Effects of Temperature and pH on Equine Skeletal Muscle Mitochondrial Membrane Potential

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Introduction:

Mitochondrial inner membrane potential (ΔΨmt) is the potential difference across the inner mitochondrial membrane and is the electric component of the proton motive force (pmF) which drives oxidative phosphorylation (Zorova et al., 2018). Current literature shows that high intensity exercise in horses can induce conditions of acidosis and hyperthermia (Byrd et al., 1989; McCutcheon et al., 1992; Lindinger, 2014). However, despite its significant contribution to metabolism, the relationship between membrane potential and these variations in physiological condition have not been investigated.

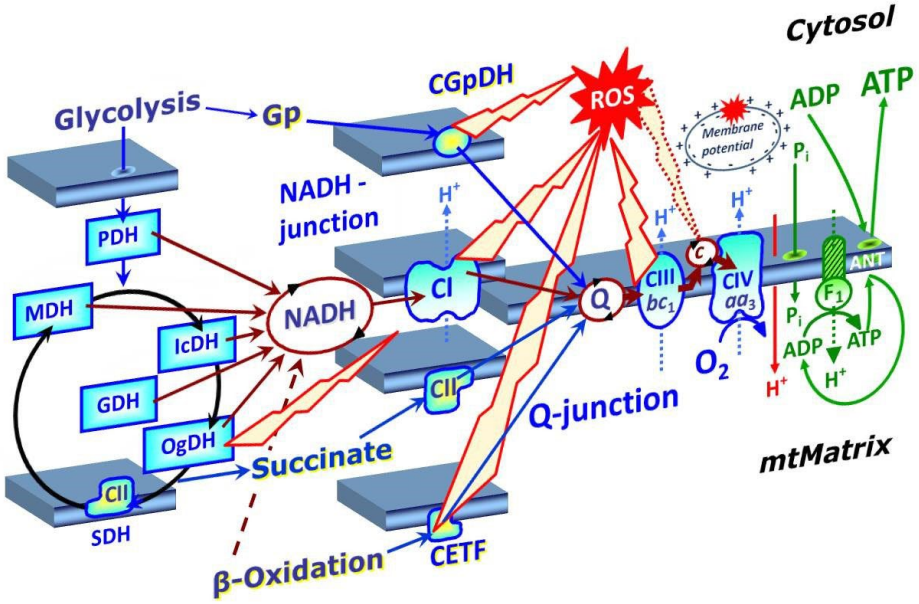


Figure 1: Schematic of mitochondrial respiration*. Adapted from Gnaiger E (2020). Demonstrates the complex interactions which contribute to ATP synthesis. See text for relevant details.*

Maximum phosphorylating respiration results from complex interactions of chemical reactions occurring in series with one another and simultaneously, with several electron sources meeting at the quinone junction of the mitochondrial electron transfer system (ETS), and further reactions in the process occurring in series through Complex III, cytochrome c, Complex IV, and ATP synthase (Figure 1). This system is tightly coupled except for ATP synthase. All other components are necessary to generating pmF to produce ATP most efficiently, but this pmF can be dissipated and decreased by avenues other than ATP synthesis. Energy losses in this system, demonstrated as a decrease in ΔΨmt, can contribute to a decrease in ATP synthesis, reducing the energy available to the exercising horse and potentially contributing to fatigue. Additionally, the production of reactive oxygen species is associated with oxidative stress and muscle damage in exercising horses. Current literature indicates that increased ROS production is associated with a high mitochondrial membrane potential (Korshunov et al., 1997; Turrens 2003), but also holds that hyperthermic conditions increase membrane permeability, leading investigators expect lower ROS production (Brooks et al., 1971; Davis et al., 2021).

A recent study at Oklahoma State University showed that hyperthermic conditions increased reactive oxygen species (ROS) production, in contrast to the study's expected hypothesis (Davis et al., 2022). Davis et al., (2022) also showed that acidosis decreased ROS production, and another recent OSU study, (Barrett et al., 2022), showed that both hyperthermia and acidosis decreased ATP synthesis. It is expected that decreased ATP synthesis would be associated with decreased membrane potential. Therefore, further study which elucidates the relationship between mitochondrial membrane potential and conditions of hyperthermia and acidosis are warranted to clarify the Davis et al. (2022) and Barrett et al. (2022) findings. This study will use high resolution respirometry (HRR) to test the hypothesis that conditions of hyperthermia and acidosis will result in decreased mitochondrial membrane potential.

