Biomarkers of multi-organ injury in a pre-clinical model of exertional heat stroke

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Running Title: A preclinical model of exertional heat stroke

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Abstract:

It is likely that the pathophysiology of exertional heat stroke (EHS) differs from passive heat stroke (PHS), but this has been difficult to verify experimentally. C57Bl/6 mice were instrumented with temperature transponders and underwent 3 weeks of training using voluntary and forced running wheels. An EHS group was exposed to environmental temperatures (Tenv) of 37.5, 38.5 or 39.5°C, at either 30, 50, or 90% relative humidities (RH) while exercising on a forced running wheel. Results were compared to sham-matched exercise controls (EXC) and naïve controls (NC). In EHS, mice exercised in heat until they reached limiting neurological symptoms (loss of consciousness). The symptom-limited maximum core temperatures achieved were between 42.1 and 42.5°C, at 50% RH. All mice that were followed for four days survived. Additional groups were sacrificed at 0.5, 3, 24, and 96 h, post EHS or EXC. Histopathology revealed extensive damage in all regions of the small intestine, liver and kidney. Plasma creatine kinase, blood urea nitrogen, alanine transaminase, and intestinal fatty acid binding protein-2 were significantly elevated compared to matched EXC and NC, suggesting multiple organ injury to striated muscle, kidney, liver and intestine, respectively. EHS mice were hypoglycemic immediately following EHS but exhibited sustained hyperglycemia through 4 days. The results demonstrate unique features of survivable EHS in the mouse that included loss of consciousness, extensive organ injury and rhabdomyolysis.
Introduction:

Exertional heat stroke (EHS) and related exertional heat injuries can occur when there is an inability to adequately dissipate the heat load induced by physical exertion, resulting in extreme elevations in core temperature and damage to multiple organ systems (10). Although EHS is more likely to occur in hot and humid environments, individuals can be at risk over a wide range of environmental conditions (57). EHS also can occur in seemingly healthy individuals in comparison to passive heat stroke (PHS), which often transpires in subjects who are very young, elderly or have significant comorbidities (23). Recent data demonstrate that hospitalizations due to EHS and related exertional heat injuries are frequent and have been on the rise in the U.S. civilian population (51). EHS is considered the third leading cause of sudden death in high school athletes (37) and the US armed forces experienced 323 cases of hospitalization due to EHS in 2013, with greater than 1,710 cases of heat injury, most of which are associated with exertion in the heat (4).

To date, there have been a limited number of published pre-clinical animal models of EHS (16, 33, 34). Although important findings have arisen from existing models, particularly from Hubbard et al. in a rat model (32, 33, 35), the existing approaches prove to be unsatisfactory as they have significant limitations, including superimposed stress responses induced by shock avoidance, stressful types of instrumentation such as rectal thermistors, and very high mortality rates. Other models have stopped at pre-defined maximum core temperatures (Tc,max) that are more likely to induce heat injury rather than heat stroke (16). In contrast, animal models of PHS in unrestrained and unanesthetized rodents have been well developed (46, 47, 56). PHS in mice results in a characteristic thermoregulatory profile (46) and displays disorders of the central nervous system, hepatic, renal, gastrointestinal, and musculoskeletal systems (45, 47). However, the extent to which PHS models translate to conditions of EHS is unknown, making parallel development of models of EHS essential to understanding mechanisms, for understanding co-morbidities or for developing new preventative strategies or treatment options.
Though human studies of the physiology of exercise in hyperthermia provide valuable information regarding adaptive mechanisms, they can only go part way to understanding underlying pathophysiology of this life threatening EHS condition.

EHS is of particular importance to study because it is the predominant form of heatstroke in the military, athletes, and occupational workers. Theoretical differences also exist in the pathophysiology of EHS compared to PHS. In previous models in the rat, Hubbard et al. showed that EHS has a greater impact on multiple organ injury at lower core temperatures, compared to PHS (35). During exposure to hyperthermia, the shunting of blood towards the body surface for heat exchange can lead to under-perfusion of splanchnic vascular beds and is likely to involve a greater degree of ischemic injury rather than thermal injury to the intestine and other internal organs (42). This is potentiated during exercise in the heat because additional cardiac output is shunted to the exercising muscles (59). These and other integrative physiological interactions are also likely to promote brain hypo-perfusion and facilitate the onset of severe neurological symptoms, one of the specific hallmarks that distinguishes heat stroke from heat injury (3).

Exercise also presents an additional stress on metabolic pathways. Heat alone increases glucose utilization (60) and exercise in the heat can pose an even greater challenge to sustaining metabolic substrates for brain, heart and muscle function. EHS induces rhabdomyolysis, which is rarely seen in PHS. The susceptibility of muscle to rhabdomyolysis is greatly enhanced during heat exposure (5). Rhabdomyolysis contributes to acute kidney failure through secondary reactions of myoglobin released into the circulation (62), and it can promote coagulopathy, believed to occur from release of tissue clotting factors from damaged muscle (66). Thus, the additional stressors that exercise presents during a hyperthermic challenge elevate the potential for multiple organ injury and dysfunction in EHS.

In this study we report a new model of EHS in the mouse that carries with it many of the pathophysiological characteristics seen in humans. Our primary purpose was to create a tool for future
development of treatment and prevention strategies that directly apply to humans with EHS. We also
determined 1) the relative impact of a range of different temperatures and humidities on the
characteristic EHS response in mice, 2) the typical core temperature profile observed in response to EHS
and recovery over 24 h, and 3) the extent and range of multiple organ injury and metabolic disorders
induced by EHS. We demonstrate that many pathophysiological characteristics of EHS in mice are
similar to those observed in mice exposed to PHS, including multi-organ injury. However, we
demonstrate that EHS is highly distinguishable from PHS due to the profound neurological symptoms at
the maximum core temperatures achieved, extensive rhabdomyolysis and injury over the entire expanse
of the small intestine.

Methods:
All animal protocols were approved by the University of Florida (UF) Institutional Animal Care and Use
Committee. Because of the stressful nature of inducing heat stroke in animals, the early pilot
experiments were monitored carefully in real time by the UF Veterinary Medical Staff and met their
approval criteria for humane animal testing. Eighty one mice were used for data collection in this study.
All were C57BL/6J males (Jackson Laboratories, Bar Harbor, ME) weighing an average of 30.2 ± 2SD
grams with an approximate age of 4 months. They were housed in groups until they were implanted
with telemetry devices, after which they were individually housed in 7.25”W x 11.75”D x 5H” cages,
maintained on a 12x12 light cycle at 20-22°C and 30-60% relative humidity (RH). A standard chow diet
and water were provided ad libitum until the EHS protocol. Experiments were performed in the
morning of the light cycle.

