Journal of Veterinary Behavior 37 (2020) 86–92

Contents lists available at ScienceDirect

Journal of Veterinary Behavior

journal homepage: www.journalvetbehavior.com

Canine Research

Effects of dietary protein and fat concentrations on hormonal and oxidative blood stress biomarkers in guide dogs during training

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ARTICLE INFO

Article history:
Received 7 June 2019
Received in revised form
17 October 2019
Accepted 5 December 2019
Available online 13 March 2020

Keywords:
guide dog
diet
training
stress
hormonal biomarker
oxidative index

ABSTRACT

We compared the effects of different feeding strategies on hormonal and oxidative stress biomarkers in guide dogs during specialized training programs. Eight neutered adult dogs belonging to the Labrador retriever breed were divided during the training work into two homogeneous groups for sex (2 males, 2 females), age (17 months ± 1), initial body weight (26.3 kg ± 1), and BCS (4.5 of 9 ± 0.11) and fed two commercial diets with different concentration of energetic nutrients. One diet was a performance diet (HPF) characterized by low-carbohydrate/high-protein and fat content (29:39:19% as-fed) and the other a normal maintenance diet (LPF), characterized by high-carbohydrate/low-protein and fat content (50:24:12% as-fed). The trial lasted 84 days. At days 0, 28, 56, and 84, 180 min before the training work (T0) and immediately after (T1) and after 120 min (T2), blood adrenocorticotrophic hormone (ACTH), cortisol, d-ROMS (reactive oxygen metabolites–derived compounds), and BAP (biological antioxidant potential) were evaluated. Lactate was measured at T0 and T1. The statistical model included the effects of diet (HPF vs. LPF), time (from day 0 to day 84, end of the trial), and exercise (T0, T1, and T2) and their interaction. ACTH ($P = 0.002$) and cortisol ($P = 0.013$) showed higher values in the HPF than in the LPF group; there were no significant differences observed for lactate. Time showed no significant difference for any hormones or blood lactate. Exercise significantly ($P < 0.001$) influenced ACTH and cortisol concentrations, showing higher values at T1 than T0 and T2, and with lactate higher ($P < 0.0001$) at T1 than T0. Diet did not influence biomarkers of oxidative stress. Time did significantly ($P < 0.05$) influence BAP results but not d-ROMs. Exercise had no effect on BAP results, but d-ROMs were higher at T0 than T2 ($P = 0.001$). There was no interaction effect. The pituitary-adrenal (HPA) axis response and the oxidative stress indices could represent an objective method to identify optimal dietary protocols for creating a successful guide dog during the early training period.

Introduction

Guide Dogs for the Blind (GDB) play an important role in society by providing independent mobility to people with visual impairment. Their service comes at high cost (approximately 25,000 euros) owing to the resources devoted to the housing, husbandry, and training required to train such animals (Guide Dogs NSW/ACT, 2010). Success rates ranged between 50% and 56% for dogs in training (Ennik et al., 2006), contributing to large production costs.

A nutritional plan suitable for the animals trained as guides for blind people represents a trivial fraction of the monetary investment because it must take into account not only the animal training work, which increases nutrient and energy requirements, but also the metabolic disorders consequent to stress derived from the changes of life style (work and kennels condition) which may cause impairments in health and well-being.

Research surrounding optimal feeding patterns for canine athletes has been investigated since the early 1930s with significant advances in feeding endurance canine athletes (Hill, 1998; Toll et al., 2010; Wakshlag et al., 2014). Ober et al. (2016) observed that

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https://doi.org/10.1016/j.jveb.2019.12.003
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working Labradors consuming a nutritionally complete and balanced performance diet with high protein and fat levels had a statistically and physiologically significant reduction in serum cortisol concentration, representing a basis for reduced metabolic stress. Others report that exercise and training program could represent a stress factor also, capable of causing endocrine and metabolic changes in guide dogs (Mizukoshi et al., 2008; Tomkins et al., 2011), agility dogs (Pastore et al., 2011), sled dogs (Angle et al., 2009), and search and rescue dogs (Vassallotti et al., 2017). Other explanations can be related to the possible involvement of the potential changes of blood lactate, according to training programs and related time points. The concentration of lactate, a metabolite derived from anaerobic pathways, represents an indicator of the fitness and level of the exercise and the oxidative capacity (Billat et al., 2003). Oxidative damage induced by physical exercise has been reported in dogs (Baskin et al., 2000; Piercy et al., 2000). Exercises and training programs have been shown to increase the production of reactive oxygen species (ROS) to a point that can exceed antioxidant defenses and cause oxidative stress (Alessio et al., 2000; Watson et al., 2005), increase muscle fatigue and muscle fiber damage (Powersand Jackson, 2008), and eventually lead to impairment of the immune system (Gleeson, 2007). It is difficult to quantify ROS in practice (Sechi et al., 2017); owing to their high reactivity, ROSs react with practically every organic molecule they meet, producing reactive oxygen metabolites (ROMs), which are more stable than the ROS and are therefore easier to quantify. Conversely, the biological antioxidant potential (BAP) matches the total antioxidant capability of plasma and includes either exogenous or endogenous components that can oppose the oxidant action of reactive species (Benzie and Strain, 1996). For these reasons, the laboratory assessment of the oxidative status should take into account both the pro-oxidizing component and the antioxidant component (Alberti et al., 2000).

Dietary manipulation may limit the exercise-induced muscle damage in athletic dogs (Baskin et al., 2000; Hinchcliff et al., 2000; Piercy et al., 2000). Very few studies have used blood stress biomarkers in animals during training to predict the value as guide for the blind.

The hypothesis was that the pituitary-adrenal (HPA) axis response and the oxidative stress indices could represent an objective method for identifying the optimal dietary protocol for a good successful guide dog during the early training period.

Research was addressed to compare the effects of different feeding strategies on hormonal and oxidative stress biomarkers in guide dogs during specialized training programs.

