**Application**

Results from this work will provide insight into cellular changes that occur before performance horses fatigue, leading to better diagnostics to reduce incidence of fatigue-induced injury.

**Introduction**

Racehorses are considered amongst the elite of domesticated athletic species. Like human athletes, specific feeding, training, and management protocols should be implemented to ensure equine athlete health and welfare. A focus of these protocols should be prevention of fatigue, as fatigue enhances an individual’s susceptibility to injury. However, defining objective measures of fatigue remains elusive, presenting a significant barrier in prediction of injury susceptibility. We hypothesized that gluteus medius mitochondrial metrics would decrease and production of H2O2, a reactive oxygen species, would increase prior to horses experiencing decreased performance, as evidenced by a decrease in run time to fatigue.

**Materials and Methods**

All experimental protocols were reviewed and approved by the [redacted for review] Institutional Animal Care and Use Committee prior to the start of the study (AUP 24-074). Gluteus medius samples were collected from 12 mature, unfit Thoroughbred geldings (mean ± SD 8 ± 2 yr; 510 ± 35 kg) on d 0, 2, and 4 prior to performing standardized exercise tests (SET) to exhaustion on a high-speed treadmill on d 1 (SET1), 3 (SET2), and 5 (SET3). Exercise tests began at 0700 each d. To facilitate completing SETs within a 2-h window, horses were randomly and evenly split into 2 groups then staggered to perform SETs on alternating days (6 horses/d). Samples were not collected on d 6 as the hypothesis to be tested was that mitochondria would become impaired \*before\* horses demonstrated a decrease in performance. Concordantly, SET3 on d 5 was necessary to determine if horses demonstrated a decrease in run time to fatigue with repeated SETs. The SET parameters were 4 min at 4 m/s followed by elevation of the treadmill to 6% and rapid increase in speed to 10 m/s. Belt speed was then increased 1 m/s every min until the horse was unable to maintain position on the treadmill with gentle encouragement. Mitochondria were isolated from the muscle samples and analysed for oxidative phosphorylation capacities (P) and production of adenosine triphosphate (ATP) and H2O2 using high-resolution fluororespirometry. Data were analyzed by one-way ANOVA with repeated measures or by mixed linear models in the case of incomplete datasets with the fixed effect of day using GraphPad Prism v10.4. Significance was declared as *P*$\leq $0.05 and trends declared when 0.05$<$*P*$\leq $0.1.

**Results**

One horse was excused from the study due to a hoof abscess developed prior to SET3. Unexpectedly, not all horses fatigued by SET3 on d 5 (defined as decreased run time to fatigue). We, therefore, divided the horses into either responders (RESP, n = 5), those horses that had a decreased run time to fatigue by SET3 (*P*=0.03), or non-responders (NON, n = 6), those horses that did not decrease their run time to fatigue by SET3 (*P*=0.6). In RESP horses, mitochondrial leak respiration, P with complex I only (PCI), maximal P with complexes I and II (PCI+II), and P with complex II only (PCII) decreased from d 2 to 4 (*P<*0.05); PCI+II and PCII also tended to be lower at d 4 than d 0 (*P*<0.1; Table 1). No P metric was impacted by d in NON horses (*P*>0.05; Table 1). The rate of H2O2 production, a reactive oxygen species, relative to O2 flux tended to increase from d 0 to 4 during leak respiration in RESP horses (*P*=0.1)but H2O2/O2 did not differ in any other respiratory state in RESP horses, nor in NON horses at all (*P*>0.05). Production of ATP during PCI tended to decrease from d 2 to 4 in RESP horses (*P*=0.1) but ATP production did not differ in any other respiratory state in RESP horses, nor in NON horses (*P*>0.05).

**Table 1**. Mean ± SEM isolated mitochondrial respiratory capacities from the gluteus medius of unfit Thoroughbred geldings whose run time to fatigue remained unchanged through SET3 (*P*=0.6; NON, n = 6) or whoserun time to fatigue decreased by SET3 (*P*=0.03; RESP, n = 5).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Variable,pmol O2 • sec-1 • mL-1 | Treatment | d 0 | d 2 | d 4 | *P*-value |
| Leak | NON | 9.3 ± 1.8 | 8.2 ± 1.7 | 6.1 ± 1.7 | 0.355 |
|  | RESP | 5.3 ± 0.8 | 6.0 ± 0.6 | 2.9 ± 0.3\* | **0.026** |
| PCI | NON | 27.3 ± 6.5 | 31.9 ± 4.2 | 25.4 ± 4.0 | 0.600 |
|  | RESP | 18.7 ± 3.7 | 25.5 ± 1.4 | 9.0 ± 1.8\* | **0.010** |
| PCI+II | NON | 46.8 ± 9.8 | 44.1 ± 1.4 | 46.0 ± 7.3 | 0.880 |
|  | RESP | 33.4 ± 5.9 | 42.1 ± 1.6 | 19.0 ± 2.2\*$†$ | **0.012** |
| PCII | NON | 31.2 ± 5.6 | 33.5 ± 3.7 | 31.5 ± 5.5 | 0.876 |
|  | RESP | 21.8 ± 3.1 | 26.8 ± 0.8 | 13.4 ± 1.4\*$†$ | **0.008** |

P, oxidative phosphorylation capacity; CI, complex I; CII, complex II. \*Within treatment, d 4 differs from d 2 (*P*<0.05). $†$Within treatment, d 4 differs from d 0 (*P*<0.1).

**Conclusions**

With repeated exercise tests to exhaustion resulting in fatigue, mitochondria appear to become impaired, evidenced by decreased respiratory capacities, increased reactive oxygen species production, and decreased ATP production. Importantly, horses which did not decrease their run time to fatigue maintained mitochondrial health. Mitochondrial metrics may be useful predictors of susceptibility to fatigue, which may be utilized to decrease fatigue-induced injury.