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ASSICURA AL CANE UN'ADEGUATA INTEGRAZIONE NUTRIZIONALE E I SUOI GENI TI RINGRAZIERANNO

Una svolta nell'approccio scientifico alla nutraceutica veterinaria

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Teknofarma

Idea geniale!

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Un elevato standard di idoneità fisica è una caratteristica essenziale per i cani specializzati nella ricerca della droga perché influenza non solo la loro capacità di sostenere livelli di attività fisica elevati, ma anche le loro capacità di concentrazione e l'efficienza olfattiva.

L'obiettivo di questo studio è stato quello di valutare gli effetti della supplementazione con Iken Up in un gruppo di cani da lavoro sottoposti a due prove sperimentali: a) prova di ricerca della droga, realizzata in ambientazioni diverse, per mettere alla prova abilità di complessa padronanza acquisite dai cani durante il corso di addestramento alla ricerca della droga; b) prova di resistenza su treadmill elettrico (*tapis roulant*) per valutare la performance fisica dei cani.

Per la sperimentazione sono stati impiegati 14 cani di razza Pastore Tedesco, 10 maschi e 4 femmine, tra i 2 e i 3 anni di età, allevati e addestrati per la ricerca di sostanze stupefacenti presso il Centro di allevamento e addestramento cani della Guardia di Finanza di Castiglione del Lago.

La **prova di ricerca della droga** è stata condotta in due trial distinti, a distanza di un anno circa.

Il primo trial è stato condotto con lo scopo di identificare un gruppo di geni associati all'abilità di ricerca della droga.

Tali geni rientrano nel pathway neuroplastico, ovvero il processo dinamico che porta a cambiamenti funzionali nell'espressione genica neuronale in risposta ad una stimolazione ambientale (intesa come un progresso sensoriale, fisico e nutrizionale): la plasticità neuronale consente al sistema nervoso, centrale e periferico, di acquisire nuove competenze e riorganizzare le reti neuronali in risposta a stimoli ambientali.

È noto, infatti, che ogni organismo è fenotipicamente determinato dall'interazione tra geni e ambiente, in quanto l'espressione di un gene è finemente regolata da modificazioni epigenetiche dovute ai cambiamenti provocati dai fattori ambientali. In questa sperimentazione il corso di addestramento alla ricerca di droghe rappresenta per i cani un valido modello di arricchimento ambientale, grazie alla vasta gamma di stimolazioni olfattive ed esperienze sociali cui sono esposti.

Nel secondo trial della prova di ricerca della droga è stato valutato l'effetto della supplementazione nutrizionale con Iken Up quale fattore aggiuntivo di stimolazione ambientale sulle espressioni del gruppo di geni individuato nel primo trial. Durante gli studi tutti i cani sono stati mantenuti in box singoli, hanno ricevuto la medesima dieta di mantenimento e avuto sempre accesso all'acqua *ad libitum*. Nel secondo trial i 14 cani sono stati assegnati casualmente a due gruppi di pari numerosità: 7 cani (gruppo "Iken Up") hanno ricevuto una supplementazione nutrizionale con Iken Up per 12 settimane (2 cpr/10 kg p.a./die durante la prima settimana; 1 cpr/10 kg p.a./die durante le successive 11 settimane), mentre gli altri 7 (gruppo "Controllo") non hanno ricevuto nessuna integrazione.

Le analisi sperimentali che hanno permesso di determinare l'effetto di Iken UP sono: l'osservazione della performance di ricerca, la valutazione della performance fisica e la determinazione molecolare dei geni coinvolti nella neuroplasticità.

Nella **prova di resistenza fisica su treadmill**, la performance fisica dei cani è stata valutata mediante registrazione dei tempi di ricerca e ritrovamento della droga e rilievo di alcuni parametri fisiologici. A tal fine sia per il gruppo "Iken Up" sia per il gruppo "Controllo" a) prima di iniziare il trattamento, b) prima e dopo la prova di treadmill (esercizio submassimale della durata di 27 minuti con diverse velocità e difficoltà) e c) dopo un periodo di recupero di 30 minuti, sono state monitorate la frequenza cardiaca (mediante Polar® System), la temperatura rettale (con un termometro digitale) e raccolti campioni di sangue per l'analisi dei parametri biochimici e per la valutazione dell'espressione dei geni correlati alla ricerca antidroga.

In entrambi gli studi, i due gruppi hanno riportato le stesse performance operative.

Dalla valutazione della performance fisica è emerso che nei cani che avevano ricevuto la supplementazione con Iken Up si riscontrava un più rapido ripristino della frequenza cardiaca basale nel periodo di riposo dopo la prova di treadmill rispetto al gruppo "Controllo". Inoltre, dopo la prova, il gruppo "Iken Up" presentava valori minori di creatina chinasi (CK), aspartato aminotransferasi (AST) e acidi grassi non esterificati (NEFA) rispetto al gruppo "Controllo", suggerendo una riduzione nei cambiamenti di permeabilità e affaticamento muscolare nei cani del gruppo "Iken Up". Quindi, dalla valutazione della performance fisica, possiamo affermare che **la supplementazione nutrizionale con Iken Up si è dimostrata efficace nel migliorare la "fitness fisica" dei cani da lavoro, intesa come la capacità dell'organismo durante uno sforzo intenso di mantenere i vari equilibri interni il più vicino possibile allo stato di riposo, nonché di ripristinare prontamente dopo l'esercizio tutti gli equilibri che sono stati perturbati**.

L'aspetto molecolare dei geni coinvolti nella neuroplasticità relativo al secondo trial della prova di ricerca della droga ha evidenziato come il gruppo "Iken Up", rispetto al gruppo di "Controllo", mostri l'assenza di una sovraregolazione genica durante la prova di ricerca: probabilmente, ciò può essere spiegato come la mancanza della necessità di stimolare una maggior produzione di tali geni. Gli autori ipotizzano che **la supplementazione nutrizionale abbia modificato il metabolismo sistemico e, di conseguenza, i livelli di mRNA di tali geni, a tal punto da renderne superflua una maggiore espressione: ciò significa che i cani trattati con Iken Up si "adattano" alla fatica fisica e mentale con minor sforzo** rispetto al gruppo "Controllo", eseguendo il test con le stesse prestazioni operative. Probabilmente, i cani trattati con Iken Up non avevano bisogno di attivare una maggior espressione genica, che si sarebbe tradotta in una maggior disponibilità proteica, poiché avevano raggiunto una maggiore resilienza alla performance.

In conclusione, si può affermare che **la supplementazione con Iken Up è in grado di fornire un supporto metabolico ed energetico che permette un migliore adattamento dell'animale, esercitando effetti benefici sul recupero delle risorse dell'animale, sul metabolismo energetico e sui biomarcatori del danno muscolare, in modo che uno sforzo fisico prolungato non rappresenti un evento stressante**. I risultati hanno importanti applicazioni gestionali e incoraggiano l'uso di routine di mangimi complementari nel regime alimentare dei cani da lavoro.

La supplementazione nutrizionale con Iken Up potrebbe rappresentare un'importante risorsa, oltre che per i cani impiegati per la ricerca della droga, anche per i cani da ricerca comunemente impiegati in interventi di soccorso in caso di calamità naturali (eventi sismici, valanghe, ecc.), che prevedono tempi di ricerca molto lunghi e un impegno fisico e mentale che può in alcuni casi risultare estenuante, poiché potrebbe aumentare le loro capacità di ricerca.

SCIENTIFIC REPORTS

OPEN

A cross-talk between blood-cell neuroplasticity-related genes and environmental enrichment in working dogs

Received: 11 January 2019
Accepted: 17 April 2019
Published online: 06 May 2019

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This study aims to identify a panel of blood-cell neuroplasticity-related genes expressed following environmental enrichment stimulation (EE). The Drug detection (DD) training course was an excellent model for the study of EE in the working dog. This research is divided into two experimental trials. In the First Trial, we identified a panel of blood-cell neuroplasticity related-genes associated with DD ability acquired during the training course. In the Second Trial, we assessed the EE additional factor complementary feeding effect on blood-cell neuroplasticity gene expressions. In the First and Second Trials, at different time points of the DD test, blood samples were collected, and NGF, BDNF, VEGFA, IGF1, EGR1, NGFR, and ICE2 blood-cell neuroplasticity related-genes were analyzed. As noted in the First Trial, the DD test in working dogs induced the transient up-regulation of VEGFA, NGF, NGFR, BDNF, and IGF, immediately after the DD test, suggesting the existence of gene regulations. On the contrary, the Second Trial, with feeding implementation, showed an absence of mRNA up-regulation after the DD test. We suppose that complementary feeding alters the systemic metabolism, which, in turn, changes neuroplasticity-related gene blood-cell mRNA. These findings suggested that, in working dogs, there is a cross-talk between blood-cell neuroplasticity-related genes and environmental enrichment. These outcomes could be used to improve future treatments in sensory implementation.

Recent scientific research in learning and memory neurobiology emphasizes the existence of multiple strategies that affect neurons with short- to long-lasting functional changes through genetic regulatory mechanisms responding to neuroplasticity[1]. Neuroplasticity is a dynamic process leading to functional changes in gene expression in response to changes in environmental experience[2–4]. Neuronal plasticity allows the nervous system to learn new skills, to consolidate and retrieve memories, and to reorganize neuronal networks in response to environmental stimuli[5].

Bloomsmith *et al.*[6] provided a useful categorization of animal environmental enrichment (EE) as a sensorial, physical, nutritional, and sociable advancement. EE gives animals a multisensorial stimulation through the molecular epigenetic mechanism bases of neuroplasticity[7]. Recent evidence suggests how epigenetic mechanisms could create a permissive state for learning to transform experiences into long-term memories by facilitating encoding in cortical sensory processes[8]. Increasing research supports the opinion that genes related to synaptic plasticity could change in expression during a period of EE[9]. One potential mechanism by which enrichment may improve learning, memory, and synaptic plasticity, is through the regulation of transcription factor activators[10] CREB (cAMP response element binding proteins). However, epigenetic changes behind EE persist unclearly, and the impact of EE on dog's brain plasticity and peripheral effects remains disputed.

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The mainstay of our study was the Guardia di Finanza (GdF)DD training course made available to us for investigation. For the dogs the course represents a high standard model of environmental enrichment due to the vast range of stimulations and experiences that provide cognitive challenges, social opportunities and promote the acquisition of new skills. The skills honed during training, and the intensive practice, force the dogs to adapt to EE with a related-genes remodeling "the genes learn from experience"[11]. An essential characteristic that gives value to our study, among the most salient and desirable behavioral attributes of working dogs, is the trainability and higher sensitivity to odorous molecules[12].

To date, no scientific literature concerning canine blood-cell neuroplasticity-related genes and specific brain regions is available, and ethical reasons hinder feasible canine brain access. Recent scientific literature, however, puts forward the view that peripheral blood mRNA could be used as a biological signature for brain disorder[13–17]. Sullivan *et al.* suggest that the cautious and attentive use of peripheral gene expression may be a useful surrogate for gene expression in the CNS when it has been determined that the relevant gene is expressed in both.

Our research aims to identify, using scientific literature and bioinformatic tools, a panel of neuroplasticity related-genes, and evaluate their expression levels in the blood-cell[18]. The question, therefore, arises as to whether, and to what extent, gene expression in peripheral blood samples is comparable to gene expression in the brain[19]. In this research, we divided the experimental design into two trials, and in both, the DD training course acted as the EE factor. In the Second Trial, we introduced the complementary feeding, given during the detection training course, as EE supplementation factors. In the First and Second Trial, immediately after the DD test, we assessed blood-cell neuroplasticity-associated mRNA levels and dog behavioral evaluation of drug detection performance.

