Impact of Successive Exertional Heat Injuries on Thermoregulatory and Systemic Inflammatory Responses in Mice

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Keywords

Heat stroke, heat shock protein, inflammation, hyperthermia, cytokines, stress

Abstract

The purpose of the study was to determine if repeated exertional heat injuries (EHIs) worsen the inflammatory response. We assessed the impact of a single EHI bout (EHI0) or 2 separate EHI episodes separated by 1 (EHI1), 3 (EHI3), and 7 (EHI7) days in male C57BL/6J mice (N = 236). To induce EHI, mice underwent a forced running protocol until loss of consciousness or core temperature reached ≥ 42.7°C. Blood and tissue samples were obtained 30 minutes, 3 hours, 1 day or 7 days after the EHI. We observed that mice undergoing repeated EHI (EHI1, EHI3, and EHI7) had longer running distances prior to collapse (~ 528 meters), tolerated higher core temperatures (~0.18°C higher) prior to collapse, and had higher minimum core temperature (indicative of injury severity) during recovery relative to EHI0 group (~2.18°C higher; all $P < .05$). Heat resilience was most pronounced when latency was shortest between EHI episodes (i.e., thermal load and running duration highest in EHI1), suggesting the response diminishes with longer recoveries between EHI events. Furthermore, mice experiencing a second EHI exhibited increased serum & liver HSP70, and lower corticosterone, FABP2, MIP-1β, MIP-2, and IP-10 relative to mice experiencing a single EHI typically at 30-min to 3-hr after EHI. Our findings indicate that an EHI event may initiate some adaptive processes that provide acute heat resilience to subsequent EHI conditions. Data and code are available at Open Science Framework repository:
https://osf.io/n5ahf/?view_only=bca7ccb1b1554e1192ae776e6a7584d3

New & Noteworthy

Mice undergoing repeated exertional heat injuries, within 1 week of an initial heat injury, appear to have some protective adaptations. During the second exertional heat injury mice were able to run longer and sustain higher body temperatures prior to collapse. Despite this, the mice undergoing a second exertional heat injury were more resilient to the heat as evidenced by attenuated minimum body temperature, higher HPS70 (serum and liver), lower corticosterone, and lower FABP2.

INTRODUCTION

Exertional heat illnesses are conditions characterized by an increased core body temperature (T_c) due to an inability or compromised ability to adequately dissipate heat generated by physical exertion and generally affect physically active populations (e.g., military, athletes, and occupational workers) (1). Heat illnesses are categorized along a spectrum of severity from heat exhaustion (mild) to heat injury (moderate) and then heat stroke (most severe) (39). Exertional heat injury (EHI) is a more recently recognized condition characterized by organ (e.g., gut, skeletal muscle, kidney, spleen, liver) dysfunction and hyperthermia in the absence of significant neurological disturbances or impairment of mental status (19, 20). In contrast, exertional heat stroke (EHS) is a life-threatening condition associated with profound central nervous system (CNS) dysfunction (e.g., delirium, agitation, stupor, seizures, or coma), hyperthermia, and organ damage, (39). If left untreated, EHS can often prove to be fatal within 30 minutes to 72 hours of the event (66).

A retrospective study of Army soldiers suggests that military personnel may experience ~2-fold increased risk from heart, kidney, or liver failure within ~30 years of heat illness hospitalization and treatment (73). Similar findings were reported in 2-year follow-up studies of civilian populations experiencing heat illness during annual heat waves (4). A growing body of evidence indicates prior heat illness predicts subsequent heat illness event(s) (53, 55), and a premature return to activity following a heat illness increases the risk for another heat illness (53). Furthermore, it is common for military, athletic, and occupational workers to return to duty, activity, or work following a heat illness episode (55). While the American College of Sports Medicine (ACSM: (55)) and Technical Bulletin: Medical 507 issued by the U.S. Army and Air Force (TBMED 507: (1)) prescribe guidelines for returning from exertional heat illnesses, these are currently based on a best guess. It is also uncertain whether an abrupt recurrence of EHI poses the risk of compounding tissue and organ damage (51).

