Fatigue-resistant endurance exercise is achieved by developing sustainable patterns of substrate provision to the working muscle and of waste product elimination from the working muscle. Endurance sled dogs demonstrate marked fatigue resistance at high levels of exercise. The studies of sled dog metabolism demonstrated several noteworthy patterns of substrate delivery to the muscle, including extremely high capacity for muscle uptake of glucose, fatty acid, and lactate. In the case of glucose and fatty acid uptake, the increase in transport capacity appears to be due to increased intrinsic capacity of these transporters, rather than synthesis of additional transporter.
Fatigue-resistant endurance exercise is achieved by developing sustainable patterns of substrate provision to the working muscle and of waste product elimination from the working muscle. Endurance sled dogs demonstrate marked fatigue resistance at high levels of exercise. The studies of sled dog metabolism demonstrated several noteworthy patterns of substrate delivery to the muscle, including extremely high capacity for muscle uptake of glucose, fatty acid, and lactate. In the case of glucose and fatty acid uptake, the increase in transport capacity appears to be due to increased intrinsic capacity of these transporters, rather than synthesis of additional transporter, thus increasing the efficiency of insulin- and contraction-stimulated substrate uptake.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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(b) Papers published in non-peer-reviewed journals (N/A for none)

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(c) Presentations
Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

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TOTAL:

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(d) Manuscripts

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TOTAL: 1
Books

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TOTAL:

Patents Submitted

Patents Awarded

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Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: ...... 3.00
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The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: ...... 0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): ...... 0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: ...... 0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense ...... 1.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: ...... 0.00

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Inventions (DD882)

1 a. Colorado State University - Ft. Collins

Sub Contractor Numbers (c):
Patent Clause Number (d-1):
Patent Date (d-2):
Work Description (e): Stable isotope tracer analysis
Sub Contract Award Date (f-1): 1/31/13 12:00AM
Sub Contract Est Completion Date(f-2): 9/30/13 12:00AM

1 b. 2002 Campus Delivery

Sub Contractor Numbers (c):
Patent Clause Number (d-1):
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Work Description (e): Stable isotope tracer analysis
Sub Contract Award Date (f-1): 1/31/13 12:00AM
Sub Contract Est Completion Date(f-2): 9/30/13 12:00AM
Whole body substrate kinetics:
To our surprise the Alaskan Husky was reliant on CHO energy sources during low-to-moderate intensity exercise. It was assumed, based largely on a dietary composition in which fat is the predominant calorie source, that long-distance racing sled dogs sustain exercise by high oxidation rates of free fatty acids. In addition, we assumed, given the highly aerobic nature of the canine and especially the Alaskan Husky, that there would be adaptations that allow for sustained rates of fatty acid oxidation. In previous studies, substrate partitioning in Labrador Retrievers was compared to pygmy goats as an example of an evolutionarily evolved aerobic species (Canis lupus) versus a specie with low aerobic capacity (Capra hircus). The hypothesis was that the highly aerobic canines would have greater rates of fat oxidation than the goats. Although the researchers found that the canines had a higher absolute rate of fat oxidation than the goats at the same relative exercise intensity, this was only due to the higher overall flux rate in the dogs. In the current study, we confirmed this CHO dependence during exercise in the Alaskan Husky.

There are several interesting aspects to the CHO dependence of the Alaskan Husky. Previous studies in yearling Alaskan Huskies have reported an average VO2max of 198.7 ml/kg/min after 4 weeks of moderate intensity training, although in our observations this value could be much higher in highly trained racing dogs. In the current study, the exercise was completed at 54.9±2.1 ml/kg/min and 59.5±3.1 ml/kg/min for non-raced and raced, respectively. Therefore, the exercise task was completed at approximately 30% of the VO2max values reported for lightly-trained dogs, and this percentage may be lower if more intensively-trained dogs develop higher VO2max values as has been anecdotally described in other, more intensively trained dogs. Further, our exercise task was completed at 10.5 km/hr, while during the Iditarod, dogs regularly sustain speeds of 13-16 km/hr for up to 6-8 hrs while pulling a sled. Therefore, it is clear that the experimental exercise task was a low percentage of the maximal work capacity of the sled dogs. It is well established that lipid oxidation sustains exercise at low intensity exercise and this switches to a dependence on CHO oxidation at higher intensities. Completion of the exercise task at such a low percentage of maximal capacity of the race dogs makes the dominance of CHO oxidation a novel finding. During racing conditions, the dog is fed a diet that is approximately 33% protein, 50% fat, and 16% CHO (Eagle Ultrapack, NPAL Analysis). If we assume 10,000 kcal/day intake during racing, this equates to 3300 kcal protein, 5000 kcal fat, and 1600 kcal CHO, or 804 gm protein, 568 gm fat, and 390 gm CHO. By energy percent, the 16% CHO is below what is considered a high CHO diet, which can reach values of 64% in highly trained cyclists during competition. However, the value of 390 gm for a 24 kg dog equates to a CHO intake of 16.3 gm/kg body weight, which exceeds the recommended CHO intake of 10–12 gm/kg for an extreme exercise program in humans. Therefore, although on a relative scale the CHO intake is low, on an absolute scale it far exceeds human endurance athlete intake and could possibly sustain the prolonged exercise during racing competition.