This study could provide information on the possibility of using blood cell gene expression as markers of EE effects to improve future treatments in sensory implementation. In addition, the fact that EE could modify the phenotype of blood cells is interesting *per se*.

Materials and Methods

Experimental protocols for the study were approved by the Ethical Committee of the University of Perugia protocol number: n. 2018–21. A standing agreement between the Italian Military Force of Guardia di Finanza and the Department of Veterinary Medicine of the University of Perugia allows ethical testing on GdF working dogs.

Animal enrolment. The dogs enrolled in the study are described in detail in the Supplementary Material S1. The dogs were physically (i.e., X-ray negative for hip dysplasia and classed in good health by a veterinarian) and behaviorally tested (i.e., the absence of behavioral pathologies identified by a veterinarian behavioral consultant) to certify their suitability for work training. It is crucial to underline that the dogs enrolled in the First and Second Trial attended the DD training course during two distinct periods but acquired the same level of expertise. For this purpose, dog-handler teams participated in a six-month DD training course at the GdF dog breeding and training center (Castiglione del Lago, Perugia, Italy). The breeding male was different in the First and Second Trial. During the treatment period, the dogs were kept in single boxes and fed with a standard maintenance diet (raw protein = 26%, crude oils, and fats = 17%, crude ash = 6.5%, crude fiber = 1.2%) and water ad libitum.

Experimental design. The research study design was divided into two experiments called First Trial and Second Trial. Both included four sampling times: T0 - baseline value at rest, 24 hours before the search test; T1 - immediately before the search test; T2 - immediately after the search test, T3 - recovery time, 30 minutes after the end of the search test, to check if the dog had recovered baseline values. In the First and Second Trials, we assessed blood biochemical parameters (T0), heart rate (T0, T1, T2, T3), rectal temperature (T0, T1, T2, T3), DD dog performance (T2) and neuroplasticity-related gene expression profiles (T0, T1, T2, T3).

The First Trial aimed to evaluate if a panel of genes, selected by the *in silico* approach (see Supplementary Material Fig. S1), are involved in the neuroplasticity pathway activated during the DD test. In this trial, the experiment was led in one group of dogs observed at the beginning, and the end of the DD training course. The Second Trial was performed one year after the First Trial with a different group of dogs. This Trial studied only the genes correlated to neuroplasticity (according to the data obtained from the First Trial). The Second Trial was designed to assess the effect of complementary feeding on neuroplasticity mediated by changes of gene expression levels during a DD test. The experimental plane of the Second Trial involved two different groups of dogs, one fed, during the DD training course, with complementary feeding, and the other, without (control).

The first trial. In this trial, we focused on evaluating a group of dogs (n = 7), before and after the DD training course, in two different types of search tests: a problem-solving test in untrained (U) dogs, and a DD search test in trained dogs (timing and group in First Trial is illustrated schematically in Fig. 1). The DD training course included a wide range of stimulations related to different experiences, and there are well-founded reasons to view them as EE[20], where the dogs acquired skills in DD and physical aptitudes to exercise. For this reason, we classified the dogs after the DD training course as Enriched Environmental (EE) dogs. The problem-solving test was meant to explore the innate search in skills of U dogs, while in EE dogs, it tested the skills acquired during the DD training course. U dogs carried out the problem-solving test indoors, inside the GdF warehouse. The problem-solving test consisted of finding hidden food under ten plastic cups, arranged in a horizontal line one meter from each other, within a 15-minute time-frame. At the end of the six-month DD training course, the EE dogs performed a DD search test. The search test consisted of three, 10-minute search sessions, in which the dogs had to find the drug in: (1) luggage moving on a tapis roulant simulating an airport situation; (2) different types of containers positioned on the floor where confounding substances were hidden in addition to the drug; (3) in a crowded situation, where the drug was hidden on a person (all procedures are described in detail in the Supplementary Material: Drug Detection (DD) test).

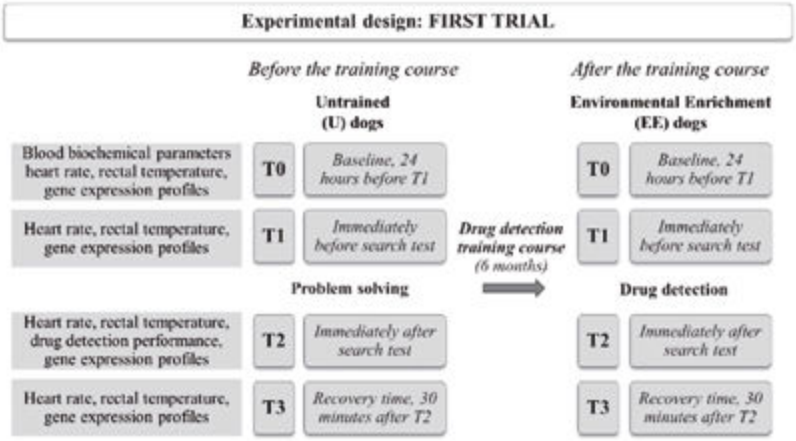

Figure 1. Experimental planning of the First Trial of the study. The figure schematizes the First Trial of the study that evaluates the dog gene expression levels at T1, T2, T3 in untrained dogs (U). In the First Trial, we assessed blood biochemical parameters (T0), heart rate (T0, T1, T2, T3), rectal temperature (T0, T1, T2, T3), observational examination of DD dog performance (T2) and neuroplasticity-related gene expression profiles (T0, T1, T2, T3).

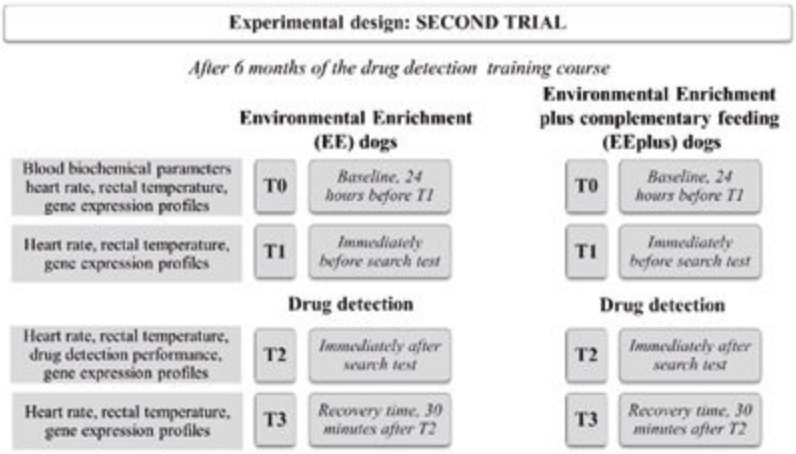

Figure 2. Experimental planning of the Second Trial of the study. The figure summarises two groups of dogs: one group on the left with the training course and the second group (right) with the training course plus complementary feeding. The timing and the parameters measured in the Second Trial are the same as in the First.

The second trial. The dogs involved in the Second Trial attended the GdF DD training course and were trained for DD. The trained dogs, in the Second Trial, were divided into two groups: Enriched Environmental plus complementary feeding (EEplus) dogs (n = 7) that received complementary feeding during the training course and EE dogs (n = 7, as a control group) (Fig. 2). The recommended doses of Complementary feeding (IKENUP® provided by Teknofarma, Italy) were two tablets/10 kg b.w./day the first week and one tablet/10 kg b.w./day for the next 11 weeks during the DD course. One tablet of complementary feeding is composed of: L-Leucine, L-Carnitine base, L-Valine, Fructose, L-Lysine, L-Alanine, D, L-Methionine, L-Isoleucine, L-Arginine, Vitamin C, Aspartic acid, Vitamin E, Magnesium, Calcium Pantothenate, Iron, Zinc, Vitamin B_1, Vitamin B_2, Vitamin B_6, Octacosanol, Vitamin B_{12} and Selenium (see Supplementary Material: Analytical content of Iken Up tablet).

Physiological parameter and blood collection. The GdF veterinarian collected blood samples by radial venepuncture, monitored heart rate (HR) in beats per minute with a stethoscope (Classic II S.E., 3 M™ Littmann®) and measured rectal body temperature (RBT) with a digital thermometer (MB TERMO 7126500, Reckitt Benckiser SpA, Milano, Italia) at each sampling time (T0, T1, T2, T3), in each dog belonging to U, and EE (First Trial); and EE, and EEplus (Second Trial). In the First, and Second Trial, blood sampling was separated into two collection tubes: one for the analysis of biochemical parameters and one for the evaluation of gene expression profiling. Blood sampling for laboratory analysis and gene expressions are described in the Supplementary Material (Gene mRNAs and laboratory analysis blood sampling).

Gene Symbol	TaqMan ID
EGR1	Cf02741635_m1
NGFR	Cf02697141_u1
VEGFA	Cf02674018_m1
BDNF	Cf02718934_g1
ICE2	Cf02632187_m1
NGF	Cf02697134_s1
IGF1	Cf02627846_m1
ACTB	Hs03023880_g1

Table 1. TaqMan Gene Expression probe used in the studies.

Assessment of drug detection performance. Dogs and their handlers waited in an adjacent structure until called to carry out the test. The test was considered successful if/when the DD dog actively signaled the presence of the target odor by barking at his/her handler and digging in-place or on the container where the drug was hidden. The drug test was considered concluded when the handler raised his/her hand to communicate that his/her dog had signaled the presence of the target odor. The maximum time allowed to find and signal the hidden drug was 10 min when the time expired, the test was stopped and considered a miss. During the trial, an operator, situated in a nearby search area, filmed the dogs using a Digital Video Camera Recorder (Canon® MD160 and SONY® DCR-SR58, Sony®). The authors recorded the following performance data: search outcome and success/failure rate as well as detection time (in seconds) at signaling, needed for the DD dogs to find the target odor.

Next, the videos were analyzed to measure both search thoroughness and to investigate potential failure reasons in dog task performance.

Gene expression levels of neuroplasticity-related genes: RNA isolation, reverse transcription, and qPCR amplification. The authors used Total RNA Purification kit (Norgen Biotek Corp™) to extract RNA to 100 µL of whole blood containing RNA Later, according to manufacturer instructions. RNA was quantified with the Qubit RNA assay (Life Technologies, Carlsbad, CA, USA) and stored at −20 °C until use. To ensure the purity of RNA from possible DNA contamination, we performed an additional step with DNase (RNase-Free DNase I Kit, Norgen Biotek Corp™). Total RNA (20 ng) was reverse transcribed in 20 µL iSCRIPT cDNA (Bio-Rad, Hercules, CA, USA), according to manufacturer suggestions. The authors included controls without reverse transcriptase to check for genomic DNA contamination. We carried out the qPCR amplification by using 4 µL of cDNA (diluted 1:20), 10 µL TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL TaqMan Gene Expression Assays (Table 1) and water in a final volume of 20 µL. Agarose gel electrophoresis verified sample amplification fidelity. Thermocycling conditions on an iCycler iQ qPCR (Bio-Rad, Hercules, CA) consisted of an initial denaturation of 10 min at 95 °C, followed by 45 cycles of 15 seconds at 95 °C for denaturing double-stranded DNA and 1 min at 60 °C for annealing/extension steps. Bio-Rad software plotted the fluorescence intensity against the number of cycles and provided the cycle threshold (Ct) value using the automatic method. Each sample was run in triplicate and results were averaged. In parallel, to assess a genomic DNA contamination, no RT control for every RNA sample was evaluated. QPCR amplification efficiency and qPCR conditions were determined as described by Diverio *et al.*[21]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of the target genes[22].