The sequelae of heat illness are thought to be a consequence of high tissue temperatures and under perfusion of the vascular beds that induce oxidative/nitrosative stress and cellular damage (40, 68). These responses in combination with excessively high organ temperatures (heat shock >41ºC, 105.8ºF) induce ischemia, tissue/cellular damage (e.g. necrosis), coagulopathy, and a systemic inflammatory response syndrome (SIRS) that often culminates in multi-organ dysfunction (9). However, as King et al. (36) note, the observed increases in plasma levels of cytokines (IL-6) and chemokines (G-CSF, KC, MIP-2, MIP-1β, and MCP-1), may cause the induction of cell repair pathways necessary for recovery from thermal injury. Therefore, some positive adaptations may occur immediately following an EHI that protect against future heat stress (36).

To elucidate the recovery process from EHI and to determine the consequences of returning after short recovery following a prior EHI, the objective of this study was to employ an EHI model to: (1) determine the impact of prior EHI on thermoregulatory responses and exercise performance during a subsequent EHI event, (2) determine the impact prior EHI has on multi-organ damage or dysfunction, and (3) delineate the inflammatory signaling pathways associated with mediating organ injury following multiple EHI events. To our knowledge, this is the first study examining the effects of two consecutive EHI events in a rodent model. We hypothesized that prior EHI would decrease thermoregulatory performance in the heat when compared to those that did not experience prior EHI. Furthermore, we predicted that multi-organ damage or dysfunction would be exacerbated following repeated EHI, and prior EHI would intensify the inflammatory signaling pathways responsible for the cytokine-induced stress response compared to those that experienced a single EHI.

METHODS

Ethical approval. All procedures were approved by the Institutional Animal Care and Use Committee. In conducting this research, the investigators adhered to the *Guide for the Care and*

Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

Animals. Male C57BL/6J mice (6-8 weeks old, 24±1.6 g on average, Jackson Laboratories, Bar Harbor, ME) were individually housed in Nalgene polycarbonate cages (11.5 in x 7.5 in x 5 in) fitted with HEPA-filter cage tops and Shepherds Specialty Blend bedding (ScottPharma, Marlborough, MA) under standard laboratory conditions (25 ± 2°C and ~30% relative humidity [RH]; 12:12h light-dark cycle, lights on at 0600 h). Rodent laboratory chow (Harlan Teklad 7012; Madison, WI) and water were provided *ad libitum* except during training sessions and the forced running protocol. For environmental enrichment, each cage was supplied with a Nalgene Mouse House (Nalgene Nunc, Rochester, NY), in-cage running wheel (4-inch diameter by 2-inch wide, Starr Life Sciences Corp., Inc., Oakmont, PA), and wood gnawing block (3.81 cm cube, Bio-Serv, Flemington, NJ). In order to limit or standardize thermoregulatory disruption, clean cages, fresh food, and fresh water were provided every two weeks or as needed.

Radiotelemetry transmitter implantation. As described previously (43), under isoflurane anesthesia (3% induction: 2% maintenance in 100% O_2), mice were intraperitoneally (IP) implanted with radiotelemetry transmitters (1.1 g, model G2 Emitter; Starr Life Sciences Corp., Inc., Oakmont, PA) to measure body core temperature (T_c; ± 0.1°C) and general activity (counts). Following surgery, all animals continued to be individually housed. Surgical analgesia was provided with a subcutaneous buprenorphine injection (0.05 mg/kg) just prior to surgery and every 8-12h during the first 48h of recovery. The mice recovered from surgery in approximately 7 days as assessed by a return to pre-surgical body weight (BW), normal food & water intake (FI and WI), and stable circadian T_c and activity rhythms (47). T_c and activity were continuously monitored at 30 sec intervals throughout surgical recovery and experimentation using the VitalView system (Starr Life Sciences Corp., Inc., Oakmont, PA).