Animal preparation and training:
Under isoflurane anesthesia mice were implanted with temperature telemetry transmitters (TA-
E-Mitter, Starr life Sciences, Oakmont, PA), placed in their abdominal cavities for real time monitoring of
core temperature (Tc). The mice were then allowed to recover with subcutaneous buprenorphine injections, every 12 h for 48 h and then monitored for a minimum of 2 weeks. At that time, exercise wheels and enrichment huts (Silent Spinner and Small Animal Igloo Hideaways, PETCO, San Diego, CA) were introduced into the cages for 3 weeks. During this period, mice had ad libitum access to the running wheel throughout the day and night. On the third week, additional exercise training was implemented to familiarize the mice to the environmental chamber in the laboratory (Thermo-Forma 3940 Incubator, Thermo-Fisher, Waltham, MA) and to the customized running wheel system (Lafayette Model 80840, Lafayette, Ind.). The forced running wheel was adapted to operate directly via a manual power supply, and the positions of the running wheel (4 cm lower) and motor (15 cm higher) were altered to eliminate interference from the wheel motor and improve the fidelity of the E-Mitter signal. The wheel speed was continuously monitored. The first exercise session consisted of 15 min of free-wheeling where the mouse was free to run in the spoked wheel without a forced pace and explore their surroundings. This was followed by a short recovery period (<5 minutes). Then mice were started at an initial speed of 2.5 m/min, which was increased by 0.3 m/min, every 10 min, for 60 min. Training sessions on the next two consecutive days consisted of only the incremental protocol on the forced wheel, lasting for 60 min each. The fourth and final session used the same protocol; however, exercise time and incremental speed were elevated until the animals exhibited fatigue. Fatigue was defined as refusal to run or walk with the wheel for >5 s. No shock or any other manual stimuli were used to maintain running speed.

Exertional Heat Stroke:

Following the last training session, mice were given two days of rest, with free access to the running wheel in their cage. The following morning they were brought to the laboratory in their own cage and allowed to rest for a minimum of 2 h while Tc was monitored. A data acquisition system was used to collect continuous Tc, averaged over 30 sec intervals (VitalView, Starr Life Sciences). After at
least 2 h of resting data in the environmental chamber, each mouse was monitored until Tc dropped to
< 37.5°C for greater than 15 min. Laboratory and environmental chamber conditions were kept
constant during this time, similar to animal housing facilities. Then the environmental temperature
(Tenv) and relative humidity (RH) were increased to the target values. As soon as the environmental
chamber equilibrated to the target Tenv, the chamber was opened and the animal quickly placed in the
running wheel. The forced running wheel protocol was then initiated. The mouse’s behavior was
monitored continuously with a video camera. Running speed began at 2.5 m/min and increased 0.3
m/min every ten minutes until the mouse reached a Tc of 41°C, which served as a threshold beyond
which the speed was kept at a steady-state for the remainder of the protocol. The study design
considered 42.5°C, as a humane end point. This endpoint was selected based on the minimum
threshold temperature that induces symptoms of heat stroke in previous studies using an established
PHS model (46). At the end of the protocol, Tenv was adjusted back to room temperature, the chamber
doors opened, and the mouse carefully watched until it regained consciousness. At this time, it was
weighed and returned to its cage, while Tc continued to be monitored for 24 h of recovery or until
sacrifice at an earlier time point. The 12-hr light dark cycle was maintained in the laboratory during the
recovery period.

In each experiment, “thermal area” was used as an estimate of “thermal stress” or “thermal
dose” and calculated as defined by Leon et al. (43). Mathematically this equals approximately the area
under the curve of the temperature profile for all points at which Tc was >39.5°C (units =°C • min). The
baseline reference Tc (39.5°C) was chosen because in PHS models (e.g. as used in the control PHS in this
this study, described below) the chamber Tenv is maintained at 39.5°C. Therefore, this is a point where
mice are unable to remove excess heat by radiation alone. In this current study we used Tenv ranging
from 37.5-39.5°C for EHS, but kept the 39.5°C as the cut off for comparison of thermal area results
between PHS and EHS at the various environmental temperatures.
Experimental Series 1: The first series of experiments were designed to study the Tc profiles and survival of mice exposed over a range of Tenv and RH values during exercise. Thirty mice were studied in series 1, with 5 groups of 6 mice per group. Three groups were exposed to 37.5, 38.5, or 39.5 °C at 50% RH and 2 groups were exposed to 30% or 90% RH, studied at 37.5°C. After 24 h, all mice were returned to the UF Animal facility in their own cages, where they were allowed to recover for a total of four days, to evaluate short term survival. After four days, animals were placed under isoflurane anesthesia and blood samples were obtained by transthoracic cardiac stick. Soleus, gastrocnemius, diaphragm, heart, liver, kidney, spleen, intestine, and brain were removed for later biochemical or histological analyses. Animals were exsanguinated and a thoracotomy with heart removal was performed for euthanasia under deep anesthesia.

Experimental Series 2: Following the establishment of EHS model and the effects of changes in Tenv and RH, three groups of additional mice were studied (n=6-8 per group) to determine the time course of multi-organ injury. All groups in series 2 experienced EHS at 37.5°C-50% RH and were sacrificed at either 0.5, 3, or 24 h after EHS for blood and tissue sample collection. Three other groups of sham (fully instrumented and trained) exercise control mice (EXC) were sacrificed at the same time periods after undergoing matched intensities and durations of exercise (max speed: 5.2 m/min, duration: 113 min) at ≈23°C and 50% RH.

Experimental Series 3: Tissue and blood samples from a second control group of mice which did not undergo any exercise training or any interventions (n=6) served as untreated mouse controls, i.e. naïve controls (NC). Another group of animals was also studied in this series, which were exposed to a PHS protocol (n=6). These mice were exposed to 39.5°C at 30% RH, similar to previous approaches described by Leon and colleagues (46). However, to mimic the heat exposure in the 37°C/50% RH EHS group in Series 1 and 2, mice stopped after reaching a Tc,max of only 42.1°C. Because extensive data
has been collected in similar models of PHS, we studied only this single time point, 3 h after reaching 
Tc,max, which corresponds to a time where there is considerable evidence of organ injury.

**Biochemical analyses:** Blood was collected with heparin, spun at 2,000 RCF and plasma (250 μl) was taken from the sample and stored at -80°C. Plasma was sent to UF College of Veterinary Medicine Diagnostic Laboratories for determination blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), and creatine kinase (CK) (Siemens Dimension Xpand Plus Integrated Chemistry System).