Materials and Methods:

This study was approved by the Oklahoma State University Institutional Animal Care and Use Committee. Five healthy Thoroughbred horses, including one mare and four geldings (median age 14, range 10-19 years), were used. Horses were unfit at the time of the study with no compulsory exercise for at least 6 months. Body condition was maintained with free access to pasture, supplemented with hay and commercial grain rations as needed to maintain a score of 5/9.

A 12ga percutaneous UCH biopsy needle was used to obtain skeletal muscle biopsies from the semitendinosus muscle of each horse using sterile technique, light sedation, and local anesthesia. Multiple passes of the biopsy needle yielded 240 to 300 mg of wet muscle. Biopsy samples were transferred to ice cold BIOPS solution (2.77 mM CaK_2-EGTA, 7.23 mM K_2-EGTA, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, and 15 mM phosphocreatine, adjusted to pH 7.1) for transportation to the laboratory. Upon arrival, samples were weighed and divided in half. Mitochondria were isolated according to a commercial kit (MITOISO1 Mitochondrial Isolation Kit, Sigma-Aldrich). Isolated mitochondria were placed in solution with a skeletal muscle storage buffer (180 mM KCl, 0.5 mM Na_2-EDTA, and 10 mM Tris, adjusted to pH 7.4) resulting in approximately 80 µl of isolated mitochondrial suspension for every 100 mg of original sample. Samples were kept at 0-4°C throughout processing. All analysis was complete within 6 hours of obtaining a fresh biopsy.

High resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) were used to analyze the effects of temperature and pH on mitochondrial oxygen consumption and membrane potential. Instrument oxygen sensors and fluorometric probes were calibrated daily at the temperature to be used for analysis. Assays were conducted in duplicate at simulated baseline physiological conditions (38°C, pH = 7.0) as well as all other combinations of two temperatures (38°C or 43°C) and two pH levels (6.5 or 7.0). Respiration chambers were filled with mitochondrial respiration media (MiR05; 0.5 mM EGTA, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l fatty acid free BSA, adjusted to pH 7.1). pH was adjusted when needed by titrating 6 µl of 2 M lactic acid into the respiration chambers. 8 µl of 0.5 mM Tetramethylrhodamine methyl ester (TMRM) was used to fluorometrically assess mitochondrial membrane potential according to the methods of Ehrenberg et al. (1988). 15 µl of isolated mitochondria suspension were then added after achieving a baseline fluorescent signal.

A single Substrate/Uncoupler/Inhibitor Titration (SUIT) protocol was used for all assays. Saturating solutions of pyruvate (5 mM), glutamate (10 mM), and malate (0.5 mM) were titrated into each chamber to produce NADH and stimulate non-phosphorylating (leak) respiration. ADP was then added to stimulate phosphorylating respiration through Complex I. The addition of

succinate (10 mM) resulted in maximal phosphorylating respiration through the combination of Complex I and Complex II. Finally, 1 µl titrations of 1 mM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were added in series until fluorescent signal was no longer affected, indicating the system was fully uncoupled.

Parameters of interest were oxygen consumption (JO_2), measured as pmol O_2/(s*ml), and membrane potential (ΔΨmt), measured as mM fluorescing (unbound) TMRM. Further calibration assays must be done to attach mM TMRM to specific voltage, but the known linear relationship of unbound TMRM to membrane potential allows for a semi-quantitative model which uses relative comparison to assess changes in membrane potential under varying experimental conditions. TMRM signal is inversely proportional to membrane potential, so the highest TMRM signal (usually fully uncoupled) is equal to lowest membrane potential (ΔΨmt = 0). Highest membrane potential was assumed to be during non-phosphorylating (leak) respiration. The demonstrated maximum membrane potential ($\Delta\Psi mt_{(max)}$) for each individual assay was calculated as the absolute value of mM $TMRM_{leak}$ minus mM $TMRM_{max}$. Membrane potential during maximal phosphorylating respiration ($\Delta\Psi mt_{(OxPhos)}$) was calculated as the absolute value of mM $TMRM_{CI\&II}$ minus mmM $TMRM_{max}$.

