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Plasma NT-proBNP and Cell-Free DNA Concentrations after Prolonged Strenuous Exercise in Working Farm Dogs

H. Hunt, N. Cave, J. Bridges, K. Gedye, and K. Hill

Background: Plasma N-terminal pro–B-type natriuretic peptide (NT-proBNP) concentration is increased in dogs with myocardial dysfunction, and cell-free DNA (cfDNA) increases in numerous disease states. In humans, both of these biomarkers can be altered after endurance exercise.

Objective: To investigate the effect of prolonged strenuous exercise on circulating NT-proBNP and cfDNA concentrations in working farm dogs.

Animals: Six healthy, privately owned working farm dogs (4 Huntaways and 2 heading dogs) from the same hill country farm in New Zealand.

Methods: Prospective, nonrandomised cohort study. Venous blood samples were collected before and after the dogs worked over 4 days. Plasma NT-proBNP concentrations were measured by a commercially available ELISA assay and cfDNA concentrations were determined by fluorometry without prior DNA extraction.

Results: The baseline (before work, Day 1) median plasma NT-proBNP concentration was 664 pmol/L. A linear mixed-effects model showed that work increased plasma NT-proBNP concentrations by 101 ± 9% ($P < 0.001$), but with each consecutive day of work, NT-proBNP concentrations declined by 16 ± 4% ($P < 0.001$). The baseline median plasma cfDNA concentration was 653 ng/mL, and plasma cfDNA concentrations increased by 138 ± 45 ng/mL after work ($P = 0.004$).

Conclusions and Clinical Importance: The plasma concentration of NT-proBNP in healthy Huntaways and heading dogs after work can exceed the upper limit of the reference range. Results in dogs sampled on the day of prolonged strenuous exercise should be interpreted with caution. Plasma concentrations of cfDNA also increase with exercise, but further studies are needed to establish reference ranges in healthy dogs.

Key words: Biomarker; Cardiac; Cell-free DNA; Natriuretic peptide.

The natriuretic peptides play a regulatory role in cardiovascular function through their endocrine and paracrine actions. B-type (or brain) natriuretic peptide (BNP) is primarily produced by the myocardium, and increased synthesis and secretion of BNP occurs in response to stimuli such as cardiomyocyte stretch and ischemia.[1] Circulating BNP or its breakdown products, including NT-proBNP, can be used as a biomarker to aid in the diagnosis and prognosis of cardiac disease in humans, dogs, and cats. In particular, plasma NT-proBNP concentrations have been shown to have high accuracy in differentiating cardiac and noncardiac disease in humans, dogs, and cats presenting with dyspnoea.[2–5]

Abbreviations:

AST	aspartate aminotransferase
BNP	B-type natriuretic peptide
cfDNA	cell-free DNA
CK	creatine kinase
dsDNA	double-stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
NT-proBNP	N-terminal pro–B-type natriuretic peptide

An ELISA that detects the N-terminal fragment of BNP (NT-proBNP) in plasma has been validated for use in dogs.[6,a] Previous studies have shown that there is a significant variation in natriuretic peptides between healthy dogs of different breeds,[7] as well as a high degree of week-to-week variability in individual dogs.[8] Extracardiac factors such as renal function can also influence circulating NT-proBNP concentrations.[9] In humans, prolonged strenuous exercise significantly increases plasma NT-proBNP concentrations above the upper limit of the reference range in endurance athletes.[10] Similar studies have not been performed in dogs, although 5-minute period of submaximal exercise in healthy dogs did not significantly affect plasma NT-proBNP concentrations.[11] Prolonged strenuous exercise in people causes transient cardiac dysfunction (known as cardiac fatigue)[12] and might even induce subclinical myocardial necrosis,[13] leading to increases in plasma NT-proBNP concentrations. Skeletal muscle fiber damage can also occur with endurance exercise, leading to increases in biomarkers such as serum creatine kinase (CK) and aspartate aminotransferase (AST) activities.[14]

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This work was conducted on a sheep and beef farm in Taihape, New Zealand. Laboratory analysis of samples was performed at IDEXX Laboratories, Hamilton, New Zealand, and Massey University, Palmerston North, New Zealand.