Training protocol for exertional heat model. The protocol for the current study is based on prior studies using this model (36). One week after surgery, in-cage running wheels were placed in cages to allow mice to run *ad libitum*. VitalView Activity software was used to monitor voluntary running (Starr Life Sciences Corp., Inc., Oakmont, PA). Six days prior to EHI, mice underwent exercise training sessions (60 min of incremental exercise) in forced running wheels (model 80840; Lafayette Instrument, Lafayette, IN) within an environmental chamber (model 3950; Thermo Forma, Marietta, OH) maintained at 25 ± 2ºC, relative humidity of ~30%. The training exercise consisted of six speed intervals starting at 2.5 m/min and increasing 0.5 m/min every 10 minutes. Training exercise sessions were repeated each day for 4 consecutive days, followed by two wash-out days with no training.

Exertional heat injury protocol. While in their home cages, mice were placed into a floor-standing environmental chamber (model Forma 3940; Thermo Fisher, Marietta, OH) at 25 ± 2°C and ~30% RH the day before heat exposure in order to acclimate to incubator noises, lighting, and smells. Cage filter tops and running wheels were removed to permit air circulation and to prevent differences in the amount run on the night prior to EHS, respectively. Between 0600 and 1000h the next day, mice with baseline T_c<36.5°C were selected for the heat stress protocol as this temperature is an indication that mice are in a resting, baseline state prior to testing and are not stressed (usually indicated by elevations in core temperature). Mice were removed from their home cage, weighed, and physically placed in the motorized running wheels inside the incubator prior to initiating the forced running protocol. The environmental temperature (T_{env}) of the incubator was increased to 37.5 ± 0.2°C with humidity remaining at ~30%. Mice were allowed to rest in the motorized wheels until the incubator reached T_{env} (~35-min), at which point the forced running protocol was initiated. The initial speed was 2.5 m/min and incrementally increased by 0.3 m/min every 10 minutes until the mice reached T_c= 41°C; at that point, wheel speed was maintained until the mice lost consciousness or $T_c \geq$ 42.7°C (Average T_{cmax} = 42.2°C); 4 mice in the EHI1 group reached 42.7°C upon their 2nd EHI exposure. This maximum

set point was chosen as the majority of mice in previous studies that reach 42.7°C do not survive in recovery. When either of these conditions was met, mice were physically removed from the heat, weighed, provided *ad libitum* food and water, and allowed to recover undisturbed at ambient temperature (T_a)=25 ± 2°C in their home cages until sample collection or exposure to a second EHI either 1 day, 3 days, or 7 days later. If mice were sacrificed at ≥1 day following the first EHI, running wheel (~4-hr post EHI), mouse house (~24-hr post EHI), and gnawing block (~24-hr post EHI) were returned to the cage and remained until the time of sacrifice or subsequent EHI. Exercise control (EXC) animals underwent the same forced running protocol at T_a=25 ± 2°C (Dehydration: 8.3 ± 1.1 %) until reaching a maximum speed of 5.8 m/min and a running duration of 160 minutes (Distance: 697 ± 50 m). All sample collection was time-matched to the EHI groups.

Experimental groups. Mice exposed to the EHI protocol (N = 148) were allocated into 4 treatment groups: EHI0 (one exposure only; n=39), EHI1 (two EHI exposures separated by 1 day; n=40), EHI3 (two EHI exposures separated by 3 days; n=33), and EHI7 (two EHI exposures separated by 7 days; n=36). Each EHI group had a matched EXC group (N = 139) designated as EXC0 (n=33), EXC1 (n=35), EXC3 (n=29), or EXC7 (n=30). Mice were either sacrificed at 30-min, 3-hr, 1 day, or 7 days after their first (EHI0 group only) or second EHI (EHI1, EHI3 and EHI7; Figure 1).

