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14. ABSTRACT

Canine exercise performance in hot environments is limited by the ability of the dog to dissipate metabolic heat, which is in turn limited by the availability of body water for evaporation. Estimated daily energy expenditure of off-leash explosive detection dogs is approximately 6000 kcal/day, requiring the evaporation of up to 5 liters of water/day. The purpose of this project was to evaluate dietary manipulations that would increase the availability of water for evaporation without adversely affecting performance and to develop technical methodology that would dissipate metabolic heat without the expense of body water. Neither an increase in dietary salt nor decrease in dietary protein altered exercise tolerance, although the decrease in dietary protein did decrease total body water as a % of body weight. Proof-of-concept experiments in support of heat dissipation through footpad cooling showed promise, but ultimately did not provide significant benefit during controlled exercise studies. Supplementary projects to assess the effects of environmental heat on olfactory performance and to assess the adaptation of muscle to prolonged hyperthermia were suspended prior to completion due to regulatory issues associated with research dog procurement and husbandry.

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Building a Better Canine Warrior

Final Technical Report

Abstract

Canine exercise performance in hot environments is limited by the ability of the dog to dissipate metabolic heat, which is in turn limited by the availability of body water for evaporation. Estimated daily energy expenditure of off-leash explosive detection dogs is approximately 6000 kcal/day, requiring the evaporation of up to 5 liters of water/day. The purpose of this project was to evaluate dietary manipulations that would increase the availability of water for evaporation without adversely affecting performance and to develop technical methodology that would dissipate metabolic heat without the expense of body water. Neither an increase in dietary salt nor decrease in dietary protein altered exercise tolerance, although the decrease in dietary protein did decrease total body water as a % of body weight. Proof-of-concept experiments in support of heat dissipation through footpad cooling showed promise, but ultimately did not provide significant benefit during controlled exercise studies. Supplementary projects to assess the effects of environmental heat on olfactory performance and to assess the adaptation of muscle to prolonged hyperthermia were suspended prior to completion due to regulatory issues associated with research dog procurement and husbandry.

Executive Summary

Athletes have increased daily requirements for water intake as a result of the need to use evaporation to dissipate metabolic heat and prevent heat-related injuries. Canine athletes are the most prone to heat-related injuries due to their exceptional capacity to generate metabolic heat (4-5x that of a human) combined with an inability to sweat in order to dissipate that heat. Previous worked funded through ONR resulted in improved physical conditioning programs for explosive detection dogs used by the US Marine Corps, but practical implementation of those programs and the resulting increase in athletic capacity was limited by the necessity for a corresponding increase in the capacity for metabolic heat dissipation. Therefore, we undertook a project to determine whether dietary manipulation could improve water availability for evaporative cooling and increase resistance to dehydration, and whether ancillary methods of heat dissipation would reduce the reliance on body water for evaporative heat dissipation in working dogs. Additional objectives were added during the project to evaluate the effect of environmental heat on olfactory performance in dogs and determine whether athletic conditioning improved heat tolerance in dogs.

Two dietary manipulations were studied: increased dietary intake of electrolytes and decreased dietary intake of protein. Dietary electrolyte supplementation failed to significantly affect any studied parameter related to electrolyte and water balance in dogs during an 8-week conditioning program and had no effect on water requirements during exercise. Dietary protein restriction resulted in lower total body water as a % of body weight and increased water requirements during exercise with ad libitum water, presumably due to a lower capacity for renal concentration of urine, but did not affect water balance during dehydration. Further testing was not believed have a high likelihood of success and the Objective was terminated.

The construction of the ancillary cooling device was successful at the proof-of-concept phase of the study, but critical evaluation of the system demonstrated variable, and overall very modest, benefit in mitigating metabolic heat build-up in dogs during exercise. Further testing was not believed have a high likelihood of success and the Objective was terminated.

The quantitative evaluation of olfactory performance and the establishment of olfactory thresholds in individual dogs proved to be extremely time-consuming, both from a methodological aspect as well as emerging regulatory issues related to research in working dogs. Data suggested that the effect of high environmental temperatures on olfactory performance in dogs was dependent upon the overall ability of the dog, with low performing dogs being adversely affected by high temperatures but high-performing dogs improving in olfactory detection. However, these results should be viewed with caution due to unexplained superior olfactory performances of some of the dogs that appeared to exceed the physical chemistry boundaries of the study. Due to excessive delays we were unable to complete this Objective within the contracted period of performance.

Regulatory issues similarly affected the efforts to evaluate heat tolerance of conditioned dogs. The experimental plan was to use privately-owned athletic dogs as research subjects, as had been done for over two decades leading up to this project (including the previously-funded ONR project and the first two years of this project). However, prior to the completion of the planned studies on heat tolerance, USDA-APHIS personnel issued a ruling that effectively limited IACUC discretionary authority with regards to approval of alternative husbandry practices that are standard for privately-owned athletic dogs. As a result, we were unable to complete the planned studies.

Introduction

Adaptation to increased metabolic output in the form of sustained exercise requires distinctly different approaches to nutrition to achieve homeostasis. Compared to baseline, sustained exercise results in greater caloric demand and increased requirements for dissipation of heat, and can result in increased protein catabolism, urinary water and electrolyte loss, and oxidative stress. Depending upon the level of adaptation to sustained exercise, the subject can undergo physiological stress, tissue damage, and in severe cases, organ system dysfunction. However, full adaptation to levels of metabolic output 3-4 fold greater than baseline is achievable and results in physiological parameters indistinguishable from a healthy resting dog. In a previous ONR-sponsored contract (IDD 2.0), we identified methods of athletic conditioning that enabled IED detection dogs to perform high levels of repeated daily exercise without debilitating injury and stress. However, the practical limitations of this improved exercise capacity was the increased level of metabolic heat stress and the resulting increased daily requirement for water. This project began with two broad objectives: Identify specific areas of nutritional optimization that could improve hydration and resistance to dehydration in working dogs, and determine whether ancillary methods for dissipating metabolic heat were practical and effective in improving exercise tolerance of athletic dogs under conditions of environmental heat stress.

A primary area of nutritional optimization was to determine whether additional dietary salt would improve hydration and water retention during conditioning and improve resistance to dehydration during periods of water deprivation. In contrast to other athletic mammals for which exercise increases daily dietary electrolyte requirements due to losses of those electrolytes in sweat, dogs are generally not regarded to have increased dietary requirements for electrolytes since most of their evaporative cooling occurs through the respiratory tract and thus without electrolyte loss. However, several studies of conditioning have found that highly-trained canine athletes experience changes in body electrolyte levels that are believed to be the result of exercise-induced renal losses secondary to hydration management. Therefore, we performed a study using 2 different levels of dietary salt supplementation to determine whether an increase in dietary salt beyond what is normally received through increased amounts of kibble would improve hydration and water conservation during conditioning and multiday exercise.

A second area of nutritional optimization will be the role of dietary protein on daily water requirements. The current NRC requirement for protein in dogs undergoing endurance exercise is 90 g per 1,000 kcal intake, or 35% of ME as protein (National Research Council, 2006). Protein requirements during exercise and exercise training increase due to increases in both protein synthesis and catabolism. Protein synthesis accounts for muscle fiber hypertrophy, increases in plasma protein content, and increased synthesis of enzymes associated with energy metabolism. Protein catabolism can produce energetic substrates for the animal during exercise (Le Moyec *et al.*, 2014; Oliveira *et al.*, 2015). In most cases, commercial diets provide sufficient protein for maintenance of body tissues, and inadequate dietary protein has been associated with an increased rate of musculoskeletal injuries during exercise. Dietary protein requirements for athletic dogs were initially based on several early studies that have reported episodes of sports anemia when fed a low protein diet (National Research Council, 2006). Reynolds reported that Alaskan sled dogs in exercise training fed crude protein at a rate of 19% of metabolic energy (ME) were more likely to have soft tissue injury compared to dogs consuming more than 24% of ME as protein. These authors also report that hematocrit and total blood volume increased linearly with increasing protein content in the diet, up to 36% of ME as protein (Reynolds *et al.*, 1999). However, the use of the same diet to support the higher caloric requirements during sustained exercise results in overfeeding of protein since a 3-4 fold increase in caloric requirements typical of sustained exercise does not also result in a 3-4 fold increase in protein requirements. Nevertheless, the dietary protein is digested and absorbed, resulting in an increase in blood and urinary nitrogen load and an obligatory water loss to rid the body of excess nitrogen (J. Appl. Physiol. 83(3): 824–829, 1997). It follows that more careful titration of dietary protein relative to overall metabolic output can ensure sufficient intake to avoid musculoskeletal injury while minimizing water requirements. The present study was designed to determine the effects of protein intake throughout a period of exercise training and a 5-day exercise test, on water conservation and overall health. It was hypothesized that compared to dogs who were fed increasing amounts of both energy and protein, dogs fed only their pre-training protein intake level would have lower daily water requirements, and be more resistant to dehydration.

A second objective was to more completely define and partition the sources and flux avenues of heat in the IDD. In all mammals, extreme increases in internal body temperatures hasten fatigue onset. Elevations in body temperatures can result from elevated internal heat production during exercise, diminished heat loss capacity due to hot environmental conditions, or a combination of the two. Metabolic activity is the primary source of internal heat in IDD, and excess internally produced heat must be lost to the environment. It is generally appreciated that a hot deployment environment impedes the elimination of internal heat and, in extreme hot environments (like those common to many of the current deployment environments), it is possible – even likely – that the environment can serve as a heat source rather than a heat sink. At ambient temperatures greater than body temperature, normal heat flow patterns can be reversed and heat can be absorbed from, rather than dissipated to, the environment. Although the dog's insulating haircoat may minimize heat transfer from the surrounding air, ground-source heat may significantly affect body thermal load because the large volume of blood that perfuses the foot pads serves as a direct thermal conduct between the internal and external environments of the IDD. Ground temperatures greater than body temperature will, at a minimum, reduce heat loss capacity and at more extreme temperatures can result in a net heat transfer into the foot pads. A means to reduce absorption of external heat and facilitate dissipation of internal heat could delay fatigue onset in IDD working in extreme thermal environments.

Two more Objectives were added to the project during the period of performance, replacing the two original objectives. Objective #1 was cancelled after the 2 listed dietary interventions after investigators concluded (based on the results of these experiments) that dietary interventions were unlikely to have a significant impact on hydration and resistance to dehydration in working dogs. Objective #2 was cancelled after investigators concluded that ancillary cooling methods were unlikely to be sufficiently effective to have practical applications. The remaining period of performance was spent addressing two new Objectives: Objective #3 was directed at establishing a means of robust quantification of olfactory detection performance and using that methodology to test the effect of environmental temperatures on canine olfactory performance. Objective #4 was to assess oxidative phosphorylation in canine skeletal muscle and determine whether conditioning improved the tolerance of these physiological processes to increased metabolic heat.

Objective #1, Task #2: Determine the effect of supplementation of electrolytes on water balance, metabolic heat management, and post-exercise rehydration measures.

Eighteen dogs (racing sled dogs, 1-4 yr old) were used for this study. Previous studies of sled dogs and dogs enrolled in the IDD program demonstrated similar physiological responses to endurance conditioning when the conditioning stimuli were matched, making sled dogs a scientifically suitable and more economical study population. At the time of the study, the dogs had been consuming the baseline diet (Eagle Power Pack, 3.9 kcal/gm, 31% protein and 13% fat as fed) for at least 3 months and had not been receiving any compulsory exercise in order to reduce their overall fitness to a basal starting point.

Total body water was measured and blood and urine samples collected at three points at the start of the study: before a 24 hr water deprivation test, after the water deprivation test but prior to allowing voluntary rehydration, and after an 8 hr voluntary rehydration period. Body weight was measured at each timepoint, as well as the volume of water consumed during the rehydration period. Blood and urine samples were tested for electrolyte values using a Radiometer Flex-80 (urine was diluted 1:5 with sterile water) and creatinine was measured using a Heska Model 700 dry chemistry analyzer. Urinary specific gravity was measured using a handheld refractometer and fractional excretion of electrolytes were calculated using standard formulas.

Table 1: Baseline values for water and electrolyte balance in unconditioned athletic dogs. *significantly different from baseline, $p < 0.05$ using paired Student's t-test; †significantly different from post-dehydration, $p < 0.05$ using paired Student's t-test.

Parameter	Baseline	24 hr dehydration	8 hr rehydration
Body weight (BW, kg)	21.40 ± 3.2	20.61 ± 3.2	21.50 ± 3.4
Total body water (TBW, kg)	12.97 ± 2.40	12.91 ± 2.42	13.73 ± 2.83*†

Total body water (TBW, % BW)	0.59 ± 0.02	0.61 ± 0.02*	0.62 ± 0.03*
Serum creatinine (mg/dL)	0.68 ± 0.19	0.65 ± 0.16	0.72 ± 0.13†
uCr/sCr	102.8 ± 42.2	97.2 ± 46.5	54.8 ± 37.5
Urine specific gravity	1.037 ± 0.011	1.050 ± 0*	1.035 ± 0.015†
Serum Na+ (meq/L)	144 ± 5.8	148 ± 6.7*	140 ± 4.6*†
Serum K+ (meq/L)	4.1 ± 0.3	3.9 ± 0.2*	4.1 ± 0.3†
Serum Cl- (meq/L)	113 ± 5.4	116 ± 6.1*	108 ± 4.9*†
FE Na+ (%)	0.58 ± 0.29	1.40 ± 0.59*	0.47 ± 0.24†
FE K+ (%)	10.6 ± 7.34	24.7 ± 11.3*	7.7 ± 4.5†
FE Cl- (%)	1.1 ± 0.53	1.9 ± 0.83*	1.1 ± 0.96†
Water consumed (L)			1.62 ± 0.49
Water consumed (ml/kg BW)			74 ± 19
ΔBody weight (% baseline)		96.2 ± 1.3*	100.4 ± 2.4†

The results of the pre-study testing demonstrate that athletic dogs such as endurance racing sled dogs are highly resistant to dehydration when sedentary and housed in temperate conditions. During the 24 hr water deprivation period, dogs lost just approximately 600 ml of water through a combination of urination and respiratory evaporation. The concurrent loss of body mass that was of lower water content than body tissues (i.e., feces) resulted in a statistically-significant increase in total body water when expressed as ml/kg of bodyweight. There was not a significant change in serum creatinine, indicating that during this period of water deprivation glomerular filtration was largely preserved. However, the dogs produced more concentrated urine (all urine samples exceeded the range of the refractometer) with increased fractional excretion of electrolytes. During the voluntary rehydration period there was a restoration of renal function that had been altered during the period of water deprivation, but this was in the face of relative overhydration, with increased total body water compared to baseline and decreased serum electrolyte concentrations. These results suggest that rehydration of athletic dogs after a period of water deprivation using ad libitum access to water without additional electrolytes may result in deviations from normal physiology.