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Cell-free DNA (cfDNA) is another biomarker that has been proposed as a novel indicator of exercise-induced muscle injury and overtraining.[15,16] Cell-free DNA consists of double-stranded fragments of DNA in plasma that are not bound to cells. In the past, cfDNA was thought to originate largely from cells undergoing apoptosis or necrosis, but in recent years, it has been suggested that DNA might also be released from living cells.[17,18] Plasma concentrations of cfDNA are increased in various disease states in dogs,[19] including mammary tumors,[20,21] lymphoid neoplasia,[22] pulmonary thromboembolism,[23] sarcomas, trauma, and sepsis.[24] However, as for NT-proBNP, the effect of prolonged strenuous exercise on plasma cfDNA concentrations in dogs is unknown.

The influence of exercise on the interpretation of circulating NT-proBNP and cfDNA concentrations in dogs is particularly important when considering the diagnostic use of these biomarkers in working dogs. In New Zealand, working dogs on sheep farms routinely work 2–8 hours per day, multiple days in a row. Furthermore, a common breed of working dog, the Huntaway, is predisposed to the development of dilated cardiomyopathy.[25] If a working dog develops exercise intolerance, fatigue, or dyspnoea during work, veterinarians might use plasma NT-proBNP concentrations to differentiate cardiac from noncardiac disease. However, if plasma NT-proBNP concentrations increase with prolonged periods of work, this could result in the misdiagnosis of cardiac disease. The aim of this study was to determine whether repeated periods of prolonged strenuous exercise influences circulating plasma NT-proBNP and cfDNA concentrations in healthy working dogs.

Materials and Methods

Animals

The study protocol was approved by the Massey University Animal Ethics Committee. The study population consisted of 6 unrelated adult working farm dogs belonging to the same shepherd on a large sheep and beef property near Taihape, in the Rangitikei region of New Zealand. Healthy adult dogs primarily used for work with livestock were considered eligible for inclusion. Exclusion criteria included auscultation of a heart murmur or arrhythmia (with the exception of sinus arrhythmia), or the presence of any injury or disease process which would prevent the dog from performing a full day of work. The study population included 4 Huntaways and 2 Heading dogs, with a mean age of 4 years (range 1.25–7 years) and equal numbers of males and females (3 of each). Sampling took place in January 2015 and for the 2 weeks before sampling, the dogs had been rested and largely confined to kennels. They had not performed any stock work during this 2-week period and were only let out of the kennels for a short duration each day when they were allowed to walk around in the immediate vicinity of the kennels. Each dog had an electromechanical activity monitor[b] mounted on their collar on the first day, and the time of starting and finishing work was recorded daily. The owner of the dogs was contacted approximately 6 and 12 months after the collection of samples to provide follow-up on the ongoing health of the dogs.

Sample Collection and Analysis

On Day 1, a complete physical examination was performed on all dogs before starting farm work. A venous blood sample (3 mL) was collected from the cephalic vein of each dog and submitted to a commercial veterinary diagnostic laboratory[c] for a complete blood count and serum biochemistry panel. Venous blood samples (1.5–2 mL) for NT-proBNP and cfDNA analysis were collected immediately before the dogs starting work and within 30–90 minutes of the dogs finishing work each day. It was not possible to sample dogs immediately after exercise as they were usually working in remote locations on the farm. At the time of sample collection, cardiac auscultation was also performed on each dog, and heart rate was recorded. Blood samples were collected by needle and syringe and then transferred immediately into tubes containing ethylenediaminetetraacetic acid (EDTA). Within 60 minutes of collection, samples were centrifuged at $5000 \times g$ for 15 minutes to separate plasma. The plasma samples were frozen at −20°C for up to 4 days before analysis at a veterinary diagnostic laboratory[c] for NT-proBNP concentrations by the Cardiopet ELISA, as well as CK and AST activities.

