 3-fold increase in natural killer (NK) cell activity when NK cell activity was assessed using CCK-8 assay to measure cell cytotoxicity against YAC-I cells. The authors concluded "these results strongly suggest that water plays a critical role in cellular metabolism, more than previously understood." This view is elegantly supported in Ball's [4] perspective on the roles of SW in living systems. Similar lines of evidence using cell systems were reported by Lee et al. [16]. Sharma et al. [17] reported increased rate of seed germination, sapling growth and development when chickpea seeds were incubated, and saplings grown, in SW compared to controls.

The present study used multi-frequency bioelectrical impedance analysis (BIA) for the non-invasive assessment of hydration and cellular integrity in horses [18-20]. At present, the limits of detecting changes in horses are similar to that in humans: it is not possible to detect changes smaller than 3% of total body water (TBW) or extracellular fluid volume (ECFV). Water contents are calculated from measures of impedance to current flow, and reactance, over a range of different frequencies. It is likely that direct measures of reactance (the delay in conduction as a result of capacitance by cell membranes and tissue interfaces) which is directly related to membrane capacitance, is able to provide an index of hydration and cellular integrity at higher resolution than calculated water volumes [19,20].

Racehorses live in a relatively stressful environment comprising little daily turnout, periodic withholding of water, bouts of near-maximal exercise, transport to racetracks and being housed in unfamiliar surroundings. In association with these stressors, many racehorses have mild to moderate upper airway inflammation which is associated with poor performance [21] and that often progressively deteriorates through a horse's racing career [22]. With efforts to reduce the use of medications by many racing jurisdictions [23], alternative approaches are being sought to more naturally support health and performance of racehorses. The purpose of the present study was to determine, using a group of actively training and racing Thoroughbred horses if daily drinking of SW would improve indicators of health including hydration, tissue conductivity, upper airway health and heart rate variability. It was hypothesized that daily consumption of 10 L of a SW product increases hydration, improves upper airway health and increases heart rate variability.

2. Materials and Methods

2.1 Structured water

The Defiance structured water - DSW (Defiance Brands, Inc., Nashville, TN, USA) was made using purified water (reverse osmosis) to which has been added a very small quantity (less than 0.01%) of salt (potassium bicarbonate, silica) in order to stabilize the water structure. This "mineral" water is then subjected to magnetic and light radiation in a specific sequence similar to that described by Lorenzen [11,12].

Aquaphotomics analysis [24] of the SW (referred to as "magnetized water" in Figure 1) included water boiling (to determine stability and to determine states of the water at different temperatures during cooling), infrared spectroscopy (with spectral analysis), electrical conductivity, dissolved oxygen and pH. The analysis showed that the DSW had a unique molecular structure accompanied by increased electrical conductivity, pH and dissolved oxygen, with enhanced absorbance of specific water molecular conformations evident from infrared spectroscopic analysis (Figure 1). Peak points on the spectrogram (Figure 1) indicated protonated water clusters (1379.5nm), trapped water molecules (1395nm), free water molecules (1411nm), hydrated water (1417, 1426.5nm) and water dimers (1442.5nm). The summary from the report stated that DSW was very stable over time (1 month) even after boiling. The sample when first tested was already 2.5 months and was re-tested 3.5 months.

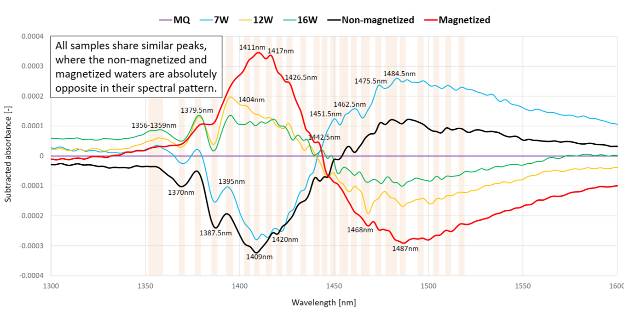

Figure 1. Spectrogram of near infrared analysis of the Defiance structured water (indicated by the "magnetized" red line, and four other water samples of which three are structured waters (7W, 12W, 16W). The reference (environmental control) was MQ (tap water purified by reverse osmosis). On the red line the wavelengths indicate peaks of interest: 1379.5nm, protonated water clusters; 1411nm, free water molecules; 1417 and 1426.5nm hydrated water; 1442.5nm, water dimers

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2.2 Animals

The study was performed in accordance with the Animal Protection Laws of the State of Florida (USA) and the United States Department of Agriculture. Ethical review was performed by the animal care and use committee of The Nutraceutical Alliance in accordance with the guidelines of the Canadian Council on Animal Care. Horses were cared for according to standard practices for a Thoroughbred race training center. The research was performed at a training yard in Palm Beach County from mid-January to mid-March 2020. Average day time temperatures were 22°C with about a 10-15°C diurnal variation. Owner informed consent was obtained prior to recruitment of horses into the study.

Horses were individually housed in 4 m X 4 m box stalls with no ceiling and continuously open door (chained entry way). Horses were fed about 3kg of timothy hay twice daily and one to 2 kg of a grain mix twice daily, once after the morning workout at about 11 am and then at about 4 pm. Feed was portioned with respect to meeting the energy and electrolyte needs and maintaining healthy, race-performance body condition. The diet of each horse in the study was not changed during the course of the study and all horses always consumed their entire grain and hay rations.

The grain was a premium race formula all grain sweet feed (T913JOC, Jockey, Lexington, KY, USA 40523) that comprised 13% crude protein (min), 6% crude fat (min), 75% carbohydrate, 12% ADF(max), 21% NDF (max) and minerals and vitamins. Horses had ad libitum access to filtered (activated charcoal) deep (about 150 feet) well water provided in 15 L buckets. Horses performed a standard race-training workout in the morning 6 days / week, with one of these at near race-pace (breezing).

Prior to recruitment into the study all horses received a veterinary examination that followed the format of the American Association of Equine Practitioners and that included endoscopic examination of the upper airway. Horses were examined in their stall and restrained using a nose twitch. A one meter long, 10 mm diameter flexible endoscope was passed up the right nostril to the nasopharynx and into the trachea to the level of the carina. The assessments were performed by a single veterinarian. Observation of locations of redness and swelling were recorded, and scoring of tracheal mucous and blood (markers of upper airway health) were as described by Salz et al. [21]. At the time of recruitment the following were in the normal range for resting horses: blood hematology and biochemistry, heart rate, respiratory rate, rectal temperature, locomotion, eyes, mucus membranes, g.i. sounds (intestinal motility). The characteristics of the horses in each group are shown in Table 1. There were no significant differences between the two groups of horses in watering practices, feeding practices, stall locations with respect to water supply, handling practices, medications, performance, age and gender.