Results for each parameter of interest were calculated for individual respirometer chambers, then duplicates were averaged by subject for each experimental condition. Data were analyzed separately using ANOVA Two Factor with Replication (Excel® Analysis Toolpak, Version 2203 (16.0), Microsoft Corporation, Redmond, WA, USA) with horse as the repeating measure. $P<0.05$ was considered significant.

Results:

Hyperthermia and acidosis decreased mitochondrial membrane potential both individually and in combination (Figure 2. The greatest ΔΨmt was achieved during baseline physiological conditions and decreased under hyperthermic and acidic conditions both alone and in combination. Acidosis decreased ΔΨmt as compared to baseline physiological conditions (38°C, pH=7.0) during non-phosphorylating respiration by 56% (p=0.0005), hyperthermia by 69% (p=0.0000), and the combination of both by 88% (p=0.0452). During maximal phosphorylating respiration, acidosis decreased ΔΨmt by 70% (p=0.0014), hyperthermia by 90% p=0.0000), and the combination of both by 96% (p=0.0053).

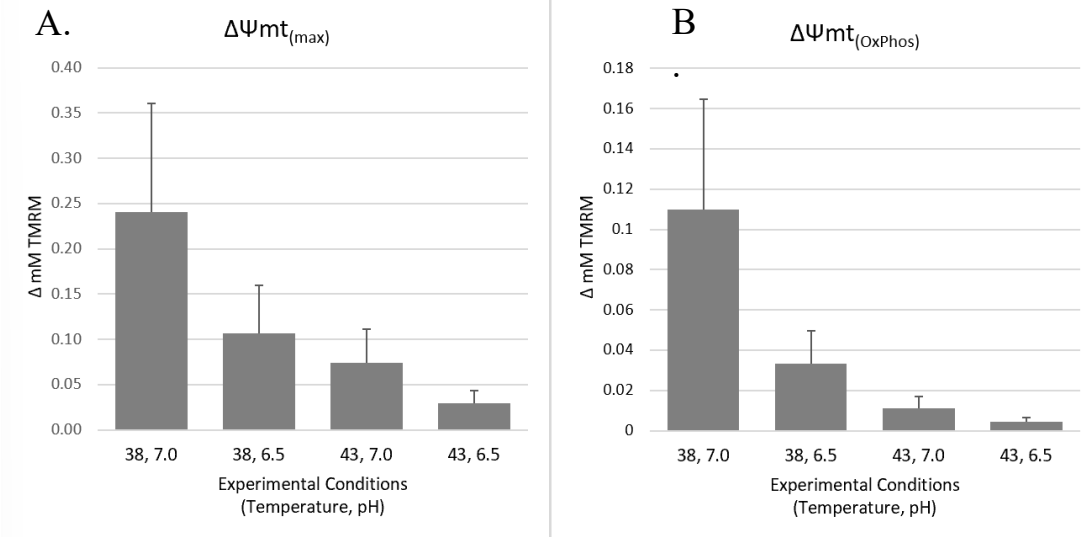
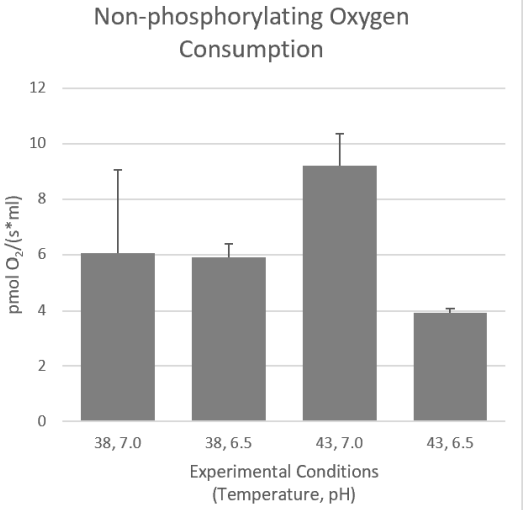