After the initial laboratory analysis, plasma samples were refrozen for transport to Massey University and then thawed for determination of cfDNA concentration. A total of 20 μL of each plasma sample was used for cfDNA quantification with the Qubit dsDNA HS Assay Kit and Qubit 2.0.[d] The Qubit assay uses a fluorescent dye selective for double-stranded DNA, and the amount of fluorescence recorded is proportional to the amount of double-stranded DNA in the sample. Before each run, the Qubit 2.0 was calibrated with the standards provided, and the concentration of cfDNA in each sample was calculated by the manufacturer's dilution algorithm. All plasma samples from the dogs were run as a single batch. A suggested reference range of 512–654 ng/mL for plasma cfDNA concentration (mean ± 2 standard deviations) was calculated using data from 24 healthy dogs presented in a recent paper by Burnett et al.,[19] which used the same analytical methods.

Statistical Analysis

Statistical analyses were performed by commercially available software.[e] Exploratory data analysis and Shapiro-Wilk tests revealed that plasma cfDNA concentrations were normally distributed, whereas plasma NT-proBNP concentrations and CK and AST activities were not, so log transformations of these variables were performed to achieve normality. Linear mixed-effects models by a restricted maximum likelihood approach were used for statistical analysis. The dogs were considered a random effect, whereas work (before or after) and day were considered as fixed effects. Duplicate models were run with day as a categorical variable and a numeric variable to enable trends between days and overall trends to be analyzed. Separate models were fitted for each variable (NT-proBNP, cfDNA, CK, and AST). Coefficient values and standard errors from models of log-transformed variables were back transformed using the formula $(\exp^{\text{value}} - 1) \times 100$ to give the percentage change in the dependent variable per day or with work. For plasma NT-proBNP concentrations, the estimated standard deviation of the between-day component (intercept) and the within-day component (residual) was used to calculate the intraclass correlation. Pearson's product-moment correlations were used to explore correlations between after work variables, including plasma NT-proBNP concentrations and CK and AST activities. The recorded heart rates of all dogs before and after work across all sampling points were averaged, and the means compared by a paired *t*-test.

Results

All dogs were normal on physical examination at the start of the study. One dog was kicked by a cow on day 3 and sustained a traumatic tibial fracture and was therefore excluded from further analysis from Day 3 onwards. All other dogs remained in good health and continued working for the duration of the study and over the following 12 months. Each day during the sampling period, the dogs performed stock work for a total of 10–12 hours, including short periods of rest. Stock work primarily involved mustering ewes and cattle on steep hill country and over rough terrain. Because of technical difficulties, a complete set of activity data was only collected from one dog. Activity counts for this dog were as follows: 26,396 (Day 1); 45,056 (Day 2); 37,616 (Day 3); and 30,344 (Day 4). Counts approximate the number of footfalls by the dog, and as all dogs worked as a team, for the same duration and over the same terrain, it is suggested that these counts are representative of all dogs in the study.

The overall mean of the after work heart rates (taken within 30–90 minutes of finishing work) was 114 beats per minute, with a standard deviation of 13. This is significantly higher than the before work mean of 93 beats per minute, with a standard deviation of 11. A paired t-test showed this difference in means was statistically significant, with a T value of -7.47 and $P < 0.001$. No cardiac murmurs were auscultated, and the femoral pulse was strong and synchronous in all dogs at each time point. Three of the dogs exhibited an increased heart rate on inspiration relative to expiration, consistent with physiological sinus arrhythmias. No pathological arrhythmias were noted.