The dogs were divided into 3 groups of equal sizes: Control, Low Supplemented, and High Supplemented. Control dogs received only the baseline diet. Low-Supplemented dogs received a mixed electrolyte (Na, K, Cl) supplement that provided 50% of the electrolytes contained in the recommended daily intake of the Control diet so that the Low Supplemented dogs received a total daily electrolyte intake of 150% of control. High-Supplemented dogs received the same mixed electrolyte supplement equal to 100% of that contained in the recommended daily intake of the Control diet so that the High Supplemented dogs received 200% of the daily electrolyte intake of the control dogs. Supplements were manufactured using proprietary technology to minimize adverse taste and fed as a top-dressing on the food. Starting caloric intake during the 2 week acclimatization period prior to the start of the study was 147kcal/kg $BW^{0.75}$, and was increased to 246kcal/kg $BW^{0.75}$ following the measurement of water balance and conservation in anticipation of increased energetic demands during conditioning. From that point, diets were adjusted individually on a weekly basis to maintain starting body weight. Conditioning consisted of regularly-scheduled off-leash walks through forests, trails, and fields surrounding the study kennel in Two Rivers, AK, with increasing duration of the walks used to provide progressive conditioning stimulus. At 4 weeks and 8 weeks of conditioning, total body water and plasma volume will be measured before and after a 24 hr water deprivation test, and again after an 8 hr voluntary rehydration period. At the conclusion of the 8 week conditioning period (prior to the conduct of the water deprivation/rehydration test), a 5 day simulated deployment exercise (derived from the IDD 2.0 program, and verified using GPS recordings on the individual dogs) was conducted, during which dogs were exercised at an

intensity and duration to mimic a typical dismounted patrol lasting 5 days. Daily mileage (Table 2) and body water turnover (Table 3) were measured during this exercise.

Table 2: Exercise test mileage (miles, mean ± SD). N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

	Day 1	Day 2	Day 3	Day 4	Day 5	Total
N	28.2 ± 4.3	26.1 C 3.4	27.2 ± 3.6	25.1 ± 2.8	23.5 ± 2.4	130.2 ± 16.1
L	27.7 ± 2.8	25.6 ± 1.7	25.4 ± 2.0	24.4 ± 1.1	23.3 ± 0.7	126.3 ± 6.7
H	25.9 ± 4.3	23.8 ± 2.7	24.9 ± 3.6	23.5 ± 2.3	22.7 ± 1.5	120.9 ± 13.4
p-value	0.5723	0.3295	0.4199	0.4766	0.6797	0.4567

Table 3: Exercise test water turnover (mean ± SD). N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

	Water turnover (L/day)	Water turnover/kg (L/kg/day)	Water per mile (L/mile)	Change in TBW (kg)	Change in BW (kg)
N	2.903 ± 0.798	0.128 ± 0.020	0.112 ± 0.030	1.237 ± 0.579	-1.42 ± 0.61
L	2.598 ± 0.397	0.113 ± 0.017	0.104 ± 0.016	0.747 ± 0.400	-1.32 ± 0.46
H	2.959 ± 0.583	0.121 ± 0.007	0.123 ± 0.026	1.257 ± 0.396	-1.20 ± 0.49
p-value	0.6117	0.3379	0.4436	0.1343	0.7794

Table 4: Water and electrolyte parameters (mean ± SD) during conditioning and dehydration/rehydration. There was no statistical effect of dietary salt supplementation, so value have been pooled for different diets

Parameter	Week	Baseline	24 hr dehydration	8 hr rehydration
BW	0	21.40 ± 3.2	20.61 ± 3.2	21.50 ± 3.4
	4	21.79 ± 3.35	21.15 ± 3.32	22.03 ± 3.38
	8	22.69 ± 3.60	22.29 ± 3.46	22.47 ± 3.61
TBW	0	12.61 ± 2.2	12.74 ± 2.3	13.37 ± 2.6
	4	13.51 ± 2.36	12.92 ± 2.14	13.57 ± 2.36
	8	14.30 ± 2.63	13.38 ± 2.33	14.40 ± 2.50
TBW%	0	0.59 ± 0.0	0.61 ± 0.0	0.61 ± 0.0
	4	0.62 ± 0.03	0.61 ± 0.03	0.62 ± 0.03
	8	0.63 ± 0.03	0.60 ± 0.03	0.64 ± 0.04
Serum creatinine (mg/dL)	0	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.1
	4	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
	8	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
uCr/sCr	0	102.8 ± 42.2	97.2 ± 46.5	54.8 ± 37.5
	4	79.1 ± 22.0	114.0 ± 36	75.8 ± 19.3

	8	85.6 ± 23.0	82.8 ± 27.6	62.8 ± 35.5
Urine specific gravity	0	1.037 ± 0.011	1.050 ± 0.000	1.035 ± 0.015
	4	1.045 ± 0.007	1.049 ± 0.004	1.044 ± 0.011
	8	1.047 ± 0.007	1.050 ± 0.002	1.039 ± 0.010
Serum Na+ (meq/L)	0	144 ± 6	148 ± 7	140 ± 5
	4	134 ± 8	148 ± 9	137 ± 10
	8	136 ± 10	147 ± 4	146 ± 6
Serum K+ (meq/L)	0	4.1 ± 0.3	3.9 ± 0.2	4.1 ± 0.3
	4	4.1 ± 0.3	4.0 ± 0.3	4.0 ± 0.4
	8	3.9 ± 0.4	4.2 ± 0.3	4.3 ± 0.2
Serum Cl- (meq/L)	0	113 ± 5.4	116 ± 6	108 ± 5
	4	102 ± 8	116 ± 7	105 ± 9
	8	105 ± 9	115 ± 4	113 ± 5
FE Na+ (%)	0	0.58 ± 0.29	1.40 ± 0.59	0.47 ± 0.24
	4	1.60 ± 0.44	1.87 ± 0.50	1.59 ± 0.51
	8	0.8 ± 0.3	1.2 ± 0.4	0.9 ± 0.4
FE K+ (%)	0	10.6 ± 7.34	24.7 ± 11.3	7.7 ± 4.5
	4	23.83 ± 5.33	20.57 ± 6.70	24.65 ± 9.17
	8	25.5 ± 7.1	26.1 ± 8.3	24.4 ± 10.8
FE Cl- (%)	0	1.1 ± 0.53	1.9 ± 0.83	1.1 ± 0.96
	4	1.32 ± 0.95	1.95 ± 1.05	0.58 ± 0.81
	8	2.1 ± 0.5	2.2 ± 0.7	2.7 ± 1.9

Table 5: Statistical testing of the effects of conditioning (ordinal), water availability, and dietary salt supplementation and interaction of factors.

Variable	Cond	Suppl	Hydr	Cond x Suppl	Cond x Hydr	Suppl x Hydr
BW	0.00000	0.68084	0.00001	0.11792	0.01980	0.88609
TBW	0.00000	0.68655	0.00000	0.33841	0.00263	0.33938
TBW%	0.01071	0.91814	0.00060	0.00681	0.00000	0.54691
sCr	0.00000	0.02329	0.27546	0.00389	0.00026	0.40928
uCr/sCr	0.52947	0.12386	0.00057	0.61461	0.01063	0.59027
SG	0.15978	0.37955	0.00005	0.84607	0.02115	0.76071
sNa+	0.16342	0.78951	0.16015	0.67378	0.00113	0.56588
sK+	0.02235	0.64633	0.12637	0.17353	0.00093	0.50805
sCl-	0.11965	0.61872	0.09809	0.64869	0.00016	0.49046
FENa+	0.00000	0.14491	0.00000	0.34787	0.00003	0.34910

FEK+	0.01623	0.08073	0.00000	0.37798	0.00000	0.72859
FECl-	0.17345	0.51577	0.00411	0.04922	0.00137	0.55189

Table 6: Statistical testing of the effects of conditioning (continuous), water availability, and dietary salt supplementation and interaction of factors

Variable	Cond	Suppl	Hydr	Cond x Suppl	Cond x Hydr	Suppl x Hydr
BW	0.00000	0.61132	0.00001	0.06846	0.01489	0.89418
TBW	0.00000	0.73986	0.00000	0.98344	0.00189	0.33988
TBW%	0.05291	0.71136	0.00282	0.21467	0.00004	0.61146
sCr	0.00000	0.21181	0.00044	0.00066	0.00012	0.40474
uCr/sCr	0.60617	0.31593	0.00746	0.51483	0.23380	0.61572
SG	0.16812	0.71307	0.00052	0.68953	0.03498	0.76756
SNa+	0.65777	0.71471	0.00352	0.41447	0.00196	0.64170
sK+	0.00854	0.46769	0.08309	0.35641	0.00247	0.51276
sCl-	0.73854	0.93527	0.00246	0.41165	0.00061	0.59145
FENa+	0.45494	0.01591	0.02162	0.68588	0.02464	0.59048
FEK+	0.00777	0.24568	0.01299	0.34197	0.00017	0.81780
FECl-	0.13759	0.31055	0.00647	0.29968	0.02361	0.54362

Table 7: Effect of salt supplementation on electrolyte balance (mean ± SD) after 4 weeks of conditioning. B: Baseline (preconditioning) values; N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter	Group	Baseline	24 hr dehydration	8 hr rehydration
Serum Na+ (meq/L)	B	144 ± 6	148 ± 7	140 ± 5
	N	134 ± 5	151 ± 12	133 ± 11
	L	136 ± 8	147 ± 6	136 ± 11
	H	134 ± 12	145 ± 7	144 ± 5
Serum K+ (meq/L)	B	4.1 ± 0.3	3.9 ± 0.2	4.1 ± 0.3
	N	4.0 ± 0.2	4.0 ± 0.3	3.7 ± 0.4
	L	4.1 ± 0.3	3.9 ± 0.2	4.0 ± 0.4
	H	4.1 ± 0.4	4.1 ± 0.3	4.2 ± 0.2
Serum Cl- (meq/L)	B	113 ± 5.4	116 ± 6	108 ± 5
	N	102 ± 4	119 ± 9	101 ± 11
	L	104 ± 8	116 ± 5	104 ± 10
	H	101 ± 11	113 ± 6	110 ± 4

Serum creatinine (mg/dL)	B	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.1
	N	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
	L	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
	H	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Urine specific gravity	B	1.037 ± 0.011	1.050 ± 0.000	1.035 ± 0.015
	N	1.045 ± 0.009	1.048 ± 0.006	1.046 ± 0.010
	L	1.046 ± 0.006	1.049 ± 0.004	1.043 ± 0.012
	H	1.045 ± 0.008	1.050 ± 0.001	1.044 ± 0.011
FE Na+ (%)	B	0.58 ± 0.29	1.40 ± 0.59	0.47 ± 0.24
	N	1.25 ± 0.38	1.40 ± 0.51	1.50 ± 0.54
	L	1.54 ± 0.28	1.86 ± 0.70	1.46 ± 0.45
	H	1.95 ± 0.40	1.87 ± 0.50	1.85 ± 0.51
FE K+ (%)	B	10.6 ± 7.34	24.7 ± 11.3	7.7 ± 4.5
	N	21.69 ± 6.05	21.14 ± 6.79	24.65 ± 9.17
	L	22.68 ± 6.04	22.60 ± 8.15	22.27 ± 10.66
	H	26.76 ± 2.92	19.74 ± 5.70	22.42 ± 5.40
FE Cl- (%)	B	1.1 ± 0.53	1.9 ± 0.83*	1.1 ± 0.96†
	N	0.52 ± 0.65	1.42 ± 0.92	0.24 ± 0.31
	L	1.58 ± 0.84	2.19 ± 1.08	0.76 ± 1.11
	H	1.73 ± 0.98	2.49 ± 0.80	0.77 ± 0.91

Table 8: Effect of salt supplementation on electrolyte balance (mean ± SD) after 8 weeks of conditioning. B: Baseline (preconditioning) values; N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter	Group	Baseline	24 hr dehydration	8 hr rehydration
Serum Na+ (meq/L)	B	144 ± 6	148 ± 7	140 ± 5
	N	134 ± 11	148 ± 4	146 ± 2
	L	136 ± 9	146 ± 5	142 ± 5
	H	140 ± 12	147 ± 4	149 ± 8
Serum K+ (meq/L)	B	4.1 ± 0.3	3.9 ± 0.2	4.1 ± 0.3
	N	3.9 ± 0.5	4.2 ± 0.3	4.2 ± 0.2
	L	3.9 ± 0.2	4.2 ± 0.4	4.2 ± 0.1
	H	4.0 ± 0.4	4.2 ± 0.1	4.5 ± 0.2
Serum Cl- (meq/L)	B	113 ± 5	116 ± 6	108 ± 5
	N	102 ± 10	116 ± 3	113 ± 3
	L	105 ± 9	115 ± 5	110 ± 6
	H	107 ± 10	114 ± 3	116 ± 6
Serum creatinine (mg/dL)	B	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.1