Figure 2: Mean Mitochondrial Membrane Potential during Non-Phosphorylating and Maximal Phosphorylating Respiration*. Mitochondria isolated (MITOISO1 Mitochondrial Isolation Kit, Sigma-Aldrich) from 5 fresh Thoroughbred semitendinosus muscle biopsies. High resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) equipped with*

fluorescent probes were used to analyze effect of temperature and pH on mitochondrial membrane potential. TMRM was used to measure relative membrane potential during all combinations of temperature (38 and 43°C) and pH (7.0 and 6.5). Each sample was measured in duplicate with results calculated individually for each chamber, and then averaged together. A. Average membrane potential during non-phosphorylating respiration with standard error of the mean. Represents ΔΨm(max) Calculated as the absolute value of mM TMRMleak minus mM TMRMmax. B. Average membrane potential during maximal phosphorylating respiration with standard error of the mean. Represents ΔΨm(OxPhos) Calculated as the absolute value of mM TMRMCI & CII minus mM TMRMmax.

Maximal phosphorylating respiration averaged 31.18 pmol O_2/(s*ml), under baseline physiological conditions (38°C, pH = 7.0). There was no effect of hyperthermia alone or in combination with acidosis (p=0.0853, p=0.7210, respectively), but acidosis decreased oxygen consumption by 32% at 38°C. (p = 0.0206). Non-phosphorylating respiration under baseline physiological conditions averaged 6.040 pmol O_2/(s*ml). Acidosis decreased leak respiration alone and in combination with temperature (p=0.0014, 0.0022, respectively). Compared to baseline physiological conditions, acidosis alone decreased non-phosphorylating respiration by 3% alone and by 35% in combination with hyperthermia. Temperature alone had no significant effect on non-phosphorylating respiration (p=0.4154).

A.



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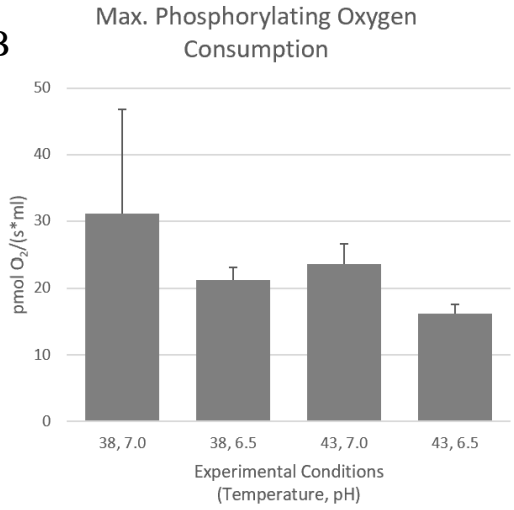

***Figure 3**: **Mean Oxygen Consumption during Non-Phosphorylating and Maximal Phosphorylating Respiration.** Mitochondria isolated (MITOISO1 Mitochondrial Isolation Kit, Sigma-Aldrich) from 5 fresh Thoroughbred semitendinosus muscle biopsies. High resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) were used to assess oxygen consumption at all combinations of temperature (38 and 43°C) and pH (7.0 and 6.5). Each sample was measured in duplicate with results calculated individually for each chamber, and then averaged together. A. Average oxygen consumption as measured in pmol O_2/(s*ml) during non-phosphorylating, leak respiration with standard error of the mean. B. Average oxygen consumption as measured in pmol O_2/(s*ml) during maximal phosphorylating respiration with standard error of the mean.*

Respiration per unit of membrane potential (JO_2/ΔTMRM) was significantly increased by hyperthermia during both non-phosphorylating and phosphorylating respiration (p=0.0000 and p=0.0004, respectively). Acidosis only affected maximal phosphorylating respiration (p=0.0284) and had no effect on leak respiration (p=0.1219). There was no significant interaction between the two variables during either non-phosphorylating or maximal phosphorylating respiration (p=0.7925 and p=0.1020, respectively).