Not only were the dogs CHO dependent, but compared to non-raced dogs the raced dogs increased CHO oxidation during an exercise bout at the same absolute intensity. It is known that CHO stores are limited and that glycogen depletion is correlated with fatigue. How then do the racing dogs sustain CHO oxidation and how do the dogs accomplish this with relatively little glycogen depletion previously observed? To appreciate the potential mechanisms that sustain CHO oxidation, it is worth comparing substrate flux values between the dogs and humans. During exercise glucose Ra and lactate Rd both exceed flux rates in humans exercising at roughly equivalent relative exercise intensities. What is more remarkable is that the glycerol Ra and Rd greatly exceeded, by approximately 5-10 times, the rates observed in humans. Glycerol Ra is the best measure of lipolysis. In addition, glycerol Rd largely represents glycerol disposal in the liver because glycerol kinase is needed to reactivate glycerol for triglyceride synthesis. Also, at the liver, glycerol can be used as a gluconeogenic substrate. The highly gluconeogenic capacity of dogs, especially from glycerol precursor, has been noted previously. In these studies, it was determined that when provided an exogenous glycerol infusion, dogs increased the rate of gluconeogenesis to match glycerol Rd. In addition, the increase in gluconeogenesis continued unabated so that the rates of conversion of glycerol to glucose far exceed those found in humans. In the current study, we calculated that a maximum of 62% of the glucose Ra could be accounted for by glycerol Rd. The data also indicate that the raced dogs have decreased contribution of glycerol to gluconeogenesis. Although the rate decreased in raced dogs, both raced and non-raced dogs have much higher rates than humans at comparative exercise intensity. However, the change in glycerol Rd from non-raced to raced suggests alternative mechanisms to sustain CHO oxidation. Although we propose that gluconeogenesis is the main fate of glycerol Rd, it is possible that dogs have a high rate of triglyceride cycling, where hepatic glycerol Rd would be used for triglyceride synthesis. It is also possible that this futile cycling could be a source of heat production to maintain body temperature in the cold. Further studies are needed to confirm the fate of glycerol Rd.
Prior to study, it was thought that lactate oxidation could represent a significant source of CHO during exercise. During exercise there was no change in lactate concentration compared to resting conditions. In addition, lactate Ra and Rd only showed a trend (p=0.09) to increase. However, lactate Rox doubled in the raced dogs compared to the non-raced dogs. This increase in oxidation suggests that part of the decrease in gluconeogenesis from glycerol could be offset by an increase in lactate oxidation, thus decreasing glucose need. The tradeoff between lactate and glycerol is interesting since lactate is another gluconeogenic substrate that has been shown to be a preferred gluconeogenic substrate and muscle fuel. It is worth noting that although the changes in lactate kinetics were relatively unimpressive, the rates of lactate turnover still exceed those found in humans at similar relative exercise intensities. Most importantly, the rate of lactate oxidation and glucose Rd cannot fully account for the amount of carbohydrate oxidized as measured by RER. Therefore, there is the possibility that these dogs did not spare glycogen, or alternative fuels, such as ketones, may change RER values.