As illustrated in Figure 1A, plasma NT-proBNP concentrations were consistently higher after work than before work on the same day and this frequently resulted in values above the suggested reference range in dogs (<900 pmol/L). Two male Huntaways had plasma NT-proBNP concentrations above this range on Day 1 before work, which increased further after work on Day 1, but normalized in subsequent before work samples. A linear mixed-effects model with day as a

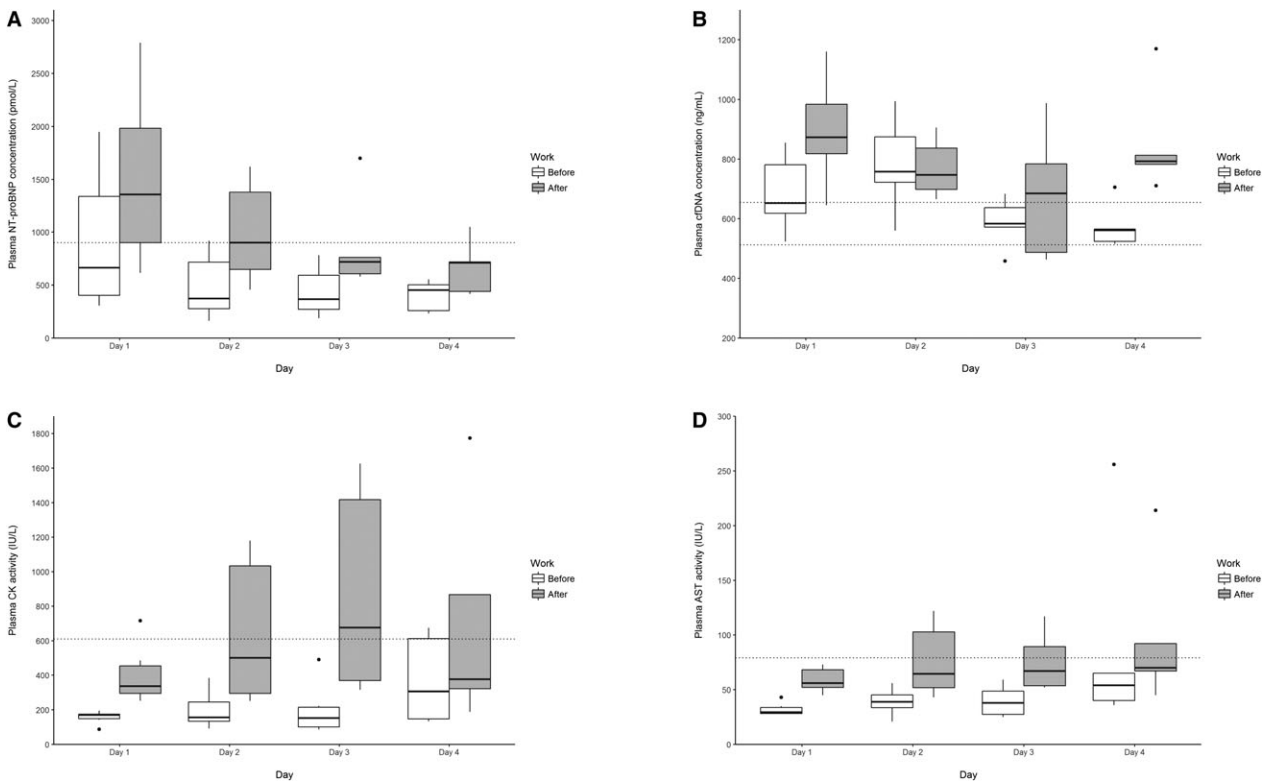

Fig 1. Boxplots showing the distribution of before (white boxes) and after work (gray boxes) concentrations of selected plasma biomarkers in dogs over 4 consecutive days from the study on plasma NT-proBNP and cell-free DNA concentrations after prolonged strenuous exercise in working farm dogs. **(A)** Plasma NT-proBNP concentrations, measured by the Cardiopet ELISA. The dotted horizontal line at 900pmol/L indicates the upper limit of the reference range in dogs **(B)** Plasma cfDNA concentrations, with one outlying value (Dog 3, before work day 3) not shown. Dotted lines indicate the upper and lower limits of a reference range for plasma cfDNA concentrations in healthy dogs calculated from a recent paper.[18] **(C)** Plasma CK activities, reference range 0–609 IU/L (upper limit indicated by dotted line). **(D)** Plasma AST activities, reference range 0–79 IU/L (upper limit indicated by dotted line). One outlying value removed (Dog 4, day 3, after work).