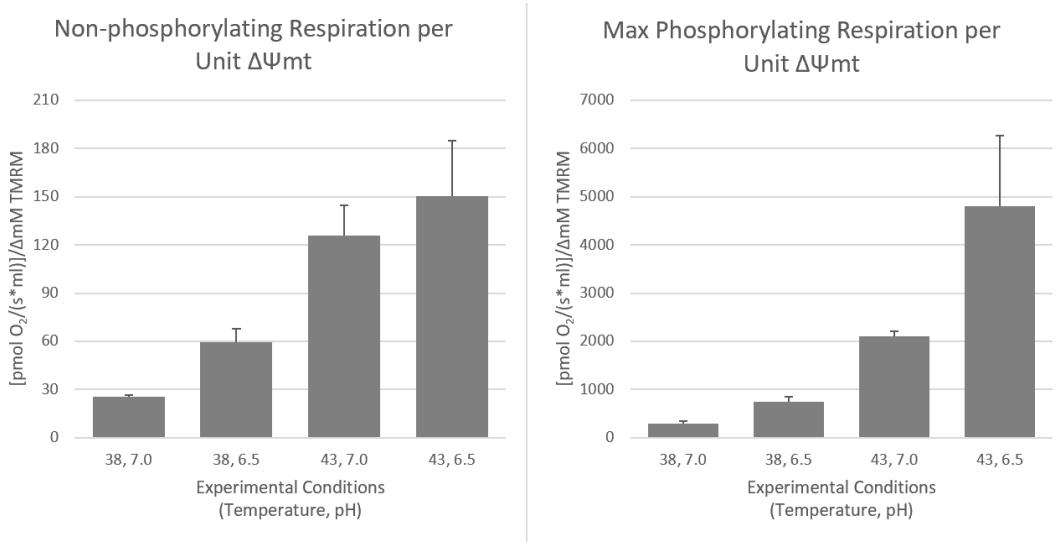

Figure 4: Amount of Oxygen consumed per unit Membrane potential. *Mitochondria isolated (MITOISO1 Mitochondrial Isolation Kit, Sigma-Aldrich) from 5 fresh Thoroughbred semitendinosus muscle biopsies. High resolution respirometers (Oxygraph O2K, Oroboros Instruments, Innsbruck, Austria) were used to assess oxygen consumption at all combinations of temperature (38 and 43°C) and pH (7.0 and 6.5). Each sample was measured in duplicate with results calculated individually for each chamber, and then averaged together. A. Average oxygen consumption as measured in pmol O2/(s*ml) divided by ΔmM TMRM during non-phosphorylating respiration with standard error of the mean. B. Average oxygen consumption as measured in pmol O2/(s*ml) divided by ΔmM TMRM during maximal phosphorylating respiration with standard error of the mean.*

Discussion:

Mitochondria use a proton motive force (pmF) created by a proton gradient across the mitochondrial inner membrane to synthesize ATP. ΔΨmt is a component of proton motive force. As protons are pumped across the inner mitochondrial membrane, it creates a chemiosmotic difference as well as an electrical potential difference. A decrease in membrane potential indicates an inability of the components of the electron transfer system to effectively maintain this gradient. Hyperthermia and acidosis both decreased ΔΨmt. However, the results of this study show that they affect membrane potential to different degrees, and they do not have an equal effect on the consumption of oxygen. This likely suggests that although the overall result is a decrease in membrane potential, they are detrimentally affecting different parts of the electron transfer system.

The greatest ΔΨmt occurs during non-phosphorylating respiration as reducing agents are produced and electrons move through the electron transfer system, but the energy is not utilized to produce ATP. Both hyperthermia and acidosis greatly decreased the maximum ΔΨmt the cell was able to generate (Figure 2), however, hyperthermia resulted in a greater percent decrease than acidosis. ΔΨmt is much less during maximal phosphorylating conditions as protons move in and out of the mitochondrial matrix and the potential energy is used to produce ATP. In this state, hyperthermia also resulted in a greater decrease than acidosis. ΔΨmt can be decreased either when the mitochondria are still respiring but the electron transfer system is unable to maintain the proton gradient due to increased permeability or when damage to one aspect of the system is so severe that the mitochondria cease to respire through it. These results alone do not say how ΔΨmt was decreased, only that it was. The design of this study does not allow for the precise quantification of ΔΨmt in terms of voltage across the inner membrane. However, the linear relationship described in the methods is sufficient to allow the comparison of differences.