**Analysis of histological samples.** The procedure for obtaining and grading of fixed histological samples of the small intestine has been previously described (52) and follows the method derived from Chiu et al. (14, 55). Briefly, 1 cm transverse sections from the duodenum, jejunum, and ileum were taken for cutting and hematoxylin-eosin staining at the UF College of Medicine Clinical Pathology Laboratory, with a section thickness of 4 μm. Histology slides were then graded and modified to apply to randomly selected individual villi. Two trained and blinded raters separately graded samples from each slide. The average score per sample across raters was reported. Villus height and width were measured using calibrated microscope image analyses. In some tissues a small number of fixed samples from randomly selected control and EHS animals were used for qualitative histopathological assessment. These were taken at time points where the corresponding biomarkers suggesting injury had peaked.

**ELISA.** Plasma ELISA kits for detection of Fatty acid binding protein 2, intestinal,(FABP2) were purchased from USCn Life Science Inc. (Wuhan, Hubei). Briefly, a microtiter plate provided with the kit was pre-coated with an antibody specific to FABP2. Plasma samples were added to the wells with a biotin-conjugated antibody specific to FABP2. Then Avidin conjugated to Horseradish Peroxidase (HRP) was added to each well and incubated. After a TMB substrate solution was added, wells that contain FABP2 biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color proportional to the amount of FABP2 present. Color change was measured spectrophotometrically at a
wavelength of 450 nm. The concentration of FABP2 in the samples is determined by comparing the optical density of the samples to the standard curve using Microsoft Excel.

*Plasma glucose.* Blood glucose was obtained at the time of sacrifice using a handheld glucose meter (OneTouch, Lifescan Inc.).

**Statistical analyses:** Statistical analyses were performed using SAS JMP (Cary, NC). One way ANOVA was used to determine differences between groups, with post hoc Tukey’s test when data was parametric. Kruskal-Wallis or Wilcoxon were used for non-parametric comparisons with central tendency expressed as medians ±25-75% quartiles. Small durations of noise in the Tc data collected from the transmitters was considered an artifact due to transmitter reception difficulties (a rare event) and were treated as missing data and extrapolated over the appropriate interval. Where appropriate, effects of multiple sampling were determined by using comparisons that met an acceptable false discovery rate (FDR = 0.15) by performing the Benjamini-Hochberg procedure (8).

**RESULTS**

**Effects of Environmental Temperature**

All animals that were followed over the four-day recovery period survived the EHS protocol and all animals that were sacrificed at intermediate time points survived up to their targeted end points. At 24 h, the animals were categorized as to their level of brightness, alertness, and responsiveness. There were no signs of morbidity (righting reflex) after the first few h of recovery. Body weights returned or exceeded pre-EHS levels by 4 days. The exercise-hyperthermia protocol was stopped in all but 3/42 EHS animals because of loss of consciousness or stupor that was indistinguishable from loss of consciousness. This was characterized by the mice laying on their back or side, without the ability or willingness to right themselves and by being unresponsive to tactile stimuli. After the short period of unconsciousness of <5 min, the animals quickly recovered upright mobility, a return to responsiveness
to gentle tactile stimuli and a return to quiet resting and grooming behavior in their home cage. The
other 3 mice reached the humane end-point of a $T_{c,\text{max}} = 42.5^\circ C$, based on previous end points in a PHS
models in mice by Leon et al. (46). No mice were stopped because they refused to run further due to
exhaustion. Therefore, in this EHS model, the physiologic endpoint was “symptom limited” by central
nervous system dysfunction.

Typical thermal profiles at each temperature are shown in Fig. 1. During pre-incubation, prior to
the beginning of the exercise protocol, the mice that were exposed to 37.5°C, 50% RH (Fig. 1A) allowed
$T_c$ to rise from the normal daytime baseline of 36-36.5°C to approach the $T_{env}$ of 37.5°C. Similarly,
animals exposed to 38.5 or 39.5°C (Fig. 1B or C) allowed resting $T_c$ to elevate to within 0.5°C of $T_{env}$.
Therefore at 50% RH, the animals started exercising at different $T_c$ values, initially maintaining only very
small gradients with $T_{env}$. One interpretation of this is that the capacity (or willingness) of mice to
remove metabolic heat within this range is extremely limited and they seem to be programmed to
thermoregulate only enough to maintain $\approx 0.5^\circ$ gradient with the $T_{env}$. Interestingly, at the beginning
of the exercise protocol, at each $T_{env}$, there was an immediate elevation in $T_c$, bringing all animals to
approximately the same $T_c$ of $\approx 40^\circ C$, independent of $T_{env}$. This means that throughout the exercise
period all mice were in a state in which their $T_c$ had risen high enough to produce an initial net gradient
of 0.5-2.5°C from $T_c$ to $T_{env}$, allowing for some radiant heat loss. During exercise, the initial metabolic
heat production in each $T_{env}$ condition was approximately the same, because each animal was put
through the same initial incremental exercise protocol. Based on the $T_c$ profiles, it appears that the
animals exposed to $T_{env}$ of 37.5 or 38.5°C initially made reasonable attempts at defending a $T_c$ of $\approx 40^\circ C$
in the face of increasing metabolic heat production until it became too great to dissipate. At that point
$T_c$ began to rapidly rise, leading to heat stroke symptoms. In contrast, at 39.5°C the rise in $T_c$ was
almost linear with the initiation of exercise, suggesting that these animals simply could not disperse the
excess heat from the metabolic load against the small gradient between $T_c$ and $T_{env}$. 
Near Tc, max, there was a rapid rise in Tc, shortly before neurological symptoms. Tc,max was often reached shortly after neurological symptoms became limiting and the running protocol was stopped. After reaching the endpoint of the EHS protocol, the door of the environmental chamber was kept open, the animals returned to their cages, and the set point of the chamber lowered to 24°C (room temperature) and 50% RH. During this period a rapid fall in Tc into a hypothermic range was observed during the first h of recovery. These changes are qualitatively similar to PHS model in unanesthetized mice (46). We could not detect a secondary fever during recovery after the hypothermic period that was previously shown in PHS (46), though we only followed these animals over a 24 h recovery (insets in Fig 1A-D). In EXC animals, there was a reproducible elevation in Tc of ≈2°C during the exercise protocol to an average maximum of 38.4 ± 0.1 SD °C (Fig. 1F).