The control group (n = 15, Table 1A) comprised 13 3-year-old horses, 1 aged 4 and 1 aged 6; two were mares and 13 were geldings. All horses received daily omeprazole and sulfacrate (to treat / prevent gastric ulcers), one received dantrium (to prevent post-workout muscle cramping), one received Equisul (333 mg sulfadiazine and 67 mg of trimethoprim / ml; used for treatment of lower respiratory tract infection); one received Regumate (Altrenogest 2.20 mg / ml; used to control estrus); one received Metronidazole (antibiotic). Six horses were routinely on medication to support airway health, and this was administered only prior to weekly breezing: furosemide (3 to 5 cc) and amicar (1 at 0, 3 at 10cc or 2 at 20 cc).

The DSW group (n = 17, Table 1B) comprised 15 3-year-old horses, 1 aged 4 and 1 aged 6; two were mares and 15 were geldings. All horses received daily omeprazole and sulfacrate, one received dantrium, and one received an amino acid supplement. Four horses were routinely on medication to support airway health, which was administered only prior to weekly breezing: furosemide (3 to 6 cc) and amicar (2 at 10 cc and 2 at 20 cc).

2.3 Research Design

The present descriptive, small-scale, clinical field trial was designed to accommodate horses in a race-training situation and that were available for up to 34 days of continuous use. The design therefore required closely matched control and treatment group horses similar in all key physiological, feeding, drinking, training and racing parameters. A cross-over design could not be accommodated because of the restricted availability of horses (34 days) and because the duration of a required "washout" period was unknown, as it remains unknown how long-lasting the effects of DSW are. The number of horses required in each group was determined using a power calculation with BIA phase angle, resistance and reactance as the key variables of interest, using data from Ward et al. [20]. The minimum number of horses required in each group was 18, and because this was a field study using race horses in active training and competition it was anticipated there would be significant attrition over the 5-week period from recruitment to study completion.

A total of 40 horses were recruited for the study and assigned to two groups so that each group was similar with

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Table 1A. Characteristics of control group horses recruited into the study at the recruitment stage. Body condition score of all horses was 3.5

Horse	Age	Sex	H (cm)	Medications other than omeprazole and sulfacrate	speed (m/s)	Upper airway score	Upper airway endoscopy results
3	4	G	169		17.35	2	slight right side asymmetry, function normal
4	3	F	168	equisul	17.75	1	
6	3	F	165	dantrium, furosemide, amicar	17.91	1	
8	3	G	170	furosemide, amicar	17.78	2	grade 3 pharyngitis
12	3	G	164		16.97	1	
14	3	G	167		17.24	1	
15	3	G	164	furosemide, amicar	17.67	1	flaccid epiglottis, grade 2 pharyngitis
16	3	G	173	Regumate, furosemide, amicar	17.56	1	
18	3	F	167		na	1	slightly flaccid epiglottis
21	3	F	167		17.76	1	
23	3	G	174	furosemide, amicar	17.66	4	left arytenoid paralysed, appears to have been tied back
25	3	G	167		17.46	1	
27	6	G	171	metronidazole	17.98	1	
28	3	G	165		17.41	1	
29	3	G	168		na	1	
30	3	G	170		na	1	
		mean	**168.1**		**17.58**	**1.31**	
		SD	**2.98**		**0.28**	**0.79**	

Table 1B. Characteristics of Defiance structured water group horses recruited into the study at the recruitment stage. Body condition score of all horses was 3.5

Horse	Age	Sex	H (cm)	Medications other than omeprazole and sulfacrate	speed (m/s)	Upper airway score	Upper airway endoscopy results
1	3	G	168	furosemide, amicar	17.53	1	
2	6	G	172	omeprazole, sulfacrate, furosemide, amicar	17.84	1	
5	5	G	168		18.32	1	
7	3	G	171		17.31	2	slight right side asymmetry, good Function
9	3	F	169	dantrium	17.77	1	
10	3	G	172		17.72	1	slightly short epiglottis
11	3	F	163		na	1	
13	3	F	165		17.74	1	slightly short epiglottis, grade 2 pharyngitis
17	3	G	170		16.60	1	
19	3	G	170	body builder	17.65	1	slightly flaccid epiglottis
20	3	G	169		16.97	1	
22	3	G	169		17.24	1	
24	3	G	166		17.46	1	
26	3	G	168		16.79	1	thin, slightly short epiglottis
31	3	G	170		na	2	grade 4 pharyngitis
32	3	G	170	furosemide, amicar	17.95	2	short epiglottis
33	3	Male			na	1	
34	3	Male	164		16.98	1	
35	4	Male	169		17.35	2	slight right side asymmetry, function normal
36	3	Female	165	dantrium, furosemide, amicar	17.91	1	
37	3	Male	170	furosemide, amicar	17.79	2	grade 3 pharyngitis
38	3	Male	165		17.42	1	
39	3	Female	168	equisul	17.75	1	
40	3	Female	167		na	1	slightly flaccid epiglottis
		mean	**168.2**		**17.51**	**1.21**	
		SD	**2.47**		**0.43**	**0.41**	

Notes: H = height; G = gelding; F = female (mare); na = not available

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respect to all parameters (as noted above) at the start of the study. The control group was assigned 16 horses and the DSW group 24 horses. One horse was lost from the control group and 7 horses were lost from the DSW group. Reasons included: horse moved off-property (6), illness (1), lameness (1). Because attrition was not balanced with respect to characteristics, the two groups varied slightly, as described below.

2.4 Study Execution

Following recruitment into the study the horses were housed in one shed row with large eaves that provided shading while permitting ample air movement. The study comprised a baseline period of 4 days, followed by 28 days of receiving placebo or DSW. On two separate days during the baseline period blood samples were taken from the jugular vein and bioelectrical impedance readings were taken.

During the 28 days of the trial, the horses in the DSW group received 10 L of DSW in their normal water bucket as the first water they consumed after the morning workout. This was followed by consumption of normal filtered groundwater *ad libitum* throughout the remainder of the day. Horses in the control group drank only the filtered deep well water, *ad libitum*. It was not practical nor possible to measure individual water intakes, nor was this necessary with the experimental design. Water intake is only one part of the fluid balance equation, which also includes significant losses of water through the respiratory tract, skin and kidneys in athletic horses.

During the baseline period, and throughout the following 28 days, when a horse was to perform a breezing workout (weekly near race-pace gallop), it was first fitted with a heart rate monitor (Polar H10 with the equine strap; (Polar Electro Oy, Kempele, Finland). The H10 monitor was connected via Bluetooth wireless to the Polar Equine application (Polar Electro Oy, Kempele, Finland) using android smartphones. This enabled the collection of heart rate data to the smartphone while the horse was resting, warming up, galloping and cooling down. Breezing was performed by all horses typically weekly, but not all horses performed a breezing workout each week. All horses performed a daily submaximal workout, with one rest day per week.

Some horses from each group (n = 7 DSW; n = 6 control) went off-site for 1 to 4 days to compete in races; individual horses did not compete in more than one race during the 28 days. When horses went off-site they drank the local water, and DSW group horses did not receive the DSW product during this time.