	N	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
	L	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
	H	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
Urine specific gravity	B	1.037 ± 0.011	1.050 ± 0.000	1.035 ± 0.015
	N	1.050 ± 0.001	1.050 ± 0.000	1.039 ± 0.010
	L	1.050 ± 0.000	1.049 ± 0.003	1.036 ± 0.010
	H	1.043 ± 0.010	1.050 ± 0.002	1.043 ± 0.012
FE Na+ (%)	B	0.58 ± 0.29	1.40 ± 0.59	0.47 ± 0.24
	N	0.53 ± 0.15	1.01 ± 0.34	0.72 ± 0.38
	L	1.05 ± 0.40	1.39 ± 0.62	0.93 ± 0.53
	H	0.95 ± 0.18	1.14 ± 0.32	1.06 ± 0.39
FE K+ (%)	B	10.6 ± 7.34	24.7 ± 11.3	7.7 ± 4.5
	N	25.7 ± 9.4	24.8 ± 9.6	21.1 ± 14.3
	L	27.7 ± 7.5	27.7 ± 8.2	27.7 ± 9.0
	H	23.5 ± 4.2	26.0 ± 8.4	25.5 ± 4.4
FE Cl- (%)	B	1.1 ± 0.53	1.9 ± 0.83*	1.1 ± 0.96†
	N	2.19 ± 0.58	2.08 ± 0.70	2.88 ± 2.33
	L	2.08 ± 0.59	2.27 ± 0.84	3.04 ± 1.92
	H	2.12 ± 0.30	2.23 ± 0.68	1.93 ± 0.87

Table 9: Effect of conditioning on baseline electrolyte balance (mean ± SD). N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter	Group	Week 0	Week 4	Week 8
Serum Na+ (meq/L)	N	144.2 ± 5.8	134 ± 5	134 ± 11
	L		136 ± 8	136 ± 9
	H		134 ± 12	140 ± 12
Serum K+ (meq/L)	N	4.1 ± 0.3	4.0 ± 0.2	3.9 ± 0.5
	L		4.1 ±0.3	3.9 ± 0.2
	H		4.1 ± 0.4	4.0 ± 0.4
Serum Cl- (meq/L)	N	112.9 ± 5.4	102.0 ± 4.4	102 ± 10
	L		103.7 ± 7.7	105 ± 9
	H		100.8 ± 11.0	107 ± 10
Serum creatinine (mg/dL)	N	0.7 ± 0.2	0.5 ± 0.1	0.48 ± 0.08
	L		0.6 ± 0.1	0.54 ± 0.09
	H		0.6 ± 0.1	0.55 ± 0.08
Urine specific gravity	N	1.037 ± 0.011	1.045 ± 0.009	1.050 ± 0.001
	L		1.046 ± 0.006	1.050 ± 0.000

	H		1.045 ± 0.008	1.043 ± 0.010
FE Na+ (%)	N	0.58 ± 0.29	1.25 ± 0.38	0.53 ± 0.15
	L		1.54 ± 0.28	1.05 ± 0.40
	H		1.95 ± 0.40	0.95 ± 0.18
FE K+ (%)	N	10.59 ± 7.35	21.69 ± 6.05	25.7 ± 9.4
	L		22.68 ± 6.04	27.7 ± 7.5
	H		26.76 ± 2.92	23.5 ± 4.2
FE Cl- (%)	N	1.10 ± 0.53	0.52 ± 0.65	2.19 ± 0.58
	L		1.58 ± 0.84	2.08 ± 0.59
	H		1.73 ±0.98	2.12 ± 0.30

Table 10: Effect of conditioning on electrolyte balance (mean ± SD) after 24 hr water deprivation. N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter	Group	Week 0	Week 4	Week 8
Serum Na+ (meq/L)	N	148.8 ± 6.7	151 ± 12	148 ± 4
	L		147 ± 6	146 ± 5
	H		145 ± 7	147 ± 4
Serum K+ (meq/L)	N	3.9 ± 0.2	4.0 ± 0.3	4.2 ± 0.3
	L		3.9 ± 0.2	4.2 ± 0.4
	H		4.1 ± 0.3	4.2 ± 0.1
Serum Cl- (meq/L)	N	116.4 ± 6.1	118.7 ± 9.2	116 ± 3
	L		115.7 ± 5.2	115 ± 5
	H		112.5 ± 6.2	114 ± 3
Serum creatinine (mg/dL)	N	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
	L		0.6 ± 0.1	0.5 ± 0.1
	H		0.7 ± 0.1	0.6 ± 0.1
Urine specific gravity	N	1.050 ± 0.000	1.048 ± 0.006	1.050 ± 0.000
	L		1.049 ± 0.004	1.049 ± 0.003
	H		1.050 ± 0.001	1.050 ± 0.002
FE Na+ (%)	N	1.40 ± 0.59	1.40 ± 0.51	1.01 ± 0.34
	L		1.86 ± 0.70	1.39 ± 0.62
	H		1.87 ± 0.50	1.14 ± 0.32
FE K+ (%)	N	24.71 ± 11.29	21.14 ± 6.79	24.83 ± 9.54
	L		22.60 ± 8.15	27.68 ± 8.22
	H		19.74 ± 5.70	26.03 ± 8.42
FE Cl- (%)	N	1.95 ± 0.83	1.42 ± 092	2.08 ± 0.70
	L		2.19 ± 1.08	2.27 ± 0.84

	H		2.49 ± 0.80	2.23 ± 0.68

Table 11: Effect of conditioning on electrolyte balance (mean ± SD) after voluntary rehydration. N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter	Group	Week 0	Week 4	Week 8
Serum Na+ (meq/L)	N	140.4 ± 4.6	133 ± 11	146 ± 2
	L		136 ± 11	142 ± 5
	H		144 ± 5	149 ± 8
Serum K+ (meq/L)	N	4.1 ± 0.3	3.7 ± 0.4	4.2 ± 0.2
	L		4.0 ± 0.4	4.2 ± 0.1
	H		4.2 ± 0.2	4.5 ± 0.2
Serum Cl- (meq/L)	N	107.8 ± 4.9	100.7 ± 11	113 ± 3
	L		104.0 ± 9.7	110 ± 6
	H		110.0 ± 4.0	116 ± 6
Serum creatinine (mg/dL)	N	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
	L		0.7 ± 0.1	0.7 ± 0.1
	H		0.7 ± 0.1	0.7 ± 0.1
Urine specific gravity	N	1.036 ± 0.015	1.046 ± 0.010	1.039 ± 0.010
	L		1.043 ± 0.012	1.036 ± 0.010
	H		1.044 ± 0.011	1.043 ± 0.012
FE Na+ (%)	N	0.48 ± 0.24	1.50 ± 0.54	0.72 ± 0.38
	L		1.46 ± 0.45	0.93 ± 0.53
	H		1.85 ± 0.51	1.06 ± 0.39
FE K+ (%)	N	7.72 ± 4.54	24.65 ± 9.17	21.1 ± 14.3
	L		22.27 ± 10.66	27.7 ± 9.0
	H		22.42 ± 5.40	25.5 ± 4.4
FE Cl- (%)	N	1.09 ± 0.96	0.24 ± 0.31	2.88 ± 2.33
	L		0.76 ± 1.11	3.04 ± 1.92
	H		0.77 ± 0.91	1.93 ± 0.87

Table 12 Effect of salt supplementation on water balance (mean ± SD) after 4 weeks of conditioning. B: Baseline (preconditioning) values; N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter		Baseline	24 hr dehydration	8 hr rehydration
Body weight (kg)	B	21.4 ± 3.2	20.6 ± 3.2	21.5 ± 3.4
	N	21.2 ± 4.0	20.7 ± 4.2	21.5 ± 4.1
	L	21.1 ± 1.1	20.5 ± 1.2	21.3 ± 1.2
	H	23.1 ± 4.2	22.3 ± 4.0	23.3 ± 4.2

Total body water (kg)	B	12.97 ± 2.40	12.91 ± 2.42	13.73 ± 2.83
	N	13.59 ± 3.19	12.96 ± 2.98	13.49 ± 2.92
	L	12.97 ± 1.07	12.60 ± 1.27	13.07 ± 1.40
	H	13.96 ± 2.65	13.20 ± 2.22	14.15 ± 2.78
Total body water (%BW)	B	59 ± 2	61 ± 2	62 ± 3
	N	64 ± 5	62 ± 4	63 ± 4
	L	62 ± 3	62 ± 4	61 ± 4
	H	60 ± 1	59 ± 2	61 ± 2
Water consumed (L)	B			1.62 ± 0.49
	N			1.34 ± 0.28
	L			1.56 ± 0.22
	H			1.96 ± 0.63
Water consumed (ml/kg BW)	B			74 ± 19
	N			66 ± 21
	L			74 ± 15
	H			84 ± 18
ΔBody weight (% baseline)	B		96.2 ± 1.3	100.4 ± 2.4
	N		97.6 ± 1.4	101.3 ± 1.1
	L		97.1 ± 1.4	101.0 ± 0.6
	H		96.4 ± 0.7	101.0 ± 0.5

Table 13: Effect of salt supplementation on water balance (mean ± SD) after 8 weeks of conditioning. B: Baseline (preconditioning) values; N: No supplement; L: Low supplement (150% N); H: High supplement (200% N).

Parameter		Baseline	24 hr dehydration	8 hr rehydration
Body weight (kg)	B	21.4 ± 3.2	20.6 ± 3.2	21.5 ± 3.4
	N	21.9 ± 4.4	21.6 ± 4.2	21.9 ± 4.5
	L	22.3 ± 1.0	21.9 ± 0.6	21.9 ± 0.7
	H	23.8 ± 4.4	23.4 ± 4.3	23.6 ± 4.4
Total body water (kg)	B	12.97 ± 2.40	12.91 ± 2.42	13.73 ± 2.83
	N	14.08 ± 3.37	13.07 ± 2.80	14.27 ± 3.39
	L	13.78 ± 1.02	13.04 ± 0.91	14.01 ± 1.04
	H	14.96 ± 3.01	13.97 ± 2.86	14.86 ± 2.69
Total body water (% BW)	B	59 ± 2	61 ± 2	62 ± 3
	N	64 ± 4	61 ± 4	63 ± 4
	L	62 ± 3	60 ± 3	61 ± 4

	H	63 ± 2	60 ± 3	61 ± 2
Water consumed (L)	B			
	N			
	L			
	H			
Water consumed (ml/kg BW)	B			
	N			
	L			
	H			
ΔBody weight (% baseline)	B		96.2 ± 1.3	100.4 ± 2.4
	N		98.5 ± 1.5	99.7 ± 1.6
	L		98.0 ± 2.6	98.2 ± 2.0
	H		98.3 ± 1.5	99.1 ± 1.4

There was significant interaction between conditioning and the effects of water deprivation and rehydration for all measured parameters (**Table 5** & **Table 6**), with the specific interaction being variable depending on the parameter. Conditioning resulted in increases in total body water, body weight, and total body water as a percentage of body weight, and as would be expected, water deprivation caused a decrease in body weight and total body water, both in as an absolute measurement as well as % body weight (**Table 4**). Voluntary rehydration was vigorous at all stages of conditioning, with voluntary rehydration resulting in increases in body water, body weight, and total body water as a function of body weight that exceeded the baseline measurements. The interaction of body weight between conditioning and hydration status was the result of the relatively smaller change in this parameter during the dehydration/rehydration challenge conducted at week 8 compared to week 4 and baseline. The interaction of total body water between conditioning and hydration status was the result of the unexplained increase in total body water during the 24 hr dehydration challenge conducted during at the start of the study.

The effects of conditioning, water deprivation/rehydration, and dietary salt supplementation on serum creatinine appears complex, with the statistical model finding significant effects of conditioning and dietary salt supplementation as independent effects (**Table 5** & **Table 6**), as well as interactions of conditioning with both hydration status and dietary salt supplementation. However, with post-hoc testing only conditioning maintained a statistically-significant effect on serum creatinine, with conditioning resulting in a progressive reduction in this parameter (**Table 4**).

Urine water recovery as quantified by the ratio of urinary creatinine to serum creatinine, and urine specific gravity, were affected by water deprivation and rehydration, both as an independent variable as well as having an interaction with conditioning (**Table 5** & **Table 6**). In the case of urine specific gravity, the expected pattern of increased specific gravity during dehydration and lower specific gravity during rehydration was more pronounced at baseline relative to the conditioned dogs (**Table 4**). In the case of urine water recovery, the pattern present at baseline and after 8 weeks of conditioning of water recovery decreasing both during dehydration and rehydration was contrasted by the very marked amount of water recovery observed during dehydration after 4 weeks of conditioning (**Table 4**).

Serum sodium and chloride were similarly affected by an interaction between conditioning and hydration (**Table 5** & **Table 6**), with 4 and 8 weeks of conditioning associated with relatively lower values prior to dehydration. Following rehydration, serum Na+ and Cl- concentrations returned to the relatively low baseline values after 4 weeks of conditioning, but remained high after rehydration at 8 weeks of conditioning (**Table 4**). There was

minimal effect of the study on serum K+, with a single interaction of high post-hydration serum potassium after 8 weeks of conditioning.