Materials and methods

Operative procedures and animal care were carried out in compliance with guidelines of Good Clinical Practices (EMEA, 2000) and international regulations (Directive 2010/63/EU). On the basis of the Italian regulation Legislative Decree26/2014, article 2, the research received the institutional approval by the Ethical Animal Care and Use Committee of the Department of Veterinary Science, of the University of Messina on October 19, 2016, Codex 006/2016 bis.

Animals and diets

The study was conducted on eight neutered adult dogs belonging to the Labrador retriever breed, clinically healthy, housed at the Regional Centre Helen Keller (https://it-it.facebook.com/centrohelenkellermessina) of the Italian Blind and Guide Dog School Union, in Messina (Italy). The Centre is unique in Italy. It is a member of the International Guide Dog Federation (IGDF) and, as such, accredited to the highest international standards. The Centre mostly provides Labrador retriever dogs, characterized by a markedly peaceful temperament with an innate sense of direction, and also commonly provides golden retriever and German shepherd.

Dogs admitted to the study were divided into two homogeneous groups for sex (2 males, 2 females), age (17 months ± 1), initial body weight (26.3 kg ± 1), and BCS (4.5 ± 0.11). The first group, called HPF group, received a "performance" diet, characterized by low-carbohydrate/high-protein and fat diet (29:39:19% as-fed), whereas the second group, called LPF group, received a "normal maintenance" diet characterized by high-carbohydrate/low-protein and fat diet (50:24:12 % as-fed).

Dogs were individually housed in pens of six square meters, adjacent to a large outdoor space to which they had access during rest, and food was provided two times a day, in an individual bowl.

The trial was preceded by 7 days of adaptation period to the experimental diets. During the adaptation period, the quantity of administered diet was the same as previously adopted by the breeder.

For the trial, three lots of feed were used. Each lot was sampled and analyzed, separately as described by Chiofalo et al. (2019a).

Both of the experimental diets provided by Farmina Pet Foods line contained lamb meal as the main protein source and, qualitatively, the same ingredients, analytical compounds, nutritional additives, and antioxidants (tocopherol-rich extracts of natural origin). The information on the chemical composition of "performance" and "normal maintenance" diets is reported in Table 1.

The amount of feed daily administered to each dog was calculated on the ratio between the calculated metabolizable energy requirements, as proposed by Hand et al. (2010), for dogs that perform work, characterized by a moderate duration and frequency:

$$DER = 2 \times RER\ (70\ Kcal * BW^{0.75})$$

where DER is the daily energy requirements, RER is the resting energy requirements, and the caloric density of metabolizable energy (ME) reported in the label (NRC, 2006), of each diet (HPF and LPF).

To respect the nutritional needs of dogs, each subject was fed to maintain body condition between a body condition score of 4 or 5 out of 9 and to maintain body weight from the beginning of the dietary trial until the end with calorie consumption never varying more than ±15% of the calorie intake at the beginning of the dietary trial. Dog weights were recorded each week, on fasted animals, at

Table 1
Chemical composition and metabolizable energy of the diets administered to dogs before and during the trial[a]

Diet	Anonymous[b]		HPF[c]		LPF[c]	
	Mean	SD	Mean	SD	Mean	SD
Moisture, g/100g as-fed	9.0	ND	5.42	0.48	6.12	0.57
CP, g/100g as-fed	26	ND	39.24	0.84	24.40	0.32
Fat, g/100g as-fed	15.50	ND	18.69	0.51	11.78	0.29
OM, g/100g as-fed	ND	ND	86.83	0.27	86.50	1.20
TDF, g/100g as-fed	2.80	ND	11.59	1.13	13.03	1.46
Ash, g/100g as-fed	4.9	ND	7.91	0.23	7.51	0.55
ME[3], kJ/100g as-fed	1633	ND	1813	ND	1433	ND

CP, crude protein; OM, organic matter; TDF, total dietary fiber; ME, metabolizable energy; ND, not determined; Anonymous = super-premium pet food, administered before the trial; HPF = high-protein and fat content diet, administered during the trial; LPF = low-protein and fat content diet, administered during the trial.
[a] Values are given as means ± standard deviation (SD).
[b] Values as reported in the label.
[c] Values determined analytically.

8:00 amusing a digital scale. Diets were adjusted to ensure that the dogs did not lose or gain more to 5% of their body weight.

Conditioning protocol

All dogs were trained and conditioned for training program activities one month before the dietary study. All dogs were between 1 and 2 years of age and had been trained by the training program to be guide dogs in the area described below (see paragraph Training program) and they fed a typical maintenance ration (see paragraph Animals and diets). Conditioning and training protocols remained the same for all dogs during the dietary trials each of which lasted 12 weeks. Thus, each dog served as its own control during the dietary trials. Moreover, the dogs were habituated to the blood collection; before the beginning of the trial, hematological and biochemical analyses were carried out on each subject to evaluate their health. All dogs had a physical examination before the beginning of the trial, to assess their clinical status (Ciaramella, 2014).

Training program

The method of participant recruitment is described in the study by Lloyd et al. (2008).

The training consisted of a various-phase program in which the dog gradually learned more guide work. This included leading a person in a straight line, stopping at any change in ground elevation, as well as overhead obstacles and obstacle avoidance. Food rewards were used in the guide dogs for the blind training program as a powerful motivation and reinforcement tool for learning and maintaining desired behavior. During each training session (at day 0, day 28; day 56 and day 84), dogs were introduced to specific guide-work behaviors:

- Stopping at streets, regardless of the type of curb or wheelchair ramp;
- Clearing the space around the handler on the right and left sides as well as above the dog's head;
- Crossing streets on a line that efficiently reaches the up curb on the other side;
- Maintaining consistent pace and drive with the verbal cue "forward";
- Responding to the various uses of the "hop-up" verbal cue for resuming or increasing pace; moving closer to a stopping point; or refocusing;
- Stopping and standing calmly after the verbal cue "halt";
- Leading the handler in a 90-turn to the right and picking up the new travel line on "right";
- Leading the handler in a 90-turn to the left and picking up the new travel line on "left."