2.5 Blood Analyses

Blood hematology and biochemistry was performed in order to determine if 28-day consumption of DSW affected any clinical parameters associated with these blood variables. Blood samples were collected into 3 cc vacutainer tubes containing EDTA for CBC / hematology (CELL-DYN 3700, Abbot Diagnostics, Lake Forest, Ill, 60045 USA) or without additive for analysis of serum biochemistry (Envoy 500, ELITech Group Inc., Smithfield, RI 02917 USA) at On Track Lab Inc. (Lake Worth, FL 33449 USA) within 2 hours of sampling.

2.6 Bioelectrical Impedance Analysis (BIA)

Bioelectrical impedance measures were obtained on two separate days mid-way through the study (days 13 to 15, between 3:00 and 4:30 pm) when the horses were resting, post-prandial in their stalls prior to the afternoon feeding. All BIA measures were performed by the same individuals to ensure consistency of electrode placement and very calm handling of the horse. The people performing the BIA measures were blinded to the treatment. Horses were not restrained prior to and during measurements. At the end of the study blood samples were obtained about 30 minutes prior to BIA, followed by BIA measurement on each of days 28 and 29. Heart rate data continued to be collected until day 31.

Horse height was measured to the top of the withers using an equine height measuring stick as described previously [25,26]. Body condition score was the same in all horses, being a 4 on the 9-point scale [27] - these were lean, well-muscled, very fit horses. Bioelectrical impedance analysis was performed using an Equistat 5000 multifrequency bioelectrical impedance analyzer (Bodystat Ltd, Douglas, Isle of Man, IM4 4QJ, British Isles) as described previously [25,26]. The hair coat on all horses was very short due to the climate, and very clean. Two 10 cm^2 carbon fibre electrode pairs (10 cm between centers) were dabbed with an adequate amount of conductive gel to penetrate to the skin, then were placed above the left fore- and hindlimb above the knee and hock, respectively, overlying the prominent muscle. On the forelimb, the electrode pair was positioned proximal to the carpal joint on the lateral portion of the radius directly over the common digital extensor, ulnaris lateralis and radial carpal extensor muscles. On the hindlimb, the electrode pair was placed on the tibia directly over the long digital extensor and lateral digital extensor muscles. The electrodes were held in place using a cuff secured by Velcro straps. Shielded leads connected the electrodes to the BIA instrument. BIA was performed using a tetra-polar arrangement in which an 800 μA alternating current was

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applied through the body using the 2 distal electrodes, and voltage drop measured by the 2 proximal electrodes. The instrument recorded impedance (Z), resistance (R), reactance (Xc) and calculated phase angle (PA) at 7 frequencies: 5, 16, 24, 50, 140, 200 and 280 kHz [26].

Measurements were repeated two to six times (depending on whether the horse moved during measurement), without removing the electrodes, until similar impedance values were obtained at least twice in sequence. Occasionally, when a horse moved a limb, one or more electrodes had to be adjusted in order to re-establish good contact between skin and electrodes and obtain a repeatable measurement series. The entire procedure for two scans required less than 2 minutes. The instrument's algorithms were developed using primarily Standardbred and Thoroughbred horses, and were used to calculate body mass, TBW, ECFV and ICFV [26]. The average values of two readings acquired when horses were standing quiet and still were used for subsequent data analysis.

The raw impedance parameters R and Xc were used to corroborate the algorithm-computed hydration parameters; this is relevant because R and Xc were not used in these algorithms [26]. The bioelectrical impedance vector analysis (BIVA) approach developed by Piccoli et al. [27] has been used extensively in assessment of acute changes of hydration [27,28,29].

2.7 Post-breezing Nasopharyngeal Endoscopy

On days when horses were breezed, they received a nasopharyngeal endoscopy within 60 minutes of finishing breezing. One veterinarian performed the procedure on all the horses during the study duration. The veterinarian was blinded with respect to the treatment and assessed the upper airway. Observations were graded as described by Salz et al. [21]. The amount of mucus in the trachea was graded using a score of 0-4 (0, no mucus; 1, small, singular threads or droplets of mucus; 2, larger, confluent droplets of mucus. The amount of blood in the trachea was similarly graded using a score of 0 to 4 (0, no blood detected in the pharynx, larynx, trachea or main-stem bronchi; 1, presence of one or more flecks of blood or two or less short, narrow streaks of blood in the trachea or main-stem bronchi). No horses had mucus greater than grade 2 or blood greater than grade 1. The upper airway was also observed at the same time for presence of chondritis, dorsal displacement of soft palate, and inflammation (swelling and redness) of the left arytenoid.

2.8 Heart Rate Variability (HRV)

Data for each horse for each day that they were breezed were organized by treatment (Control, DSW), study duration (0, 1, 2, 3, 4 weeks) and activity (rest, canter, full gallop, recovery). Raw txt files downloaded from the Polar Equine app were imported into an HRV analysis program (Kubios HRV Standard 3.3.1, Kupio, Finland) which filtered the data to remove artifacts. Approaches similar to that described previously [29-31] were used to select the time periods for HRV. Data for analysis of HRV were selected from the entire heart rate trace during a 3-minute period at rest, for a 3-minute period when the horse was in the cantering phase of the warm-up, for 30 s when at full gallop, and for a 5 minute period in recovery.

2.9 Statistics

The person that performed the data entry and statistics were blinded to the treatment group. The statistics program (SigmaPlot 14, Systat Software Inc.) tested the data for normality and kurtosis (Shapiro-Wilk test). Treatment and time effects for the endoscopy data, BIA data, and blood data were assessed using two-way analysis of variance. Within-treatment effects were examined using one-way, repeated measured analysis of variance. For the HRV data, complete data sets were obtained from only three horses in each group in all of the three categories of treatment, week and activity. Missing data were the result of inadequate signal detection, illness, off site to race, and attrition from the study. Thus 2-way RM-ANOVA with respect to treatment and study duration was not possible. The entire data set was used for the analysis in order to provide adequate data for each week and for each activity level. Comparisons were assessed using a 2- way ANOVA (treatment, week). Activity was a confound because of the large variation from rest to full gallop. For effects within each activity level a 2-way ANOVA was performed with respect to treatment and week. When a significant F-ratio resulted, then a 1-way RM-ANOVA (horse as subject) was performed within treatment and activity. The Bonferoni post-hoc test was used for because of its ability to handle missing data. Data are presented as mean $\pm$ SD. Significance is at $p < 0.05$ at a power of 0.8 or greater.

3. Results

3.1 Upper Airway

The results of the upper airway endoscopic examination showed that in the control group there was no change over time. There was a significant difference between the control and DSW groups. In the DSW group there was an increase in the number and percentage of horses that had an improved upper airway result (Table 2, Figure 2).