The Tc,max values achieved in each experimental group are shown in Fig. 2A. These were not statistically different between groups studied at the different levels of Tenv, while in 50% RH, the average ranging from 42.1-42.5°C. However, the exercise time required to attain symptom-limited EHS was significantly different between groups (Fig. 2B), requiring only ≈61 min at 39.5°C-50% RH, but ≈113 min at 37.5°C-50% RH. In addition, both the distance run in meters, and the maximum running speed attained during the exercise protocol were much greater at the lower temperatures (Table 1). Thermal areas (Fig. 2C), i.e. the integration of temperature >39.5°C, over time (°C · min), as described by Leon et al. (46), were significantly elevated as the level of Tenv was decreased. The highest thermal area was in the 37.5°C group (144 ± 21°C · min), but was still substantially lower than values observed in models of PHS in awake mice exposed to 39.5°C, i.e. ≈275-356 °C · min (46). All animals lost between 6-8 % of body weight during the test (Fig. 2D), and there were no significant differences between groups. Other major outcome variables are summarized in Table 1. Briefly, there were no differences in baseline measurements between groups. The average minimal core temperature attained over 30 sec (Tc,min)
and the profiles during the hypothermic phase of recovery were not significantly altered by the different Tenv exposures at 50% RH exposure.

**Effects of Environmental Humidity**

In general, the responses at 37.5°C-30% RH resembled the responses of 37.5°C-50% RH and were not significantly different for any variable. However, the responses to 37.5°C, 90% RH were unexpectedly altered. The mice reached symptom-limited Tc,max at an average of 41.5±0.2°C, significantly lower than observations at both 30 and 50% RH (Fig 3A). Surprisingly, the 37.5°C, 90% RH group were able to do this while running for longer distances (Fig. 3B) at faster running speeds (Table 2). Of note, the 90% RH group had a similar total thermal area (Fig 3C) and percent weight loss compared to the other two RH groups (Fig 3D). However, hypothermia depth was significantly shallower, possibly corresponding with the lower Tc,max reached. Subjectively, the fur of all the mice exposed to 90% RH (but not 30% or 50% RH) was saturated with moisture at the end of the exercise period.

The temperature profile of the 90% RH mice had other attributes that were different from the 50% or 30% RH groups. Compared to the other groups exposed to 37.5°C at lower humidities (Fig. 1A and D), the 90% RH animals showed a rapid rise in Tc during the pre-incubation period, again reaching a Tc of ≈40°C prior to the initiation of exercise, very similar to the animals exposed to 39.5°C (Fig. 1C). This means that at high humidity the animals were willing to allow Tc to elevate again to this common 40°C threshold, but providing them a 2.5°C gradient to dissipate heat, but with a limited ability to dissipate it through evaporative processes. The striking difference in response is that once they achieved this 40°C set point they were able to defend it for an extended period, far exceeding the duration of the EHS protocols in all other conditions. We suggest that this reflects the ability to dissipate heat by radiation across the wet fur.

**Histological evidence for injury to liver, kidney and intestine**
In order to evaluate organ injury in this model, random samples of liver, kidney, and small intestine were obtained at time points where corresponding damage biomarkers were most evident (discussed below). These measurements were made in the animals exposed to 37.5°C and 50% RH. The liver, kidney, and small intestine were selected because they have been known to be highly susceptible to heat injury (62).

Kidney damage was measured at the 0.5 h recovery time point (Fig. 4 A & B). The tissues from EHS mice showed visible vascular congestion, hemorrhage and thrombi. Typical liver tissue histopathology, measured at 24 h post EHS, is shown in Figure 4, C & D. These images also suggest hemorrhage, vascular congestion, and apparent thrombus formation in large vessels. None of these changes were apparent in EXC controls.

We analyzed intestinal injury at 0.5 h into recovery, based on evidence of injury in previous studies at this time point using PHS models (52, 54). As shown by examples in Fig. 5, visible evidence of intestinal injury was prominent in all three segments of the small intestine. The results of quantitative analyses from all animals are shown in Fig. 6 (A-C). There were consistently higher levels of injury in all three areas of the small intestine in EHS mice compared to EXC mice (Fig. 6A). Villus length, used as a measure of the process of restitution, usually appears quickly after villus injury (18). At the 0.5 h time point there were no significant differences in villus length between EXC and EHS (Fig. 6B), which is in contrast to other models of PHS in anesthetized mice (52, 54). Across all three regions, there were small but significant elevations in villus width by main effects in ANOVA (P < 0.02), (Fig. 6C) but within individual areas of the intestine, this did not reach statistical significance by post hoc analyses. Villus width is generally indicative of acute swelling and vascular congestion (69).

**Biomarkers for organ damage**

A variety of common plasma biomarkers were used to detect damage to specific organ systems over the course of recovery from EHS. Biomarkers were chosen because of their ability to quantify estimates of specific injury over time and because of the potential for translation to clinical applications.
ALT, a marker of liver injury, was significantly elevated to the same extent at 0.5 h in both EHS and EXC (Fig. 7A). However, by 3 h, while EXC levels returned to normal, ALT continued to rise, reaching median peak value of >100 U/L at 24 h. The 95% CI for ALT in a large population of adult C57bl/6 mice was 17-25 U/L (22), a range very similar to our NC group. Therefore, the elevation in ALT is consistent with significant liver injury. In most animals, ALT was still elevated at 4 days (mean value = 132 ± 153 SD U/L), but this did not reach statistical significance compared to NC mice.

Creatine kinase was used as a marker of skeletal and/or cardiac muscle damage and displayed a unique time course (Fig. 7B). Damage was not evident at 0.5 h of recovery, but was significantly increased at 3 h compared to EXC and NC. Surprisingly, it then returned to values similar to baseline or NC at 24 h, followed by a marked late-phase response at 4 days (96 h) that was significantly elevated compared both EXC and NC.

Kidney function was evaluated using BUN (Fig. 7C) and its relationship to creatinine (Fig 7D). Elevations in BUN were markedly higher than both EXC and NC at 0.5 h post EHS, and were still higher in EHS compared to NC at 3 h. However, by this time point, these values generally fell within what is considered the normal range for mice (22). Interestingly, at 24 h, we observed a significantly lower median BUN value (17 mg/dL) compared to both EXC and NC mice. Elevations in BUN can indicate acute renal failure, increased amino acid catabolism, dehydration, and/or gastrointestinal bleeding; while reductions in BUN are typical of liver failure (23). Since all of these conditions can be present in heat stroke, we attempted to further clarify the interpretation of BUN by measuring creatinine and calculating the BUN/creatinine ratio (Fig. 4D). Creatinine levels were never significantly elevated post EHS, but at 3 h, the median level for plasma creatinine was 0.1 mg/dL, significantly lower than NC (P < 0.002) and EXC (P < 0.05) (data not shown). Creatinine was similar in all groups at 0.5 h, 24 h and 4 days. The 95% CI for creatinine in mice is 0.49-0.55 mg/dL (22). The BUN/creatinine ratios (Fig. 4D) were significantly higher at 0.5 h compared to NC for both EHS and EXC groups, but in EHS the ratio continued
to rise, generally reaching peak values at 3 h. High BUN/creatinine ratios can indicate acute renal failure but are also elevated in shock, severe dehydration, and/or gastrointestinal bleeding (23). At 24 h into recovery, BUN/creatinine dropped below NC levels (p < 0.005). A low ratio can indicate low levels of urea formation from reduced protein intake, liver disease, and/or rhabdomyolysis (23), all likely to be present and therefore not particularly specific for renal injury.