Blood and organ collection. At sacrifice, mice were anesthetized with isoflurane (5% induction and maintenance in 100% O_2), and between 500-1000 µL of whole blood were collected *via* cardiac puncture. Blood was divided as follows: 200 µL in a lithium heparin (LiH) tube with the remainder aliquoted into two 500 µL ethylenediaminetetraacetic acid (EDTA) tubes. Complete blood counts (CBC) were determined on EDTA blood with a VetScan HM5 Hematology Analyzer (Abaxis, Union City, CA). Aspartate Aminotransferase (AST), Blood Urea Nitrogen (BUN), and Creatine Kinase (CK) were determined on LiH blood using a Vetscan VS2 Chemistry Analyzer (Abaxis, Union City, CA). The remaining blood was kept on ice until plasma

separation by centrifugation (4°C; 5 min, 3000 g). The plasma volume was approximately half of the collected blood volume. Plasma aliquots were stored at -80°C until analysis. Following exsanguination, organs were excised, rinsed with cold 0.9% saline, and snap-frozen in liquid nitrogen and stored at -80°C.

Plasma cytokine and chemokine measurements. Plasma cytokines (interferon gamma – IFN-γ; interleukins – IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, & IL-17; tumor necrosis factor alpha – TNF-α) and chemokines (granulocyte colony-stimulating factor – G-CSF, granulocyte-macrophage colony-stimulating factor – GM-CSF, interferon gamma-induced protein 10 – IP-10, keratinocytes-derived chemokine – KC, monocyte chemoattractant protein-1 – MCP-1, and macrophage inflammatory proteins – MIP-1α, MIP-1β, & MIP-2) were determined using a MILLIPLEX MAP Mouse Cytokine/Chemokine Panel 25-Plex (Millipore, Burlington, MA) on a Bio-Plex 200 system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Sample size was 4 to 9 mice per group.

Plasma ELISA assays. Plasma ELISA kits were used for the determination of fatty acid-binding protein 2 (FABP2, Cloud-Clone, Katy, TX), corticosterone (Enzo Life Sciences, Farmingdale, NY), and heat shock protein 70 (HSP70, Enzo Life Sciences, Farmingdale, NY) in mouse plasma samples. Assays were performed according to the manufacturers' instructions.

Liver protein extraction and ELISA assays. For liver HSP70 measurement, ~25 mg frozen liver samples were homogenized using the Fastprep-24 (MP Biomedicals, Santa Ana, CA) in 180 µl tissue protein extraction reagent (T-PER, Thermo Fisher, Waltham, MA) containing protease inhibitor cocktail (P8340-5ML; Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail 3 (P0044-5ML; Sigma-Aldrich, St. Louis, MO). Homogenates were centrifuged at 10,000 g for 5 min at 4°C. Supernatant total protein concentration was determined using the BCA Protein Assay (23225; Thermo Scientific, Rockford, IL) by calculating sample protein concentration based on absorbance values on a standard curve with absorbance versus Known bovine serum albumin (BSA) concentrations measured in ug

197 protein/uL . Volumes for each sample containing 25 µg of total liver protein were used to
198 quantify HSP70 levels using the HSP70 High Sensitivity ELISA kit (Enzo Life Sciences,
199 Farmingdale, NY). The assays were performed according to the manufacturer's instructions.
200 *Calculations*. BW was measured on a Sartorius balance (± 0.1g; Fisher Scientific,
201 Waltham, MA) immediately prior to heat exposure and at collapse to determine percent
202 dehydration, calculated as the following:

203 $$\%\ Dehydration = \frac{(BW_{start} - BW_{@Tcmax})}{BW_{start}} \times 100\%$$

204 T_{cmax} and T_{cmin} (i.e., hypothermic depth) were the maximum and minimum T_c observed,
205 respectively. Thermal load (°C·min; measured as thermal area) was calculated as the following
206 ∑ [time intervals (min) × 0.5 (°C above T_c = 37.5°C at the start of the interval + °C above T_c =
207 37.5°C at the end of the interval)]; 37.5°C was set as the threshold temperature for calculations
208 as this was the ambient temperature in the chamber for the EHS protocol. Hypothermia was
209 defined as T_c <34.5°C with hypothermia duration being the total time (min) T_c was below 34.5°C
210 (44).
211 *Statistical analysis*. Thermoregulatory and running performance variables were
212 compared using Welch's One Way ANOVA with Games-Howell's Post-Hoc Test on GraphPad
213 Prism 8.3.0 (GraphPad Software, Inc., La Jolla, CA). Due to significant skew of the distributions
214 (visually confirmed through plots of the residuals), biomarkers measured in the plasma and
215 organs were log transformed as recommended by Cole (16) and the residuals were visually
216 inspected to confirm appropriate model fit. Biomarker comparisons were then made using a
217 linear mixed model, PROC MIXED, in SAS 9.4 (SAS Institute, Cary, NC). Comparisons between
218 EHI and EXC conditions were made with specific contrasts using the LSMESTIMATE
219 statement. In order to control for multiple comparisons, a Holm-Bonferroni correction was
220 applied to the pairwise comparisons. Statistical significance was determined at alpha <0.05.
221 Data are expressed as mean ± SD, unless otherwise indicated. In our data visualizations, the