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Table 2. Results of nasopharyngeal endoscopy performed 30 - 45 minutes after breezing. Results are only from horses that completed the entire study

DSW Horses (n = 17)					
	Week 0	Week 1	Week 2	Week 3	Week 4
Mucus - 1	4	5	2	2	3
Mucus - 2	2	1	3	0	1
blood - trace	4	1	2	0	1
blood 1	1	0	0	0	0
SLC	2	0	2	1	1
chondritis	0	1	1	0	0
ddsp	0	0	1	0	0
OK	7	5	7	11	10
n =	16	14	16	14	14
% with clear airway	43.8	35.7	43.8	78.6	71.4
Control Horses (n = 15)					
	Week 0	Week 1	Week 2	Week 3	Week 4
Mucus - 1	3	3	3	4	3
Mucus - 2	1	0	1	0	0
blood - trace	1	1	3	2	4
SLC	1	3	0	2	0
chondritis	0	0	0	0	0
ddsp	0	0	0	0	0
OK	7	7	7	7	2
n =	12	12	12	13	8
% with clear airway	58.3	58.3	58.3	53.8	25.0

Notes: ddsp = dorsal displacement of soft palate

SLC = slight inflammation of the left arytenoid (slight left cord)

DSW - Defiance structured water

OK indicates the absence of adverse observations (clear airway).

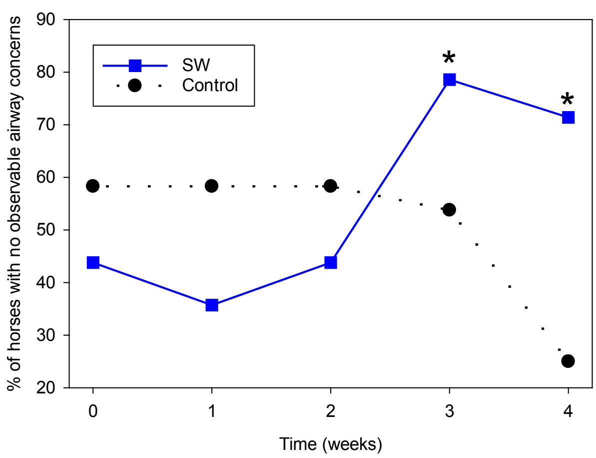

Figure 2. Percentage of horses that had no observable indications of impaired airway health (see Table 2) upon examination after breezing in the control (n = 15) and DSW (n = 17) groups. * indicates significantly greater than in the control group and significantly greater that at week 2 in the structured water (SW) group

3.2 Performance Indices

Training speeds did not change over time and there was no significant difference between groups (Control 12.4 ± 0.3 seconds / furlong; DSW 12.4 ± 0.3 s/ f). Breezing distances ranged from 3 to 6 furlongs. For horses from each group that competed in a race, speeds were also not significantly different (Control 11.9 ± 0.2 s / f; DSW 11.9 ± 0.5 s / f) over distances ranging from 6 to 8.5 furlongs. Note that horses only raced once in a 4-week period and races occurred during each of these 4 weeks so it is not possible to determine a treatment effect.

3.3 BIA - Hydration Parameters

In the control group TBW increased from 333 ± 6 L to 348 ± 6 L at 2 weeks, then decreased 342 ± 19 L at 4 weeks (Figure 3). In the DSW group TBW continued to increase from baseline (336 ± 19 L) to 2 weeks (353 ± 19 L) to 4 weeks (360 ± 6 L); the increase in TBW in the DSW group was significantly greater than in controls. At 2 weeks, in both groups, the increase in TBW was primarily due to increased ICFV, as ECFV did not increase significantly (Figure 3). In the control group the decrease in TBW from week 2 to week 4 was mirrored by the decrease in ICFV, but ECFV was increased at 4 weeks. In the DSW group there was no change in ECFV, while ICFV continued to increase from 2 to 4 weeks. Baseline values for ECFV were 124 ± 5 and 124 ± 6 in the control and DSW groups, respectively. Baseline values for ICFV were 211 ± 16 and 216 ± 14 in the control and DSW groups, respectively.

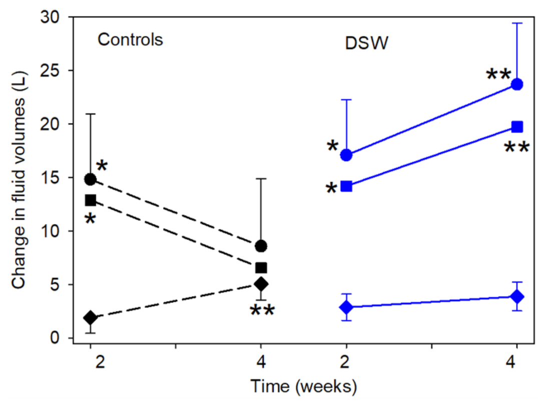

Figure 3. Changes in BIA-predicted whole body hydration parameters from baseline (0) to 2 and 4 weeks in total body water (TBW, circles) intracellular fluid volume (ICFV, squares; error bars omitted for clarity) and extracellular fluid volume (ECFV, diamonds) in the control group (black, n = 15) and the DSW group (blue, n = 17). Values are mean + SD. DSW = Defiance structured water; * indicates significant increase from baseline. ** indicates significant increase from baseline and compared to the opposing group

Plasma volume (PV) was similar at baseline in the control (18.7 ± 1.3 L) and DSW (18.9 ± 1.1 L) groups. PV increased significantly more in DSW at 2 weeks (20.2 ± 1.1 L) and 4 weeks (20.6 ± 1.2 L) than in controls at 2 weeks (19.4 ± 1.3 L) and 4 weeks (19.0 ± 1.4 L). In the DSW group the increase at 4 weeks represented a 9.2% increase in PV, contributing to increased circulating blood volume.

In controls, the increase in fluid volumes were accompanied by increases in calculated body mass as follows from 466 ± 14 kg (baseline) to 490 ± 14 ($p < 0.05$) and 487 ± 15 kg at 2 and 4 weeks, respectively. In the DSW group, calculated body mass increased from 479 ± 12 kg (baseline) to 505 ± 12 ($p < 0.01$) and 513 ± 13 kg at 2 and 4 weeks, respectively. At 4 weeks the increase in calculated body mass in the DSW group was greater than in controls ($p < 0.01$). At each time point the increase in TBW was 65 to 70% of the increase in calculated body mass.

3.4 BIA - Bioelectrical Parameters

The mean impedance-frequency relationships (IFR) for both groups at baseline, 2 and 4 weeks are shown in Figure 4. At baseline there was no significant difference between groups in the IFR. At 2 weeks in the control group there was a modest and significant decrease in impedance at each frequency, but no significant difference between baseline and 4 weeks and no significant difference between 2 and 4 weeks. In the DSW group there was a significant decrease in impedance at each frequency from baseline to 2 weeks, and a further significant decrease to 4 weeks, with no significant difference between 2 and 4 weeks.

Resistance (R) and reactance (Xc) were measured and phase angle (PA) calculated by the instrument at only the 50 kHz frequency. At this frequency relatively little current passes through cells. Resistance is the decrease in voltage reflecting conductivity through ionic solutions. Reactance is the delay in the flow of current measured as a phase-shift, reflecting dielectric properties mainly attributed to capacitance of cell membranes and tissue interfaces [20,29].