The guide dogs for the blind were trained every day. Each training session started every day at 11:00 AM and terminated at 12:00 PM (Chiofalo et al., 2019a).

Physical examination

To evaluate the performance of the studied dogs, from day 0 (start of the administration of the new food) to day 84, all dogs underwent physical examination weekly (Ciaramella, 2014), including mentation/level of consciousness, posture and gait, hydration status, rectal temperature (C°), pulse rate, respiratory rate and breath character, perfusion indicators.

Measurement of hormonal and lactate concentrations

To evaluate the hormonal changes of dogs, blood samples were taken monthly (day 0, day 28, day 56, and day 84) 180 min before the training session (8:00 AM), immediately at the end of the training session (T1 at 12:00 PM), and 120 min after the end of the training session (T2 at 2:00 PM). Each training session started every day at 11:00 AM and terminated at 12:00 PM.

Before the trial, the dogs were habituated for the blood collection procedure (see paragraph Conditioning protocol). All samples were collected with identical evacuated tubes (Venoject EDTA K_3, Terumo® Europe, Rome, Italy) and were immediately refrigerated at 4°C after collection. The first samples were subsequently (within 1 h) centrifuged for 15 min at 1500 × *g*, and the second samples were used to assess hematological parameters. All serum samples were collected and stored at −20°C until their analyses. Plasma adrenocorticotrophic hormone (ACTH) and serum cortisol concentrations were assayed in duplicate using the fluorescence enzyme immune assay, marketed by Tosoh Bioscience (TB), Belgium-Japan (AIA 360), validated for use in dogs (Scott-Moncrieff et al., 2003; Higgs et al., 2014). This analyzer uses a competitive fluorescent enzyme immunoassay, which is performed entirely within small, single-use test cups containing all necessary reagents. The analyte presented in the sample competes with enzyme-labeled hormone for a limited number of binding sites on hormone-specific antibodies, immobilized on magnetic beads. The beads are washed to remove the unbound enzyme-labeled hormone and then incubated with a fluorogenic substrate, 4-methylumbelliferyl phosphate (4MUP). The amount of enzyme-labeled hormone that binds to the beads is inversely proportional to the hormone concentration in the test sample. Calibration, daily check, and maintenance procedures were carried out as described in the System Operator's Manual. The intra-assay and interassay coefficients of variation for cortisol and ACTH concentrations were 7% and 15%, respectively. To evaluate dogs training workload, blood lactate concentrations were measured 180 min before (T0) and immediately after (T1) exercise, using Accusport tester (Boehringer Mannheim, GmbH).

Measurements of oxidative stress indices

To measure the oxidative stress with d-ROMS (reactive oxygen metabolites–derived compounds) and BAP (biological antioxidant potential) tests by spectrophotometer, blood samples were withdrawn monthly for the variable time at day 0, day 28, day 56, and day 84 before the exercise; for the variable exercise, immediately before (T0), immediately after (T1), and 120 min after exercise (T2).

In the d-ROMs test, reactive oxygen metabolites (primarily hydroperoxides) in a biological sample, in the presence of iron released from plasma proteins by an acidic buffer, are able to generate alkoxyl and peroxyl radicals, according to the Fenton reaction. Such radicals can then oxidize an alkyl substituted aromatic amine (N, N-diethylparaphenylenediamine), thus producing a pink-colored derivative which is photometrically quantified at 505 nm (Albertiet al., 2000). The d-ROMs concentration is directly proportional to the color intensity and expressed as Carratelli units (1 CARR U = 0.08 mg hydrogen peroxide/dL). In the BAP test, the addition of a plasma sample to a colored solution, obtained by mixing ferric chloride solution with a thiocyanate derivative solution, causes a discoloration, whose intensity was measured photometrically at 505 nm and was proportioned to the ability of the plasma to reduce ferric ions (Benzie and Strain, 1996). The results are expressed as mole/L of reduced ferric ions. Both tests were validated for canine species (Pasquini et al., 2008).

Table 2
Effect of the diet on hormonal and oxidative blood stress biomarkers in the trial[c]

Groups	ACTH (pg/mL)		Cortisol (μg/dL)		BAP test (μmol/L)		d-ROMs test (CARR U)		Lactate (nmol/L)	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
HPF	30.25[b]	1.11	2.24[b]	0.12	3099	101	83.47	2.88	1.78	0.16
LPF	33.06[a]	1.22	2.51[a]	0.17	2825	123	88.04	3.53	1.74	1.15

ACTH = adrenocorticotropic hormone; BAP = biological antioxidant potential; d-ROMs = reactive oxygen metabolites derivatives; CARR U = Carratelli units; HPF = high-protein and fat content diet; LPF = low-protein and fat content diet.
[a,b]Means within a column with different superscripts indicate a significant difference at $P < 0.05$, using the Tukey test.
[c] Values are given as least square mean (LSM) ± standard error of the mean (SEM).

Statistical analyses

A mixed-model analysis of variance (XLSTAT, 2014) with the fixed effects of time (day 0, day 28; day 56 and day 84), diet (HPF vs. LPF), and exercise (T0: 180 min before exercise, T1: immediately after exercise, and T2: 120 min after exercise) was chosen for analysis. The interaction (diet*time *exercise) was forced into every model. Random effects in the model were individual dog. Residuals were examined for normality and in each of the case residuals were normally distributed. Least squares means and standard error of the mean were calculated. Comparison between least squares means were performed using the Tukey test. Differences were considered significant for $P < 0.05$.