EXC data are represented by a uniform gray bar (mean +/- SD) due to the homogeneity of the biomarker data across all control groups.

RESULTS

Thermoregulatory response and running performance in the heat. EXC mice developed ~2°C increase in T_c during 160 min of forced running at the normal housing temperature (Figure 2). EHI1, EHI3, and EHI7 mice ran for a significantly longer time and distance and remained in the heat ~1h longer than EHI0 mice before collapsing (Table 1; $P<0.05$). The increased heat exposure time and running performance resulted in EHI1 and EHI3 attaining significantly higher T_{cmax} than EHI0 mice (Table 1; $P<0.05$), whereas T_{cmax} of EHI7 mice was virtually identical to the EHI0 group (Table 1). All mice that experienced two heat exposures, regardless of the recovery time between them, developed significantly greater dehydration than EHI0 mice (Table 1) although there were no differences in body weights for each mouse between EHI events. EHI0 mice showed the typical hypothermic response during recovery but this response was significantly blunted in EHI1, EHI3 and EHI7 mice (Figure 2 and Table 1; $P<0.05$). There were 9 animals in each EXC and EHI group at each time point except for EHI3-7D (n=6) and EHI7-7D (n=6). With the exception of 4 mice, all animals completed their respective protocols, and there were no fatalities resulting from either the EXC or EHS protocols. For the 4 mice that did not complete the protocol: 2 failed to run, 1 did not get at or below 36.5°C, and 1 had a wheel malfunction during the protocol.

Cytokine and chemokine assays. Although all cytokine and chemokine results were analyzed, we have chosen to only report the analyses that were significantly increased or decreased by heat stress following one or both EHI events.

Circulating HSP70 and corticosterone response during recovery. EHI0 mice showed virtually identical circulating HSP70 levels as EXC mice at all time points of recovery (Figure 3A). EHI1 mice showed a significant increase in plasma HSP70 compared to EHI0 mice that

remained elevated at 3-hr of recovery but returned to baseline within 1 day (Figure 3A; $P<0.05$). EHI3 and EHI7 mice displayed an increase in plasma HSP70 at 30-min recovery that was significantly less than that observed in EHI1 mice (Figure 3A; $P<0.05$). By 1 day of recovery, the plasma HSP70 response of all EHI groups was virtually identical to the EXC group (Figure 3A).

At 30-min of recovery, all EHI groups showed a significant increase in plasma corticosterone compared to the EXC group (Figure 3B; $P<0.05$). EHI0, EHI3 and EHI7 mice maintained elevated plasma corticosterone through 3-hr of recovery with return to EXC group levels by 1 day of recovery (Figure 3B). On the other hand, EHI1 mice showed a more rapid recovery, or possibly suppression, of corticosterone within 3-hr of recovery and this response was maintained through 7 days (Figure 3B).