The significant difference between groups and the time course of change in the IFR led to an analysis of the time course of change in R, Xc and PA. Hydration parameters can change in the absence of change in Xc and PA, and this remained to be determined. Figure 5 shows the time course of change in R and Xc in control and DSW horses. In controls, there was a small significant decrease in R at 2 weeks but not at 4 weeks, and there was no change in Xc or PA (not shown). In the DSW group, there were significant decreases in both R and Xc at 2 and 4 weeks, with no change in PA. The PA of horses was not significantly different between groups nor at any time point; pooled data are described as 14.7 ± 0.6 (range 13.1 to 16.5; median 14.7).

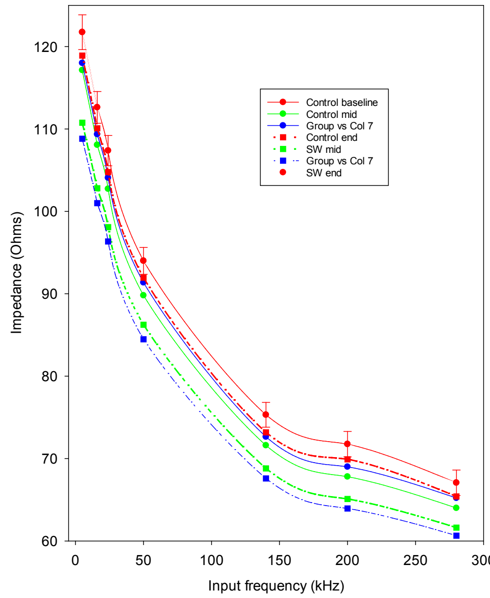

Figure 4. Impedance-frequency relations in DSW and control groups horses at baseline, mid- study (2 weeks) and end-study (4 weeks). There was a significant decrease in impedance at each frequency in DSW horses at mid-study (2 weeks) that was maintained at end-study (4 weeks)

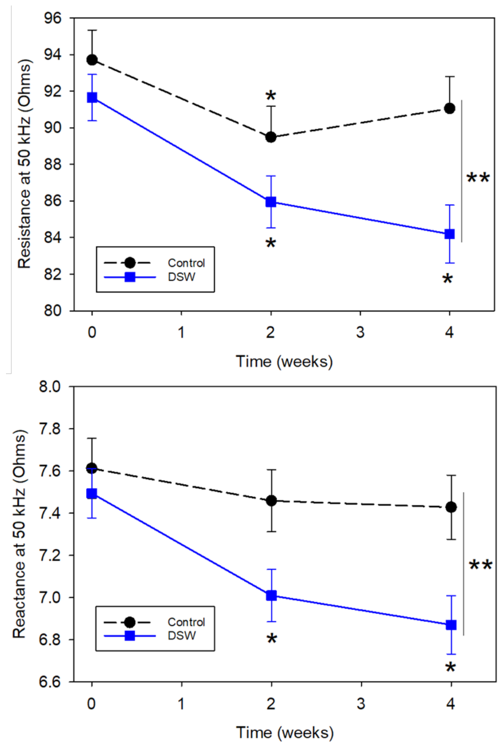

Figure 5. Time course of whole body resistance (top panel) and reactance (bottom panel) measured at a frequency of 50 kHz. DSW = Defiance structured water; * indicates significantly different than baseline; ** indicates significant difference between groups

Analysis of the combined data sets for control and

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DSW horses yielded significant linear relationships between predicted fluid volume parameters (only shown for TBW, Figure 6) with respect to R and Xc (Figure 6 top and middle panels), and a significant linear relationship between Xc and R (Figure 6, bottom panel). When examined this way, there was no significant difference between groups, and the data are used to illustrate the relationships between bioelectrical and physiological parameters.

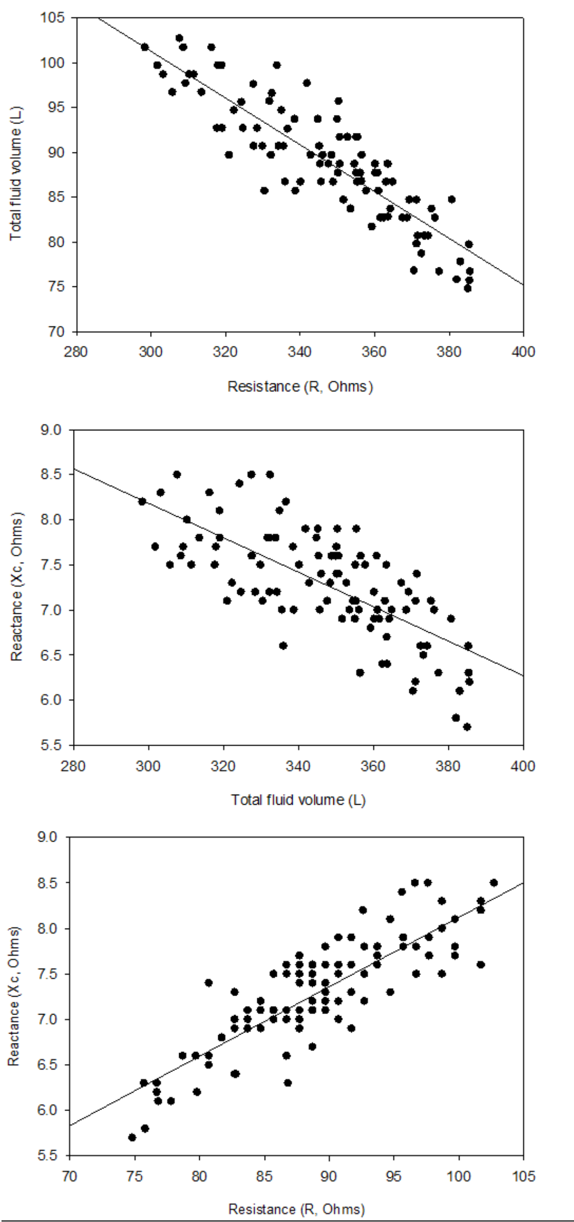

Figure 6. Top panel: the relationship between resistance (R) and total body water (TBW): R = 179.5 - TBW x 0.261; $r^2 = 0.787$; $p < 0.001$. Middle panel: the relationship between reactance (Xc) and TBW: Xc = 13.92 - TBW x 0.0191; $r^2 = 0.524$; $P < 0.001$. Bottom panel: the relationship between reactance (Xc) and resistance (R) at the frequency of 50 kHz. Data from all horses at all time points. Xc = 0.490 + R x 0.0763; $r^2 = 0.721$; $P < 0.001$