Statistical analysis. The authors chose to apply nonparametric tests because it is most appropriate with small sample sizes, and when the data does not show normal distribution. The qPCR data ($2^{-\Delta\Delta Ct}$) were analyzed with the Kruskal-Wallis test, and the Mann-Whitney test was used to compare drug detection performance. Range, medians (Mdn) and interquartile ranges (IQR) of DD times were reported. The post hoc analyses were performed using Dunn's post hoc test of multiple comparisons following a significant Kruskal-Wallis test. We performed both analyses with GraphPad Prism Software (GraphPad, San Diego, CA, USA) and used $P < 0.05$ as the cut-off for statistical significance.

Results

Biochemical blood parameters and physiological data. The blood biochemical parameters evaluated at T0 (24 hours before T1) First Trial (U and EE dogs) and Second Trial (EE and EEplus dogs) were comprised within the canine reference range values (Table 2). These results allow us to exclude the presence of limiting and evident dysmetabolic states that would have led to the exclusion of some subjects from the study. At T0 (First Trial and Second Trial), the mean heart rate (70–155 bpm) and rectal temperature (38.6–39.1 °C) were within the normality range, in accordance to the physiological state of the dogs (rest and work activity).

Dog behavioral evaluation of drug detection performance. All trained dogs, both in the First (EE dogs) and Second (EE dogs and EEplus dogs) Trial, detected the presence of the drug within the maximum time allowed (10 minutes). With regards to the effect of the complementary feeding, no significant differences were found in detection time. All the dogs were able to indicate where the drug sample was hidden with a detection range of 13–80 seconds for EE in the First Trial (Mdn = 46, IQR = 14–75 seconds). In Second Trial, the detection range was 13–80 seconds for EE (Mdn = 38, IQR = 16–47 seconds) and 5–86 seconds (Mdn = 45, IQR = 11–75 seconds) for EEplus. In none of the trials did the authors observe signs of stress or anxiety in the dog behavior. During the search tests, the dogs remained calm and focused on their tasks.

Blood biochemical parameters	T0 First Trial	T0 Second Trial	Normal values
Chlorine (Cl) mmol/l	101.2 ± 3.9	109.5 ± 8.8	105–115
Ferro (Fe) µmol/l	24.9 ± 7.6	26.7 ± 9.2	15–40
Phosphorus (P) mmol/l	1.0 ± 0.19	1.37 ± 0.16	0.84–2
Glutamic Oxaloacetic Transaminase (GOT) U/l	24.5 ± 3.5	24.0 ± 5.7	23–44
Glutamic Pyruvic Transaminase (GPT) U/l	35.1 ± 11.1	37.0 ± 13.8	10–50
Alkaline Phosphatase (ALP) U/l	45.2 ± 3.2	37.6 ± 3.2	20–120
Glucose (mmol/l)	3.9 ± 0.4	5.5 ± 0.7	3.61–6.55
Lactate dehydrogenase (LDH)	174.7 ± 55.0	63.3 ± 10.6	45–233
Non-Esterified Fatty Acids (NEFA) mmol/l	0.44 ± 0.05	0.34 ± 0.05	0.4–0.7
Creatine Kinase (CK) U/l	80.3 ± 23.0	118.2 ± 34.3	30–120
Creatinine µmol/l	116.4 ± 15.5	104.5 ± 15.6	44.2–132.6

Table 2. Blood biochemical parameters. Averages ± standard deviations of blood parameters evaluated in the dogs at T0 (24 hours before T1) First Trial (U and EE) and T0 Second Trial (EE and EEplus).

Gene expression profiles of neuroplasticity-related genes. Total RNA yield was not significantly different among samples. RNA concentrations ranged from 4 to 6 ng/µl. Minimal variations in total RNA content were corrected during reverse transcription using fixed RNA input. RNA ratio 260/280 was close to 2.0 (1.8–2.0). The qPCR relative expression value was calculated by using the formula: $2^{-\Delta\Delta Ct}$, where ACTB is the reference gene and T0 the control gene (ΔCt target mRNA = Ct target − Ct ACTB; $\Delta\Delta$Ct = ΔCt target − ΔCt T0).

First Trial data. The qPCR data in U dogs did not show a significant difference ($P > 0.05$) comparing T1, T2 and T3 relative expression values of NGFR, VEGF-A, BDNF, EGR1, IGF, NGF, ICE2 genes. However, all the genes revealed an upregulation trend at T2. Conversely, five genes in EE dogs showed a significant increase in relative expression levels at T2 (immediately after the DD test) compared to T1 ($P \leq 0.05$ for VEGFA, and NGF; $P \leq 0.01$ for NGFR, BDNF, and IGF (Fig. 3). ICE2 and IGF1 still trended up but not significantly.

Second Trial data. In this trial, only the genes statistically different at T1 and T2 (First Trial) were analyzed (NGFR, VEGF-A, BDNF, IGF1, and NGF). Findings in the Second Trial highlighted that the T2 gene expression levels of EE dogs showed a statistically significant upregulation ($P \leq 0.05$ for NGF; $P \leq 0.01$ for NGFR, BDNF, and IGF; $P \leq 0.001$ for VEGFA) as observed in the EE group of the First Trial. Conversely, in EEplus dogs, relative expression levels did not show a statistically significant difference between timepoints. Comparing T2 EE relative expression levels, and T2 EEplus, only the genes NGFR, VEGFA, and IGF1 ($P < 0.01$, $P < 0.001$, and $P < 0.01$ respectively) showed a statistically significant upregulation (Fig. 4).

In the First and Second Trial, when comparing T1 and T3 relative expression value, there were no statistically significant differences. The gene expression levels at T1, T2 and, T3 in EE and EEplus did not indicate significant variations.

Discussion

This study focuses on how environmental enrichment factors directly influence the expression levels of blood-cell neuroplasticity-related genes in the working dog. The neural homeostasis is regulated by a complex neuroendocrine system involving many central and peripheral signals, but the signaling pathways regulating canine neuroplasticity are mostly unknown. Thereby, we do not assume that the mRNA found in the blood comes from the central nervous system (CNS), but that the enriched environment can influence the blood-cell[23–25] neuroplasticity-related gene regulation as well as the CNS. Blood-cell and CNS correlation may seem more reasonable if one considers that lymphocytes often travel through many different body regions and may be exposed to the same environment as CNS tissue. This may be particularly true for the pituitary and hypothalamus (glands); having relatively direct access to the blood, they detect and respond to its changes.

Moreover, we would like to emphasize that the tissue-specific pattern of mRNA expression may indicate main clues concerning gene function. The blood transcript levels, sharing more than 80% of the transcriptome with major tissues, are exploited as EE molecular signatures in nutritional enrichment to discriminate the individual animals that benefit from nutrient supplementation[26–29]. Besides, peripheral blood mRNA expression levels are measured as a reference for monitoring an olfactory environment[30,31]. Furthermore, the use of whole blood as a surrogate for CNS expression may be permissible in the investigation of some sets of genes; it is known that on a transcriptome level, whole blood shares significant similarities in gene expression with CNS tissues[18]. The whole blood BDNF showed the same mRNA median level as CNS (excluding amygdala, hypothalamus, cerebellum peduncles[32]). No study showed a long-lasting BDNF response; therefore after EE stimulation, the basal peripheral BDNF needs to increase. In healthy humans, the brain contributes to almost 75% of circulating BDNF, suggesting that the brain is the major, but not sole, contributor to circulating BDNF while a quarter of circulating BDNF seems to stem from peripheral sources. A source of circulating BDNF mRNA/protein is the peripheral blood mononuclear cells: T and B lymphocytes[33–35]; eosinophils[36,37], monocytes[34,35]. After the synthesis, "new BDNF" is released into the blood circulation which may, in turn, be absorbed more efficiently by central and/or peripheral tissues, where it could induce a cascade of neurotrophic and neuroprotective effects[5]. Environmental

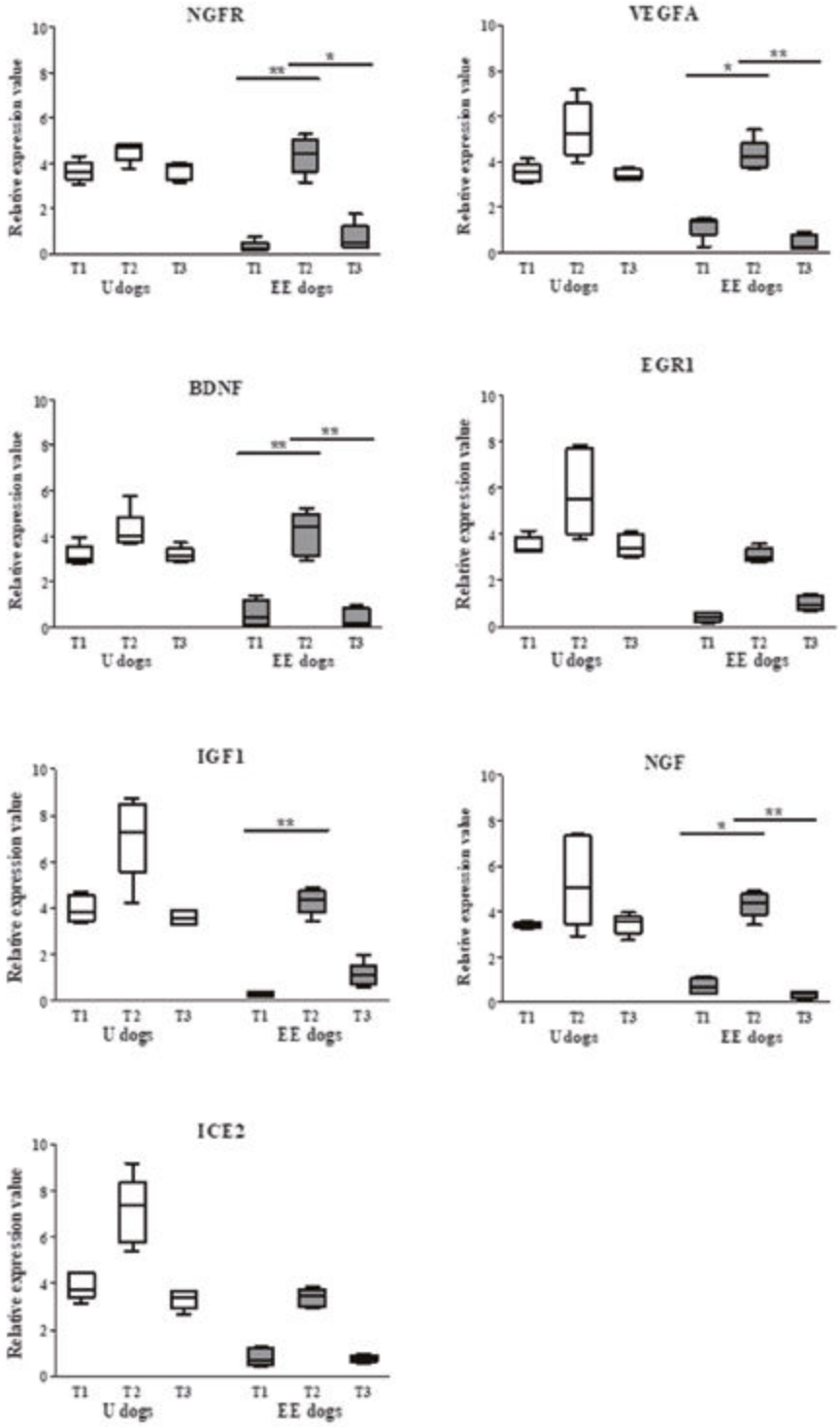

Figure 3. First Trial relative expression values of blood-cell neuroplasticity-related genes. The graph shows the relative expression levels at T1, T2, T3 in U dogs and EE dogs. T2 (white) represents U dogs values immediately after PS, whereas T2 (grey) indicates gene expression levels of EE dogs immediately after the DD test. The relative expression values of genes (y-axis) are presented as median (Mdn) and interquartile range (IQR) of $2^{-\Delta\Delta Ct}$. The EE dogs relative expression levels of genes NGFR ($P \leq 0.01$), VEGF-A ($P \leq 0.05$), BDNF ($P \leq 0.01$), IGF1 ($P \leq 0.01$) and NGF ($P \leq 0.05$) showed a significant difference between T1 *versus* T2. All genes included in the study presented that the recovery time (30 minutes) was sufficient to return to T1 baseline value because there are no statistically significant differences between T1 and T3.