Circulating tissue injury biomarkers during recovery: CK, FABP-2 and AST. EHI0 and EHI1 were the only groups to show a significant increase in plasma CK and this response peaked at 3-hr of recovery and returned to EXC values within 1 day (Figure 4A; $P<0.05$). Exercise had no impact on plasma FABP-2 levels, whereas EHI0, EHI1, EHI3, and EHI7 mice all showed a significant increase compared to the EXC group at 30-min of recovery (Figure 4B; $P<0.05$). However, this response was blunted in EHI1 mice compared to EHI0 and EHI3 mice (Figure 4B; $P<0.05$). Plasma FABP-2 levels of EHI1 mice returned to baseline within 3-hr of recovery, whereas this response remained elevated at this time point in all other groups. By 1 day of recovery, plasma FABP-2 levels were virtually identical among EXC and all EHI groups (Figure 4B). All EHI groups showed a significant elevation of plasma AST from 30-min through 3-hr of recovery with a return to EXC values (control group) within 1 day of recovery (Figure 4C; $P<0.05$). A noteworthy difference between groups was that the EHI1 group displayed significantly higher AST values than EHI3 mice at 30-min and 3-hr and higher than EHI7 mice at 3-hr of recovery (Figure 4C; $P<0.05$).

Liver HSP70 protein levels during recovery. EHI0 mice experienced an increase in liver HSP70 protein levels from 3-hr through 1 day of recovery relative to the 30-min time point

(Figure 4D; P<0.05). The rate of rise of liver HSP70 protein levels for EHI1 mice was more rapid than EHI0 mice with a peak observed within 30-min of recovery that was sustained through 1 day of recovery, at which time point the levels were virtually identical to the EHI0 group (Figure 4D; P<0.05). EHI3 and EHI7 mice also showed a more rapid increase in liver HSP70 levels compared to EHI0 (Figure 4D). By 7 days of recovery, liver HSP70 protein levels were virtually identical among all EHI mice and the EXC group (Figure 4D).

Circulating cytokine and chemokine levels: IL-6, IL-10 and IP-10. All EHI groups showed a significant increase in plasma IL-6 levels above EXC group levels that were evident at 30-min and 3-hr of recovery with no difference between EHI groups (Figure 5A; P<0.05). By 1 day of recovery, EHI7 had returned to EXC group plasma IL-6 levels, and by 7 days of recovery, plasma IL-6 levels returned to EXC group levels for all EHI groups except for EHI1, which maintained levels greater than EXC mice (Figure 5A; P>0.05).

At 30-min of recovery, EHI0, EHI1, and EHI7 mice showed a significant increase in plasma IL-10 compared to EXC mice, whereas this response was absent in EHI3 mice at this time point (Figure 5B). By 3-hr of recovery, all EHI groups showed significantly elevated plasma IL-10 levels except for the EHI1 mice, whose levels were virtually indistinguishable from EXC mice (Figure 5B). By 1 day of recovery, all EHI groups were indistinguishable from EXC mice and this was observed through 7 days (Figure 5B).

EHI0 mice displayed a significant elevation of plasma IP-10 levels above EXC mice starting at 30-min of recovery (Figure 5C; P<0.05). By 3-hr of recovery, the plasma IP-10 increase in EHI0 mice was significantly elevated above all other EHI groups and only returned to EXC levels by day 7 (Figure 5C: all P<0.05). EHI1, EHI3, and EHI7 mice also showed a significant plasma IP-10 increase starting at 30-min of recovery, but these groups returned to EXC group levels within 1 day of recovery (Figure 5C).

Other circulating chemokines: MIP-1β, MIP-2, G-CSF and KC. EHI groups showed a significant increase in MIP-1β at 30-min of recovery although this response was less

pronounced in EHI1 mice at this time point (Figure 6A; $P<0.05$). Plasma MIP-1β only remained elevated in EHI0 mice at 3-hr of recovery whereas the other groups had returned to EXC levels at this time point. However, EHI0 and EHI1 mice showed a secondary increase in plasma MIP-1β at 7 days of recovery that was not observed in the other groups (Figure 6A; $P<0.05$).

Plasma MIP-2 was significantly elevated in EHI0, EHI3, and EHI7 mice at 30-min of recovery, which is a response that was not observed in the EHI1 group (Figure 6B; $P<0.05$). In fact, the EHI1 group did not show any increase in MIP-2 at any time point during the recovery. EHI7 mice showed a return of plasma MIP-2 levels back to the EXC group within 3-hr of recovery, whereas EHI0 and EHI3 mice continued to show elevated levels at this time point. By 1 day of recovery, all EHI groups showed plasma MIP-2 levels virtually identical to EXC mice and this was sustained through 7 days (Figure 6B).