Intestinal damage was suggested by elevations in the intestinal FABP2 plasma biomarker. FABP2 is a protein found in enterocytes of the small intestinal epithelium. Upon cellular damage, it is released into the circulation (1). Peak FABP2 concentration after 0.5 h of recovery was significantly higher in EHS than both NC and EXC (Fig. 8). EXC animals also showed levels higher than NC at 0.5, 3, and 24 h. Although EHS concentrations of FABP2 decreased considerably over time, they were still significantly higher than NC at 3 h and 24 h. Interestingly, FABP2 was higher in EXC than EHS at 24 h.

Comparisons with PHS

Extensive work had been done in PHS models in the mouse by Leon and colleagues in terms of organ damage and cytokine secretion (45, 46). However, the mildest level of PHS in those studies had an average Tc,max endpoint of 42.4°C, which was 0.3°C higher than our average peak Tc,max for the groups studied for tissue injury (i.e. 37.5°C -50% RH). For this reason we wished to evaluate the types of injury that would occur at this lower Tc,max in PHS to compare directly with our EHS model. We followed the animals only to the 3 h recovery, a point in time where plasma biomarkers of injury are most often near-maximum. The mice in the PHS group (N = 6) had an average thermal area of 409 ± 71 SD (°C • min) compared to 144 ± 22 SD in the EHS group (°C • min). Therefore though the two groups reached the same Tc,max, the animals in the PHS group were exposed to over two times the heat dose. As shown in Figure 7, the median ALT, BUN, and BUN/creatinine, either met or exceeded the levels seen in EHS at the 3 h time point (none of which were significantly different from EHS). However, there was no evidence for elevations in CK at this time point, suggesting that muscle damage is not part of the
constellation of problems in PHS, at least at 3 h, and at this Tc,max. Intestinal injury was also apparent, based on the elevations in FABP2 (Fig. 6), being significantly greater than both EHS and EXC at the same point in time (P<0.01). Mean blood glucose was 64 ± 31 SD mg/dL in the PHS group, which was lower at this 3 h time point (P<0.05) compared to both EXC and EHS groups, and consistent with previous studies in the unanesthetized PHS mouse model (45).

**Blood Glucose**

Non-fasted blood glucose levels were taken at the time of sacrifice, post EHS or EXC. As shown in Fig. 9, at 0.5 h into recovery, blood glucose was significantly lower in EHS compared to EXC or NC, reaching average levels of 47±14 mg/dL compared to EXC (250±46 SD mg/dL) or NC (110±30 SD mg/dL). No differences in glucose were noted between EHS, EXC or NC at 3 h (Figure 9B). However, after 24 h of recovery, in both EHS and EXC, blood glucose values were significantly higher than NC. For the 4 day measurements (Fig. 9D), the glucose tests were only available in the EHS groups receiving 30% and 90% RH at 37.5°C. However, similar results were seen in both groups with the extreme levels of RH. Mean glucose in these groups combined was 342 ± 68 mg/dL, a non-fasting value and range that is considerably higher than could be expected from either the effects of handling stress and/or isoflurane anesthesia. Both of these possible confounders independently elevate plasma glucose in mice (7, 15).

**Discussion:**

We have identified a unique preclinical model of EHS that closely resembles the pattern or response observed in humans. A primary outcome that distinguishes this approach is that the end point is determined by neurological symptoms, i.e. transient loss of motor control and consciousness. These characteristics are closely aligned with the clinical definitions of heat stroke (3) and what is generally observed in humans in EHS (62). Another feature is that EHS appears to induce organ system damage at a lower thermal “dose” as estimated by the thermal area. At a cellular or organ system level,
the principles of tissue damage as a function of thermal “dose” (temperature and time of exposure),
have been well established (20, 71) and have been applied to conditions of heat stroke in animal models
by Hubbard et. al. (33) and Leon et al. (46). Extensive evidence of organ injury was observed in this
model of EHS at a thermal dose of less than half of that created by the PHS protocol, when animals are
brought to the same average Tc,max. This suggests that other pathological processes such as tissue
ischemia, increased metabolic load, or a more rapidly ascending temperature profile may accelerate the
injury seen in EHS compared to PHS. In addition, when thermal load is superimposed on metabolic
load, both of which induce their own patterns of gene expression or activation of cell signaling
pathways, it would be expected to alter the pattern of responses compared to either thermal stress or
exercise alone. Another unique feature, consistent with human EHS, is the presence of extensive
rhabdomyolysis, which was not present in a matched model of PHS (Fig. 7B). Since rhabdomyolysis is a
contributor to underlying secondary pathologies, such as kidney failure and disseminating intravascular
coagulation, this may be a critically important aspect of the unique integrative responses to EHS.

Origins of Multiple Organ Injury

Several different mechanisms are likely to converge to promote neurological dysfunction or
unconsciousness during EHS, including reductions in cerebral blood flow from combined requirements
of exercising muscles and heat exchange, dehydration, ongoing hypocapnia (24, 51), loss of blood-brain-
barrier integrity (41), direct neurological effects of hyperthermia (67) and hypoglycemia. Of these
mechanisms, the only variable we evaluated in this study was hypoglycemia, which was observed at 0.5
h of recovery (47 ± 14 mg/dL). This is within a range of values generally considered “moderate”
hypoglycemia in humans, but at a level associated with significant cognitive impairment (72). What this
level of hypoglycemia means in a mouse is less clear. Normal values for blood glucose in mice can have
a wide range. Some reported normal values are inordinately high when samples are taken in the awake
state, e.g. 203-271.95% CI (22), but the elevations have been shown to be proportional to the extent of
handling of the mice and its impact on stress and cortisol levels (7). In chronically cannulated awake
mice, normal non-fasting values still have a wide range, but are much lower, i.e. 155 ± 28 SD mg/dl;
these rise to 196 ± 76 in the same animals when put under mild isoflurane anesthesia (15). Our values
for EXC and for NC mice, under baseline conditions and anesthetized with isoflurane, are within the 95%
CI for this range. In PHS, Leon et al. observed glucose values of 56 ± 16 mg/dL, approximately 3 h into
recovery (45), but the average value at Tc,max was reduced by ≈13 mg/dL. This suggests that it is likely
that during the end stages of EHS, at the time of neurological symptoms, hypoglycemia in our EHS mice
may have been more severe. The low delivery of glucose for normal nervous system function may have
contributed to neurological symptoms, along with the other factors mentioned above.