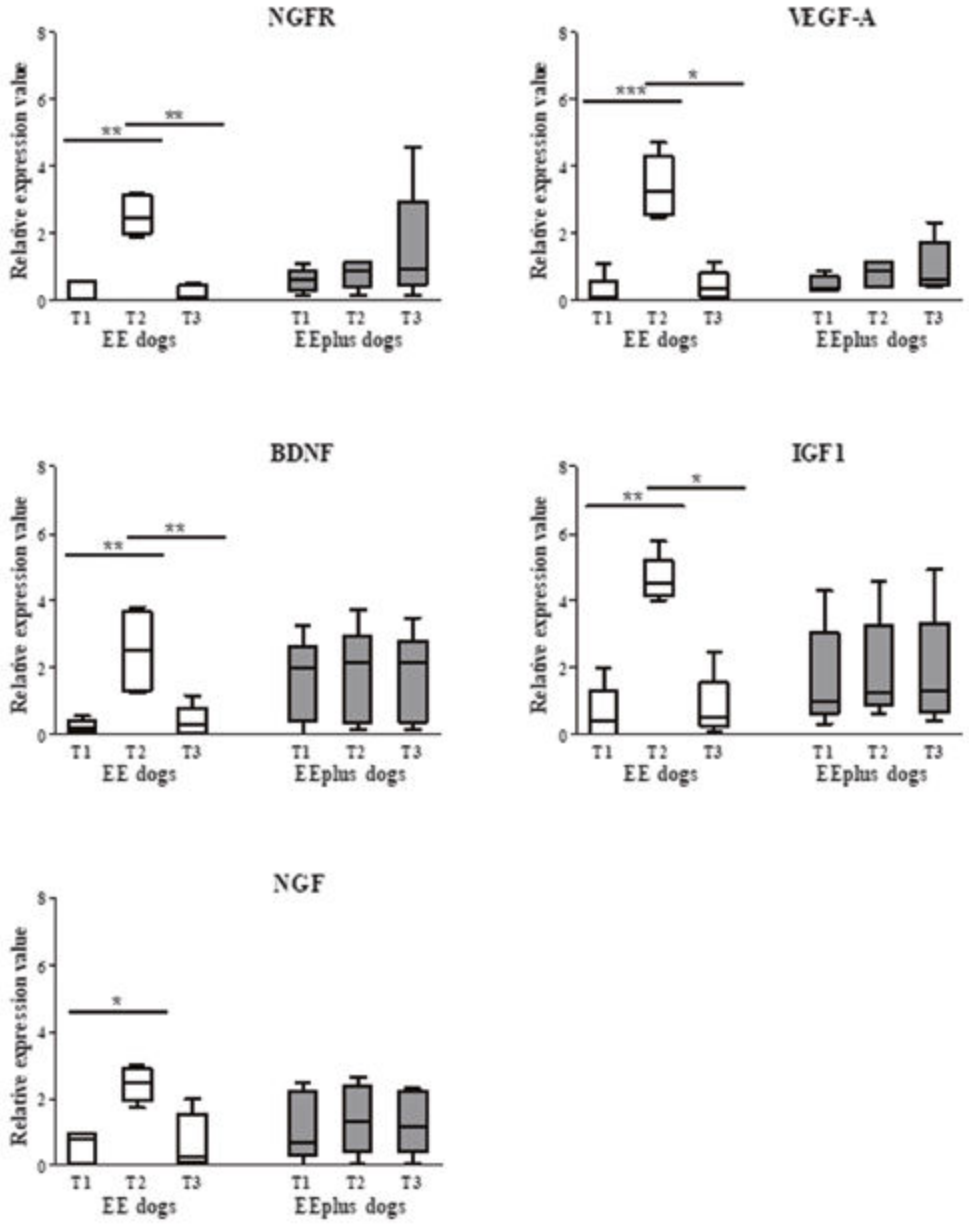

Figure 4. The Second Trial relative expression values of blood-cell neuroplasticity-related genes. The relative expression levels of NGFRP ($P \leq 0.01$), VEGF-A ($P \leq 0.001$), BDNF ($P \leq 0.01$), IGF1 ($P \leq 0.01$) and NGF ($P \leq 0.05$) genes of EE group (white) showed a significant difference between T1 *versus* T2 (as in the First Trial). While in EEplus group (grey) relative expression values revealed no significant differences between T1, T2, and T3. All genes included in the study presented a recovery time sufficient to return to baseline.

enrichment is known to exert a variety of epigenetic modifications in the brain and behavior, including neuroplasticity, improved learning, and memory. This research does not propose to evaluate which epigenetic mechanisms underlie the different regulation of gene expression, but rather the effects of environmental enrichment associated with the DD training course on circulating neuroplasticity-related genes.

The Dog Detection GdF training course ranks first in Italy and is among the best both in Europe and internationally[38]. It is based on the principle of play versus constraint in addition to favoring the animal's instinct. The GdF drug detection training course represents an optimal environmental enrichment. Moreover, it is a learning trial that includes the acquisition of olfactory expertise and a broad range of socialization skills; this enables the dogs to move freely among crowds of people without being a risk or nuisance. An additional environmental enrichment in the GdF DD training course is the physical exercise which prepares the dogs to work with less physical effort.

In this manuscript, we examined gene levels after the drug detection training course (First Trial) and after complementary feeding given during the training course (Second Trial). We deliberately avoided a comparison between U and EE dogs because the DD training course period (6 months) is too extended to compare gene

expression levels in dogs aged from 2 to 3 years old. In our opinion, during this time, a multitude of factors could induce genes to switch between activity states. In addition, we did not compare results of EE dogs-First Trial with EE dogs-Second Trial because individual differences (different parents; individual personality which could influence the way the animals perceive EE challenges) along with environmental differences (most popular drugs trafficked while the course was being held and the instructor who trained the dogs) were relevant in the analyses of a small number of dogs.

Previous studies, conducted in dogs, defined that some skills are acquired through repetitive practice to allow dogs to live in human groups[39,40]. In the DD dogs, the work skill ability has sometimes been described as a reduction in the speed of movement execution and an increase in accuracy[41]. In mice[42] and rats, repeated exposure to an odorant could increase the olfactory receptor cells sensitivity to that odorant, due to a neuroplasticity mechanism[43].

In the beginning of our study, and based on these theories, we hypothesized that the DD training course could teach the dogs how to search, enhancing their DD abilities through the increase of olfactory sensitivity and acquisition of skills through the mechanism of neuroplasticity. Mammalian studies have found evidence supporting a role for neurotrophic factors[44,45] BDNF and NGF in growth and survival of 70–80% of sensory neurons[46] during learning experiences (plasticity)[47]. The BDNF gene may be the critical protein in which transcription is enhanced during olfactory learning[48]. BDNF, NGF, NGFR and IGF genes could also be related to voluntary exercise[49,50]. Ando *et al.* (2016) studied the changes of the NGF gene in dog serum, demonstrating that epigenetic factors such as exercise, could modify NGF expression levels[51]. Regarding rodents and humans, studies have shown that exercise and intermittent fasting[52] could enhance cognitive performance and increase serum BDNF levels[53,54].

In the First Trial, the outcomes of our research paper showed a gene upregulation immediately after T2 only in EE dogs. This change in mRNA levels of neurotrophins NGF, BDNF, receptor NGF, growth factors VEGFA and IGF1 underlines the ability of EE dogs to activate blood-cell neuroplasticity-related genes during the DD test. Peckham *et al.*[11] defined epigenetic dogma as the condition in which "genes learn from experience". Conversely, gene expression in untrained dogs showed no significant difference, emphasizing that the dogs, in the absence of the DD training course, were unable to stimulate the molecular mechanism related to blood-cell neuroplasticity-related genes. To elucidate the up-regulation role of VEGF-A, BDNF, IGF, and NGF genes in EE dogs after the DD test, we need to clarify the intracellular signaling pathways that mediate gene expression. A central role in neuroplasticity has the transcription factor Cyclic AMP (cAMP)-responsive element-binding protein (CREB) because it could increase the expression of genes that modulate memory and skills[10,55,56]. CREB binding cAMP response element activated by cAMP regulates RNA polymerase activity, controls gene expression and induces transcription of growth factors (IGF-1, VEGF-A), and neurotrophins (BDNF, NGF)[10,57]. The transcriptional activity of CREB depends on its phosphorylation status and is a critical mechanism in neurogenesis regulation induced by environmental enrichment[58]. CREB could be considered as a regulator of learning, memory, and neuroplasticity[57] in the majority of species[59–61].

To the authors' knowledge, there is no literature clarifying the mechanism by which BDNF, NGF, VEGFA, IGF1 and NGF receptors are not upregulated at the end of the DD test in dogs with complementary feeding in addition to environmental enrichment. Scientific evidence, in mice, shows that food intake suppresses up-regulation in genes via a BDNF-dependent mechanism[62,63]. However, it is difficult to delineate the precise effects of nutrients or bioactive food components in each epigenetic modulation because nutrients also interact with genes, other nutrients, and other lifestyle factors. Furthermore, each epigenetic phenomenon also communicates with the others, adding complexity to the system and probably, complementary feeding affected epigenetic mechanisms at multiple levels[64].

First, nutrients act as a source of methyl groups or as co-enzymes for one-carbon metabolism that regulates methyl transfer[65]. Second, nutrients and bioactive food components could directly affect enzymes that catalyze DNA methylation and histone modifications[66]. Third, diet is the last input determining systemic metabolism which modifies cellular milieu leading to alterations in epigenetic patterns. We suppose that one of the effects of complementary feeding, used in this study, was the presence of vitamins C, E, B1, B2, B6, and B12 which reduce oxidative damage and protect reactive oxygen stress resulting in energy increase. Moreover, the reduction of oxidative stress would interact with the BDNF system leads[67] to a loss of cell function, apoptosis, or necrosis[68]. Furthermore, soluble vitamin B has an effect on DNA methylation, carries a methyl group and delivers that methyl group for the synthesis of S-adenosyl methionine, the unique methyl donor for DNA methylation reactions[64,69,70].

The authors hypothesize that the complementary feeding during a high metabolic consumption (detection test) in dogs, may render the BDNF up-regulation unnecessary. Energetic homeostasis and olfactory perception are linked. The feeding state modulates olfactory sensitivity in animals and humans owing to crosstalk between the feeding state and olfactory perception. The olfactory system is not merely a sensor of external chemical cues, but a parallel detector of internal chemical cues (the chemistry of metabolism)[71].