Plasma G-CSF levels were significantly elevated in all EHI groups from 30-min through 3-hr of recovery with a return to EXC group levels by 1 day of recovery (Figure 6C; $P<0.05$). The only significant difference among groups was higher G-CSF levels in EHI0 vs. EHI1 mice at 3-hr of recovery indicating a later peak in the former vs. latter group (Figure 6C).

All EHI groups showed a significant increase in plasma KC levels from 30-min through 3-hr of recovery with return to EXC group levels by 1 day (Figure 6D; $P<0.05$). EHI0 mice showed higher plasma KC values at 3-hr of recovery compared to the EHI1 and EHI3 groups whereas this response was similar to the EHI7 group. The return to baseline at 1 day of recovery was maintained through the 7 days recovery period (Figure 6D).

DISCUSSION

In this study we utilized an EHI mouse model to better understand the physiological responses to and consequences of repeated EHI exposures. Contrary to our *a priori* hypotheses, the primary finding was that mice were resilient in their 2nd EHI exposure, and markers of organ damage or inflammation were diminished in mice with multiple EHI exposures.

Specifically, the mice exposed to a 2nd bout of EHI performed better in the heat as evidenced by the improved running performance in terms of distance (in meters, EHI0 = 1399.75±340.1; EHI1 = 1999.04±525.2; EHI3 = 1778.28±445.1; EHI7 = 1826±437.0) and duration (in minutes, EHI0 = 239.14±35.1; EHI1 = 309.43±50.2; EHI3 = 287.12±45.5; EHI7 = 287.33±39.6), higher T_{cmax}, and less pronounced hypothermic depth (higher T_{cmin}) after EHI and faster recovery from that hypothermic depth (Table 1 and Figure 2). It could be argued that the EHI1 group, which was exposed to a subsequent EHI within 24 hours of the first, had the most favorable response to the 2nd EHI compared to the groups of mice with a longer recovery period between EHI exposures (i.e., EHI3 and EHI7). This is likely an indication of mice gaining transient heat resilience after an initial EHI exposure, the heat resilience being most apparent after a second EHI with 1 day of recovery, and heat resilience likely decaying with 3 days and 7 days of recovery following the initial EHI. In rats, HSP70 upregulation occurs in the liver, kidneys, and small intestines following passive heating (23) and in skeletal muscles following passive heat or exercise in the heat (70) while cultured cells induced to express HSP70 exhibit improved heat tolerance/resistance (48, 77). However, HSP70 upregulation after heating may be a transient event; data from rat skeletal muscles indicate muscle HSP70 expression declines at 8-48 hours following heat stress, depending on the muscle region (i.e., deep vs. superficial portions) (57).

Mice in our EHI model exhibited elevated levels of circulating inflammatory cytokines/chemokines and measures of injury relative to EXC mice. The mice undergoing the 2nd EHI exposure (EHI1, 3, and 7 groups) generally 1) had delayed onset of EHI collapse, 2) had attenuated/shallower hypothermic depths after collapse (indirect marker of reduced EHI severity), and 3) returned to baseline T_c more quickly relative to EHI0 mice. This suggests that the initial heat injury initiated an adaptive response to enable thermal resilience in the groups which received two EHI exposures (EHI1, EHI3, & EHI7). The attenuation of the hypothermic depth is critical since hypothermia following an EHI is associated with increased intestinal damage and reduced survival in a mouse model (44). Contrary to our hypothesis, EHI1 animals

(2^{nd} exposure within 24hrs), when considering all measured variables of EHI severity, fared much better than other groups in this study. The EHI1 group had the longest running time and distance run (Table 1; values not significantly different from EHI3 & EHI7), implying heat-resilience is gained following the initial EHI, and this effect is apparent with shorter durations of recovery between heat injury events. However, this is likely a transient effect as the protection is not as robust following 3 and 7 days of recovery between EHI events, likely indicating a gradual decay in heat resilience.