There are several limitations to the current study. First, our study was cross-sectional. This limitation was a practical consideration since teams are picked in the days just prior to the race. Second, our measurements only took place over 90 min of exercise in relatively rested dogs and we were likely at an exercise intensity that differed from racing conditions. During long-distance races, dogs regularly complete 4-8 hr runs at roughly 10-18 km/hr. Therefore, it is unclear how the prolonged nature of that exercise and the intensity at which the exercise is performed changes substrate preference. For example, changes in hormonal profiles could shift substrate partitioning as well as progressive glycogen depletion. Third, we were limited in the number of experimental trials we could complete, thus limiting the number of tracer protocols used. We determined tracer protocols a priori to capture the most amount of information on substrate flux, and carried out the experiments over a period of two years. Because of the relatively small number of dogs tested, the constraints placed on the timing of testing, and the extreme environmental conditions, our data, although unique and informative, are not yet complete. For example, 13C glucose and fatty acid should be used to confirm RER measurements. These studies strongly indicate that sled dogs, which are equivalent to the athletic elite of aerobic mammals, are dependent on CHO metabolism during exercise. Finally, total substrate oxidation is not fully accounted for by the tracers used in the current study. As mentioned, additional carbon-labeled tracers are needed. In addition, it is possible that alternative substrates, such as ketones, have marked oxidation rates, or that there is contribution from glycogen stores in the exercising muscle or other non-exercising muscle. Future studies will need to explore these other potential contributors.

**Sarcolemmal transporter translocation and activity**

A season of aerobic conditioning resulted in a significant increase in the resting (basal) sarcolemmal transport of both glucose and palmitate. The increased basal transport of glucose and palmitate are unlikely to be due to the effects of post-prandial insulin since at the time of examination, the dogs had not been fed for at least 12 h. It is also unlikely to be due to residual effects of acute exercise as the dogs had been rested for at least 96 h and previous studies have shown that 96 h following a prolonged, glycogen depleting exercise bout, muscle glycogen concentrations have returned to baseline values. It is possible that the increase in basal transport measured in this study is not present in vivo. Although expression of sarcolemmal GLUT4 is considered the rate-limiting step for muscle glucose uptake at rest, phosphorylation of intracellular glucose by hexokinase II is believed to be the rate-limiting step during exercise. It is possible that in highly-conditioned dogs, the latter regulatory approach predominates even at rest. In this scenario, sarcolemmal transport of glucose is limited by down-regulation or inhibition of hexokinase II, resulting in increased intracellular concentrations of glucose and loss of the trans-sarcolemmal concentration gradient. Sarcolemmal flux of glucose would thus be reduced despite increased expression of transporters on the sarcolemma. Alternatively, the measured increase in basal substrate uptake suggests an increased metabolic activity of the muscle. The nature of this increased activity, if it exists, is unknown. Lacking a recent history of strenuous exercise that might induce a conditioning response, we would not expect increased metabolic activity secondary to de novo protein synthesis. Rather, the most likely explanation would be increased energetic costs of maintenance of the conditioned phenotype, which also could also help explain why such a phenotype is not maintained when it is not needed during deconditioning. However, the magnitude of the substrate transport increases are substantial (3-fold increase in glucose transport and ~50% increase in fatty acid transport), making it unlikely to be explained entirely by increased metabolic requirements of the conditioned muscle. The effect of exercise on substrate transport was profoundly affected by conditioning. With the translocation of both GLUT4 and FAT/CD36 being contraction-mediated, resulting in increased substrate transport, the lack of an exercise effect on substrate transport in unconditioned dogs was somewhat surprising. However, the intensity of our exercise test was relatively modest by the standards of even unconditioned sled dogs, whose initial conditioning activities may last 2-3 h at speeds 50% greater than our exercise test and while pulling a load. Exercise-induced translocation of GLUT4 increases as the duration of the exercise challenge increases, so the 20 min of exercise used in this study may not have been sufficiently strenuous to stimulate increased transsarcolemmal movement of substrate. That the same modest intensity exercise resulted in large increases in substrate transport in conditioned dogs suggests that an increase in the gain of contraction-mediated substrate uptake is a major feature of endurance conditioning in dogs. Our finding of increased sarcolemmal transport of glucose during submaximal exercise in response to endurance conditioning is in contrast to a similar study in humans which found decreased sarcolemmal glucose transport. Fatty acid transport was not measured in that study, but presumably the sarcolemmal transport of fatty acids increased to fuel the exercise challenge. It is possible that the increase in sarcolemmal transport capacity of glucose is misleading as the increased permeability of the sarcolemma to glucose may be offset by insufficient hexokinase II activity to maintain a large diffusion gradient for glucose. However, a similar regulatory point for fatty acids has not been described and the increase in the gain of contraction-mediated fatty acid transport reported here is consistent with the general conclusion that endurance training increases fatty acid utilization at moderate workloads.