categorical fixed effect showed that plasma NT-proBNP concentrations increased by 101 ± 9% with work, ($P < 0.001$), and Days 2, 3, and 4 were significantly different to Day 1 ($P < 0.001$). The intraclass correlation was 0.79, indicating a large amount of variation between days compared to the variation within each day. As seen in Table 1, the model with Day as a numeric variable gives a negative, statistically significant value (−0.176775, $P < 0.001$) for Day, and back transformation of this value shows that plasma NT-proBNP concentrations decrease by 16 ± 4% with each repeated day of work. There was no correlation between heart rate and plasma NT-proBNP concentrations.

A linear mixed-effects model estimated that plasma cfDNA concentrations increased by 138.1 ± 45.2 ng/mL with work samples, as shown by the fixed effect value in Table 1 ($P = 0.004$). As seen in Figure 1B, there was a large amount of variation in plasma cfDNA concentrations between dogs at each sampling point, compared to the between-day variation (intraclass correlation of 0.085). Plasma cfDNA concentration did not change significantly over repeated days of work ($P = 0.076$). One sample (Dog 3, before work Day 3) was considered an outlier at 1930 ng/mL and was excluded from statistical analysis as prior, and subsequent samples from the same dog were considerably lower and more consistent with cfDNA concentrations recorded in the other dogs. Plasma CK and AST activities were also significantly increased after work (Table 1 and Figures 1C–D), and the largest increases were seen on Day 2 and 3 when the highest activity counts were recorded.

Discussion

Results of the present study demonstrate that prolonged strenuous exercise in previously rested dogs significantly increases circulating NT-proBNP concentrations, but the impact of exertion lessens over consecutive days of work. In Huntaways and Heading dogs, if sampling for plasma NT-proBNP concentration is performed on a day when the dogs have been working, the results can exceed the upper limit of the reference range in the absence of any clinical evidence of cardiac dysfunction. If sampling is delayed until the following day (before further work), plasma NT-proBNP concentration normalizes in the majority of cases and results might more accurately reflect the true likelihood of cardiac disease in an individual dog. Plasma cfDNA is a new biomarker in dogs and lacks reference ranges established using large-scale studies, but the results of this research show that plasma cfDNA concentrations also increase with physical work, despite marked variation between individual dogs.

Table 1. Linear mixed-effects model data from the study on plasma NT-proBNP and cell-free DNA concentrations after prolonged strenuous exercise in working farm dogs, with Day as a numerical value. NT-proBNP concentrations and CK and AST activities have been log transformed. Random effects (Dog) are not presented here. *P*-values <0.05 are considered significant.

	Fixed Effect	Value	Standard Error	*t*-value	*P*-value
NT-proBNP (log)	Work (after)	0.700	0.0863	8.109	<0.001
	Day	−0.177	0.040	−4.450	<0.001
cfDNA	Work (after)	138.100	45.248	3.052	0.004
	Day	−37.265	20.406	−1.826	0.076
CK (log)	Work (after)	1.003	0.149	6.748	<0.001
	Day	0.151	0.068	2.211	0.033
AST (log)	Work (after)	0.562	0.108	5.188	<0.001
	Day	0.162	0.049	3.294	0.002