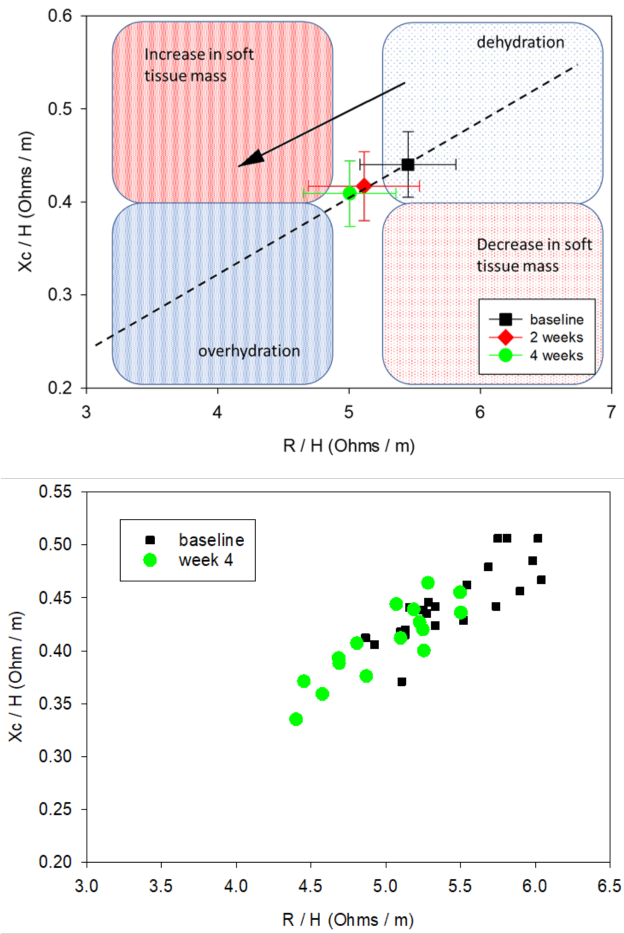

Figure 7. Top panel: RXc mean (± SD) graph for horses in the DSW group (n = 17). The dashed line indicates directional change of hydration without loss or gain of soft tissue mass. The solid arrow indicates the vector direction. Bottom panel: RXc graph of individual DSW horses at baseline (black squares) and at 4 weeks (green circles). R / H, height-adjusted resistance; Xc / H, height- adjusted reactance

In the DSW group, there was a simultaneous decrease in R and Xc without change in PA. In order to better understand this the BIVA mean graph (Figure 7, top panel) shows the vector of change in R and Xc directed downwards and to the left, thus indicating primarily an increase in hydration with perhaps some increase in soft tissue mass. Cluster presentation of individual data clearly demonstrates the downwards shift to the left (Figure 7, bottom panel).

3.5 Heart rate variability (HRV)

The only significant differences between treatment groups in HRV occurred in horses standing quietly at rest in the stall. Resting heart rate did not change over time in the control group. At baseline, week 1 and week 2 there was no significant difference between control and DSW groups (Figure 8). At weeks 3 and 4 resting heart rate in the DSW was significantly lower than at baseline, and significantly lower than in the control group.

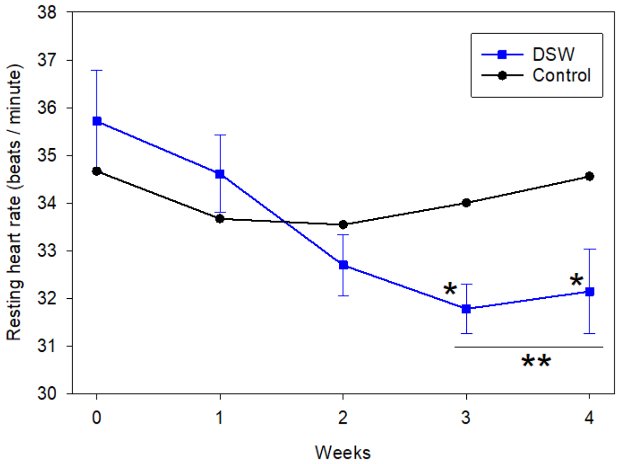

Figure 8. Resting heart rate in horses receiving 10 L / day of DSW (n = 7 to 18) and controls (n = 6 to 11). DSW = Defiance structured water; * significantly different from baseline within treatment; ** significant different between treatments

There was no significant difference between groups in the beat to beat (RR) interval due to the rather large variability in the control group (Figure 9). However, in the DSW group there was an increase in RR interval over time, so that at 3 and 4 weeks it was significantly elevated compared to baseline. The progressive lowering of resting heart rate and lengthening of RR in DSW horses was associated with an increase in the PNS Index (Figure 10) and a decrease in the SNS Index (Figure 11).

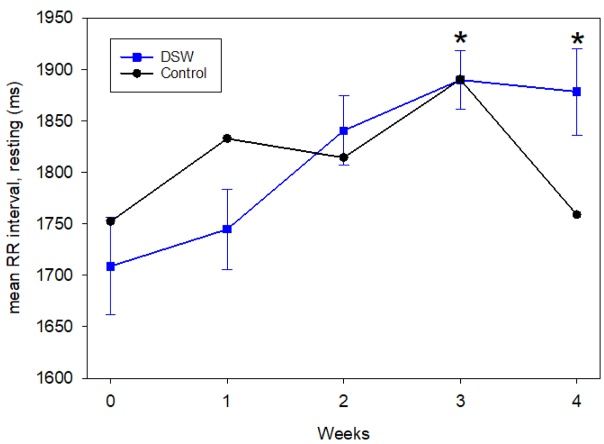

Figure 9. The beat to beat (RR) interval in horses receiving 10 L / day of Defiance structured water (DSW; n = 7 to 18) and controls (n = 6 to 11). * significantly different from baseline within treatment

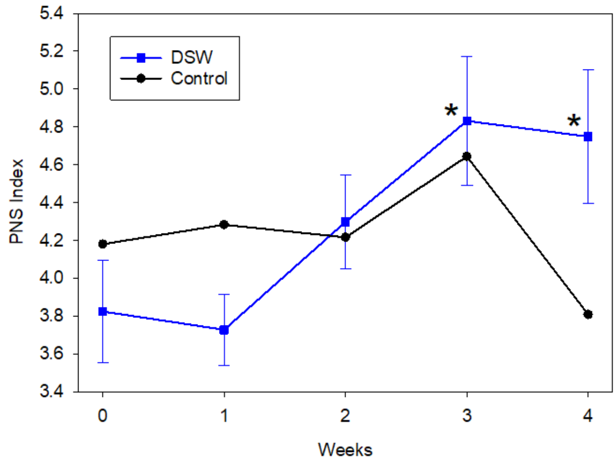

Figure 10. The index of parasympathetic nervous system activity (PNS Index) to cardiac control in horses standing at rest. DSW horses received 10 L / day of Defiance structured water (DSW; n = 7 to 18) and controls received normal water (n = 6 to 11). * significantly different from baseline within treatment

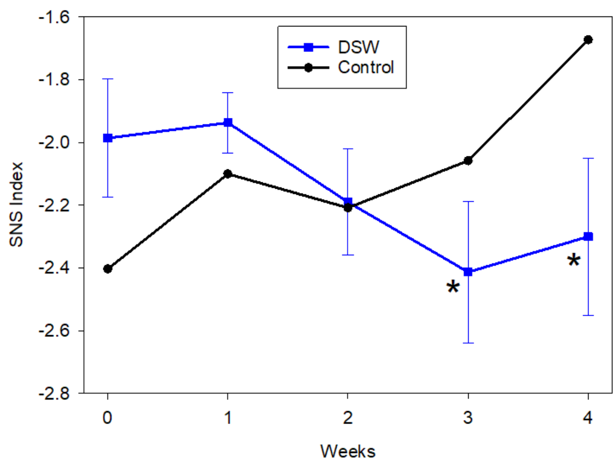

Figure 11. The index of sympathetic nervous system activity (SNS Index) to cardiac control in horses standing at rest. DSW horses received 10 L / day of Defiance structured water (DSW; n = 7 to 18) and controls received normal water (n = 6 to 11). * significantly different from week 1 within treatment

3.6 Blood biochemistry and hematology

There was no significant difference between baseline and end-study for both treatment groups, and no significant differences between treatment groups (Table 3).