In future studies, calibration assays will be conducted which allow investigators to assign specific voltage to mM free TMRM observed.

The results of this study did not indicate that temperature had a significant effect on oxygen consumption during non-phosphorylating respiration. However, all previous studies show increased temperature significantly increased leak oxygen consumption in horses (Davis et al., 2021; Davis et al., 2022) as well as rats, dogs, and humans (Brooks et al., 1971; Jarmuszkiewicz et al., 2015; Fulton, 2019; Davis and Barrett, 2021, and Fiorenza et al., 2019). As shown in Figure 3, hyperthermia alone did result in an apparent (but non-significant) increase in non-phosphorylating respiration at pH=7.0, but the opposite effect was observed in combination with acidosis, effectively cancelling each other out when assessed using ANOVA two-factor analysis. Decreased oxygen consumption by acidosis was found to be significant, even with a smaller percent change. Contrary to these results, previous studies in equine skeletal muscle have shown that acidosis does not affect oxygen consumption during non-phosphorylating respiration in equine skeletal muscle (Davis et al., 2021; Barrett et al., 2022; Davis et al., 2022). Hyperthermia almost certainly increased leak respiration, but in this study was eclipsed by a previously unobserved effect of acidosis.

Expressing data as $JO_2/\Delta TMRM$ helps illustrate whether a decrease in $\Delta\Psi mt$ is due to impaired respiration or increased permeability of the mitochondrial inner membrane. As stated earlier, previous work shows that acid had no effect on leak respiration and hyperthermia does. These results are reversed from what the oxygen consumption results of this study shows but can be explained by examining $JO_2/\Delta TMRM$. Calculating respiration per unit of membrane potential accounts for the direct inhibition of TCA and/or Electron Transfer System elements that may have been damaged by experimental conditions and therefore prevent respiration by the affected avenue. Acidosis did not affect the relationship between oxygen consumption and $\Delta\Psi mt$, demonstrating that decreased pH impaired respiration rather than increasing membrane permeability. In contrast, hyperthermia greatly increased the amount of oxygen consumed to maintain one unit of $\Delta\Psi mt$ showing the mitochondria were still respiring but the electron transfer system had to do more to maintain the proton gradient. During experimental assays at pH=6.5, mM free TMRM during Complex I was often greater than or equal to the fully uncoupled system after the addition of CCCP. This effect could result from insufficient amounts of NADH or a damaging effect to Complex I itself. If lack of NADH were the problem, such a large effect would have to be the result of malate dehydrogenase as it is the greatest NADH contributing dehydrogenase during substrate saturating conditions. While the effect of acidosis during leak was uniquely significant in this study, these results are consistent with Barrett et al. (2022) in demonstrating that acidosis did decrease maximal phosphorylating respiration.

$JO_2/\Delta TMRM$ and the other results from this study show hyperthermia has the greatest effect on $\Delta\Psi mt$, but it does not significantly alter oxygen consumption itself during phosphorylating respiration. Past literature associates increased ROS production with increased $\Delta\Psi mt$ and believes it is proportional to mitochondrial respiration. Davis et al. (2022) demonstrated that ROS increased with hyperthermia, but hyperthermia decreased oxidative phosphorylation efficiency (the ratio of ATP produced to oxygen consumed) without affecting phosphorylating respiration itself. This likely indicates that hyperthermia-induced increases in ROS production are not proportional to respiration but more likely due to inefficiencies within the electron transfer system which are more pronounced under hyperthermic conditions. As fewer electrons are passed properly through the electron transfer system, the proton gradient decreases and there is less potential energy across the membrane (decreased $\Delta\Psi mt$).

Hyperthermia and acidosis are both responsible for decreasing mitochondrial membrane potential with varying effects on oxygen consumption. Although it is likely that they affect mitochondrial function in different ways and to varying degrees, it almost certainly results in decreased ATP synthesis, contributing to the development of fatigue in exercising horses. Understanding how hyperthermia and acidosis affect $\Delta\Psi mt$ illustrates how they may contribute to the development of fatigue in exercising horses.

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