The transient increases observed in circulating NT-proBNP concentrations with endurance exercise in dogs in the present study are similar to what has been described in humans participating in endurance exercise events such as marathons, ultramarathons, and professional cycling events.[13,26,27] In humans, the duration of exercise, but not the intensity, is associated the with peak plasma NT-proBNP concentrations after exercise.[10,28,29] Because of the small sample size and the practical limitations of dogs working in remote areas on the farm, it was not possible to quantify exercise intensity in the present study. In regard to the total amount of exercise daily, the absolute activity counts recorded from one of the dogs are not in themselves quantitatively informative, but they show differences in activity between the days. As the dogs in this study worked as a team of dogs, it is suggested that the monitored dog acted as an indicator for the relative activity of all the dogs. The highest increases in after work plasma NT-proBNP concentrations were seen on Day 1 and declined over subsequent days, whereas the highest activity counts were seen on Day 2, followed by Day 3. This suggests that increases in plasma NT-proBNP concentrations in dogs after exercise are not solely determined by the total amount of physical activity performed. The exact mechanism responsible for increases in plasma NT-proBNP concentrations seen after endurance exercise is unclear, although prolonged exertion might induce reversible diastolic left ventricular dysfunction,[26] or decreased myocardial contractility,[12] referred to as cardiac fatigue. Increases in specific biomarkers of cardiomyocyte necrosis, such as plasma cardiac troponin T concentrations, have been found to be significantly correlated with increases in plasma NT-proBNP concentrations after exercise, indicating that subclinical myocardial injury and necrosis,[13] or cardiomyocyte membrane leakage,[30] might also contribute to elevated plasma NT-proBNP concentrations with endurance exercise.

There is also the possibility that the changes in plasma NT-proBNP concentrations seen after work and over consecutive days in the present study are a result of altered clearance of the peptide, rather than changes in expression and release. NT-proBNP is thought to be removed from circulation by organs with high blood flow rates, including the kidneys and liver, compared to

the biologically active carboxy-terminal of BNP, which is also cleared by active receptor-mediated binding.[1] During endurance exercise, the glomerular filtration rate usually decreases,[31] which could reduce renal clearance of NT-proBNP and increase plasma concentrations of the peptide, similar to what is seen in dogs with renal dysfunction.[9] However, the glomerular filtration rates of the dogs in the present study were not assessed.

Of particular interest in the present study, the magnitude of increases in NT-proBNP concentrations seen with exercise declined over the 4-day study period. In humans, BNP might play a role in myocardial adaption to endurance training.[30] Healthy male Army recruits had increased resting plasma BNP concentrations and increased left ventricular mass after a 10-week endurance training program,[32] whereas endurance athletes with a longer history of training did not have elevated plasma BNP and NT-proBNP concentrations at rest,[33,34] suggesting that with prolonged endurance training, the myocardium might adapt to prolonged periods of exertion. The dogs in the present study had a long history of endurance training as they had been working for 6–12 hours daily (including short periods of rest) in the months before the study. However, in the 2 weeks immediately before the sampling period, the dogs had been rested and largely confined to the kennels, and it is not known what effect this might have had on any prior myocardial adaption to endurance exercise. To understand the significance and physiology of this apparent trend for repeated exercise to have a diminished effect on plasma NT-proBNP concentrations in dogs, further studies are needed, involving larger numbers of working dogs over a longer sampling period, with differing levels of prior work.

Two of the dogs included in the present study, both male Huntaways, had plasma NT-proBNP concentrations on the first day that would usually be associated with an increased likelihood of cardiac disease (>900 pmol/L). These values increased further with work, but normalized over the course of the sampling period, and over the following year neither dog developed any clinical signs of cardiac dysfunction. Because of the small sample size in the present study, it might be coincidental that the 2 dogs with elevated NT-proBNP concentrations initially were both male Huntaways, whereas the 2 female Huntaways and the heading dogs had resting values within the reference range. However, studies in retired racing greyhounds have found that plasma NT-proBNP concentrations in this breed at rest are significantly higher than non-Greyhound controls, with 11 of 24 healthy greyhounds having NT-proBNP concentrations above the upper limit of the reference range.[35] A similar situation might exist in Huntaways, and there might also be an influence of sex on plasma NT-proBNP concentrations at rest, although in humans, females tend to have higher concentrations than males.[36] The high before work NT-proBNP concentrations on Day 1 could also be related to the dogs' anticipation of imminent work after a fortnight of rest. All the dogs were restless and excited on Day 1 before work, and epinephrine and norepinephrine can induce BNP synthesis in human cardiomyocytes in vitro.[37] It might therefore be possible that activation of the sympathetic nervous system in these dogs contributed to elevated plasma NT-proBNP concentrations in Day 1 before work samples. A larger sample size would be needed to further investigate any breed or sex-specific effect on circulating NT-proBNP concentrations in working Huntaway dogs, and sampling without a lengthy period of inactivity before the start of the study could help to minimize the influence of circulating catecholamines.