The hyperglycemia that emerged late in the recovery process was an unexpected finding. The
fact that it remained unresolved after 4 days of recovery suggests that it may reflect an underlying
metabolic disorder, possibly due to liver, intestinal, muscle, or pancreatic injury, all of which provide
hormonal signals or processes that directly control normal blood glucose. The pancreas is susceptible to
ischemia in heat stroke due to reductions in splanchnic blood flow (29) and in animal models exposed to
extreme hyperthermia (1 h at 45°C) the pancreas exhibits extensive histopathological damage (63).
However, to our knowledge, pancreatic injury has not been carefully evaluated in documented heat
stroke in humans or in models of survivable heat stroke. Exercise in the heat has been shown to
increase the rate of glucose utilization (60) and this could put additional strains on pancreatic
metabolism and on other organ systems involved with normal glucose homeostasis (liver and intestine).
Furthermore, in exercise in the heat, as the splanchnic region is hypoxic, the resulting increases in
lactate concentration in the liver can induce hyperglycemia (60). Whether this would be extended
beyond the early recovery period would depend on what systems were damaged. Clinically, the
hyperglycemia we observed after EHS is of interest, because in humans, heat stroke frequently presents
with hyperglycemia at the time of admission (17, 31), which has proven to be among the best predictors
of long term heat stroke mortality (17). The hyperglycemia could also reflect artifacts of other underlying processes such as inflammation and stress. When a host responds to a pathogen there is an initiation of the inflammatory response resulting in background hyperglycemia (2). In addition, both catecholamines and corticosteroids are generally increased transiently over the first 24 h in heat stroke (28, 45), which can theoretically contribute to profound hyperglycemia. However, after four days, there is little evidence that corticosteroids or catecholamines would still be elevated (though this has not been studied extensively). Alternatively, it is also possible that following a traumatic event like EHS, mice are in a “vulnerable” psychological state that makes them extremely sensitive and more susceptible to the psychological stress from successive handling or the process of induction of anesthesia. However, based on measured effects of handling stress and/or isoflurane anesthesia on glucose and corticosteroids in the blood of control mice, the values we observed at 4 days are much higher than expected, suggesting an underlying loss of glucose homeostasis (7, 15).

Although we demonstrate that multiple organ damage was present by histological measurements in liver, kidney and intestine at brief time windows of recovery, we depended on plasma biomarkers to evaluate the time course of ongoing injury. It is important to recognize that these measurements need to be viewed with caution, because elevations can reflect multiple ongoing processes that may or may not be a result of organ injury per se. The accumulation of biomarker proteins in the plasma is a net result of both production and clearance, so if clearance is reduced, such as might occur with kidney or liver damage, there may be an accumulation that is disproportionate to the extent or timing of injury.

Striated muscle injury was also a unique feature of this model compared to PHS and is consistent with the constellation of disorders typical of EHS in humans. Though elevations in CK are also evident in cardiac injury, in general, CK is still the most commonly used clinical indicator of skeletal muscle rhabdomyolysis (36). The time resolution and the interpretation of accumulated plasma CK
measures are influenced by the long half-life, being 15 h for muscle isoforms in humans (43) but only 
\[ \approx 1.5 \text{ h in mice (65). This may partially explain the somewhat unexpected time-course of the response} \]
observed in mice, which demonstrated rather sharp increases and decreases in magnitude over time.
No significant elevation was seen at 0.5 h, but at 3 h the accumulation in plasma was significant. By 24 h
the elevation was greatly attenuated, but then the signal reappeared at 4 days (Fig. 7B). The later rise in
CK at 4 days has some similarity to the delayed responses in CK seen after eccentric exercise (38), and
may reflect some form of delayed muscle inflammation. Therefore, this late response raises the
question as to whether some aspect of the type of exercise being performed in forced running wheels
may have had a unique impact on CK release. Anecdotally, we have observed the behavior of the mice
running on the forced running wheel to be quite different from behaviors on the treadmill. The running
behavior includes considerable stretching and grasping of the spoked wheel, which could result in some
eccentric motor movements, making delayed muscle injury possible. We did see very modest but
significant elevations in CK in the EXC group performing exactly the same exercise, but without
hyperthermia.

The liver is a particularly vulnerable organ in hyperthermia. Recent models estimating
temperature distributions in body compartments during heat stroke in rats have predicted that liver
temperatures may be as high as 2°C above Tc (58). Previous studies by Hubbard et al. (34) also have
suggested that liver enzymes may be among the most sensitive markers of heat injury, and it is thought
to be the result of both hypoxia and direct heat damage (39). These markers are also commonly
elevated in exhaustive exercise. For example, Hubbard et al. demonstrated that 24 h after rats were run
to exhaustion, elevations in ALT were modestly increased, even when exercising in the cold (5°C) but
rose as a proportion to the Tenv during the run (34). We also saw small elevations in ALT at 0.5 h in EXC
animals but the response quickly resolved to baseline by 3 h (Fig 4A). Hubbard also observed that when
rats are passively heated to 41.5°C the increase in ALT exceeded what was seen in exercise in the heat
suggesting a complex relationship between exercise and hyperthermia in producing a given degree of liver damage. In contrast to Hubbard et al., we continued to see ALT elevations suggesting ongoing liver damage extending up to 4 days in 3 of the 6 mice studied. It has been hypothesized that even though liver damage generally peaks about 24 to 48 h after heat stroke, it may take much longer (i.e. weeks or months) to entirely repair this damage (9, 44, 50, 61). The interpretation of the timing of the liver injury as reflected by ALT measurements is complicated by the long half-life in plasma, being 47 h in normal man (40). The half-life is less quantified in mice but decay following chemically-induced liver injury suggests it is much less than 24 h (70).