Results

Physical examination and body weight

During the trial, the dogs presented normal general appearance, normal level of consciousness, no abnormalities in the posture, adequate hydration status, rectal temperature within the normal physiological ranges (38.4°C ± 0.32). The femoral artery pulse was strong and within the reference range for dogs (92 bpm ± 14) (Ciaramella, 2014). The mean of respiratory rate, determined visually or by auscultation as count either inspirations or expirations, was within the normal physiological ranges (18–29 ± 3) (Ciaramella, 2014). Mucous membrane color was pink, and capillary refill time was less than 2 seconds. Diet influenced the animal performances in relation to their different protein, fat, and carbohydrate levels, showing a significantly higher ($P < 0.001$) body weight in the HPF group than in the LPF group (25.40 vs. 23.44 kg).

Hormonal response

There were higher mean values of ACTH ($P = 0.002$) and cortisol ($P = 0.013$) concentrations (Table 2) in the LPF group compared to the HPF group. Nevertheless, the hormonal response was not significantly influenced by the time (Table 3), ACTH ($P = 0.607$), or cortisol ($P = 0.326$) concentrations. ACTH and cortisol concentrations were significantly influenced by exercise (Table 4). The *P*-values for both ACTH and cortisol concentrations were paired to $P < 0.0001$. Plasma ACTH concentrations showed higher ($P < 0.01$) mean values at T1 than those observed at T0 and at T2. Higher cortisol values were observed at T1 compared to T2 ($P = 0.02$) and T0 ($P < 0.01$) values.

The interaction diet*time*exercise showed no significant differences for ACTH ($P = 0.745$) or cortisol ($P = 0.381$) concentrations.

Lactate

No significant differences in relation to the diet ($P = 0.776$; Table 2) and time ($P = 0.696$; Table3) variables were observed for lactate. Exercise (Table 4) significantly influenced blood lactate concentrations on the whole trial period, with higher ($P < 0.001$) mean values at T1 (immediately after exercise) than those observed at T0 (before exercise).The interaction diet*time*exercise showed no significant ($P = 0.995$) differences for blood lactate concentrations.

BAP and d-ROM test

Diet (Table 2) did not influence either oxidative biomarker (BAP [$P = 0.092$]; d-ROMs [$P = 0.321$]).

Significant ($P = 0.02$) differences were observed in relation to the variable time (Table 3) for the BAP test, with significant higher mean values at day 0 ($P = 0.011$) and day 84 ($P = 0.033$) than those observed at day 56. The d-ROM test showed no significant ($P = 0.062$) differences in relation to the time.

As regards to the variable exercise (Table 4), no significant ($P = 0.808$) differences were observed for BAP, whereas d-ROM showed a significantly ($P = 0.001$) higher value before the exercise (T0) than that observed at 120 min after exercise (T2).

The interaction diet*time*exercise during the trial showed no significant differences for BAP ($P = 0.492$) as well as for d-ROM tests ($P = 0.350$).

Table 3
Effect of the time on hormonal and oxidative blood stress biomarkers in the trial[c]

Time[d]	ACTH (pg/mL)		Cortisol (μg/dL)		BAP test (μmol/L)		d-ROMs test (CARR U)		Lactate (nmol/L)	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
Day 0	31.80	3.71	2.41	0.20	3243[a]	158	90.75	4.52	1.80	0.21
Day 28	35.47	3.00	2.46	0.22	2937[ab]	158	91.26	4.52	1.69	0.28
Day 56	33.12	3.06	2.31	0.18	2520[b]	158	71.13	4.52	1.85	0.22
Day 84	33.41	3.07	2.20	0.21	3149[a]	158	85.89	4.51	1.68	0.19

ACTH = adrenocorticotropic hormone; BAP = biological antioxidant potential; d-ROMs = reactive oxygen metabolites derivatives; CARR U = Carratelli units.
[a, b]Means within a column with different superscripts indicate a significant difference at $P < 0.05$, using the Tukey test.
[c] Values are given as least square mean (LSM) ± standard error of the mean (SEM).
[d] Time = blood sampling at day 0, day 28, day 56, and day 84, before the exercise.

Table 4
Effect of the exercise on hormonal and oxidative blood stress biomarkers in the trial[c]

Exercise[d]	ACTH (pg/mL)		Cortisol (μg/dL)		BAP test (μmol/L)		d-ROMs test (CARR U)		Lactate[e] (nmol/L)	
	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM	LSM	SEM
T0	23.21[b]	1.24	1.62[c]	0.08	3019	137	96.69[a]	3.92	1.06[b]	0.36
T1	47.95[a]	2.07	3.20[a]	0.11	3011	137	85.77[ab]	3.92	2.45[a]	0.29
T2	29.19[b]	1.71	2.22[b]	0.09	2856	137	74.80[b]	3.92	ND	ND

ACTH = adrenocorticotropic hormone; BAP = biological antioxidant potential; d-ROMs = reactive oxygen metabolites derivatives; CARR U = Carratelli units.
[a,b]Means within a column with different superscripts indicate a significant difference at $P < 0.05$, using the Tukey test.
[c] Values are given as least square mean (LSM) ± standard error of the mean (SEM).
[d] Exercise = blood sampling immediately before (T0), immediately after (T1), and 120 min (T2) after exercise.
[e] ND: not done because the blood lactate concentrations are restored at the baseline values within 60 min.

Discussion

A diet for the dog during periods of normal maintenance (PG = 20%-23%; LG = 10%-12%) does not meet the requirements during its training work. Use of large amounts of feed is not recommended. The consumption of the "performance" diet, characterized by low-carbohydrate/high-protein and fat diet (29%:39%:19% as-fed), seems to be more appropriated for light prolonged exercise than the ingestion of a normal maintenance diet rich in carbohydrates (Chiofalo et al., 2019b). This diet limited weight loss in the HPF group, as observed for the dogs of the LPF group (−18%). Nevertheless, all the animals during the trial lost weight; this could be due to the training work for the service guide for the blind. Weight loss is normal in guide dogs during the training, according to the exercise and the life in kennel (Chiofalo et al., 2019a). Moreover, considering that Labrador retrievers may be genetically predisposed to obesity and osteoarticular diseases (Raffan et al., 2016), and considering the important role of GDB, they should maintain moderate body weight during the training program.