To explain the lack of differences in the recorded DD times among the EE and EEplus dogs, we could hypothesize that the potential DD skills of the trained dogs, at the end of the training course, were expressed, in all dogs, at their maximum working capacity, therefore overriding the additional positive effect of complementary feeding. It is possible that food supplementation becomes valuable when dogs need to cope with more difficult psycho-physical conditions, such as in the case of avalanche searches and rescue situation, who often face long working periods in extreme environmental conditions. The social impact of working dogs needs a more profound understanding of all the factors that could interfere with their successful performance. This study has limitations and thus, encourages future investigations. Firstly, the size of the sample studied; although this depends on the limited number of dogs accepted in the DD training course. Besides, the First Trial used a different test for U and EE dogs; however, it was impossible to do otherwise because the dogs acquired skills only after the training

course. To achieve well-defined goals, further investigation will be required to study the limiting factors concerning the performance of working dogs, including diet and health.

Conclusion

In this study, we demonstrated that blood-cell neuroplasticity-related mRNA responds to enriched environmental signals. In the First Trial, we found a significant gene upregulation after DD. In the Second Trial, the blood-cell neuroplasticity-related genes associated with DD showed the absence of transient upregulation only in dogs with complementary feeding. Presumably, the complementary feeding allowed the dogs to carry out the test effortlessly with the same DD performance. We suppose that complementary feeding alters the systemic metabolism, which, in turn, changes BDNF and blood-cell neuroplasticity-related gene mRNA levels. The research outcomes suggest that there is a cross-talk between blood-cell neuroplasticity-related genes and environmental enrichment. The advancement of blood-cell neuroplasticity-related genes understanding should lead to improving future treatments in sensory implementation.

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Acknowledgements

We would like to extend our sincere gratitude and appreciation for all of the work and dedication provided by all military the GdF personnel (Walter Di Mari, Roberto Pulicani and Massimo Teodoro, and all the Dog Trainers and Handlers), and the LEBA collaborators (Claudia Enas, Federica Scotti, Roberta Gobbi, Danilo Zampini) for their precious and outstanding contributions. Medical writing assistance was provided by Diana Cella, writer on CLA University of Perugia. We gratefully acknowledge Teknofarma S.p.A. particularly Dr. Giovanni Valle, whose long-standing commitment to improving animal health and wellbeing through research. Teknofarma S.p.A. had no role in the study design, collection, analysis, and interpretation of data, or in writing the manuscript and the decision to submit it for publication.

Author Contributions

G.G. and S.D. made substantial contributions to conception and design; acquisition of data was undertaken by G.G., A.B.C., L.M.[1], M.B., C.S., P.C., M.M.S.; data analysis and interpretation were undertaken by G.G. and S.D. the manuscript was drafted by G.G., M.B. and S.D., and all authors critically revised the manuscript for important intellectual content. S.D. obtained funding. L.M.[3]provided biochemical laboratory analysis.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-43402-4.

Competing Interests: The authors declare that the research was conducted in the absence of any financial and non-financial competing of interests that could be construed as a potential conflict of interest, including specific interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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RESEARCH ARTICLE

Benefits of dietary supplements on the physical fitness of German Shepherd dogs during a drug detection training course

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OPEN ACCESS

Citation: Menchetti L, Guelfi G, Speranza R, Carotenuto P, Moscati L, Diverio S (2019) Benefits of dietary supplements on the physical fitness of German Shepherd dogs during a drug detection training course. PLoS ONE 14(6): e0218275. https://doi.org/10.1371/journal.pone.0218275

Editor: Juan J Loor, University of Illinois, UNITED STATES

Received: January 31, 2019

Accepted: May 29, 2019

Published: June 14, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: The study was partially financed by Teknofarma Spa. The funder Teknofarma Spa had no role in the study design, collection, analysis and interpretation of data, or in writing the manuscript and the decision to submit it for publication.

Competing interests: We have the following interests: The study was partially funded by Teknofarma Spa. We have used as diet integration

Abstract

A high standard of physical fitness is an essential characteristic of drug detection dogs because it affects not only their ability to sustain high activity levels but also their attention and olfaction efficiency. Nutritional supplements could improve physical fitness by modulating energy metabolism, oxidative processes, and perceived fatigue. The aim of this study was to investigate the physiological and biochemical changes induced by submaximal exercise on drug detection dogs (German Shepherd breed) and to assess whether a dietary supplement improves their physical fitness. During a drug detection dog training course, seven dogs were fed with a basal diet (Control Group) for three-month period, while a further seven dogs were fed with a basal diet as well as a daily nutritional supplement containing branched-chain and limiting amino acids, carnitine, vitamins, and octacosanol (Treatment Group). At the end of this period, individual physical fitness was assessed by making each subject take a graded treadmill exercise test. A human heart rate monitor system was used to record the dog's heart rate (HR) during the treadmill exercise and the subsequent recovery period. The parameters related to HR were analysed using nonparametric statistics. Blood samples were collected before starting the nutritional supplement treatment, before and after the treadmill exercise and following recovery. Linear mixed models were used. The dietary supplements accelerated HR recovery, as demonstrated by the lower HR after recovery ($P<0.05$) and Time constants of HR decay ($P<0.05$), and by the higher Absolute HR Recovered ($P<0.05$) recorded in the Treatment group compared with the Control dogs. The supplemented dogs showed the lowest concentrations of creatine kinase (CK; $P<0.001$), aspartate aminotransferase (AST, $P<0.05$) and non-esterified fatty acids (NEFA; $P<0.01$) suggesting a reduction in muscle damage and improvement of energy metabolism. These data suggest that this combined supplement can significantly enhance the physical fitness of drug detection dogs.

a commercial product of the Teknofarma (Iken Up). There are no patents, products in development or further marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

Introduction

Since ancient times, working dogs have been considered as extremely accurate and flexible extensions of mankind's senses and capabilities. Despite modern technological advancements, machines fail to match the competence of dogs trained for a range of tasks, i.e. for detecting explosives and narcotics or searching for missing people and avalanche victims [1–5]. For this reason, drug detection dogs are used by law-enforcement authorities all over the world [6].

However, narcotic detection is a complicated and unexplored science. The dog's skills and disposition must match with appropriate training, with the handler's abilities as well as with the entire dog-handler team. There are multiple physical and behavioural traits that are essential for detection dogs. Detection work requires dogs to have social-cognitive skills, innate characteristics and maintain engagement and motivation [6]. However fitness and physical training are equally essential requisites [6–8]. Detector dogs should be athletic and be trained to work long hours in physically challenging and complex search environments. They should work quickly but without missing the intended targets or exhausting themselves prematurely [6,8]. Indeed, extreme physical activity and high temperatures that result in overheating divert the dogs' attention away from the scent detecting task (sniffing) towards maintaining their body temperature within normal limits through panting. Since dogs are unable to sniff and pant simultaneously, panting decreases sniffing rate and consequently olfaction and detection efficiency [6,8]. From a behavioural point of view, fatigue can negatively affect the strategies used by dogs in response to task demands, thus reducing the effectiveness of problem-focused coping and attention and amplifying emotion-focused coping at the same time [9].

Similarly to athletes, nutritional supplements can improve the fitness of working dogs. A recent meta-analysis by Bermingham et al. [10] showed that energy requirements of working dogs are only marginally lower than those of racing dogs. However, they did not consider specific requirements such as micronutrients and did not distinguish between the various breeds of working dogs. Some studies have evaluated the effect of supplementation on aged dogs [11,12], dogs with particular pathologies or deficiencies [13–15] or on specific aspects such as poor reproductive performance [16,17]. Vassalotti et al. [18] recently suggested that the athletic performance of search and rescue dogs may benefit from n-3 PUFA supplementation. However, previous studies have mainly been carried out on sled dogs or racing Greyhounds [19–22] while few studies have focused on the importance of quality nutrition and supplementation for working dogs [10].

Physical fitness has been defined as "the ability of the organism to maintain various internal equilibria as closely as possible to the resting state during strenuous exertion and to restore promptly after exercise any equilibriums that have been disturbed" [23,24]. This study mainly focuses on exercise-induced physiological changes as indices of physical fitness. Heart rate is a good indicator of fitness and has been used to evaluate the physical fitness of dogs in several experimental settings [1,4,7,23,25–27]. Some mathematical models of heart rate kinetics in response to exercise [28], have not only been used to evaluate animal fitness and cardiovascular health but also for assessing physical performance and the effectiveness of sports and fitness training programs. For example, the anaerobic threshold measures the ability to perform at high intensity for prolonged periods of time [29] while the Absolute HR Recovery (HRR) and Time constants of the post-exercise HR decay (τ20) are indices of heart rate recovery [4,30,31]. Many biochemical parameters are also useful for assessing the degree of physical exertion, wellbeing and recovery potential of dogs after exercise. Increases in plasma muscle enzymes such as creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) can indicate muscle damage and fatigue [4,5,7,23,32–37]. Indeed, exercise could result in increased membrane permeability, linked to the increase in oxidant species, or membrane

breakage at the level of the sarcolemma and Z-disks. [35,37]. These localized damages result in the release of muscle enzymes into the circulation that, in healthy subjects, could be transitory and reflect the physiological response to an incremental exercise test [32,36,37]. However, the degree to which serum enzymes increase is affected by several factors such as age, breed, gender, exercise type, training and also nutritional interventions [37–39]. Then, they have been used in the dog not only for the diagnosis of pathological conditions such as myopathies [38,40], but also to study physiological adaptations [5,33,36]. Moreover, changes in glucose and non-esterified fatty acids (NEFA) can indicate glycogen storage capacity in muscle and, more generally, the animal energy balance [4,5,23].

In the present study, we hypothesize that a dietary supplementation, modulating the energy metabolism, oxidative processes and perception of fatigue, could improve the physical fitness and ultimately help drug detection dogs to sustain their training course and their future work. We assume biochemical and cardiovascular changes during an incremental submaximal exercise test as indicators of dogs' physical fitness.

Then, the aim of this study was to determine the effects of a nutritional supplement, containing branched-chain and limiting amino acids, carnitine, vitamins, and octacosanol, on the physical fitness of drug detection dogs. For this purpose, an experimental protocol was developed to evaluate the physiological and biochemical responses of these dogs to treadmill exercise.

Materials and methods

The experimental protocol of the study was approved by the Ethical Committee of the University of Perugia protocol number: n. 2018–21 and complies with the laws established by the Italian Ministry of Health. There is a standing agreement between the Italian Military Force of GdF and the Department of Veterinary Medicine of Perugia University for the ethical testing and study of GdF working dogs.

Animals and diet

Fourteen drug detection dogs were enrolled in this study. All of them were German Shepherd dogs, 4 females and 10 males (S1 Table) which were aged between 2–3 years and weighed between 21.7–30.5 kg. All of the dogs were born and reared in the same place, at the GdF (Guardia di Finanza), Dog Breeding and Training Centre, Castiglione del Lago, Perugia, Italy. The dogs were individually housed in indoor pens (2.9 m x 2.4 m x 2.3 m) and walked and/or trained on a daily basis for approximately 4 hours. All of the dogs were physically (i.e. found to be in good health by a veterinarian and x-rayed to confirm the absence of hip dysplasia) and behaviourally tested (i.e. absence of behavioural pathologies assessed by a veterinary behaviour consultant) in order to determine their suitability as detection dogs. All of the dogs–and their handlers–participated in the same drug detection training course. The dog's training course, lasting six months, included sessions of basic obedience, physical exercises, socialization with people and environmental cues and drug search learning tasks carried out in several operational environments. The training course is based on the motivation of the dog to play and essentially on the principles of conditioning and learning focused on the search, detection and discrimination of drugs through the sense of smell. We would like to underline that, to protect the safety of the dogs, during the training only *pseudodrugs* are always used. These are chemical derivates of real drugs (marijuana, hashish, heroin, etc.), synthesized in a secret laboratory authorized by the Italian Ministry of Health, maintaining the same smell of the drugs but not having any effect. Under operational conditions, the fact that dogs are motivated to search for

drugs to obtain their "prize" (a sleeve or a ball) to play with and not for the drug itself, significantly reduces any risk of contact contamination or inhalation of real drugs.