Renal failure is commonly present in heat stroke patients and BUN accumulation can be an indication of kidney damage or necrosis (50). Unfortunately, BUN, creatinine, and BUN/creatinine ratios are not particularly specific for renal injury or failure even though these are among the most common clinical indicators used (23). We were surprised to find that creatinine values were essentially equal to normal controls, with the exception of the 3 h time point, where they were significantly lower than both NC and EXC. Although plasma creatinine is commonly used to indicate acute renal injury in steady state clinical conditions such as hypertension in humans (48) it is not recommended as an indicator for critically ill patients where hydration and organ function are not stable (49). In sepsis, which has many parallels to the pathophysiology of heat stroke, creatinine production from muscle is markedly reduced, making it of limited use in examining acute kidney injury (21). Furthermore, in dogs with fatal heat stroke, creatinine concentrations have been found to be normal at the time of admission, even with existing evidence of pathological renal lesions (13). It is of note that previous studies have also questioned the reliability of the creatinine values using common autoanalyzers, whereas BUN values have been validated (26). For this reason we relied on BUN measurements as our primary biomarker for renal function in this setting with the understanding of the extensive limitations to its interpretation.
The intestinal barrier has long been considered a major target for injury during heat stroke (12, 25, 30). Exertional heat stroke may be particularly susceptible to intestinal injury since in humans (68) and in mice (27), heavy endurance exercise alone can result in evidence for loss of the intestinal barrier function and enterocyte injury. The effects of intestinal injury can have important consequences for the progression of heat stroke. It can lead to the release of endotoxin or other inflammatory mediators into the blood, which can drive the immune system to initiate a systemic inflammatory response syndrome (SIRS), believed to contribute to organ injury and mortality in severe heat stroke (12, 45). Though the liver is usually responsible for the clearance of endotoxin, as the exercise intensity and/or the heat load increase, less blood flow to the liver and liver injury can potentially compromise this function (62). In our previous work in a PHS model in anesthetized mice, we found marked elevations in intestinal permeability, that were more or less isolated to the duodenum (52, 53). In contrast, in this EHS model, damage is clearly present in all three segments of the intestine in essentially every sample we observed. The other marker of small intestinal damage we examined was FABP2. Intestinal enterocytes express FABP2 during early damage of the epithelial cell and the protein is released rapidly into the circulation (19). In PHS the level of FABP2 was extremely high, suggesting extensive injury in the unanesthetized PHS conditions as well.

Another difference we observed between the histological results we saw in this experiment compared to our previous analyses in anesthetized PHS models is that at this 0.5 h recovery window, there was no evidence of shortening of the intestinal villi, i.e. restitution. Restitution, or sloughing off of the tips of the damaged villi, is one of the chief mechanisms in the early stages of intestinal repair. In all of our previous analyses, this process was nearly complete within this time window. One possibility is that PHS protocols require 1-2 h of greater heat exposure to reach Tc,max. Therefore the restitution process may have not had time to develop in this EHS model. Secondly, the injury may be so extensive
in EHS that the epithelial cells lining the intestinal wall may not have recovered sufficiently to begin the
repair process effectively.

Significance of the mouse model development

This is the first model of EHS in the mouse that resembles human EHS. It was developed to
provide a preclinical instrument to evaluate treatment and prevention paradigms for EHS in humans,
which remains a serious and unpredictable medical condition. Improvements in preventative and post
EHS-care, particularly rapid cooling and appropriate diagnosis and monitoring no doubt have raised the
likelihood of survival in humans in recent years (6, 10). However, patients who survive may have long-
term medical complications and susceptibilities to other causes of mortality that are still poorly
understood (64). Therefore, improving survival rates may not be enough. New approaches to
prevention, prediction of susceptibility, and development of new forms of treatment are greatly
needed. Numerous questions remain unanswered such as why specific individuals succumb to EHS on
specific occasions, comorbidities that are not currently recognized, the involvement of the immune
system in both organ dysfunction and repair and the influence of exercise conditioning and acute
exercise on the evolution of the organ injury (62). These are all easiest to address in animal models,
prior to human studies. Furthermore, there are technical advantages to having a mouse model,
including the availability of mouse-specific assays and the ability to rapidly test mechanistic questions
through genetically altered strains. The use of mice, rather than rats or other species, may also have
been fortuitous, because once trained, mice will run willingly in the heat until they lose consciousness.
This may reflect the fact that mice have a greater tendency for flight in response to threats (such as
severe heat exposure), compared to the domesticated laboratory rat, which shows a greater tendency
to freeze in response to threatening environments (11). This also suggests that even though we went to
great lengths to avoid the use of electrical shock or startle stimuli to induce continued running, the mice
probably experienced a very stressful state, and they responded by running to escape continued
exposure. However, these natural stressors that were placed on the mice may be analogous to conditions humans often experience in EHS, where they push themselves beyond their normal capabilities due to competitive drive, coupled with psychological stressors inherent to combat or competition. Finally, the predictability of the mouse model across a variety of temperatures and humidities and the 100% survival rates in the face of relatively severe organ injury and dysfunction, will facilitate identification of comorbidities and risk factors in future studies.
Acknowledgements: This research was entirely supported by a contract from the US Army Research Institute for Environmental Medicine, Natick Massachusetts, with supplemental support from the BK and Betty Stevens Endowment (TC). The authors wish to thank the Veterinary Staff at the University of Florida Animal Care Services, for their hands-on oversight of the animal welfare aspects of the model development, specifically Karl Andrutis, DVM and Maggie Struck, DVM, and for the cooperation of the University of Florida Veterinary Clinical Pathology Core and the College of Medicine Molecular Pathology Laboratories. In conducting the research described in this report, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals" as prepared by the Committee for the Update of the Guide for the Care and Use of laboratory Animals of the Institute for Laboratory Animal Research, National Research Council. The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.


Table 1: Effects of environmental temperatures on EHS

<table>
<thead>
<tr>
<th>Tc Response</th>
<th>39.5°C</th>
<th>38.5°C</th>
<th>37.5°C</th>
<th>P value (ANOVA)</th>
<th>PHS 39.5/50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Weight (g)</td>
<td>30.7±1.6</td>
<td>29.4±2.5</td>
<td>30.7±2.4</td>
<td>0.500</td>
<td>28.1±1.1</td>
</tr>
<tr>
<td>Time (min) to reach Tc Min</td>
<td>178.8±26</td>
<td>204.6±24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250.9±29.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001*</td>
<td>483±64</td>
</tr>
<tr>
<td>Tc Min Temp (°C)</td>
<td>32.8±0.9</td>
<td>32.2±1.3</td>
<td>32.6±1.2</td>
<td>0.112</td>
<td>28.7±1.0</td>
</tr>
<tr>
<td>Distance (meters)</td>
<td>196.2±39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>316.0±32.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>444.9±89.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.0001*</td>
<td>n/a</td>
</tr>
<tr>
<td>Hypothermia Depth (°C)</td>
<td>33.2±0.9</td>
<td>32.6±1.4</td>
<td>33.0±1.1</td>
<td>0.679</td>
<td>29.2±1.1</td>
</tr>
<tr>
<td>Hypothermia Length (min)</td>
<td>141.2±75.9</td>
<td>199.6±100</td>
<td>193.8±51.8</td>
<td>0.387</td>
<td>n/a</td>
</tr>
<tr>
<td>Hypothermia Transition Time (min)</td>
<td>74.8±53.7</td>
<td>127.1±77.1</td>
<td>99.2±56.2</td>
<td>0.382</td>
<td>n/a</td>
</tr>
<tr>
<td>Ascending Thermal Area (°C·min)</td>
<td>48.9±13.7</td>
<td>69.7±16.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>96.5±14.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.0002*</td>
<td>223±90</td>
</tr>
<tr>
<td>Descending Thermal Area (°C·min)</td>
<td>9.5±2.5</td>
<td>6.7±2.1</td>
<td>6.1±3.0</td>
<td>0.079</td>
<td>12.4±3.7</td>
</tr>
<tr>
<td>Max Speed (meters/min)</td>
<td>3.7±0.4</td>
<td>4.6±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3±0.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.0001*</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Values are means +/- SD. N = 6 in each group. ANOVA for MAIN effect of Tenv.