ACTH, cortisol (Kaneko et al., 2010; Larsson et al., 2015), and blood lactate (Kaneko et al., 2010; Fazio et al., 2015; Ober et al., 2016) concentrations were in agreement with physiological ranges reported in literature. The slight variations might be ascribed to differences among laboratories using different methods and instruments and are the subject of speculation. Some differences may also be explained by different experimental training programs, as the intensity and severity of the workload and/or of training status (Rovira et al., 2008), and varying dietary contents (Larsson et al., 2015; Ober et al., 2016).

Significant changes in energy metabolism occur during exercise and are maintained for some hours thereafter, revealing challenges for energetic homeostasis. Exercise corresponds to a stress model followed by some endocrine modifications that occur to counterbalance its effects on thermogenesis and substrate metabolism (Mastorakos and Pavlatou, 2005). At each sample time, mild stressors, both positive and negative, can be incredibly helpful in training. In fact, the act of training itself is mildly stressful for dogs. Reward-based training methods promote positive stress, also known as "eustress," which encourages betterment of oneself.

HPA hormonal concentrations differed for HPF and LPF groups. These findings are consistent with those of other studies indicating that the higher dietary protein and fat levels could reduce HPA response to chronic stressors in animals (Hennessy et al., 2002). Nevertheless, it is not clear whether this finding is related to stress-reducing influence or a more-direct metabolic effect of the different diets.

The HPA axis response is linked to a wide range of arousing or aversive and threatening events, but HPA activation is not necessarily reflected in behavior. Although behavioral signs of stress often correlate positively with measures of HPA activity, it is also possible for hormonal concentrations to be elevated while animals appear calm (Helmerich et al., 2012) or that behavioral and cortisol can become dissociated to stress response (Elder and Menzel, 2001).

In this study, having collected no behavioral data, we are not able to distinguish the emotional component from the physical effort for the GDB during the whole training period. Hill et al. (2009) reported that a low-carbohydrate/high-protein and fat diet had the potential to be beneficial to working dogs because it is not only closer to the ancestral diet (Case, 2005) but also appeared to confer advantages to working dog performance subjected to prolonged bouts of exercise requiring a sustained energy source (Davenport et al., 2001). Kronfeldet al. (1977) suggested that carbohydrates are not necessary in endurance dog diet.

With relation to time, although this variable did not influence the hormonal response, both ACTH and cortisol concentrations showed slightly higher values at day 28 compared to the other days, in all dogs. These slight differences could be related to the training work at the first period of these activities for all dogs, involving aerobic activity with low energy requirement. The training exercise clearly stimulated ACTH release and related consistent increase of circulating cortisol concentrations, mostly immediately after exercise, in both HPF and LPF groups. These increases confirmed that exercise stress induces both metabolic rate and energy consumption increase through the activation of the HPA axis (Sapolsky et al., 2000). Increased activity of the HPA axis, in response to a wide range of threatening, arousing, or aversive events and the amplitude of hormone response may be correlated with the severity, predictability, and control of the stimulus (Beerda et al., 1998; Fazio et al., 2015), as shown by data observed during the overall training program.

What is more, the physiological cortisol range observed 120 min after exercise is in line with recent data showing whether a glucocorticoid action permits, stimulates, or suppresses an ongoing stress-response (Sapolsky et al., 2000). Data attempt to assimilate these heterogeneous glucocorticoid actions into a physiological whole, as previously observed by Sapolsky et al. (2000).

The highest blood lactate concentration observed at T1 of each training week could be due, first, to superimposed effects of training load plus metabolic outcome, as expected in untrained younger dogs; second, it could be explained on the basis of the initial and consequently intense activity of these subjects, according to the access to the outdoor area, independently of the intensity and duration, confirming previous data obtained after standardized exercise on the treadmill (Ober et al., 2016). It is possible that the initial training activity, in young dogs, induced a substantial fatigue occurring after training program, according to the consistent requirement/utilization of O_2 store (Billat et al., 2003).

The results of BAP test are in accordance with those obtained by Dohi et al. (2005) on dogs and they are slightly over the referral range (2069–2554 μmol/L) validated by Pasquini et al. (2008) in canine species. Data on d-ROMs test are in accordance with those observed by Iamele et al. (2002) and Trotti et al. (2002) on a sample of dogs (56.4 to 91.4 CARR U) and by Pasquini et al. (2008) in canine species (67.1–91.5 CARR U).

There was no diet effect observed for oxidative stress indices (BAP and d-ROMs tests); these results testify a balanced content of essential nutrients (vitamins and minerals) in both commercial diets and is responsible of the general health status of dogs considering that the BAP matches the total antioxidant capability of plasma including endogenous (e.g., protein) components that can oppose the oxidant action of reactive species (Benzie & Strain, 1996). This effect was particularly felt for the BAP test at the end of the trial (day 84); therefore, after three months, the dogs received experimental diets, showing a good adaptive response to the diets.

In relation to the exercise, the high d-ROMs mean value (96.69 CARR U) observed immediately before the beginning of the exercise (T0), above the upper range level (92–95 CARR U) defined by Pasquini et al. (2010) and by Sechi et al. (2015) as "threshold borderline," could be the excited state of the dogs before working. This value falls within the "normal" range values (50 to 90 CARR U) immediately after exercise and 120 min after exercise, demonstrating the recovery of normal oxidative status (Michailidis et al., 2007) and/or the adaptation to exercise that may decrease oxidative stress (Leeuwenburgh and Heinecke, 2001; Watson et al., 2005; Apor and Radi, 2006; Jackson, 2008).