An analysis was performed using all horses that had elevated concentrations of muscle enzymes, although the concentrations were not markedly elevated and in the expected range for regularly exercised Thoroughbred horses in training. This analysis was performed to determine if

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daily consumption of DSW would reduce the appearance of muscle enzymes. All horses that had an AST of 400 IU/L and higher were selected for analysis, and correlations analyzed with respect to days since breezing and concentrations of each of the 4 enzymes. There was no relationship between days since breezing and enzyme concentration. There were no significant differences between baseline and end-study for muscle enzymes in the two treatment groups.

4. Discussion

The present study showed that horses that drank DSW displayed improved hydration by two weeks, with significant increases in TBW, ECFV, ICFV and plasma volume. In 50% of the DSW group horses having signs of upper airway health concerns at the start of the study, there was an improvement (reduction of number and severity of marker) of airway health. There were no effects on of performance, blood biochemistry and hematology, not any adverse events. By 3 weeks, the DSW group horses showed an altered autonomic response characterized by lower heart rate, increased RR interval, increased PNS index and reduced SNS Index. The present study indicates that it is not necessary to drink all water as “beneficial”

Table 3. Blood biochemistry and hematology in horses in the control group (n = 15) and Defiance structured water (DSW) group (n = 17). Standard deviation = SD

		Control Baseline		Control End		DSW Baseline		DSW End	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Glucose	mg / dl	116.25	8.08	116.45	7.98	112.73	8.60	113.85	8.29
BUN	mg / dl	15.36	1.70	14.76	1.68	15.73	2.10	14.74	2.30
CRE	mg / dl	1.56	0.18	1.51	0.18	1.55	0.16	1.48	0.19
BUN/CRE	ratio	9.92	1.51	9.85	1.15	10.23	1.42	10.02	1.26
Na	mmol/L	137.9	3.4	137.8	2.9	137.6	3.3	137.1	2.9
K	mmol/L	3.87	0.24	3.75	0.28	3.72	0.30	3.79	0.29
Cl	mmol/L	99.14	2.85	98.76	3.03	98.82	2.77	99.65	2.39
TCO2	mmol/L	29.07	1.22	29.17	1.26	29.07	1.07	29.15	0.99
CA	mg / dl	12.11	0.58	11.87	0.54	12.13	0.58	12.07	0.62
P	mg / dl	4.02	0.32	3.92	0.33	4.02	0.30	3.99	0.31
TP	g / dl	6.18	0.31	6.01	0.29	6.13	0.35	5.90	0.76
ALB	g / dl	4.05	0.16	3.93	0.17	3.95	0.16	3.90	0.41
GLB	g / dl	2.13	0.21	2.08	0.23	2.18	0.27	2.23	0.40
A/G	ratio	1.92	0.20	1.91	0.24	1.83	0.23	1.80	0.25
BILIRUB	mg / dl	2.50	0.73	2.60	0.68	2.53	0.70	2.65	0.77
ALK/PHOS	IU / L	158.43	15.43	155.38	23.99	153.09	28.10	151.91	21.70
CK	IU / L	340.57	433.47	359.03	431.05	256.77	90.18	335.62	353.82
AST	IU / L	486.14	387.94	458.93	320.59	424.00	280.31	504.88	423.13
GGT	IU / L	35.86	13.05	36.66	13.15	37.41	9.25	41.18	15.47
WBC	x10^3 / ul	8.53	1.46	8.48	1.37	8.63	1.58	8.57	1.54
RBC	x10^6 / ul	9.51	0.66	9.55	0.68	9.57	0.58	9.55	0.62
HGB	g / dl	14.36	1.02	14.40	1.00	14.51	0.83	14.48	0.91
HCT	%	41.3	3.0	41.4	3.1	41.7	2.5	41.6	2.8
MCV	fL	43.2	0.92	43.3	0.79	43.19	0.88	43.45	0.64
RDW	%	25.7	1.1	25.7	1.1	25.9	0.9	25.8	1.2
PLT	x10^3 / ul	153.6	26.5	163.0	36.4	175.8	35.4	172.2	38.9
SEG.NEU	%	55.3	8.9	56.3	6.2	55.3	8.6	55.8	10.7
LYM	%	43.2	9.0	42.4	6.2	42.9	8.4	42.4	10.4
EOS	%	0.9	0.7	0.8	0.7	1.0	1.1	1.0	1.2
MONO	%	0.5	0.6	0.4	0.5	0.8	0.7	0.7	0.6

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SW, so long as an adequate amount is ingested, however high-quality dose-response studies have yet to be performed.

Limitations of the present study are related to the fact that this was a field trial that permitted minimal researcher invasiveness to routine racehorse practices at this training facility. The purpose of the control group was therefore to control for those independent variables that could not be controlled, notably the inability to measure 24/7 water intake and losses (respiratory, skin, renal) and body mass using a weigh beam. Despite the confounds of some horses going off-site to compete, the duration off-site, the inability to consume DSW when off-site and the loss of horses from study groups, the results demonstrated that DSW has physiological effects which may be deemed as beneficial in racehorses. These limitations may also be taken as a positive feature in that the study was performed in actual field conditions and the results are thus readily applicable to horses in active training programs.

Chronic stress, such as that experienced by racehorses in training and competition, may result in chronic inflammation in many tissues and impair cell health and performance. An unexpected finding of the present study was the reduction of signs commonly associated with mild-to moderate upper airway inflammation in racehorses [21-23]. None of the horses in the study had more than mild upper airway symptoms, however 50% of the horses that consumed DSW that initially had upper airway symptoms showed an absence of symptoms at 3- and 4 weeks. This may be of practical significance because airway health has been reported to progressively worsen during the racing career of horses [22], and the presence of airway symptoms is causally related to impaired racing performance [21].

It is generally recognized by the scientific community, although not generally recognized by horsemen involved in training, that competitive horses in training undergo periods of what may be considered as mild dehydration [25]. There are many reasons for this, the main ones include withholding of water prior to morning workouts, no provision of water during transport to competition, and withholding of water at competition. Again, in science, this is counterintuitive because it is known that health and performance are correlated with hydration [33], although this has not been systematically studied in horses. The standard operating premise is that (race) horses are able to tolerate some level of dehydration (up to 5%) without adverse effects, and that the associated loss of body water (mass) enables the horse to run faster or sustain speed for a longer time. However this aspect of equine physiology requires systematic study.