All measures of fractional excretions of electrolytes were affected by hydration status (**Table 5** & **Table 6**), with a nearly uniform pattern of increasing urinary fractional electrolyte excretion during the water deprivation challenge, followed by a decrease in the calculated fractional excretion following rehydration (**Table 4**). The one exception to this pattern was FEK+, which decreased during dehydration after 4 weeks of conditioning (resulting in a significant interaction between conditioning and hydration status for this parameter). In addition, there was an independent conditioning effect for both FENa+ and FEK+, with FENa+ being significantly higher at 4 weeks of conditioning and FEK+ being significantly lower at the start of conditioning.

Additional breakdown of data as a function of supplement level is shown in Table 7, Table 8, Table 9, Table 10, Table 11, Table 12, & Table 13.

Discussion

The results of this study demonstrate that aerobic conditioning affects electrolyte and water balance in athletic dogs, but that salt supplementation during conditioning of up to double the normal dietary intake has no effect on electrolyte and water balance.

Dehydration/rehydration

The challenge of 24 hr water deprivation and voluntary rehydration produced the expected changes in hydration and renal function, with all measured parameters significantly affected. As was found in the baseline examinations, 24 hr water deprivation caused decreased total body water. Water losses occur continuously through the respiratory, urinary, and gastrointestinal tracts. Respiratory losses are essentially ion-free due to evaporation of water, and in a 22 kg dog at rest can be up to 2-3 liters/day if minimal metabolic heat is dissipated through non-evaporative mechanisms (i.e., if the dog is living in a hot environment with minimal thermal gradient between its body and its environment). Urinary losses are more variable, depending on hydration status and diet and whether the kidney is prioritizing water conservation, electrolyte conservation, or both. The ratio of urine creatinine to serum creatinine provides an indicator of how much glomerular filtrate water is recovered by the kidney since creatinine is freely filtered at the glomerulus but not recovered by renal tubule transport. The inverse of the uCr/sCr ratio can be considered the fraction excretion of water (FEH_2O) and is analogous to the fractional excretion of electrolytes. A uCr/sCr of 102.8 (initial measured value for dogs at the start of the study) corresponds to a water fractional excretion of 0.97%. This value can be compared to the fractional excretion of other components of the glomerular filtrate to provide a perspective on the relative priorities of the kidneys and, by inference, the body conditions that are stimulating the reno-modulatory pathways. In this example, concurrent measurements of FENa+, FEK+, and FECl- of 0.58%, 10.6%, and 1.1% indicate that at the time of sample collection, the kidneys were more aggressively conserving sodium, less aggressive in conserving potassium, and were conserving chloride at about the same rate as water. After 24 hr of water deprivation (during which the dogs lost an average of only 60 ml of body water), renal conservation of water was essentially unchanged (FEH2O 1.03%), and renal conservation of electrolytes was reduced (1.40%, 24.7%, and 1.9%, for FENa+, FEK+, and FECl-, respectively), indicating that during this period, there was a greater emphasis on water conservation than electrolyte conservation relative to the euhydration baseline. This would be consistent with a relatively greater loss of water compared to electrolytes, as would occur through respiratory evaporation. The reduced emphasis on electrolyte conservation was appropriate given the higher serum concentrations of the main extracellular electrolytes. The increase in FENa+ - or more precisely the lack of a decrease in FENa+ - suggests that blood pressure was maintained sufficiently to minimize additional activation of the renin-angiotensin-aldosterone system which would have resulted in more aggressive conservation of sodium and decreased FENa+. During the rehydration period, the FEH2O was 1.82% - still quite low, but increased compared to baseline as the ad libitum access to water allowed for rehydration and less need for water conservation. Electrolyte excretion returned to normal (pre-water deprivation) values, indicating that relative to baseline, there was no detection of excessive electrolyte concentrations and no alteration in blood pressure. These patterns, and by inference the underlying physiology and regulatory stimulis, were preserved throughout the conditioning period, but were not affected by dietary salt intake.

Conditioning

Eight weeks of progressive aerobic conditioning resulted in an increase in total body water, increase in body mass, and increase in lean body mass (body water as a function of body mass). This is a commonly reported response, resulting both from loss of fat mass as well as an absolute increase in body water. The latter is generally regarded as a mechanism to increase plasma volume and improve cardiac diastolic filling, with resulting increased cardiac output as a component of increased aerobic capacity. Paired with this increase in body water was a decrease in serum creatinine. A change in creatinine could reflect either decreased production and release of creatinine into the blood or increased clearance through increased glomerular filtration. There is no previously-described occurrence of decreased production and release of creatinine as a function of aerobic conditioning – indeed, the conditioning-induced increase in muscle mass could increase production and release of creatinine. Conversely, increased creatinine clearance is conceivable as a function of increased plasma volume and increased rate of glomerular filtration.

The conditioning-associated increase in FENa+ at 4 weeks that corresponded to low serum Na+ and aggressive water recovery during dehydration. There is no scenario associated with aerobic conditioning that explains these findings. Inappropriate release of vasopressin, leading to increased blood pressure and renal water recovery, could explain these findings, but an underlying cause for this process remains unknown.

Interaction between conditioning and hydration

The well-known effect of conditioning on body water raises the possibility that conditioned dogs will have altered responses to water deprivation and rehydration. This study provides some tentative evidence in support of this hypothesis, insofar as the water deprivation challenge conducted after 8 weeks of conditioning resulted in smaller changes in body water compared to the water deprivation challenges at the beginning of the study and after 4 weeks of conditioning. It was less clear whether this effect was merely due to the dogs having more total body water or a change in renal function, as the significant interactions detected in the renal concentrating ability (urine specific gravity, uCr/sCr) and serum electrolytes were inconsistent. Further study will be required to determine whether there is a conditioning-associated change in renal function that helps preserve body water during water deprivation.

Effect of dietary salt supplementation

There was no statistically-significant effect of dietary salt supplementation on any measured parameters in this study. It is possible, perhaps even likely, that some changes in physiology were not detected due to insufficient statistical power. Indeed, when an alternative approach to statistical analysis was applied, in which the conditioning factor was treated as a continuous variable, there was a conditioning-associated effect on FENa+, as would be expected from increased salt intake. However, the analysis of the critical primary endpoints concerning the dogs' exercise performance was straightforward and there were no compelling trends that would support an effect – positive or negative – of salt supplementation on these endpoints. It can be concluded by these data that in contrast to our original hypothesis, increased dietary salt intake does not improve athletic performance of dogs nor their hydration balance during challenges of modest water deprivation. Similarly, these data indicate that an increase in dietary salt that occurs for other reasons should not raise a concern about an adverse effect on athletic performance or water balance.

Objective #1, Task #2: Determine the effect of reduced dietary protein on water requirements and markers of skeletal muscle integrity and amino acid turnover in exercising dogs

Material and Methods

All procedures were approved by Oklahoma State University's Animal Care and Use committee (A3722-01). Alaskan sled dogs were used in this study as a model for athletic canines that are capable of endurance exercise. Sixteen mixed-breed elite racing sled dogs (22.0 ± 2.5 kg BW) were used in this study that was conducted during the summer of 2013 (June – July). During this time period, the Fairbanks, Alaska area had warmer than normal temperatures, with July having a mean temperature of 64.3°F and 9 days that were 80°F or warmer. All dogs had been unexercised for 2 months prior to the start of the study. Dogs had been maintained on a

commercial dog fed formulated for athletic activity (31.4% crude protein, 12.7% crude fat, on an as fed basis) in amounts sufficient to maintain body weight.

Exercise conditioning consisted of guided walks, during which the handlers traveled (walking) 3 hrs (with rests) 3 times per week in week 1, and increased to 5 hr of travel (with rests) 4 times per week in week six (**Table 14**). During these walks, dogs ran off-leash. Individual distance traveled per dog was recorded by GPS (Garmin DC40 GPS tracking collar). Daily and weekly distances were recorded for each dog.

Table 14: Weekly conditioning program. *with sufficient rests to avoid overheating

WEEK	DURATION	FREQUENCY
Week 1	3 hr of activity*	3X per week
Week 2	3 hr of activity*	4X per week
Week 3	4 hr of activity*	3X per week
Week 4	4 hr of activity*	4X per week
Week 5	5 hr of activity*	3X per week
Week 6	5 hr of activity*	4X per week

During exercise conditioning period the calorie intake for 8 dogs (Control) was increased by feeding the standard commercial kibble in increasing amounts to maintain the dog's body weight. Body weight was assessed weekly and feed offered was adjusted accordingly to maintain body weight. Feed intake was held constant during the 5-d exercise test (described below).

The remaining low protein dogs (Low) were also fed to maintain body weight. However, the initial commercial kibble amount fed during week 1 was maintained throughout the exercise conditioning period. Additional calories required to maintain body weight (assessed weekly) were provided using table sugar and vegetable oil to obtain a 50:50 ratio of remaining calories from carbohydrate and fat. The Low dogs were also supplemented with the vitamin-mineral premix from the commercial feed at a similar inclusion rate to ensure other nutrients were being met similarly to the Control dogs. Daily metabolizable energy intake and daily protein intake was calculated (National Research Council, 2006). The diets resulted in similar calorie intakes (181.3 ± 20.0 and 205.7 ± 36.3 kcal/kg$^{0.75}$, for the control and low protein dogs respectively) but Control dogs had higher protein intakes, both in amount of protein ingested (6.8 ± 0.8 and 4.6 ± 0.9g/kg BW) and as a fraction of ME intake (32.2 ± 0.0 and 19.4 ± 2.4% of ME intake).

At the end of week 6, dogs completed a 5-d exercise test in which they traveled 24km per day (distance determined by the least distance covered by a single dog during the previous week). During each day of the test, handlers walked for approximately 6 hrs while the dogs traveled off leash. The dogs were monitored by GPS, and when each dog had traveled 24 km, the individual dog was picked up by the researchers and returned to the kennel using motorized transport. Total Energy Expenditure (TEE) during the 5-day exercise test was determined using the doubly-labeled water technique (D_2O, 0.3 g ^2H_2O/kg bodyweight) and ^{18}O-labeled water (0.2 g $H_2{}^{18}O$/kg bodyweight). Isotope-enriched water was administered intravenously as filtered and ultraviolet light sterilized isotonic saline. Daily serum samples were obtained each morning for measurement of specific isotope concentrations in body water, and the difference in the decay rates between D_2 and ^{18}O-labeled water was used to calculate total energy expended during the exercise-test period (Hinchcliff *et al.*, 1997; Wolfe, 1992). Samples also were analyzed for serum urea nitrogen (SUN), creatinine (Cre), and albumin (Alb) concentrations and creatine phosphokinase (CPK) activity on days 1 (first day of exercise) and the day after exercise, day 6. Samples were also analyzed for Na, K, Cl, Ca on days 0 (day before exercise test) and on day 6 (day after the exercise test).

The effects dietary protein intake on body water and renal function during water deprivation and voluntary rehydration were assessed on the day after completion of the 5-day exercise challenge. Total body water (TBW) was measured using deuterium oxide (D_2O, 0.3 g ^2H_2O/kg bodyweight) administered intravenously following a pre-dosing baseline blood sample (taken by jugular venipuncture). Four-hours post dosing, a second

blood sample was collected and TBW was calculated (Son *et al.*, 1998). TBW was determined as part of the measurement of TEE the day before the exercise test (to assess the post dietary and conditioning period) and the day after the exercise test (post-exercise test). The dogs then underwent a 24-hr dehydration test (water withheld) and TBW was determined at its completion. The dogs then underwent an 8 hour rehydration test (with ad libitum water intake recorded) followed by the last TBW determination. The blood collected at pre-dosing was analyzed for creatinine, sodium, chloride and potassium. Free-catch urine samples were obtained just prior to each administration of deuterium oxide. Urine was analyzed for creatinine, sodium and chloride. The urine creatinine-serum creatinine (CrU/CrS) ratio and the fractional excretion (FE) of Na and Cl, were also calculated.

Analysis of variance using Graph Pad Prism (GraphPad Software, La Jolla, CA) was used to determine if there were differences in calorie intake, protein intake and distances traveled between the two dietary groups during the exercise-conditioning period. Unpaired t-tests were conducted between the two dietary treatments for differences in TEE from the exercise test, the TBW at each time point (post-conditioning, post-exercise test, post-dehydration and post-rehydration) and the concentrations of Na, K, Cl, CrU/CrS, FE- Na and FE-Cl. Statistical differences were set at $P < 0.05$, while trends were noted at $P \leq 0.1$.

Results.

Conditioning period

All dogs consumed their diets daily. There were no subjective reports of abnormal feces during the study as result of the oil and sugar diet. All dogs maintained similar body weight throughout the study and no injuries or lameness occurred. Body weight did not change throughout the diet and conditioning period, nor was there a difference between dietary groups.

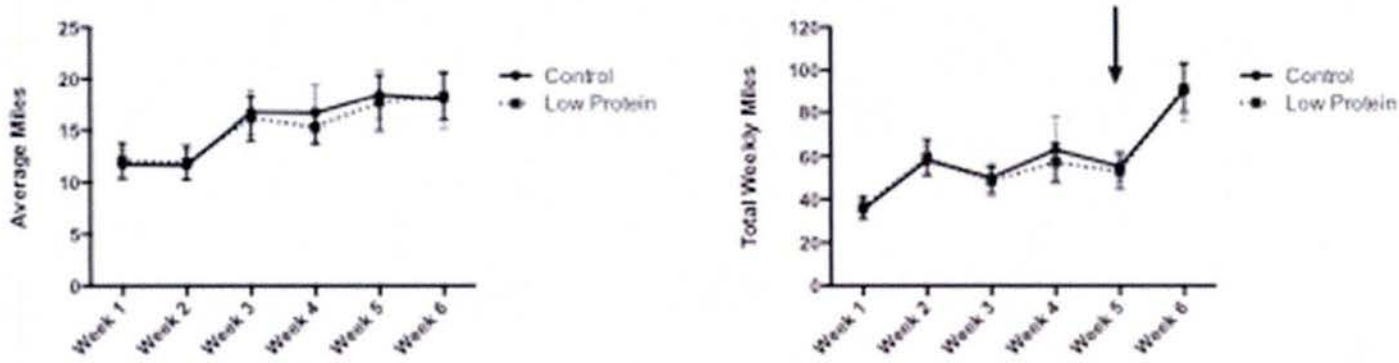

Figure 1 Average daily mileage (km) and total weekly mileage (km) for dogs on low protein or control protein diets during 6 weeks of exercise conditioning. The arrow indicates the week where forest fires prevented exercise on two days, affecting the total weekly mileage in week 5.