The trends of d-ROMs and BAP are not in accordance with those obtained by Bergero et al. (2004). These authors observed an increase in the markers of oxidation and a decrease of antioxidant capacity during exercise. The controversial effect could be due to the particular exercise of guide dogs which although are subjected to a physical and mental work, continuous and demanding, though physical exertion is not particularly intense. Another explanation could be that the oxidative stress can be contained by a good training. For this reason, the evaluation of oxidative status with specific parameters could become a cheap, rapid, and practical method to monitor activity of working dogs (Pasquini et al., 2010).

The similar values observed in relation to the interaction factors diet*time*exercise could be related to administration of a controlled and balanced antioxidant content in both diets that may be a valid approach to restoring good cell metabolism and neutralizing excess free radicals in utility dogs (Sechi et al., 2017).

Conclusion

Blood stress biomarkers are of major interest in canine sport medicine to assess health status and fitness level, as well as to monitor the stress induced by exercise. Presently, there is a lack of information on the physiological effect of moderate activities in canines, particularly in the guide dog.

Results on body weight evidenced that the low-carbohydrate/high-protein and fat diet appears to be the nutritional plan most suitable to support moderate exercise in guide dogs during their training work, the maintenance of BW and BCS, and the stressful exercise response. The dietary factors could reduce HPA response to chronic stressors in GDB involved in the training program. From a practical point of view, the evaluation of the reference values and related changes of neuroendocrine and functional variables in Labrador retrievers at the start of initially training programs represents a basic requirement for future implementation of reliable markers for the training status and the future progressive fitness. Ensuring good mental and physical condition of the dogs seems to be crucial not only to welfare but also toward the main goal of a successful activity of these animals.

The results of oxidative stress indices, which are within the reference range, may be due to the good training program to which the dogs were submitted by the dog school guides that are members of the International Guide Dog Federation (IGDF) and therefore accredited to the highest international standards.

Training programs are inevitably associated with a stress response, but this may be avoided by a proper diet, handling, and management facilities, which take the Labrador retriever temperament into account. Increases in cortisol concentrations immediately after the exercise confirm that cortisol is a marker of stress also in dogs during a specific guide work. The implication of these findings is that the exposure to training program can alter the endocrine response to stress and may thereby predispose individuals to their different future performance.

Blood stress biomarkers monitoring during training in relation to diet, time, and activity may be an effective method to identify the potential suitability subjects in advance, optimizing the dietary protocols and reducing the costs associated in guide dogs training. However, further investigation regarding optimal protein and fat calories may be worthwhile for guide dogs.

Acknowledgments

This research was supported by the Farmina Pet Foods/Russo Mangimi S.p.A. (Via Nazionale delle Puglie, 80035 Nola-NA, Italy) grant, 2016. The authors want to thank for the continuing support of the President and the Guide Dog Trainers of the Regional Centre Helen Keller of the Italian Blind and Guide Dog School Union in Messina.

Authorship statement: The idea for the paper was conceived and the experiment was designed by Biagina Chiofalo. The experiments were performed in farm by Salvatore Cucinotta and Cristina Cravana and in laboratory by Pietro Lombardi, Vincenzo Mastellone, and Cristina Cravana. The data were analyzed by Ambra Rita Di Rosa. The paper was written by Biagina Chiofalo and Esterina Fazio.

Ethical considerations

This manuscript has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. All authors will hold themselves jointly and individually responsible for the content of the manuscript.

Conflict of interest

The authors declare no conflicts of interest. The funder 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.

References

Alberti, A., Bolognini, L., Macciantelli, D., Carratelli, M., 2000. The radical cation of N-N- diethyl-para-phenylendiamine: a possible indicator of oxidative stress in biological samples. Res. Chem. Intermed. 26, 253–267.

Alessio, H.M., Hagerman, A.E., Fulkerson, B.K., Ambrose, J., Rice, R.E., Wiley, R.L., 2000. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. Med. Sci. Sports Exerc. 32, 1576–1581.

Angle, C.T., Wakshlag, J.J., Gillette, R.L., Stokol, T., Geske, S., Adkins, T.O., Gregor, C., 2009. Hematologic, serum biochemical, and cortisol changes associated with anticipation of exercise and short duration high-intensity exercise in sled dogs. Vet. Clin. Pathol. 38, 370–374.

Apor, P., Radi, A., 2006. Physical exercise, oxidative stress and damage. Orv. Hetil. 147, 1025–1031.

Baskin, C.R., Hinchcliff, K.W., Di Silvestro, R.A., Reinhar, G.A., Hayek, M.G., Chew, B.P., Burr, J.R., Swenson, R.A., 2000. Effects of dietary antioxidant supplementation on oxidative damage and resistance to oxidative damage during prolonged exercise in sled dogs. Am. J. Vet. Res. 61, 886–891.