Post hoc comparisons “a”-difference from 39.5°C, “b” difference from 38.5°C. Tc Min is the lowest average value attained over 30 sec and hypothermia depth was the lowest 1 hr average Tc during recovery. * = significantly different P<0.05
### Table 2: Effects of environmental humidity on EHS

<table>
<thead>
<tr>
<th>Tc Response</th>
<th>37.5/90%</th>
<th>37.5/50%</th>
<th>37.5/30%</th>
<th>P value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Weight (g)</td>
<td>30.0±0.6</td>
<td>30.7±2.4</td>
<td>28.8±0.6</td>
<td>0.309</td>
</tr>
<tr>
<td>Time (min) to reach Tc Min</td>
<td>242.5±22.8</td>
<td>250.9±29.7</td>
<td>246.1±16.8</td>
<td>0.755</td>
</tr>
<tr>
<td>Tc Min Temp (°C)</td>
<td>33.6±0.40</td>
<td>32.6±1.2</td>
<td>31.8±0.4</td>
<td>0.067</td>
</tr>
<tr>
<td>Distance (meters)</td>
<td>802.0±62.7</td>
<td>444.9±89.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>503.6±43.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.008*</td>
</tr>
<tr>
<td>Hypothermia Depth (°C)</td>
<td>34.2±0.4</td>
<td>33.0±1.1</td>
<td>32.2±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.039*</td>
</tr>
<tr>
<td>Hypothermia Length (min)</td>
<td>83.8±38.6</td>
<td>193.8±51.8</td>
<td>231.0±25.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.035*</td>
</tr>
<tr>
<td>Hypothermia Transition Time (min)</td>
<td>27.2±13.0</td>
<td>99.2±56.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>147.6±18.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.007*</td>
</tr>
<tr>
<td>Ascending Thermal Area (°C·min)</td>
<td>161.9±8.5</td>
<td>96.5±14.7</td>
<td>137.0±29.8</td>
<td>0.009*</td>
</tr>
<tr>
<td>Descending Thermal Area (°C·min)</td>
<td>4.8±0.5</td>
<td>6.2±3.0</td>
<td>9.5±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.033*</td>
</tr>
<tr>
<td>Max Speed (meters/min)</td>
<td>7.1±0.6</td>
<td>5.2±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.006*</td>
</tr>
</tbody>
</table>

Values are means +/- SD. N = 6 in each group. ANOVA P values are Main Effect of relative humidity. Post hoc comparisons “a”-difference from 90% RH, “b” difference from 50%.
**Figure Legends**

**Figure 1.** Thermoregulatory profiles at varying temperature and humidities. Insets are slower time scales to demonstrate late thermoregulatory responses during recovery. Broken lines represent time of beginning the elevation in temperature in the environmental chamber. Dashed line represents the beginning of exercise.

**Figure 2.** Effects of environmental temperatures on select elements of the thermoregulatory profiles in EHS. A. Maximum core temperatures attained ($T_c$,max). B. Time to max core temperature. C. Thermal area, defined as the integration temperature-time product when $T_c > 39.5^\circ C$. D. Percent body weight lost. Means ± SE, (N = 6 per group) *P<0.05, **P<0.01, ***P<0.0001

**Figure 3.** Effects of relative humidity on select elements of the thermoregulatory profiles in EHS. Maximum core temperatures attained ($T_c$,max). B. Time to max core temperature. C. Thermal area, defined as the integration temperature-time product when $T_c > 39.5^\circ C$. D. Percent body weight lost. N = 6 per group, Means ± SE, *P<0.05, **P<0.01, ***P<0.0001

**Figure 4.** Representative histopathology of kidney and liver injury in EHS. Hematoxylin and eosin staining of EXC and EHS (magnification 10x). Kidney: (A) Normal kidney (B) Typical kidney from EHS animals, showing vascular congestion/ hemorrhage and thrombi (arrows), (C): Normal liver (D) Liver vascular congestion/ hemorrhage and thrombi in EHS (arrows).

**Figure 5.** Representative histological images of regions of the small intestine in EHS vs EXC. (hematoxylin-eosin staining) Contrast and brightness of individual images were adjusted using PowerPoint, no other adjustments were made.
Figure 6. Quantification of intestinal injury in different regions of the small intestine: A: Average Injury Scores, B: Average villi height, and C: Average villi width. EHS: Exertional heat stroke, EXC: Matched exercise controls. Data are means ± SE. Villus width* = Group effect of EHS in ANOVA.)

\[ P<0.05*, P<0.01**, P<0.001***. \]

Figure 7. Responses of biomarkers of organ injury during recovery from EHS. Symbols = medians. The 25-75% interquartile ranges are represented in table format below each graph. N = 6 per group. A. Alanine Transaminase. See figure legend for symbols and lines. B. Creatine Kinase. C. Blood urea nitrogen (BUN). D. BUN/creatinine ratio. EHS: exertional heat stroke, EXC: matched exercise controls, NC: naïve controls, PHS: temperature-matched passive heat stroke controls. See text for details. * = P<0.05 EHS vs NC, #= P<0.05, EHS vs EXC, \( \nabla \) = P < 0.05 EXC vs NC. N = 6-7 per group.

Figure 8. Measurements of Intestinal Fatty Acid Binding Protein-2 (FAB2) following exertional heat stroke: Data represent medians ± 25-75% quartiles (table below) EHS: exertional heat stroke, EXC: matched exercise controls, NC: naïve controls, PHS: temperature-matched passive heat stroke controls. See text for details. * = P<0.05 EHS vs NC, #= P<0.05, EHS vs EXC, \( \nabla \) = P < 0.05 EXC vs NC. N = 6-7 per group.

Figure 9. Blood glucose levels post exertional heat stroke. EHS: Exertional hyperthermia, EXC: Matched exercise controls. A: Glucose, 0.5 hs post EHS or EXC B: 3 h post EHS, C: 24 h post EHS. D. 4 Days post EHS (see text for details) h. Data are medians ±25-75% ranges; \( P<0.05^*, P<0.01^* \)
A

**Peak Core Temperature**

B

**Exercise Duration**

C

**Thermal Area**

D

**% Weight Loss**