At the beginning of the training course the 14 drug detection dogs were randomly distributed into two groups: Treatment (n = 7) and Control (n = 7) group. During all six months of the training course, both groups were fed a nutritionally complete and balanced dry dog food (S2 Table) and had ad libitum access to water. Besides, only for the last three months of the training course, the dogs of the Treatment group also received dietary supplementation composed of commercially available branched-chain amino acids (BCAA) and other limiting amino acids (LAA), carnitine, vitamins, trace elements, octacosanol (Iken Up,Teknofarma, Torino, Italy, S3 Table). The supplementation consisted of two tablets/10 kg b.w./day the first week and one tablet/10 kg b.w./day for the following 11 weeks.

Exercise test

At the end of the training course and dietary supplementation (Treatment group), all of the dogs were submitted to a submaximal exercise test in order to assess their physical fitness level (Fig 1). All dogs had been acclimated to treadmill experience by running 15–20 min once a week for the previous 6 months. These treadmill exercises were part of the activities included in the training course, but no specific speed protocols were foreseen. Only one dog refused to walk on the treadmill and was exempted from exercise. The Exercise Test developed for the present experimentation was performed on a motorized treadmill (professional canine treadmill, Grillo, Modena, Italy) between 9:00 and 12:00 a.m.. Both the speed and incline can be set on this treadmill. The dogs were not fed for at least 2 h before the test but were allowed to drink fresh water ad libitum. During the Exercise Test all of the handlers kept their dogs on leashes in the same way. In particular, each handler positioned in front of the treadmill keeping the dog on a leash without pulling it and providing positive vocal reinforcement to the dog.

The session (total 27 min) was conducted setting the treadmill at different walking speeds and inclinations at pre-set time intervals (S1 Fig): 5 min at a 2.5% incline at 3.8 km/h (walking, Phase 1), 5 min at a 5% incline at 3.8 km/h (walking, Phase 2), 10 min at a 2.5% incline at 7.0–8.0 km/h (trotting, Phase 3), 7 min of cool down at a 2.5% incline at 3.8 km/h (walking, Phase 4).

Physiological parameters

Blood samples (5 ml) were taken from the saphenous vein of each dog before starting nutritional supplementation treatment (T0), immediately before the Treadmill Exercise Test (T1), within the first 2 minutes from exercise test termination (T2) and 30 min after Exercise test termination, considered as passive recovery period (T3; Fig 1). Glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), non-esterified fatty acids (NEFA), and creatine kinase (CK) were analysed using an automatic chemistry bioanalyser (Konelab 200, Italy). The analytical methods and reagents (CK, AST and LDH: Sclavo Diagnostic Dasit-Italy; NEFA: Randox, Crumlin, UK) and measure units adopted were designed for this instrument.

Rectal temperatures were assessed prior to treadmill exercise, immediately after and 30 min post-exercise using a digital thermometer (MB TERMO7126500, Reckitt Benckiser SpA, Milano, Italia).

Fifteen minutes before starting the treadmill exercise, the dogs were equipped with a Polar System (PolarM400's built-in GPS, Polar H7 heart rate sensor with soft elastic strap; Polar Electro Oy, Kempele, Finland). Use and reliability of the Polar system for dogs was described earlier [4,27,41,42]. On the day before the Exercise test, the coat of each dog was clipped at all

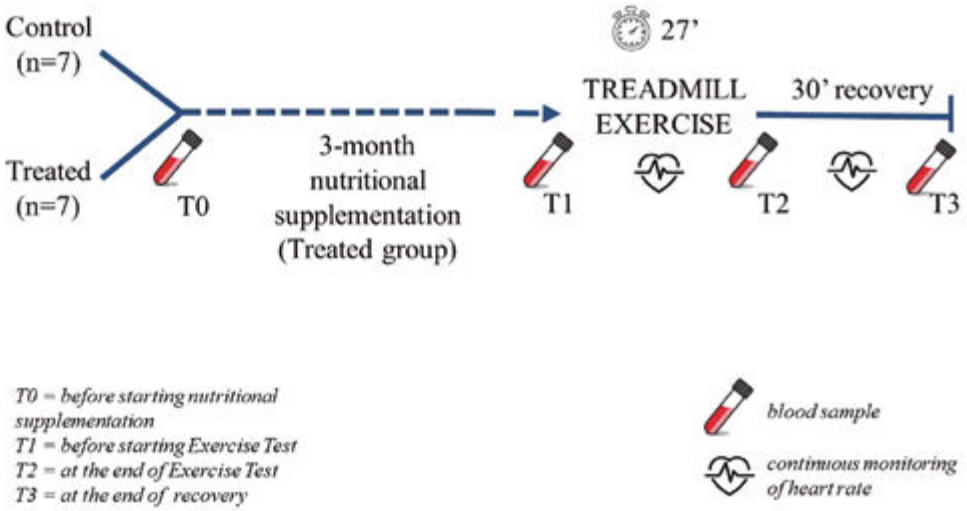

Fig 1. Experimental protocol. Fourteen dogs were randomly divided into two groups: a Control (n = 7) and a Treatment (n = 7) group. The dogs in the Treatment group were fed a dry diet with dietary supplementation for 3 months. At the end of this period, all of the dogs underwent a standardized submaximal treadmill Test conducted at four walking speeds and inclinations. Heart rate was monitored throughout the duration of the test (27 min) and recovery period (30 min). Blood samples were taken before starting the nutritional supplementation treatment (T0), immediately before starting the Exercise Test (T1), within the first 2 minutes after exercise test termination (T2) and at the end of the passive recovery period (T3).

https://doi.org/10.1371/journal.pone.0218275.g001

electrode sites, the skin was cleaned with alcohol and air-dried and Fiab electrode transmission gel (FIAB SpA–Firenze, Italy) was applied liberally to each clipped area in order to enhance conductivity. The electrode belt was strapped ventrally and the electrodes were placed on each side of the sternum. Heart rate (HR) was registered second by second and transmitted to a computer via USB cable at the end of each recording. All Polar data were provided as GPX or CSV files and transferred onto Excel spreadsheets. The Polar device was removed at the end of the 30-minute passive recovery period (T3). However, the HRs recorded during the last ten minutes of the recovery period were not considered because it was the time the dogs required for displacements. Then, we analysed 20 minutes of recovery [43].

Baseline HR was obtained from each dog the day before the treadmill exercise session (at rest), by monitoring the subjects, relaxed but awake, for 15 minutes. A five-minute interval (from min 8 to 13) was extracted and analysed [28,41].

Data processing

Heart rate (HR) measured by the Polar device was expressed as means ± SD (bpm) and highest (HR_{peak}) values.

The deflection point (HRdp) was estimated by the third-order curvilinear method (Dmax method) [44,45]. In brief, the third order curvilinear regression curve was calculated from HR values vs time (during the incremental phases of the exercise, i.e. Phases 1–3). Two end points of the curve were connected by a straight line and the most distant point of the curve to the line (Dmax) was considered as the HRdp (Fig 2) [45].

Various parameters were used to asses post-exercise heart rate recovery (HRR): HR at 20 minutes after exercise termination, Absolute Heart rate recovered (HRR), and Time constants of the post-exercise HR decay (τ20). HRR was defined as the difference between maximal HR and HR at the end of recovery [46]. End-recovery HR was calculated as the mean value of the

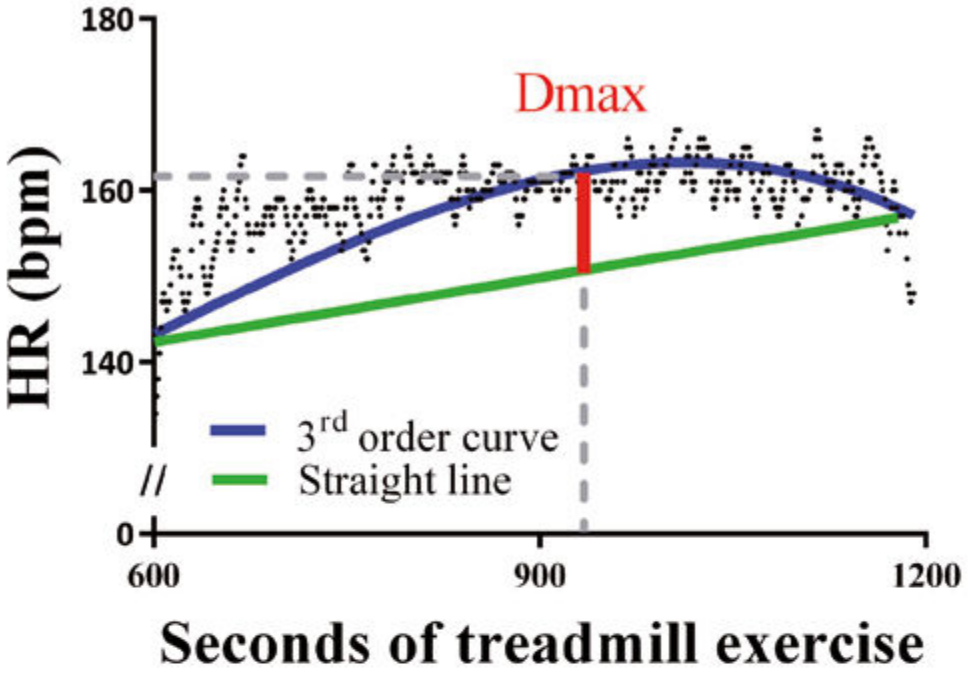

Fig 2. Single dog's deflection point (HRdp; dashed line) determined via Dmax method (Dog n° 12). For each dog, a third order curve was calculated by plotting heart rate (HR) values vs time (during the incremental exercise phase); two end points of the curve were connected by a straight line and the most distant point of the curve to the line (Dmax) was considered as the HRdp.

https://doi.org/10.1371/journal.pone.0218275.g002

20th minute of recovery. The τ20 was determined as proposed by Imai et al. [47]. The natural logarithm of the HR during the first 20 min post-exercise was plotted against the time of recovery and a linear regression analysis was applied (Fig 3). The τ20 was therefore determined as the negative reciprocal of the slope of the regression line and lower values were deemed desirable [30,31,47].

Statistical analysis

Mann-Whitney, Wilcoxon or Friedman tests were used to compare body weight, rectal temperatures and the parameters related to HR. The results were expressed as median (Mdn) and interquartile range (IQR).

Blood parameters were analysed with linear mixed model procedures. In these models, the dogs were treated as random factors while Time was treated as a repeated factor. The models evaluated the effect of Time (3 levels: T1, T2, and T3), group (2 levels: Control and Treatment groups), and interaction between time and group while the values at T0 were included as covariates. The results were expressed as estimated marginal means ± standard error (SE).

Statistical analyses were performed with SPSS Statistics version 23 (IBM, SPSS Inc., Chicago, IL, USA). Statistical significance occurred when $P < 0.05$.

Results

Body weight, Baseline HR and rectal temperature

No significant differences were observed in body weight ($P = 0.209$ and $P = 0.259$ before and after the nutritional treatment, respectively), basal heart rate ($P = 0.209$) and rectal temperatures ($P = 0.383$; S4 Table) between groups.