Travel distances are shown in Figure 1. In week 1, dogs traveled an average of 19.1 ± 2.5 km each day (weekly total 57.2 ± 7.5 km), and increased to an average of 29.0 ± 3.9 km traveled each day (weekly total 145.0 ± 19.6 km) at week 6. At the end of 6 weeks of training, there was no significant difference in average distance travelled per day during week 6 between the two groups (28.7 ± 4.4 and 29.2 ± 3.6 km for Control and Low respectively; $P = 0.55$). During week 5 there were forest fires in the area that affected total weekly mileage (Figure 1B).

Body chemistry values following the conditioning period, and before the start of the exercise challenge, are shown in Figure 2. Following the diet and exercise-conditioning period, dogs on the low-protein diet had significantly lower plasma K^+ ($P=0.02$) and serum urea nitrogen ($P=0.035$) concentrations. In addition, there was a trend for the dogs on the low-protein diet to have lower serum CPK activity (data not shown) and lower total body water (as a percentage of body weight) (Figure 6B), although these differences did not achieve statistical significance.

Exercise test

Total energy expenditure over the 5-day exercise test is shown in Figure 3. Dogs expended an average of 1491 ± 264 kcal/day (145 ± 25 kcal/$kg^{0.75}$/day), with no difference between the dietary treatments (p = 0.333). Time to complete the 24km test varied between dogs, but there was no difference between the groups. Daily water turnover during the 5-day exercise test, expressed as a % of TBW, was significantly greater in Low dogs compared to Control dogs (22 ± 3 vs. 19 ± 2%, p = 0.042).

There were no significant changes in any of the electrolytes as a result of the exercise test (data not shown). Albumin, creatinine and serum urea nitrogen data are presented in Figure 4. Albumin was lower in the control dogs following the exercise test (P = 0.0016) compared to the start. Serum urea nitrogen also decreased in the control dogs (P = 0.0012). Creatinine concentrations tended to be lower following the exercise test compared to the start for the control dogs (P < 0.1).

Water Balance

Body weight (expressed as a % of post-condition BW) and total body water (% of body weight) are shown in Figure 5 & Figure 6. There were no significant differences in body weight between the control diet and low protein diet at each time point; post-conditioning period, post-exercise test, post-dehydration and post-rehydration (P>0.05; Figure 5A). Dogs consuming the low protein diet tended to have lower %TBW following the conditioning period (pre-exercise) and post-dehydration, but this difference was only significant following the exercise test (Figure 5B). Both groups of dogs had similar %TBW after the rehydration period. Within the Control group, %TBW was higher following rehydration compared to the other time periods, while %TBW in the Low group remained similar across the time points (Figure 6). During the voluntary rehydration phase, there was a tendency for the control dogs to consume more water during the rehydration period than the low-protein dogs (1362 ± 353 ml vs. 1075 ± 375 ml, P=0.07).

After the exercise test, control dogs had higher uCr/sCr and lower fractional excretion of sodium. However following water deprivation and following voluntary rehydration, there was no effect of diet on renal performance (Table 15).

Table 15: Effect of dietary protein intake on renal function during a water dehydration/voluntary rehydration test on dogs.

	Post-Exercise			Post-Deprivation			Post-Voluntary Rehydration		
Analyte	Control	Low	P-value	Control	Low	P-value	Control	Low	P-value
uCr/sCr	72.0 ± 18.3	56.8 ± 15.4	0.047	65.7 ± 13.6	65.5 ± 14.4	0.484	49.0 ± 15.1	42.8 ± 13.4	0.199
FENa+	0.29 ± 0.05	0.43 ± 0.13	0.006	0.68 ± 0.20	0.72 ± 0.23	0.364	0.59 ± 0.26	0.53 ± 0.21	0.317
FECl-	1.89 ± 0.52	2.34 ± 0.82	0.104	2.42 ± 0.44	2.29 ± 0.88	0.361	2.19 ± 1.22	2.23 ± 1.17	0.474

Discussion

The findings of the present study suggest that while a low protein diet did not appear to negatively affect the overall health and performance of this group of highly athletic dogs, diet did affect water balance and kidney function.

As a result of the dietary treatment, the low protein group consumed significantly less protein than the control group by replacing some of the kibble (with 31.4% CP on an as fed basis) with sugar and oil (0% protein). The low protein dogs were consuming less protein then recommended by the National Research Council (National Research Council, 2006). These recommendations come from the work of Reynolds (Reynolds *et al.*, 1999) and Hill (Hill *et al.*, 2000) who reported negative performance, increased injuries or circulatory issues when dogs are fed lower levels of protein. Unlike the findings of Reynolds (Reynolds *et al.*, 1999), there were no injuries or lameness in either group of dogs in the present study. Both groups of dogs traveled the same distance during

the off-leash conditioning period and there was no difference in time to complete the 24-km run, suggesting that the performance of the low-protein dogs was not impaired. Similarly, Ober and others (Ober *et al.*, 2016) offered 18% of ME as protein to Labrador Retrievers and also reported no negative health or performance issues with exercise conditioning and a 30-min treadmill exercise test. It should also be noted that the "low" protein diet in the present study, was still relatively high compared to other species, with dogs consuming 19.4% of ME intake of protein, or 4.6 grams per kg BW per day. This value is more than twice what is recommended for human athletes (1.2 - 2g /kg BW per day, Joint Position Statement, American College of Sports Medicine, 2016).

Towards the end of the conditioning period, dogs were offered approximately 200 kcal/kg $BW^{0.75}$ when fed to maintain body weight (2,236 kcal for a 25kg dog). During the 5-day exercise test, the TEE of the dogs was approximately 65 kcal/kg BW, or 1,625 kcal/day. While these values are similar to work in other athletic activities (de Godoy *et al.*, 2014; National Research Council, 2006; Pratt-Phillips *et al.*, 2015), kilocalorie intake was higher than the TEE measured during the 5-day exercise test. This can be explained by the workload of the exercise test being limited to the level of the least active dog (24km/day and 120km/week compared to a group average of 30km/day and 150 km/week during week 6 of the conditioning period) in order to have uniform exercise intensity that would minimize further increases in conditioning during the exercise test.

Excessive protein has potential negative effects on exercise. In horses, excess protein is degraded and results in an increase in blood urea, and may affect acid-base balance during exercise (Graham-Thiers *et al.*, 1999; National Research Council, 2007). Increased urea production from protein catabolism requires excretion via urine, which requires water. Thus, a small reduction in protein intake may be advantageous for water conservation, particularly in arid climates. There was no apparent effect of dietary protein during the conditioning period, but when exercise frequency was increased to every day during the exercise test, differences in body chemistry and water balance became apparent. Compared to Control dogs, dogs consuming lower amounts of dietary protein had slightly higher serum albumin and urea nitrogen and lower body water (as a percentage of body weight) consistent with mild dehydration. Alternatively, a difference in TBW as a %BW could be due to altered body composition (i.e., lower lean body mass and higher % body fat in the Low group) or some combination of both factors.

Low dogs had a higher rate of daily body water turnover and by inference higher daily water intake during the exercise test. Since the TEE for the two groups was similar, it is likely that the water loss due to evaporation and metabolic heat dissipation was similar, suggesting that the increased daily water requirement in the Low dogs was due to increased urinary losses – a conclusion that is supported by the lower renal concentrating activity in these dogs but directly contradicting the hypothesized water conservation benefit of a lower protein diet. A possible explanation for these surprising results is that the magnitude of the dietary protein restriction was sufficient to result in decreased renal medullary urea concentrations, creating a condition of mild medullary washout and impairing renal concentrating ability. However, it is important to note that dogs had free choice access to water during the exercise test and when access to water was restricted during the water deprivation phase of the study, both groups of dogs responded similarly. Thus, it is also possible that the increased water turnover and reduced renal concentrating performance during the exercise test was driven by increased voluntary water intake, as was recorded during the rehydration phase of the study.

Unlike other studies in athletic dogs, the present study did not find an increase in CPK activity following 5-d exercise test. This is in contrast to work in Foxhounds (de Godoy *et al.*, 2014) and racing sled dogs (McKenzie *et al.*, 2008; Piercy *et al.*, 2000). While the dogs in the present study were racing sled dogs in the winter months, the present study was conducted in the off-season (summer). As a result, the distance traveled by the dogs in the present study was significantly shorter (24km/day) than other studies (eg. 160 km/day, (McKenzie *et al.*, 2008)) and likely caused less muscle damage.

In horses, higher dietary protein is associated with higher plasma urea nitrogen (Graham-Thiers *et al.*, 2003). Similarly, Ober reported decreased serum urea nitrogen concentrations in their dogs fed 18% of ME as protein vs. 27% (Ober *et al.*, 2016). Thus, the significantly higher serum urea nitrogen concentration in the present study control (higher protein) group is consistent with previous studies. We also report lower albumin in our control dogs, which was also apparent as a trend by Ober (Ober *et al.*, 2016). Conversely, Reynolds reported

lower albumin in dogs fed reduced protein diets (Reynolds *et al.*, 1999). It may be suggested that protein was being used for anabolic muscle synthesis during the exercise test, thus resulting in lower free nitrogen or albumin in the blood at the end of the test in the control group. However, the intensity of the exercise test was at or below the exercise intensity of the dogs voluntary conditioning workload, making a conditioning effect of the exercise test unlikely.

In conclusion, reduced protein intake appears to have altered body chemistry, but did not affect athletic performance in highly athletic dogs. Given that the dogs' capacity to respond to hydration stress was preserved, the alterations in body chemistry may reflect a benign change in homeostasis and not true physiological impairment.

Figures

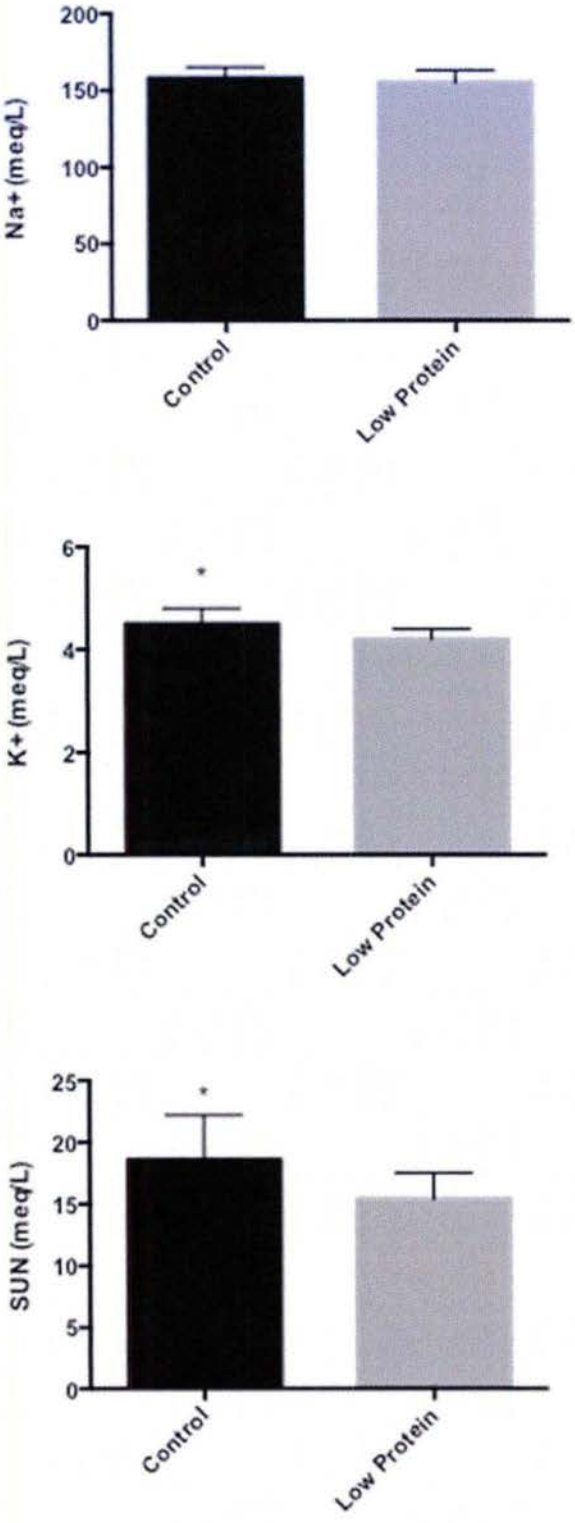

Figure 2 Post-conditioning body chemistry values for dogs consuming low protein or control protein diets over 6-weeks. * denotes P < 0.05 between low and control groups

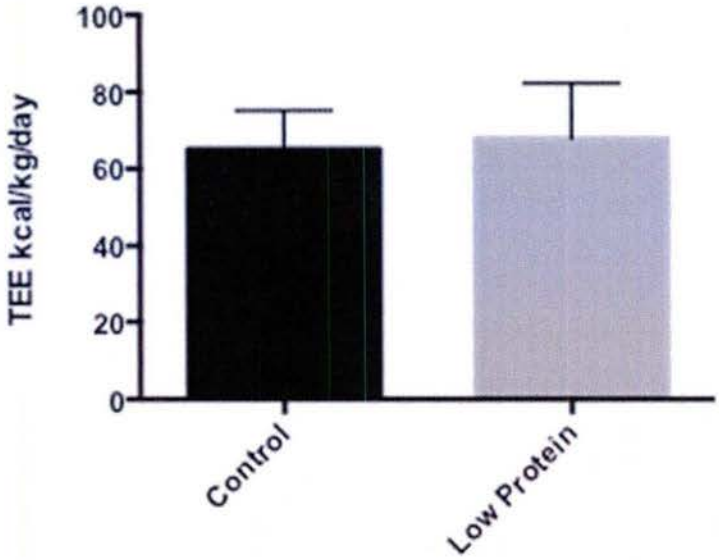

Figure 3 Total energy expenditure (TEE) expressed as kcal/kg BW/day for dogs being fed low protein or control protein diets over a 5-d exercise test.