Beerda, B., Schilder, M.B.H., van Hoff, J.A.R.A.M., De Vries, H.W., Mol, J.A., 1998. Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs. Appl. Anim.Behav. Sci. 58, 365–381.
Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power:" The FRAP assay. Anal. Biochem. 239, 70–76.
Bergero, D., Miraglia, N., Schiavone, A., Polidori, M., Prola, L., 2004. Effect of dietary polyunsaturated fatty acids and Vitamin E on serum oxidative status in horses performing very light exercise. Ital. J. of Anim. Sci. 3, 141–145.
Billat, V.L., Sirvent, P., Guillaume, Py., Koralsztein, J.P., Mercier, J., 2003. The concept of maximal lactate steady state a bridge between biochemistry, physiology and sport science. Sports Med 33, 407–426.
Case, L.P., 2005. Man and wolf: the process of domestication. In: , 2nd ed.The Dog: Its Behavior, Nutrition and Health Blackwell Publishing Professional, Iowa, pp. 3–16.
Ciaramella, P., 2014. Semiologia Clinica Veterinaria. Poletto Editore, Milano, Italy.
Chiofalo, B., De Vita, G., Lo Presti, V., Cucinotta, S., Gaglio, G., Leone, F., Di Rosa, A.R., 2019a. Grain free diets for utility dogs during training work: Evaluation of the nutrientdigestibility and faecalcharacteristics. Anim. Nutr. 5, 297–306.
Chiofalo, B., Fazio, E., Cucinotta, S., Cravana, C., 2019b. Thyroid and lipid status in guide dogs during training: effects of dietary protein and fat content. Animals 9, 597.
Davenport, G.M., Kelley, R.L., Altom, E.K., Lepine, A.J., 2001. Effect of diet on hunting performance of English pointers. Vet. Ther. 2 (1), 10–23.
Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010. Protection of animals used for scientific purposes. In: Official Journal of the European Union L 276 of the October 20, 2010, pp. 33–79.
Dohi, K., Satoh, K., Ohtaki, H., Shioda, S., Miyake, Y., Shindo, M., Aruga, T., 2005. Elevated plasma levels of bilirubin in patients with neurotrauma reflect its pathophysiological role in free radical scavenging. In Vivo 19, 855–860.
Elder, C.M., Menzel, C.R., 2001. Dissociation of cortisol and behavioral indicators of stress in an orangutan (Pongo pygmaeus). Primates 42, 345–357.
EMEA, 2000. European Agency for the Evaluation of Medicinal Products. CVMP/VICH/595/98-FINAL, Canary Wharf, London. http://www.eudra.org/emea.html.
Ennik, I., Liinamo, A.-E., Leighton, E., van Arendonk, J., 2006. Suitability for field service in 4 breeds of guide dogs. J.Vet.Behav.: Clin. Appl. Res. 1, 67–74.
Fazio, E., Medica, P., Cravana, C., Pupillo, A., Ferlazzo, A., 2015. Effect of ovariohysterectomy in dogs and cats on adrenocortical, haematologica and behavioural parameters. Acta Sci.Vet. 44, 1339.
Gleeson, M., 2007. Immune function in sport and exercise. J. Appl. Phys. 103, 693–699.
Guide Dogs NSW/ACT, 2010. Guide Dogs NSW/ACT February Newsletter. Hillfields, Burghfield Common:International Guide Dog Federation. www.igdf.org.uk.
Hand, M.S., Thatcher, C.D., Remillard, R.L., Roudebush, P., Novotny, B.J., 2010. Small Animal Clinical Nutrition, 5th ed. Mark Morris Institute, Topeka, KS.
Helmreich, D.L., Tylee, D., Christianson, J.P., Kubala, K.H., Govindarajan, S.T., O'Neill, W.E., Becoats, K., Watkins, L., Maier, S.F., 2012. Activebehavioral coping alters the behavioral but not the endocrine response to stress. Psychoneuroendocrino 37, 1941–1948.
Hennessy, M.B., Voith, V.L., Hawke, J.L., Young, T.L., Centrone, J., McDowell, A.L., Linden, F., Davenport, G.M., 2002. Effects of a program of human interaction and alterations in diet composition on activity of the hypothalamic –pituitary–adrenal axis in dogs housed in a public animal shelter. J. Am. Vet. Med. Assoc. 221, 65–71.
Higgs, P., Costa, M., Freke, A., Papasouliotis, K., 2014. Measurement of thyroxine and cortisol in canine and feline blood samples using two immunoassay analysers. J. Small Anim.Pract. 55, 153–159.
Hill, R.C., 1998. The nutritional requirements of exercising dogs. J. Nutr 128 (12 Suppl), 2686S–2690S.
Hill, S.R., Rutherfurd-Markwick, K.J., Ravindran, G., Ugarte, C.E., Thomas, D.G., 2009. The effects of the proportions of dietary macronutrients on the digestibility, post-prandial endocrine responses and large intestinal fermentation of carbohydrate in working dogs. N. Z.Vet. J. 57 (6), 313–318.
Hinchcliff, K.W., Reinhart, G.A., Silvestro, R., Reynolds, A.J., Blostein-Fujii, A., Swenson, R.A., 2000. Oxidant stress in sled dogs submitted to repetitive endurance exercise. Am. J. Vet. Res. 61, 512–517.
Iamele, L., Fiocchi, R., Vernocchi, A., 2002. Evaluation of an automated spectrophotometric assay for reactive oxygen metabolites in serum. Clin. Chem. Lab. Med. 40, 673–676.
Jackson, M.J., 2008. Free radicals generated by contracting muscle: by-products of metabolism or key regulators of muscle function. Free. Radic. Biol. Med. 44, 132–141.
Kaneko, J., Harney, J.W., Bress, M.L., 2010. In: Kaneko, J., Harney, J.W., Bress, M.L. (Eds.), Blood Analyte Reference Values in Small and Same Laboratory Animals (Appendix IX): Clinical Biochemistry of Domestic Animals. Academy Press, San Diego, p. 896.
Kronfeld, D.S., Hammel, E.P., Ramberg, C.F., Dunlap, H.L., 1977. Hematological and met-abolic responses to training in racing sled dogs fed diets containing medium, low, or zero carbohydrate. Am. J. Clin. Nutr. 30, 419–430.