Endurance conditioning did not increase the abundance of either GLUT4 or FAT/CD36 in skeletal muscle. Previous studies in humans, rats, and horses have documented increased skeletal muscle content of GLUT4 following exercise conditioning, as
were log2-transformed for DE statistics and thresholding. To facilitate the use of direct comparisons throughout the data, all
the standardized fluorescence units of the raw data were background-corrected and used to create ratios of fluorescence that
this dog could be used.

TP1 dog data (3 comparisons) was generally eliminated from the dataset, however the other three time point comparisons for
replicates were not done. One time point (TP1) for one Dog (dog9) was judged as unsatisfactory due to a trapped bubble. This
point. In addition, the experimental designed allows for any of the time points to be used for baseline comparisons. Technical
Nine (9) dogs were used. Because of the experimental design, this resulted in nine complete biological replicates for each time
10

2. Significantly DE and thresholded by criteria such as level of differential expression (e.g. 2-fold) or using probability (p =

Significantly

1

1

Effect of conditioning and exercise on skeletal muscle gene expression (transcriptomics)
A total of 36 muscle biopsies (9 dogs x 4 time points) were processed on 5/15/2009 and 6/14/2009. The average biopsy yielded
11.5 μg of total RNA (SD ± 7.6 μg) and samples with an A260/A280 ratio less than 2.0 were re-purified through a Qiagen
RNAeasy column. All samples were sufficient for labeling using the Epicentre TargetAmp 101 amino-ally-aRNA labeling kit.
Fluorescent dyes used were Alexa-dye NHS-ester linkage Probes 488, 522, 555, and 647. The scanner was an Axon 4400B.
Hybridizations were done as described previously. All experiments used four-color microarrays, such that for each individual
dog, direct comparisons could be made to all timepoints. As such, six comparisons are possible.

Because of this design it is possible to make direct comparisons between groups using time points 2, 3, or 4. However, as the
experiment is actually testing two different interventions (conditioning and exercise), only comparisons between Timepoint 1-3
(conditioning) and Timepoint 3-4 (exercise) have been critically evaluated. For every time point, genes were grouped into those
that were:

1. “Significantly” or statistically differentially expressed (DE, usually a large group)
2. Significantly DE and thresholded by criteria such as level of differential expression (e.g. “2-fold”) or using probability (“p = 0.01”)
3. UP or DOWN regulated within group 2

Nine (9) dogs were used. Because of the experimental design, this resulted in nine complete biological replicates for each time
point. In addition, the experimental designed allows for any of the time points to be used for baseline comparisons. Technical
replicates were not done. One time point (TP1) for one Dog (dog9) was judged as unsatisfactory due to a trapped bubble. This
TP1 dog data (3 comparisons) was generally eliminated from the dataset, however the other three time point comparisons for
this dog could be used.
The standardized fluorescence units of the raw data were background-corrected and used to create ratios of fluorescence that
were log2-transformed for DE statistics and thresholding. To facilitate the use of direct comparisons throughout the data, all
data were normalized globally by centralizing the ratios to a value of zero. This resulted in a near standard Gaussian distribution based on fluorescence intensity. These data showed good relative distributions of the data, and were used for thresholding at “fold-changes” in gene expression. However, to make direct comparisons any of the data sets the values in the chart above were all normalized to a standard Gaussian. Standardization loses fold-change information, but retains statistical probabilities (p-values) for DE genes. In general, both methods of normalization and thresholding were used during the data analyses, and both methods gave similar results. Although the technical aspects of the mRNA detection were successful and produced highly satisfactory results, the annotation of those results has been disappointing. The microarray vendor (Agilent) has produced a very poorly-annotated array and as a result we have been forced to spend considerable amounts of time performing these annotations in a largely piecemeal fashion. After using the limited annotation provided by Agilent, we have focused our annotation efforts on targets that were significantly altered by either conditioning or exercise by searching the National Library of Medicine databases for comparable genomic sequences to array sequences of interest. The resulting annotation lists have been formatted for KEGG analysis to provide big-picture overviews of pathway changes. Highlights of preliminary results include expected upregulation of energy-producing pathways secondary to conditioning (confirmed by separate, ongoing studies), downregulation of protein synthesis during racing (confirmed by subsequent studies), and downregulation of apoptotic capacity. More complete interpretation of these results is pending more complete annotation.

Technology Transfer

None