Similar to NT-proBNP, the concentrations and activities of other biomarkers measured in the present study (CK, AST, and cfDNA) were increased in plasma after work. The changes in plasma CK and AST activities are consistent with previous reports in dogs and humans,[14,38] as both cardiac and skeletal muscle can release CK and AST into circulation when there is muscle damage or increased muscle membrane permeability.[39] Transient increases in plasma cfDNA concentrations with exercise have been reported previously in humans and have been proposed as a marker of overtraining and exercise-induced inflammation in muscle.[15,16] The origin of cfDNA released during exercise is the subject of much debate, but hemopoietic cells might be the main source.[40] There was no significant correlation between plasma cfDNA concentrations and either CK or AST activities, which suggests that muscle leakage alone is unlikely to be an explanation for the increases seen in plasma cfDNA concentrations with work on Days 1, 3, and 4. On Day 2 of the study, the before and after work plasma cfDNA concentrations were similar, and the interval between finishing work and sampling was 90 minutes (compared to 30–45 minutes on the other days), because of the dogs working in a remote area of the farm. In people competing in a half-marathon event, plasma cfDNA concentrations returned to baseline values within 2 hours of finishing the race,[41] so it is possible that the similarity between before and after work values is because of the relative delay in sample collection. The small sample size and wide individual variation in plasma cfDNA concentrations in the present study mean that it is not possible to make any conclusions regarding the influence of exercise duration, intensity, and prior training on plasma cfDNA concentrations in dogs. Furthermore, most of the plasma cfDNA concentrations measured in these working dogs were higher than what was reported in a previous study by the same analytical technique.[19] In the previous study, most of the control dogs were colony-housed, relatively inactive dogs, so it might be that the higher values are reflective of prior endurance training or subclinical soft tissue injury. However, further studies are needed to determine a reference range for plasma cfDNA concentrations in working farm dogs.

The main limitations of this prospective study were the low number of dogs enrolled, the short duration of the study, and the lack of echocardiography. To minimize variation in animal husbandry practices and in the duration, intensity, and environment of work, all

recruited dogs belonged to the same shepherd, which limited the size of the study population. Breed differences have been documented in plasma NT-proBNP concentrations in dogs,[7] but the small study size precluded analysis of any breed-related effects. The duration of the present study (4 days) was based on the farming operation activities and the number of consecutive days of high-intensity work that the dogs were required for. This limited the detection of longer term trends in plasma NT-proBNP, cfDNA, CK, and AST with continued endurance exercise. The local veterinary clinic did not have the equipment or expertise to facilitate echocardiography of the dogs, and transporting them to the nearest veterinary hospital with these imaging capabilities was unfortunately not practical. Therefore, assessments regarding the cardiac health of the dogs had to be based on through clinical examinations and the dogs' continued ability to undertake demanding physical work daily without any difficulty.

In conclusion, the present study demonstrates that plasma NT-proBNP concentrations in healthy Huntaways and heading dogs increase with prolonged hill country work and can exceed the upper limit of the reference range. If a working dog presents with clinical signs suggestive of cardiorespiratory disease during or following a day of work, clinicians should consider delaying sampling for plasma NT-proBNP concentrations until the following day to overcome any confounding effect of recent exercise. Further work is needed to determine the effect of prior training, the intensity and duration of activity, and possible breed-related differences in Huntaways.

Footnotes

[a] Cardiopet proBNP, IDEXX Laboratories Inc., Westbrook, ME
[b] Heyrex, Wellington, NZ
[c] IDEXX Ltd., Hamilton, NZ
[d] Life Sciences, Carlsbad, CA
[e] R Development Core Team 2003, http://www.r-project.org

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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