Using BIA, the present study demonstrated in DSW horses significant increases in hydration variables by two weeks that were sustained through weeks 3 and 4. Based on the timing of BIA measurements (at 2- week intervals) it remains unknown how fast the hydration response occurs, and it is possible that a higher level of hydration may have occurred. Rehydration of exercise-dehydrated Standardbred horses showed that hydration at 18 hours increased above that seen at baseline [25], indicating the potential to further hydrate horses that were not seemingly dehydrated at the start of the experiment. This is similar to the present study, where at baseline there were no signs of dehydration, including no elevation of plasma protein concentration, and no clinical signs of dehydration in control group horses at the end of the study. The increased apparent hydration in DSW horses at 2 and 4 weeks ultimately has to be due to increased water retention in the body, as increased intake could easily be matched by increased urine output. Necessary assumptions for the correct estimation of body hydration (composition) include homogenous composition, fixed cross- sectional area and consistent distribution of current density [34]. In these healthy horses of both groups it is likely that these assumptions were achieved, and that the significant differences in hydration parameters from weeks 2 to 4 do represent an enhanced hydration with consumption of DSW.

The raw impedance parameters R and Xc were used in the BIVA approach to assess acute changes of hydration. In the present study, the integrity of R and Xc as independent indicators of hydration are supported by the fact that there were no significant differences between groups in the relationship between either the R vs TBW, the Xc vs TBW, nor for any of the other hydration variables ECFV, ICFV and PV (data not shown). The BIVA plot for horses in the DSW group (Figure 7) clearly shows the significant increase in hydration that occurred, and there was no effect in control horses.

It should be expected that the PA did not change in response to consumption of DSW. In healthy humans, the main determinants of PA are age, sex and body mass index [34], and these parameters were very similar in the two groups of the present study. PA is calculated as its arc tangent, (Xc/R) $180^{o}/\pi$, and represents both the amount and quality of soft tissue. As such, it is had been validated and used as an indicator of cellular health. Higher values reflect higher cellularity, cell membrane integrity and better cell function. In healthy humans the PA ranges between 5 and 7 and values as high as 9.5 have been reported in human athletes [35]. Mean ($\pm$ SD) of the PA of athletic horses in the present study was 14.7 $\pm$ 0.6 (range 13.1 to 16.5; median 14.7), substantially greater than in humans but less than obtained in individual muscles of the horse (range

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from 10 to 25 [19]).

Analysis of heart rate variability (HRV) provides information on the magnitudes of parasympathetic and sympathetic neural drive affecting autonomic control of physiological systems, including heart rate [32]. The only significant differences between treatment groups occurred in DSW group horses standing quietly at rest in the stall. It is likely that the intensity and variability of exercise would have overwhelmed differences present at rest. The combination of decreased heart rate, increased RR interval, increased PNS Index and decreased SNS Index indicate effects on the autonomic control of the cardiovascular system [36,37]. The autonomic nervous system (ANS) consists of two branches, the parasympathetic and sympathetic, that together controls the function of internal organs (e.g., heart rate, respiration, digestion) and responds to disturbances. The influence of the parasympathetic nervous system is represented by the PNS Index, while the sympathetic nervous system is represented by the SNS Index in HRV analysis. The parasympathetic nervous system (PNS) is the "rest and restorative" system that maintains a low resting heart rate and restorative state when sleeping or relaxing. The sympathetic nervous system (SNS) is the "fight or flight" system that responds to large disturbances by acceleration of heart rate. The present results suggest that horses regularly drinking DSW developed a slower and more restful or restorative cardiovascular response than control horses, and this effect was manifest at 3 and 4 weeks of consuming DSW.

Perspective

The mechanisms by which daily drinking of SW affects hydration and health are not understood, and the present study was not designed to address mechanism. What is known, is that how water interacts with solutes, colloids and "fixed" proteins determines the attributes and functions of all of the fluids, cells and tissues in the body. The how is influenced by the "water kind", a term used by Lenormand et al. [38] to acknowledge different structural states. Water is not simply bulk fluid within the body - rather water molecules form coherent clusters and layered sheets [4,6,7] around molecules and in association with both polar and non-polar moieties [3,39]. At present, it remains unknown how ingested SW distributes within the body, or if there is a preferential retention of SW. The polar, protic, and amphoteric attributes of water contribute to complex structural and dynamic characteristics that allow water to interact in so many ways with biomolecules [4,5]. Several models for structured or clustered water have been proposed for biological systems - we know that they are present and important [2,7], although we have yet to elucidate much about molecular-level structures and interactions [39]. We do know that these coherent associations of structured water with other molecules directly affect things like protein conformation and therefore protein function [40,41], and ultimately multimolecular networks such as membranes, signal transduction pathways and biochemical pathways [41,42]. This includes structural proteins that form cytoskeletal networks [38] as well as enzymes that are involved in the regulation of biochemical reactions. For example, the dynamics of the living cytoskeleton is "directly" linked to the "dynamics of water" such that cytoskeletal "networks are slaved in a direct fashion to fluctuations arising in intracellular water" with acknowledgment that changing "water kind" impacts living cells [38]. Angel et al. [43] proposed "that ordered waters contribute to the functional plasticity needed to transmit activation signals" from the cytoplasmic face of rhodopsin. This can be extended to observations describing interactions of water with the cytoskeleton during the process of cell volume regulation [44] although they did not explore the influence of "water kind". The unique results of the present study provide evidence, albeit indirect at this point, that ingestion of SW alters how water behaves in the body and suggests that ingested SW retains some physicochemical properties that affect interactions of this water with molecules including endogenous water kinds. Future studies are aimed at elucidating main effects using cellular physiology techniques.

The horses in the control group were clinically euhydrated, yet ingestion of DSW increased hydration in the DSW group horses without adverse effects and arguably with beneficial effects. How does hydration improve when it is deemed to be already adequate? It is known that SW, whether as small clusters or larger sheets has a greater density than bulk phase water, and these denser water structures have a greater ability to interact with molecules, and hence membranes, in biological systems. We propose that ingestion of DSW results in a re-structuring of water within the body, and the manifestation of this is an increased hydration with attendant effects on cells and tissues that presented as healthier airways and increased HRV in the present study.

5. Conclusions

When Thoroughbred racehorses drank 10 L / day of DSW, compared to control horses, they became more hydrated, had improved airway health, and a greater HRV when at rest. There were no effects on performance measures, no effects on blood hematology and biochemistry, nor any adverse events. The daily consumption of DSW conferred physiological and health benefits in competitive Thoroughbred racehorses.

Author Contributions

The research was designed by MIL and reviewed by FN. Veterinary examinations for recruitment were performed by FN. MIL analysed the data, FN assisted with the interpretation. MIL wrote the manuscript, FN assisted with and approved revisions.

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Conflicts of Interest

The Nutraceutical Alliance Inc., to which MIL is an employee, was contracted by Defiance Brands Inc. (Nashville, TN, USA) to design and execute the study. MIL does not benefit outside of this contractual work. FN declares no conflicts of interest. MIL and FN declare no other competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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