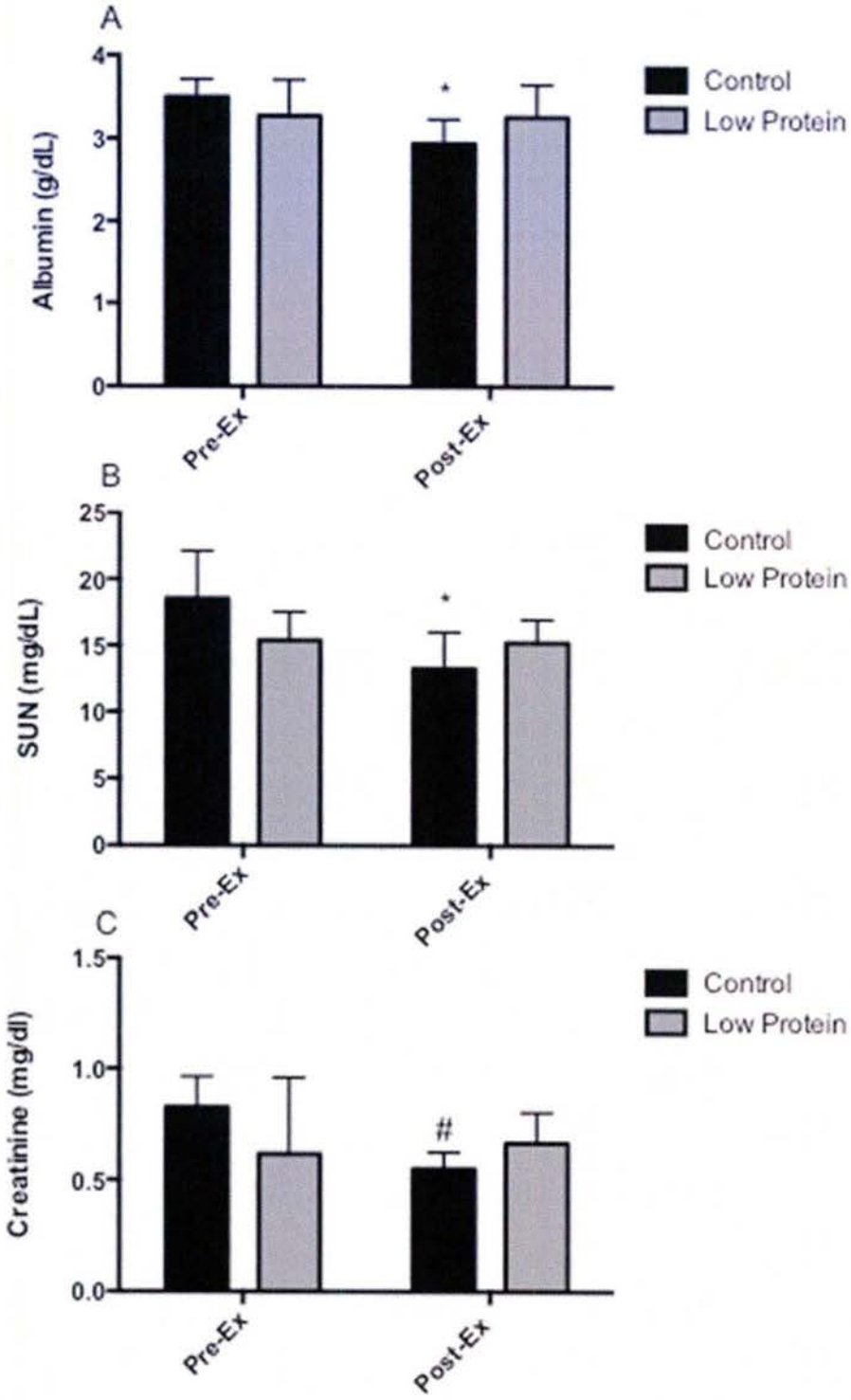

Figure 4 Effect of a 5-d exercise test dietary protein on serum albumin (A), serum urea nitrogen (SUN, B) and creatine (C). *Denotes a significant pre- vs. post-exercise difference of $P<0.05$, # denotes a trend for pre- vs. post-exercise difference at $P=0.1$.

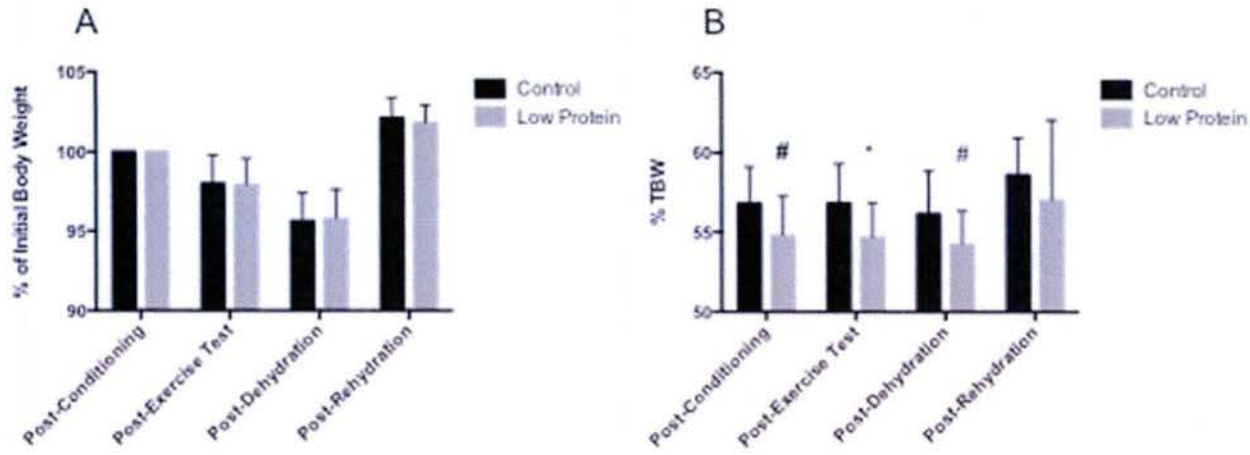

Figure 5 Body weight, expressed as a % of initial, post-conditioning body weight (A) and the % of TBW, expressed as total body water as a % of total body weight (B). # indicates a trend ($p \leq 0.1$) and * indicates a significant difference ($p < 0.05$) between the two dietary groups.

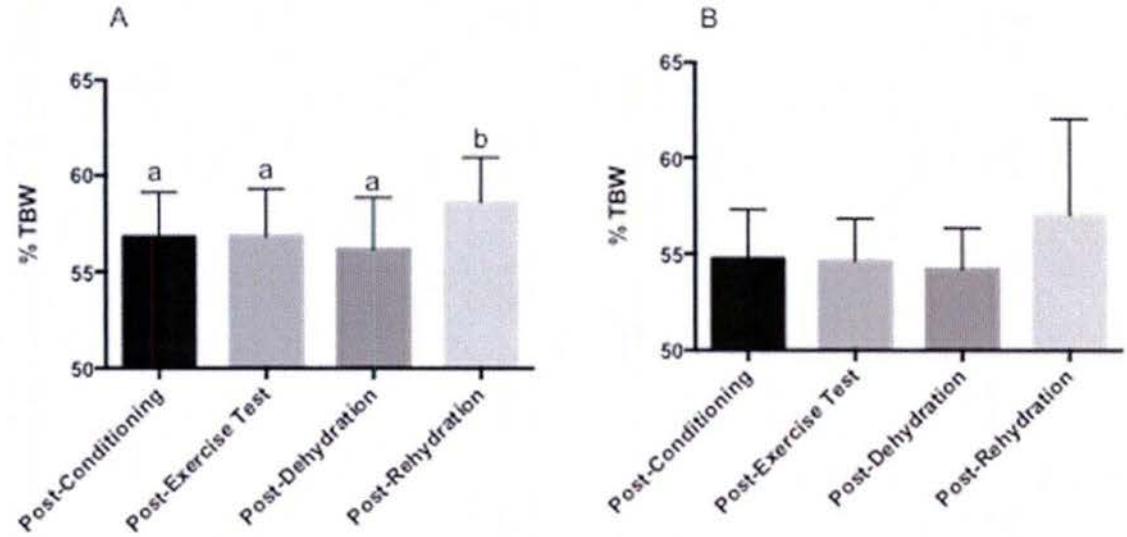

Figure 6 TBW expressed as a % of total body weight within control (A) and low protein (B) dogs. TBW was higher ($p < 0.05$) following rehydration in the control dogs.

References

de Godoy, M., Beloshapka, A., Carter, R., Fascetti, A., Yu, Z., McIntosh, B., Swanson, K. and Buff, P., 2014. Acute changes in blood metabolites and amino acid profile post-exercise in Foxhounds fed a high endurance formula. J Nutr Sci 3: e33.

Graham-Thiers, P., Kronfeld, D. and Kline, K., 1999. Dietary protein moderates acid-base repsonses to repeated sprints. Equine Veterinary Journal Supplement 30: 463-467.

Graham-Thiers, P., Kronfeld, D., Kline, K., Sklan, D. and Harris, P., 2003. Dietary protein and fat effects on protein status in Arabian horses during interval training and repeated springs. Journal of Equine Veterinary Science 23: 554-559.

Hill, R., Bloomberg, M., Legrand-Defretin, V., Burger, I., Hilock, S., Sundstrom, D. and Jones, G., 2000. Maintenance energy requirements and the effect of diet on performance of racing Greyhounds. American Journal of Veterinary Research 61: 1566-1573.

Hinchcliff, K.W., Reinhart, G.A., Burr, J.R., Schreier, C.J. and Swenson, R.A., 1997. Metabolizable energy intake and sustained energy expenditure of Alaskan sled dogs during heavy exertion in the cold. Am J Vet Res 58: 147-162.

Le Moyec, L., Robert, C., Triba, M., Billat, V., Mata, X., Schibler, L. and Barrey, E., 2014. Protein catabolism and high lipid metabolism associated with long-distance exercise are revealed by plasma NMR metabolomics in endurance horses. PLoS One 9: e90730.

McKenzie, E.C., Hinchcliff, K.W., Valberg, S.J., Williamson, K.K., Payton, M.E. and Davis, M.S., 2008. Assessment of alterations in triglyceride and glycogen concentrations in muscle tissue of Alaskan sled dogs during repetitive prolonged exercise. Am J Vet Res 69: 1097-1103.

National Research Council, 2006. Nutrient Requirements of Dogs and Cats. National Academy Press, Washington DC.

National Research Council, 2007. Nutrient Requirements of Horses. National Academy Press, Washington, D.C.

Ober, J., Gillette, R., Angle, T., Haney, P., Fletcher, D. and Wakshlag, J., 2016. The effects of varying concentrations of dietary protein and fat on blood gas, hematologic serum chemistry, and body temperatures before and after exercise in Labrador Retrievers. . Front Vet Sci 3.

Oliveira, C., Azevedo, J., Martins, J., Barreto, M., Silva, V., Julliand, V. and Almeida, F., 2015. The impact of dietary protein levels on nutrient digestibility and water and nitrogen balances in eventing horses. J Anim Sci 93: 229-237.

Piercy, R., Hinchcliff, K., DiSilvestro, R., Reinhart, G., Baskin, C., Hayek, M., Burr, J. and Swenson, R., 2000. Effect of dietary supplements containing antioxidants on attenutation of muscle damage in exercise sled dogs Am J Vet Res 61: 1438-1445.

Pratt-Phillips, S., Kutzner-Mulligan, J. and Davis, M., 2015. Energy intake and expenditure of improvised explosive device detection dogs. Comparative Exercise Physiology 11: 249-254.

Reynolds, A., Reinhart, G.A., Carey, D., Simmerman, D., Frank, D. and Kallfelz, F., 1999. Effect of protein intake during training on biochemical and performance variables in sled dogs. Am J Vet Res 60: 789-795.

Son, H., d'Avignon, D. and Laflamme, D., 1998. Comparison of dual-energy x-ray absorptiometry and measurement of total body water content by deuterium oxide dilution for estimating body composition in dogs. Am J Vet Res 59: 529-532.

Wolfe, R., 1992. MEasurement of total energy expenditure using the double-labeled water method. In: W. RR (Ed.), Radioactive and stable isotope tracers in biomedicine. Principles and practice of kinetic analysis. . Wiley-LISS, New York pp. 207-233.

Objective #2, Task #1: Develop instrumentation for thermal management of IDD

The key instrumentation that was developed during Task 2.1 was a working prototype of a system designed to extract heat from the dog through the footpads while working. This system was modeled after a similar system that was developed for human metabolic heat management during the DARPA Peak Soldier Performance program. The functional basis for the system is the fact that the glabrous (non-hairy) body surfaces of humans (palms of the hands, soles of the feet, face, and ears) and dogs (foot pads and tongues) are similar in anatomy and serve the same physiological function: to eliminate heat from the body during periods of hyperthermia. Studies in humans have demonstrated that these radiator-like vascular structures can serve as potent bidirectional heat flux pathways – absorbing heat if exposed to heat sources greater than body temperature and removing heat if exposed to material cooler than body temperature.