Larsson, C., Ahlstrom, O., Junghans, P., Jensen, R.B., Blache, D., Tauson, A.H., 2015. The oral [13 C] bicarbonate technique for measurement of short-term energy expenditure of sled dogs and their physiological response to diets with different fat: carbohydrate ratios. J. Nutr. Sci. 4, 1–10.
Leeuwenburgh, C., Heinecke, J.W., 2001. Oxidative stress and antioxidant in exercise. Curr. Med. Chem. 8, 829–838.
Legislative Decree 26/2014, 2014. GU Serie Generale n. 61 del.
Lloyd, J.K.F., La Grow, S.J., Stafford, K.J., Budge, R.C., 2008. The guide dog as a mobility aid part 1: perceived effectiveness on travel performance. Int. J. O&M 1 (1), 17–33.
Mastorakos, G., Pavlatou, M., 2005. Exercise as a stress model and the interplay between the hypothalamus–pituitary–adrenal and the hypothalamus–pituitary–thyroid axes. Horm. Metab. Res. 37, 577–584.
Michailidis, Y., Jamurtas, A.Z., Nikolaidis, M.G., Fatouros, I.G., Koutedakis, Y., Papassotiriou, I., Kouretas, D., 2007. Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. Med. Sci. Sports. Exerc. 39, 1107–1113.
Mizukoshi, M., Kondo, M., Nakamura, T., 2008. Evaluation of the potential suitability of guide dog candidates by continuous observation during training. J. Vet. Behav.: Clin. Appl. Res. 3, 193–198.
NRC - National Research Council (U.S.), 2006. Ad Hoc Committee on Dog and Cat Nutrition. In: Nutrient Requirements of Dogs and Cats. National Academies Press, Washington, DC.
Ober, J., Gillette, R.I., Angle, T.C., Haney, P., Fletcher, D.J., Wakshlag, J.J., 2016. The effects of varying concentrations of dietary protein and fat on blood gas, hematologic serum chemistry, and body temperature before and after exercise in labrador retrievers. Vet. Sci. 3, 1–4.
Pasquini, A., Luchetti, E., Marchetti, V., Luchetti, E., Marchetti, V., Cardini, G., Iorio, E.L., 2008. Analytical performances of d-ROMs test and BAP test in canine plasma. Definition of the normal range in healthy labrador dogs. Vet. Res. Commun. 32, 137–143.
Pasquini, A., Luchetti, E., Cardini, G., 2010. Evaluation of oxidative stress in hunting dogs during exercise. Res. Vet. Sci. J. 89, 120–123.
Pastore, C., Pirrone, F., Balzarotti, F., Faustini, M., Pierantoni, L., Albertini, M., 2011. Evaluation of physiological and behavioral stress-dependent parameters in agility dogs. J. Vet. Behav.: Clin. Appl. Res. 6, 188–194.
Piercy, R.J., Hinchcliff, K.W., Di Silvestro, R.A., Reinhart, G.A., Baskin, C.R., Hayek, M.G., Burr, J.R., Swenson, R.A., 2000. Effect of dietary supplements containing antioxidants on attenuation of muscle damage in exercising sled dogs. Am. J. Vet. Res. 61, 1438–1445.
Powers, S.K., Jackson, M.J., 2008. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol. Rev. 88, 1243–1276.
Raffan, E., Dennis, R.J., O'Donovan, C.J., Becker, J.M., Scott, R.A., Smith, S.P., Withers, D.J., Wood, C.J., Conci, E., Clements, D.N., Summers, K.M., German, A.J., Mellersh, C.S., Arendt, M.L., Iyemere, V.P., Withers, E., Söder, J., Wernersson, S., Andersson, G., Lindblad-Toh, K., Yeo, G.S., O'Rahilly, S., 2016. A Deletion in the canine POMC gene is associated with weight and appetite in obesity-prone labrador retriever dogs. Cell Metab 10 (5), 893–900.
Rovira, S., Munoz, A., Benito, M., 2008. Effect of exercise on physiological, blood and endocrine parameters in search and rescue-trained dogs. Vet. Med. 53, 333–346.
Sapolsky, R.M., Romero, L.M., Munch, A.U., 2000. How do glucocorticoids influence stress response? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.
Scott-Moncrieff, J.C., Koshko, M.A., Brown, J.A., Hill, K., Refsal, K.R., 2003. Validation of a chemiluminescent enzyme immunometric assay for plasma adrenocorticotropic hormone in the dog. Vet. Clin. Pathol. 32, 180–187.
Sechi, S., Chiavolelli, F., Spissu, N., Di Cerbo, A., Canello, S., Guidetti, G., Fiore, F., Cocco, R., 2015. An antioxidant dietary supplement improves brain-derived neurotrophic factor levels in serum of aged dogs: preliminary results. J. Vet. Med. 2015, 412501.
Sechi, S., Fiore, F., Chiavolelli, F., Dimauro, C., Nudda, A., Cocco, R., 2017. Oxidative stress and food supplementation with antioxidants in therapy dogs. Can. J. Vet. Res. 69, 1097–1103.
Toll, P.W., Gillette, R.L., Hand, M.S., 2010. Feeding working and sporting dogs. In: Hand, M.S., Thatcher, C.D., Remillard, R.L., Roudebusch, P., Novotny, T.L. (Eds.), Small Animal Clinical Nutrition, 5th ed. Mark Morris Institute, Topeka, KS, pp. 321–358.
Tomkins, L.M., Thomson, P.C., McGreevy, P.D., 2011. Behavioral and physiological predictors of guide dog success. J. Vet. Behav.: Clin. Appl. Res. 6, 178–187.
Trotti, R., Carratelli, M., Barbieri, M., 2002. Performance and clinical application of a new, fast method for the detection of hydroperoxides in serum. Panminerva Med. 44, 37–40.
Vassalotti, G., Musco, N., Lombardi, P., Calabrò, S., Tudisco, R., Mastellone, V., Grazioli, R., Bianchi, S., Cutrignelli, M.I., 2017. Nutritional management of search and rescue dogs. J. Nutr. Sci. 6, 1–4.
Wakshlag, J., Shmalberg, J., 2014. Nutrition for working and service dogs. Vet. Clin. North Am. Small Anim. Pract. 44 (4), 719–740.
Watson, T.A., Macdonald-Wicks, L.K., Garg, M.L., 2005. Oxidative stress and antioxidant in athletes undertaking regular exercise training. Int. J. Sport Nutr. Exe. 15, 131.
XLSTAT, 2014. Data Analysis and Statistical Solution for Microsoft Excel. Addinsoft, Paris, France.