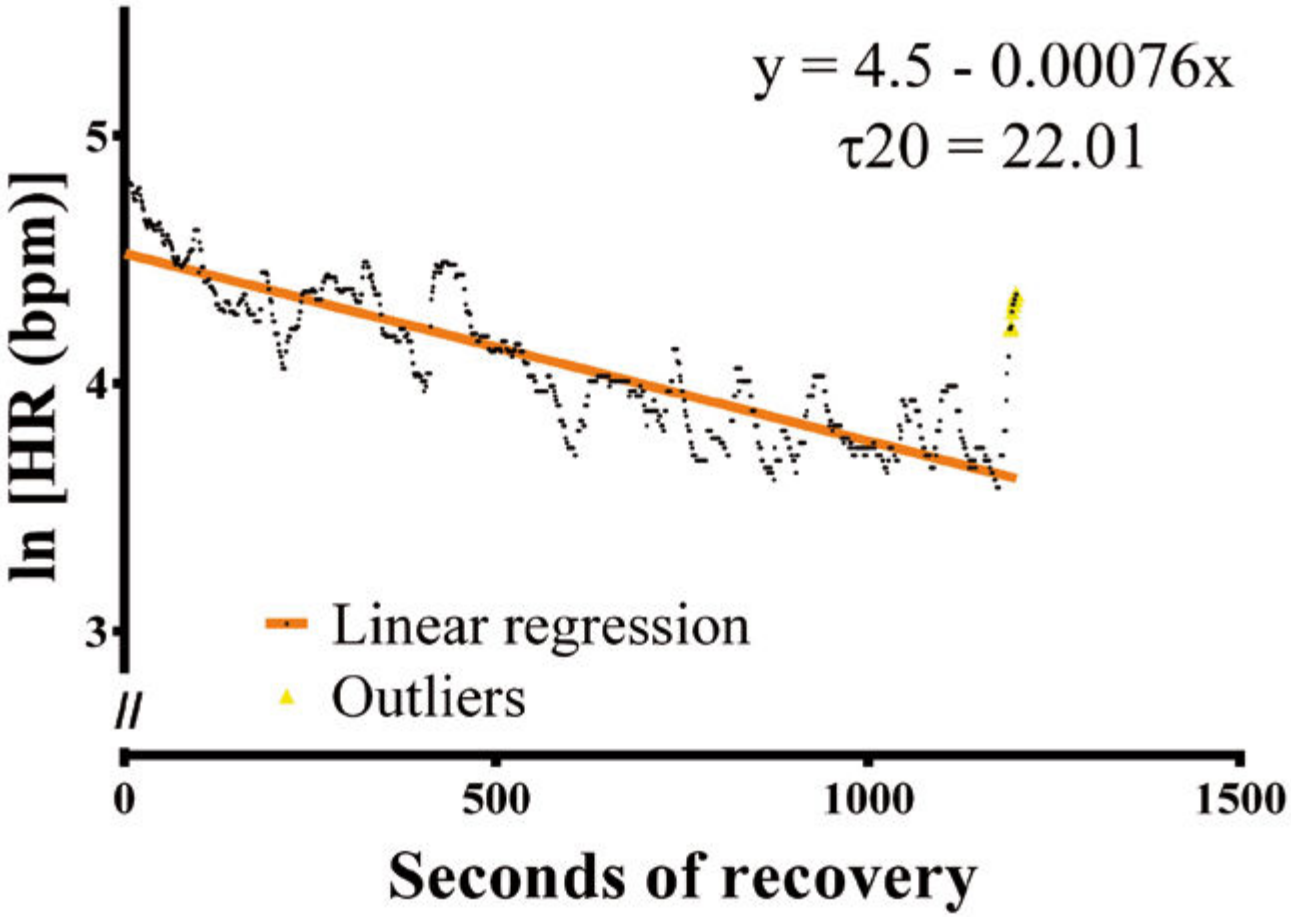

Fig 3. Single dog's time constants of the post-exercise HR decay (τ20; Dog n° 7). The τ20 was determined starting from the slope of the regression line obtained by plotting the natural logarithm of the heart rate (HR) vs time (during the first 20 min of recovery).

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The rectal temperature increased after exercise (Mdn = 38.7 C°, IQR = 38.3–39.0 C° and Mdn = 39.2 C°, IQR = 38.9–39.5 C° before and after treadmill, respectively; P <0.01) and then returned to baseline values after the recovery phase (Mdn = 38.8 C°, IQR = 38.6–39.0 C°; P <0.01). However, no significant differences were observed between the two groups (S4 Table).

HR during treadmill exercise and recovery

Regardless of group, mean HR increased from Phase 2 to Phase 3 (from walking to trotting; P = 0.01; S5 Table), while mean HR decreased from Phase 3 to Phase 4 (from trotting to walking; P = 0.003) and from Phase 4 to passive recovery (P = 0.003; S5 Table).

No significant differences were observed between study groups regarding HR means, HR_{peak} and parameters related to the HRdp obtained during the Exercise test (Table 1 and S4 Table).

The HRdp could not be calculated in two of the 13 dogs (15%) as their third order regression curves did not show the downward inflexion. However, these models fit well in most cases: the third order curves explained more than 50% of the variability of the data (R^2>0.5) in 11 out of 13 dogs (S6 Table). Regardless of group, median HRdp was 95% of the maximum average heart rate (IQR = 94–96%; mean = 94%; range = 84–98%).

Table 1. Heart rate (HR) mean, deflection point (HRdp), HR at 20 min after exercise termination, heart rate recovery (HRR) and time constants of the post-exercise HR decay (τ20) in control and treated groups. The values are medians and interquartile range.

Parameter	Group		P value
	Control	Treated	
HR mean during treadmill (bpm)	140 (110, 148)	127 (118, 152)	0.358
HR_{peak} during treadmill (bpm)	167 (136, 193)	161 (146, 190)	0.895
Percentage of HR_{peak} during treadmill (%)	80.9 (74.9, 83.9)	84.0 (78.4, 86.3)	0.181
Time on the treadmill at HRdp[1] (sec)	947 (928, 949)	883 (852, 948)	0.177
HRdp (% HR_{peak})[2]	94 (93, 95)	95 (94, 96)	0.126
HR mean during recovery (bpm)	96 (85, 124)	92 (63, 101)	0.234
HR at 20 min after exercise termination (bpm)	108 (80, 111)	84 (78, 91)	**0.012**
HRR[3] (bpm)	48 (41, 80)	81 (70, 95)	**0.035**
τ20[4] (min)	138 (105, 386)	57 (27, 83)	**0.008**

Bold P-values are significant at the 0.05 level.

[1] HRdp = Heart rate deflection point

[2] Heart rate deflection point expressed as a percentage of the maximum heart rate (HR_{peak})

[3] HRR = Absolute Heart rate recovered

[4] τ20 = Time constants of the post-exercise HR decay

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During the recovery phase, the Treatment dogs showed higher HRR (P = 0.035) and lower τ20 values (P = 0.008; Table 1) than those of the Control group. Twenty minutes after the end of the Exercise test (T3), HR was lower in the Treatment group than for the Control dogs (P = 0.012; Table 1).

Blood parameters

All biochemistry and haematology parameters fell within the normal range for both groups. However, estimated marginal means recorded in the Treatment group were lower for AST (P<0.05), NEFA (P<0.01), and CK (P<0.001) than Control (Table 2). Pairwise comparisons showed differences between groups after two months of treatment (T1) and exercise (T2) for CK and after recovery (T3) for NEFA (P<0.05; Table 2). Time effects were observed for AST (P<0.001), Glucose (P<0.001), and CK (P<0.05; Table 2 and S7 Table).

Discussion

Drug detection dogs must have good olfactory detection capabilities and behavioural traits and also need to be physically fit. Indeed, excessive physical exertion may affect concentration, olfactory capability, search duration and the performance of working dogs [8].

In our study, a graded treadmill exercise protocol (Exercise test) was developed in order to assess the effects of certain dietary supplements on the physical fitness of drug detection dogs. The Treatment dogs received a dietary supplement containing branched-chain (BCAA), limiting (LAA) amino acids, carnitine, vitamins and octacosanol during the last three months of their drug detection training course. At the end of this period, a human heart rate monitor system recorded the dogs' heart rates during a treadmill Exercise test and the subsequent recovery period. The data recorded by the device were used to calculate mean HRs and some parameters indicating cardiovascular responses to progressive incremental exercise. Moreover, recovery capacity and exercise-related degree of muscle damage and exhaustion were evaluated with conventional biomarkers. The dietary supplement proved to have some beneficial effects on

Table 2. Physiological parameters evaluated in control and treatment groups before starting nutritional supplementation (T0), before starting the treadmill exercise (T1), within 2 minutes after completing the treadmill exercise (T2) and after 30 min of passive recovery (T3). The values (raw data) are estimated means±standard error.

Parameter	Time	Group		P value		
		Control	Treatment up	Group	Time	Group x Time
AST (U/l)	T0	24.5±3.5	24.1±6.3	**0.049**	**<0.001**	0.945
	T1	25.9±0.8	24.2±0.8			
	T2	26.4±1.1	25.3±1.1			
	T3	22.8±0.8	21.1±0.8			
	Mean*	**25.0_a±0.5**	**23.5_b±0.5**			
ALT (U/l)	T0	35.1±11.1	37.9±11.1	0.102	0.137	0.968
	T1	48.2±5.6	40.1±5.6			
	T2	49.1±6.1	40.5±6.1			
	T3	38.3±4.8	32.3±4.8			
	Mean*	45.2±3.2	37.6±3.2			
Glucose (mmol/l)	T0	3.9±0.4	3.6±0.4	0.630	**<0.001**	0.467
	T1	5.7±0.2	5.9±0.2			
	T2	5.6±0.2	5.6±0.2			
	T3	5.1±0.1	5.1±0.1			
	Mean*	5.5±0.1	5.6±0.1			
LDH (U/l)	T0	174.7±55.0	139.81±26.1	0.454	0.392	0.969
	T1	83.7±11.7	79.9±11.7			
	T2	77.2±6.5	71.4±7.1			
	T3	72.2±8.6	63.6±8.6			
	Mean*	77.7±5.4	71.6±5.5			
NEFA (mmol/l)	T0	0.44±0.05	0.40±0.03	**0.002**	0.327	0.062
	T1	0.40±0.02	0.36±0.02			
	T2	0.42±0.02	0.39±0.02			
	T3	**0.51_a±0.05**	**0.31_b±0.04**			
	Mean*	**0.44_a±0.02**	**0.35_b±0.02**			
CK (U/l)	T0	80.3±23.0	84.47±53.5	**<0.001**	**0.011**	0.744
	T1	**131.5_a±13.0**	**100.4_b±11.1**			
	T2	**131.8_a±13.0**	**95.7_b±10.8**			
	T3	103.9_a±13.0	80.8_b±6.7			
	Mean*	**122.4_a±5.2**	**92.6_b±5.7**			

AST = Aspartate transaminase; ALT = Alkaline Phosphatase; LDH = Lactate dehydrogenase; NEFA = Non-esterified fatty acids; CK = Creatine kinase.

Values in the same row not sharing the same subscript are significantly different (P<0.05, Sidak correction). Bold P-values are significant at the 0.05 level.

*Estimated Marginal Means for Group effect.

https://doi.org/10.1371/journal.pone.0218275.t002

the physical fitness of the drug detection dogs by reducing their HR recovery time and indicators of muscle damage after physical exercise.