The system developed for dogs was comprised of a set of footpads with tubing connecting to a battery-operated circulating pump that was contained in a set of "saddle-bags" worn by the dog. The weight of the batteries and pump on one side of the system was offset by the use of a freezer pack on the contralateral side as the source of cold water for circulation. For the sake of initial testing, the system was fitted with footpads only on the front limbs, as it was easier to secure the associated tubing without interfering with the dog's ability to move. Attention to ergonomics was deferred while the basic design and testing was taking place.

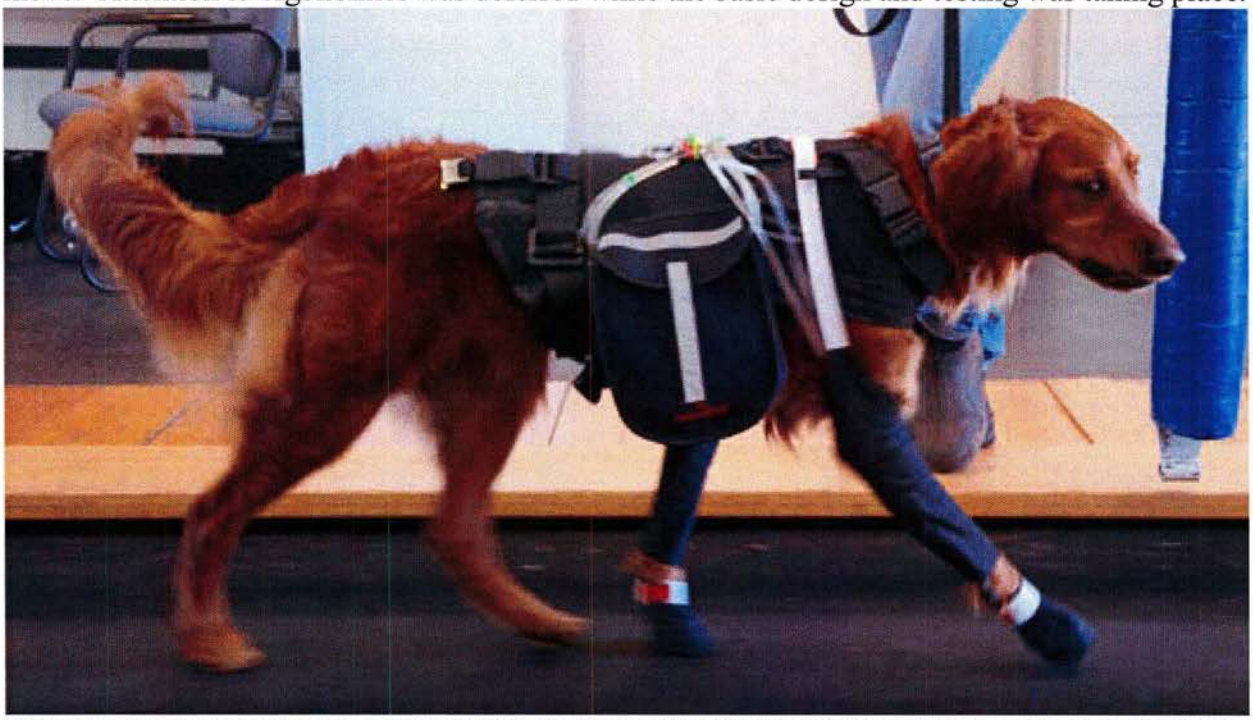

Figure 7: Photograph of a dog performing treadmill exercise with the initial prototype of the canine footpad cooling device.

An initial test of functionality of the system was performed on a single dog that was exercising at 4 mph (slow trot) on a level treadmill for 30 minutes. An ingestible radiotelemetric temperature capsule was used to record core temperature of the dogs during exercise. The dog was exercised while wearing the cooling system on two consecutive days – on Day 1 (ambient conditions 30°C, 30% RH, enthalpy 55 kJ/kg) with the circulating pump turned on, and on Day 2 (ambient conditions 22°C, 72% RH, enthalpy 53 kJ/kg) with the circulating pump turned off. It is important to note that heat is dissipated down an enthalpy gradient, and that despite the marked difference in ambient temperature, the relative capacity of the ambient air for cooling was similar due to the higher humidity. In both instances, the exercise resulted in a gradual increase in core temperature, but the rate of increase was lower (0.0203°C/min) when the cooling system was circulating compared to when it was turned off (0.0306°C/min). The primary goal of this Task was to develop a system that would remove heat from the dog during exercise, and this small study sufficiently demonstrated that the system was ready to support more rigorous testing of the hypothesis that canine metabolic heat build-up during exercise could be mitigated by extraction of heat from the footpads.

Objective #2, Task 2: Determine whether radiant shielding and active cooling of glabrous skin reduces proportional heat retention and prolongs exercise capacity.

The effect of the prototype footpad cooling device on exercise-induced heat load was tested in 8 athletic dogs. Dogs performed a standardized exercise test (7 mph, 3% grade, 30 min) under a single ambient condition (40°C produced through radiant heat sources) and two different thermal management systems (with and without active footpad cooling) in a randomized block design. Exercise sessions were separated by 48 hr to permit complete recovery and rehydration. For each exercise session, dogs were equipped with CorTemp temperature telemetry capsules to record the body heat load, and in all cases the dogs exercised with the footpad cooling system in place – the independent variable will be whether the system is turned on – to eliminate the confounding effects of the device weight and ergonomics on dependent variables. Both core temperature and rectal temperature at the conclusion of the exercise challenge were expressed as change from pre-exercise baseline values, and an effect of the cooling device was tested using a paired Student's t-test.

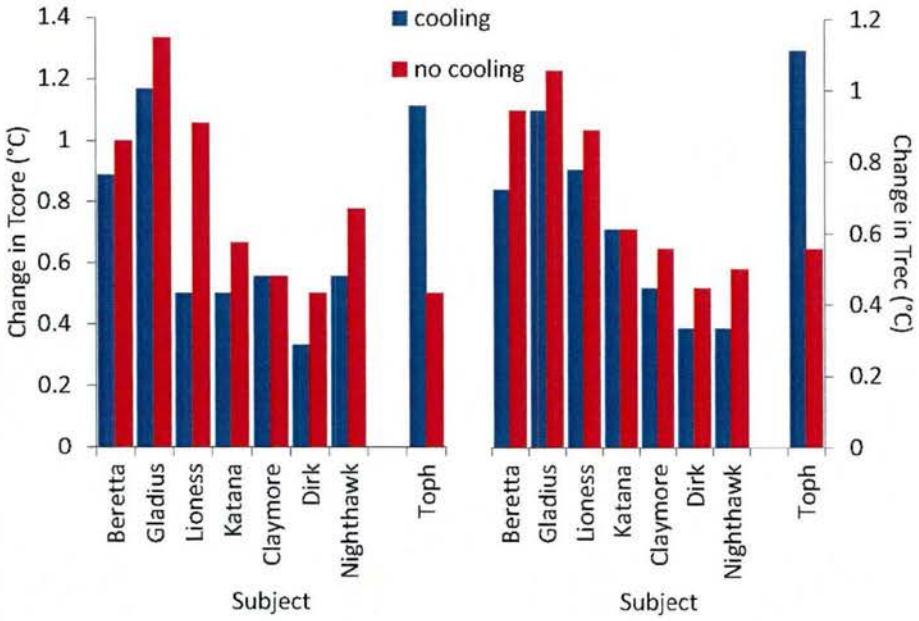

Figure 8: Effect of cooling device on exercise-induced temperature increases. Exercise was a standardized exercise test in a hot environment.

There was no significant effect of the cooling device on exercise-induced increases in core temperature or rectal temperature ($p = 0.214$ and $p = 0.351$, respectively). However, one dog was an obvious outlier, for which the exercise performed while the cooling system was active resulted in a much larger increase in heat retention than the exercise performed while the cooling system was inactive. This may have been due to device malfunction or improper operation. When this dog was eliminated from the study data, the cooling device was associated with a significant reduction in both core and rectal temperatures following exercise ($p = 0.011$ and $p = 0.002$, respectively). However, the effect of the device was modest, with a mean reduction of core temperature of only 0.2°C. Due the small benefit that appeared to be possible, combined with difficulty in achieving concurrence of all investigators with the need to improve performance of the system, the PI elected to cancel further efforts on this objective.

Objective 3: Effect of Environmental Temperature on Olfactory Performance in Dogs

There is a general appreciation that environmental conditions may affect the olfactory performance of dogs, but the exact effects have not been quantified due to considerable technical challenges relating to the precise control of the environment and its effects on the dogs and the source of the odor. With the recent construction of a large environmental chamber specifically designed to address these technical challenges, the effects of environment on olfactory performance can be quantified in a precise and reproducible manner. Twelve dogs will be trained to perform a detection routine to identify a target odorant on an odor wall equipped with a system that provided precisely controlled vapor concentration through the use of metered airflow and gas permeation tubes. Baseline performance (odor concentration detection threshold) will be established at an ambient temperature of 18°C. To quantify the combined effects of environment on both the odor source and the detector dog, then retested using the same odor concentration range under hot conditions (38°C) and cold conditions (0°C) to quantify the physiological effects of ambient temperature change the detection performance of a trained dog.

Dogs were imprinted on full-strength 1-bromo-octane and trained to sequentially search a series of 6 cans arranged along the wall of the testing chamber. For testing purposes, standards were created through progressive dilution of 1-bromo-octane with ethanol, with each dilution being 3 orders of magnitude lower than the previous dilution. Targets and blanks (100% ethanol) were created by applying 1µl droplet onto a piece of filter paper, which was then sealed in a low-density polypropylene pouch and placed in a stainless-steel perforated container. The container with the target was placed inside one of 6 empty cans arranged in a line along a wall of the testing chamber. Blanks in identical stainless steel perforated containers were placed in the remaining cans. The dog and the dog handler were blinded to the location of the target canister. Upon establishing the testing set-up, the dog was released by the handler to search the cans, with a time-limit of 30 seconds to complete the search. A positive detection was recorded if the dog provided the trained indication (sitting or lying down at a can) at the correct can within 30 seconds. A negative detection was recorded if the dog provided an indication at an incorrect can or failed to provide an indication within the 30 second time limit. Each tested dilution was evaluated 6 times daily for 3 consecutive days to establish an individual dog's baseline olfactory threshold. In order to "pass" a test for the ability to detect a specific dilution, the dog was required to have no more than one negative detection on the first day of testing for a particular odor dilution and no negative detections on the second and third days of testing for a particular odor dilution. If a dog passed a dilution, the next dilution tested would be 4 dilutions (10^{-12}) lower. The first failed dilution would be followed by testing a dilution midway between the failed dilution and the lowest passed dilution, with the following dilution being the next lowest concentration or next highest concentration, depending on whether the dog passed or failed the tested dilution. A dog's olfactory threshold was considered the lowest concentration that was passed under a given experimental condition. A diagram is provided in Figure 7 to illustrate the testing paradigm.

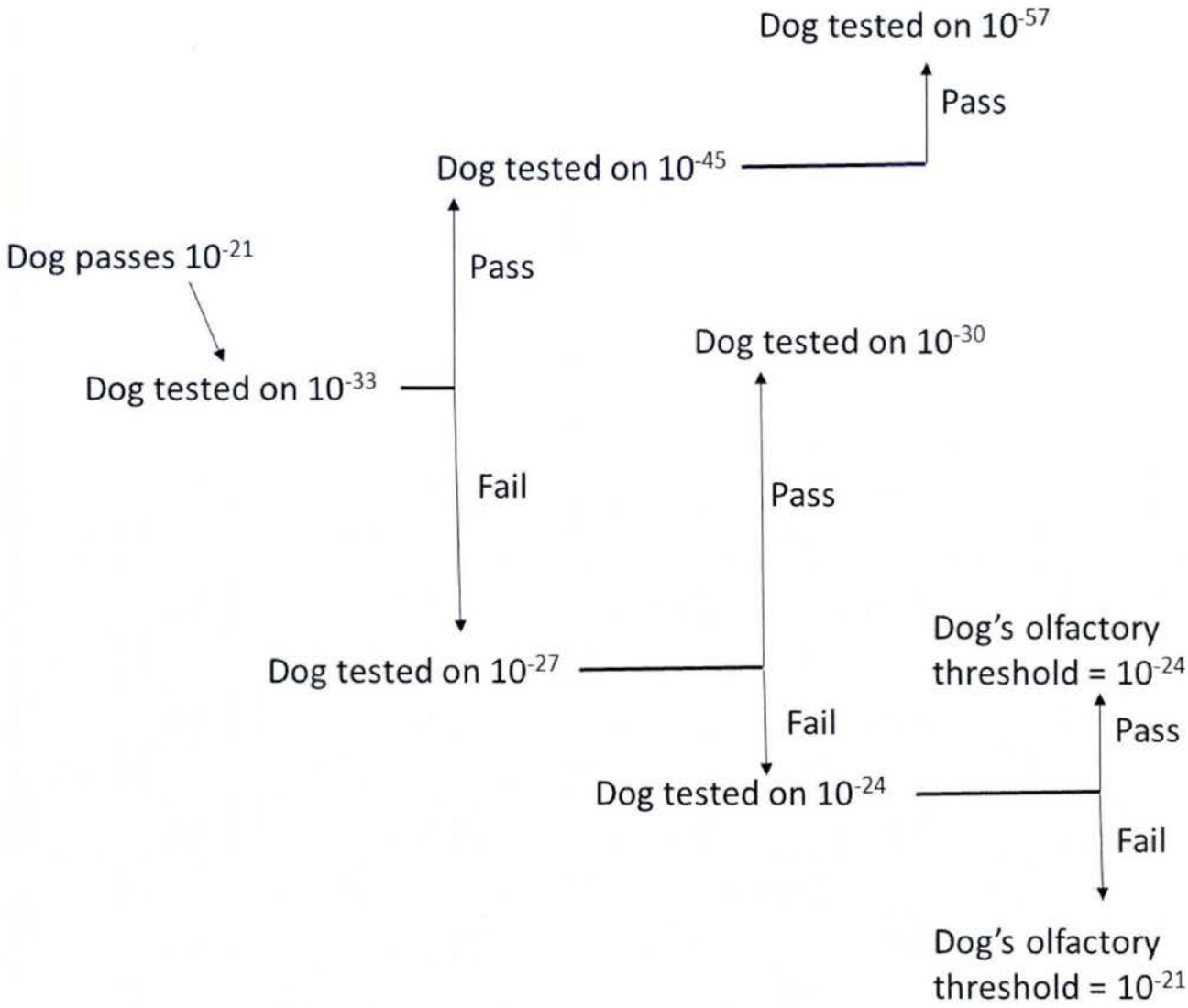

Figure 9: Diagram of testing paradigm for the determination of olfactory threshold.