The differences observed in HR means were only due to the increase in exercise intensity over time, progressively switching from walking to trotting, thus suggesting an increase in oxygen demand [4,26]. However, the incremental Exercise test enabled us to determine the HR Deflection point (HRdp) [29,48]. The HRdp is an inexpensive, non-invasive marker for implementing training programmes and assessing human endurance capacity. Indeed, it is correlated with the anaerobic threshold, the break point between the aerobic and anaerobic metabolism, which is accepted as a measure of an individual's ability to perform at high

intensity for a prolonged period of time [28,29,49,50]. The HRdp appears as a decrease in the HR-work slope during incremental exercise [29,48] and can be determined through visual inspection or mathematical models [27,28,49]. In the present study, regression techniques were used for HRdp assessment, in order to avoid subjective interpretation [29,45]. Furthermore, unlike previous studies [27,50], we have chosen to undergo animals to a submaximal exercise test. Submaximal exercise could involve less stress for the animals tested than maximal exercise and, at the same time, induce the increase in blood lactate concentration characteristic of the anaerobic threshold [51]. Indeed, the HR peaks reached by the German Shepherd dogs of the present study (136–193 bpm) were reduced compared to those obtained during maximal exercises [27,50], but similar to those of Beagles subjected to a moderate-intensity exercise (131–209 bpm) [52]. Actually, we cannot confirm the achievement of the lactate threshold in our sample because lactate concentrations during exercise were not determined. However, most of the dogs (85%) showed the downward inflexion of the time-HR curve indicating HRdp. Radin et al. [27], who carried out the first study on HRdp in dogs, reported that HRdp was on average 80% of the maximum heart rate for Border Collies. In our study, median HRdp positioned at the 95% of the peak heart rate and no significant differences were observed between groups. This value was in accordance with that recently found by Restan et al. [50] in Beagles. The concordance was not obvious as these authors submitted dogs to a maximal exercise test reaching higher maximum HR (229–291 bpm) and, further, they used visual methods to determine HRdp. In fact, differences in the breed and animal training, exercise and the method of determination may explain any discrepancies between our finding and previous studies.

Conversely, the parameters characterising the recovery phase (Absolute HR Recovered, Time constants of the HR decay and HR at the end of the recovery) showed that post-exercise heart rate recovery of the Treatment dogs was faster than for the Control dogs.

Heart rate recovery after the end of the exercise is mediated by both sympathetic withdrawal and parasympathetic reactivation, primarily by vagal reactivation, which helps the body return to normal resting state. A fast HR recovery is associated with cardiovascular fitness and endurance capacity. On the other hand, attenuated HR recovery is indicative of exhaustion due to the lack of physical fitness or training, and it is associated with increased risk of cardiovascular events and mortality [4,30,31]. At a practical level, recovery capabilities could affect the performance of working dogs. Since drug detection operations may take a long time and require much physical effort, rapid recovery may allow the dog to resume activities quickly and in optimal physiological conditions.

CK, LDH, AST, glucose and NEFA were measured to determine the degree of metabolic and muscular effort. CK and AST are important enzymes of skeletal muscle energy metabolism and indices of muscle damage following strenuous exercise, which can damage the structure of skeletal muscle cells thus increasing membrane permeability and inducing the release of these enzymes into the circulation [7,35,53]. Changes in serum levels of muscular enzymes do not necessarily imply a disease but can indicate the adaptability of muscle to physical activity as part of the physiological response. Their serum levels can be affected by several both intrinsic and extrinsic factors. In general, increases of CK reduce with the training and increase with the intensity of exercise [4,35,36]. LDH interconverts pyruvate and lactate. Elevated LDH indicates that ATP production shifts from aerobic to anaerobic processes and is a biomarker of peripheral muscle fatigue [5,32,34]. NEFA are released from the adipose tissue in response to increased energy demand in order to maintain constant blood glucose concentrations. NEFAs are generally accepted as markers of negative energy balance and are indirectly responsible for muscle fatigue as they increase progressively during exercise when muscle glycogen concentration is low [1,5,54].

In the present study, the low values of CK, AST, and NEFA suggest reductions in changes of muscle permeability and fatigue and improvements in the physical fitness of the group of supplemented dogs [5,23,55,56].

Similar changes have been observed in search and rescue dogs supplemented with n-3 PUFA, chondroitin sulphate and glucosamine [18], in hunting dogs supplemented with betaine, L-carnitine, dietary buffers, B-vitamins and yeast [55], and in racing Greyhounds supplemented with L-carnitine [22].

Regardless of the group, no significant increases in AST and CK levels were observed immediately after exercise while a decrease in these levels was evident at the end of the recovery period compared to the samples collected at the beginning of the treadmill exercise. Previous studies have reported increases in muscle enzymes in dogs after exercise but they did not investigate muscle enzymatic activity after 30 min of passive recovery [5,7,53,55]. Rovira et al. [4] and Diverio et al. [1] found that exercise induces increases in CK, AST and LDH in search and rescue dogs but these values returned to baseline after 30 min and 2 h of rest, respectively. Conversely, Cerqueira et al. [36] found the peak in CK and AST 6h after the exercise test and returning to baseline levels after 12h. In the present study, we suppose that changes in muscle metabolism did not involve significant fibrillar disruption but a long-term monitoring (up to 12 hours) would have improved the accuracy of our evaluations.

It is important to note that changes in biochemical parameters and HR may depend on many factors such as temperature, altitude, nutrition, intensity of exercise, training, age and gender [5,7,28,41,53]. However, a standardised submaximal treadmill exercise was adopted in our study and all of the dogs were born and reared under the same conditions. All of the dogs participated in the same Training course, which consisted in a 6-month period of physical and olfactory training in the detection of illegal drug substances. Moreover, the dogs were all of the same breed (German shepherd) with slight age disparities (less than a year age gap). Sample homogeneity helps to reduce potential confounding factors. We can therefore speculate that recovery capacity was enhanced by dietary supplementation.

The dietary supplement ingredients can enhance recovery capacity and fitness by exerting their influence on energy metabolism, oxidative processes, chemical damage to tissues and fatigue substances. Many studies, primarily focused on humans and rodents, have demonstrated the beneficial effects of BCAAs on endurance capacity. BCAAs reduce the perceived mental fatigue and muscle damage, supply energy to skeletal muscles, improve cognitive performance after exercise and have beneficial effects on cardiac function [56–59]. These effects appear to be mediated by changes in membrane permeability, synthesis of serotonin and cortisol and circulating ammonia [56,58]. In dogs, infusions of BCAA prevented net protein degradation during exercise [60]. Several studies carried out on human beings reported that BCAA supplementation suppresses the increase in CK (and LDH) activity induced by exercise [56,59,61].

Some dietary supplement ingredients, such as L-Carnitine and vitamins C and E reduce hypoxia and free radical damage to muscle tissue, reduce the inflammatory response after exercise-induced muscle damage, enhance fatty acid oxidation and blood flow to tissues thus mediating rapid post-exercise recovery [39,62,63]. Some studies have evaluated the effects carnitine supplementation on the physical performance of dogs and found positive benefits regarding activity intensity, muscular strength, muscle recovery and oxidative capacity [22,62,64]. Several authors have studied vitamin E supplementation in dogs, highlighting its important antioxidant properties [15,21,65–67]. Conversely, the effect of octacosanol supplementation has been investigated in rats and humans but not in dogs [68,69].

Conclusions

The supplement containing branched-chain and limiting amino acids, carnitine, vitamins, and octacosanol proved effective in improving the physical fitness of drug detection dogs by exerting beneficial effects on HR recovery, energy metabolism and biomarkers of muscle damage. The findings have important management applications and encourage the routine use of nutritional supplements in the feeding regime of working dogs.

The present study developed an evaluation protocol, including a mathematical model of heart rate kinetics, which was applied during the training period. This model could be implemented for routinely monitoring the physical fitness of detection dogs during their working activity. Indeed, improving the fitness of drug detection dogs could optimise their performance in many operating contexts as well as their wellbeing. Moreover, our protocol could be an easy, low-cost and non-invasive tool for evaluating the performance of other working dogs, such as search and rescue dogs, which often have to work in challenging physical environments.

Supporting information

S1 Fig. Phases of exercise test on treadmill.
(TIF)

S1 Table. Demographic data of the dogs.
(PDF)

S2 Table. Nutrient composition of base diet.
(PDF)

S3 Table. Nutrient composition of supplemental treatment.
(PDF)

S4 Table. Body weight (BW), rectal temperatures, baseline HR and HR means during the different phases of the treadmill in control and treated groups. Values are medians and interquartile ranges.
(PDF)

S5 Table. Heart rate means according to the phases of treadmill test and recovery.
(PDF)

S6 Table. Parameters and goodness of fit of the third order regression curves used to HRdp determination in each dog.
(PDF)

S7 Table. Estimated marginal means ± standard error (SE) and P value of time effect.
(PDF)

Acknowledgments

We would like to extend our sincere gratitude and appreciation for all of the work and dedication provided by all military the GdF personnel (Walter Di Mari, Roberto Pulicani and Massimo Teodoro, Michele Matteo Santoro, and all the drug detection dog Trainers), and the LEBA collaborators (Claudia Enas, Federica Scotti, Roberta Gobbi, Danilo Zampini, Anna Casano) for their valuable assistance and outstanding contributions. Medical writing support was provided by the English mother-tongue copyeditor Eleanor Fabri. We gratefully

acknowledge Dr. Giovanni Valle for his long-standing commitment to improving animal health and wellbeing through supporting scientific research.

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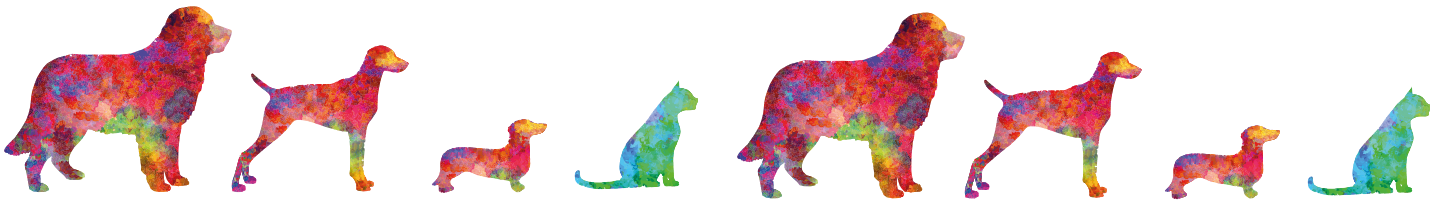

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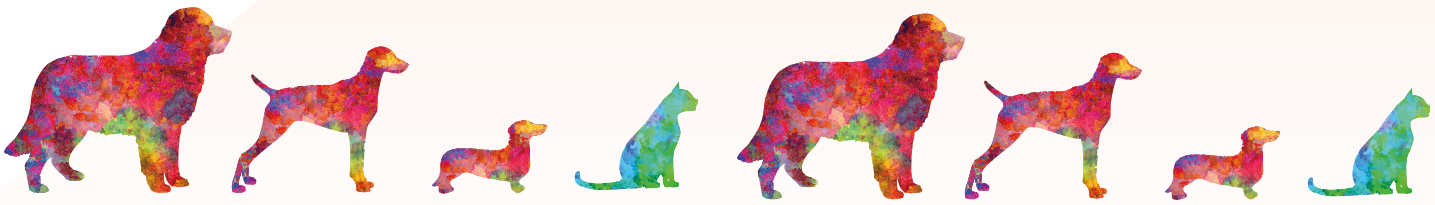

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