Completion of this objective was ultimately unsuccessful due to a variety of factors. First, olfactory threshold testing required far more time than was originally envisioned due to the unexpectedly wide range of detection capabilities within our dog population. Using dogs selected by trainers experienced in selecting dogs for olfactory performance for military applications, we nevertheless found at least a range of over 480 orders of magnitude (with the technical caveat articulated below) between the least sensitive dog (Jessie) and the most sensitive dogs (Keeper, Flora, and Nadine). The actual range of sensitivity was likely much higher, in that we were unable to identify a lower limit of detection for these latter three dogs prior to the end of the study. This unexpectedly large range of sensitivity, combined with the duration of testing required to "pass" a specific threshold and the magnitude of the increments that we elected to use in advancing dogs through the range of odor strengths meant that clearing a range of 60 orders of magnitude dilution would require a minimum of a month of uninterrupted testing. Second, it became difficult to perform uninterrupted testing due to regulatory issues. The initial group of dogs purchased for this study were determined to have not been purchased in full compliance with USDA regulations. At the onset of the study, it was recognized by both the PI and the OSU IACUC that suitable dogs would be very difficult to procure in full compliance with USDA regulations, which include a requirement that the vendor either be a USDA-licensed vendor (which in a practical sense is limited to breeders and vendors of purpose-bred research dogs) or be purchased from the original breeder of the dog. Purpose-bred research animals are generally either beagles or mixed-breed hounds, neither of which adequately represent the target population of military working dogs. Purchasing from the same sources from which military working dogs such as IED detection dogs are purchased was the preferred approach, but these dogs are

rarely still in the possession of their original breeders when they are old enough and sufficiently trained and socialized to be suitable for the experiments planed. Therefore, the OSU IACUC granted a waiver for the animal source requirements, but subsequently discovered that such a waiver was beyond their authority as determined by the USDA. By the time this determination was made, dogs (Jessie, Quinton, Chip, Princess, Boone, Nikko, Riley, Jack, Murphy, Gizmo, and Baker) had been imprinted and approximately 8 months had been spent establishing baseline olfactory sensitivity and in some cases the olfactory sensitivity at higher environmental temperatures. In response to the USDA determination, all animal work was suspended. Eventually, it was determined that two of the original dogs purchased (Baker and Murphy) were obtained in compliance with USDA regulations and we were allowed to continue working with them. The remaining dogs were not allowed to participate in any research and were eventually adopted out to private homes, partially negating 8 months' worth of work. After another 6 months, OSU's office of regulatory compliance determined that work could resume if suitable dogs could be obtained. Eight additional dogs (Buttercup, Truman, Dolce, Stella, Rita, Flora, Keeper, and Nadine) were obtained from a local breeder of champion hunting dogs and imprinted on 1-bromo-octane. Unexpectedly, these dogs as a group turned out to have far greater olfactory sensitivity, with some of them (Keeper, Flora, and Nadine) having olfactory sensitivity more than 250 orders of magnitude lower than the best of the remaining dogs. We were unable to determine the lower limit of detection of these three dogs prior to the end of the study period. Finally, we developed progressive difficulties in maintaining testing for some of the dogs due to husbandry and illness issues. For example, Murphy was repeatedly reported to be suffering from severe debilitating neck pain by the staff of Animal Resources. Despite the clinical faculty not being able to reliably reproduce or confirm this clinical sign, nor find any anatomic abnormality on examination (including CT and MRI), Murphy was repeatedly removed from research activities due to receiving medications with unknown effects on olfactory performance. Similar issues plagued Truman, who was eventually removed completely from the research dog housing facility due to repeated injury.

Table 16: Olfactory detection thresholds for dogs in baseline and hot conditions. *indicates odor dilution that was being tested when testing was stopped for the individual dog.

Dog	Baseline Threshold (v/v)	Hot Threshold (v/v)	Cold Threshold (v/v)
Jessie	1×10^{-5}	1×10^{-3}	N/D
Quinton	1×10^{-14}	1×10^{-25}	N/D
Chip	1×10^{-43}	1×10^{-40}	N/D
Princess	1×10^{-52}	1×10^{-40}	N/D
Buttercup	1×10^{-70}	Refused to work	N/D
Truman	$1 \times 10^{-85*}$	N/D	N/D
Boone	1×10^{-88}	$1 \times 10^{-88}*$	N/D
Nikko	1×10^{-103}	$1 \times 10^{-85}*$	N/D
Dolce	1×10^{-136}	$1 \times 10^{-157*}$	N/D
Riley	1×10^{-148}	$1 \times 10^{-151}*$	N/D
Jack	$1 \times 10^{-163}*$	N/D	N/D
Murphy	1×10^{-198}	N/D	N/D
Gizmo	$1 \times 10^{-198}*$	N/D	N/D
Baker	1×10^{-225}	$1 \times 10^{-234*}$	N/D
Stella	1×10^{-231}	$1 \times 10^{-251*}$	N/D
Rita	1×10^{-231}	$1 \times 10^{-251*}$	N/D

Flora	$1 \times 10^{-486*}$	N/D	N/D
Keeper	$1 \times 10^{-486*}$	N/D	N/D
Nadine	$1 \times 10^{-486*}$	N/D	N/D

Useful information can be gleaned from our results despite the difficulties, and ultimate inability, to complete the objective. In addition to the remarkably wide range of olfactory sensitivity in this population of dogs, the dogs appeared to segregate into two groups with respect to the effect of hot environment on olfactory sensitivity. Dogs with relatively high thresholds (10^{-6} to 10^{-103}) had higher thresholds at high temperatures, indicating a relative inability to perform the detection activity, with the increased threshold 3-12 orders of magnitude higher than the corresponding baseline threshold. In contrast, dogs with lower thresholds ($<10^{-136}$) had increased ability to perform the detection activity. The magnitude of this shift cannot be confirmed, as we were unable to establish olfactory thresholds for any of the higher performing dogs prior to the cessation of the study; however, in some cases dogs were successfully detecting odors that were ~20 orders of magnitude more dilute under hot environmental conditions. With limited amount of data, it is impossible to confirm what factors caused this pattern of results, but subjectively it appeared that the dog's desire to perform played a significant role. At an extreme (as in the case of Buttercup who simply refused to perform the search activities in the hot room), a dog's ability to perform the detection activities is contingent on the dog's willingness to engage in the detection "game", and it is possible that lower performing dogs simply lose interest as the "game" gets more difficult (lower odor concentrations) or less enjoyable (hot environment). In contrast, dogs that are good at the "game" may be more willing to work at solving the problem, including potentially voluntarily electing to suppress panting so that they could increase the exposure of the olfactory mucosa to the ambient air. This approach, combined with the higher availability of the odor due to the higher temperature, would be expected to result in the ability to detect lower dilutions.

A technical caveat is in order overall with respect to the dilutions and the capacity of individual dogs to detect targets from distractors. All chemicals were stored in tightly-sealed glass containers, and all dilutions were prepared with cleaned glass and metal devices. Additionally, all targets (sample and distractor) were made fresh daily and all containers used for conducting the searches were steam-cleaned between uses. Additionally, searches were conducted with the dog handler blinded to the location of the target sample, which was switched randomly throughout the testing by the dog trainer to avoid inadvertently cueing the dog as to the correct location. Nevertheless, it is necessary to consider that the dogs that appeared to be successfully detecting extremely low dilutions of the target chemical were in fact correctly identifying the sample target through some means of detection other than the 1-bromo-octane odor. With a molecular weight of 193 gm/mole and density of 1.118 gm/ml, our starting 10 ml aliquot of pure 1-bromo-octane (10^{0} dilution) contained 3×10^{22} molecules of 1-bromo-octane. Serial dilution of this standard with ethanol would result in only 3 molecules of 1-bromo-octane in 10 ml of the 10^{-22} dilution, and the next 10-fold dilution would only have a 1 in 3 chance of having even a single molecule of 1-bromo-octane, making it virtually impossible that the dogs were actually detecting 1-bromo-octane vapor during their searches. The success of the dogs makes it also virtually impossible that they were successfully guessing the correct location of the target sample. In recognition of this unexpected and apparently impossible detection activity, all devices and containers involved in the creation of the targets were thoroughly cleaned several times during the course of the study. We have been unable to identify the source of alternative target discrimination.

Objective 4: Optimal muscle temperature respirometry

The capacity to produce ATP to fuel muscle contraction is the product of a complex chain of proteins that control transport and enzymatic catabolism of substrates within the muscle cell. Like any protein, these transporters and enzymes are potentially susceptible to degradation of performance due to the environmental milieu of the cell, including (but certainly not limited to) temperature. The working muscle has the highest range of temperatures due to the fact that it is the primary source of heat and circumstantial evidence has demonstrated that it is more tolerant of heat than many other tissues. Using newly-developed instrumentation,

we propose to precisely quantify heat tolerance of working muscle energetic pathways to test two hypotheses: First, that optimal performance of the myocyte energetic pathways is at a temperature above the "standard" resting temperature of the dog, resulting in a biochemical need to "warm up" the muscle in order to function as efficiently as possible; and second, that conditioning improves heat tolerance of myocyte energetic pathways so that ATP production is preserved over a higher and wider range of tissue temperatures.

The studies to address this objective were planned for completion in August and December of 2016, but were suspended on the order of the USDA as being potentially in violation of the Animal Welfare Act. Research using dogs is governed by the Animal Welfare Act and associated regulations, intended to protect dogs (and other mammals) used in biomedical research. Exemptions are made for livestock used in production or when the research is intended to improve the production of those species. While the use of working dogs is also exempted by policy of the USDA, no exemption has been extended to working dog research activities. Thus, it is the policy of the USDA and by extension the OSU IACUC that all working dogs used in research must be managed in full accordance with the Animal Welfare Act. It is important to note that the Act provides the IACUC with the authority to waive most (but not all) regulations after review if the deviation is justified scientifically, but the OSU IACUC has been increasingly reluctant to provide such waivers for the current working dog projects.

The conflict between the regulatory authorities and the investigators has resulted in our inability to complete this objective. Since 2000, the PI has conducted multiple research projects each year using professional racing sled dogs as experimental subjects, and as a result it was the intention of the PI to use this resource for this objective. The use of these dogs is financially and logistically advantageous for studies of exercise physiology since they are privately-owned (thus, the investigators are not responsible for daily husbandry), and they are intensively trained for strenuous exercise each racing season (thus, the investigators are not responsible for arranging the time and resources for proactively altering the dogs' fitness). Because the dogs are privately-owned and the experimental activities are conducted in the field using university-owned portable facilities, these studies have been considered field studies and permission for the use of the dogs was obtained through an informed consent form signed by the dogs' owner. About 8 years ago the OSU attending veterinarian mandated that the studies instead be treated as regular studies and began listing the kennels that provided the dogs as formal OSU research sites. This resulted in the annual inspection of these kennels (not just the OSU-owned portable laboratories) annually by both the USDA and the OSU IACUC. Despite obvious conflicts between the Animal Welfare Act regulations and the industry standards for managing a professional kennel of racing sled dogs, these inspections were uniformly free of citations until June of 2015, when the USDA issued numerous citations for these deviations. Though these citations were eventually rescinded, the USDA maintained that based on the classification by the OSU IACUC, the full kennel was subject to USDA inspection when research was occurring if that research involved dogs from that kennel, and that any deviations found at that time would result in citation and possible fines. In order to address this issue, the PI submitted animal protocols for approval that explicitly listed only the OSU-owned and controlled portable research facilities as the pertinent research sites for the work originally planned for this objective as well as two other similar projects, and these protocols were approved by the OSU IACUC. Despite the fact that with all of these projects, the subjects remained in possession of the owners and no aspects of animal husbandry were dictated or provided by investigators, the USDA nevertheless insisted that the husbandry within the kennel be in compliance with the Animal Welfare Act regulations. It is important to note that in instances of privately-owned animals participating in a clinical trial of an experimental drug obtained through the university veterinary teaching hospital, no effort is made to assure that the home environment of those animals conforms to the standards outlined in the Animal Welfare Act. Negotiations between the OSU-IACUC and the USDA continued through the start of the first phase of the study in August 2016, when the USDA notified the OSU-IACUC of an immediate inspection of the on-going studies. As a result, the PI was compelled to cancel the scheduled studies and vacate the study premises to avoid sanction and fines from the USDA.