A possible change that enabled the EHI1 group to be most heat-resilient is a transient increase in plasma HSP70 (Figure 3A). However, the EHI3 and EHI7 groups did exhibit a small increase in plasma HSP70 at 30 minutes post EHI, in comparison to EXC, following the 2nd EHI exposure at the time points we measured (Figure 3A). This despite having larger thermal areas, faster recovery, and more shallow hypothermic depth compared to the EHI0 mice (Figure 2). Along the thermal curve, the increased plasma HSP70 in EHI1 approximately the time of T_{cmax} at 30-min and T_{cmin} at 3h. However, as this correlation between time point and HSP70 were not observed in EHI3 and EHI7 mice, some of the heat resilience observed in EHI3 and EHI7 groups is unlikely to be related to changes in circulating HSP70. In general though, HSP70 induction is necessary for thermal adaptation and greater HSP70 has been associated with heat acclimation in multiple organs (41). From the current study, we cannot determine the source of the increase in the serum HSP70. It is believed that increased serum HSP70 may be involved in immunoreactivity and serve to mediate cytokine and chemokine responses (63). It has also been postulated that plasma HSP70 could originate from either 1) increased HSP70 expression in circulating blood cells (62, 63) or 2) released from other organs or tissues due to stress-induced damage (12, 13). Therefore we cannot definitively conclude whether the transient increase in plasma HSP70 represents a beneficial or protective response to EHI.

Whereas serum HSP70 may mediate cytokine and chemokine responses in the bloodstream, within organs and tissues HSP70 is a protein with housekeeping functions in cells

and interacts with other chaperone proteins to fold non-native proteins during stress events (50). Particularly during heat stress, a highly inducible isoform of HSP70 is upregulated to protect the cell from injury and allow recovery from thermal stress (23). In the current study, liver HSP70 was elevated following initial heat injury in the EHI0 group 3 hours and 1-day after EHI (Figure 4D). Liver HSP70 is also elevated in EHI1 above all other groups at 30-min and 3-hr after the 2nd EHI, and this elevation persists for up to 1 day after the 2nd EHI exposure. Liver HSP70 was elevated in EHI3 at 30-min, 3h, and 1 day following the 2nd EHI (although always to a lesser degree than in EHI1); liver HSP70 was also elevated at 3h and 1 day in EHI7 mice, reflecting almost a delayed timescale of these changes compared to the other groups. Accordingly, this is similar to the general pattern for thermal load (positive relationship) and hypothermic depth (inverse relationship). The data from our mice were congruent with prior research in control or non-heat acclimatized rats conducted by Maloyan et al. (49) and Weshler et al. (76). Non-heat acclimated rats subjected to heat stress have increased cardiac HSP expression (49), and there is also increased heat resilience in the rat following an initial heat stress (76). Interestingly, cardiac HSP upregulation and heat resilience after initial stress are most pronounced at 1-2 days post-heating, but both events are transient. Cardiac HSP70 expression begins to decline by 3 days post-heating and whole-organism heat resilience is lost roughly 4-5 days following the initial heat stress. Our mice exhibited a similar pattern of heat resilience, being most pronounced in mice experiencing the 2nd heating event 1 day after the 1st. Although this resilience persisted up to at least a week from initial heating, there is a trend for heat resilience being lost in the 3 day and 7 day groups with longer latencies between the 1st and 2nd heating events. Our tentative interpretation is that observed patterns of liver HSP70 concentration indicate HSP70 increases as a response to the heat stress and confers protection against future exposures. The initial EHI (EHI0) increases HSP70 abundance in the liver such that HSP70 is initially heat-responsive. This response confers more heat resilience during a more proximal 2nd exposure and decaying resilience with the two more distal 2nd exposures. Essentially, the EHI1

mice had the greatest protection, at least from a heat shock response perspective, but the HSP70 response is transient. Deacclimation to EHI may occur rapidly, as demonstrated by the HSP70 response within out study, but epigenetic mechanisms may provide for quicker re-acclimation (27). It is unclear from our data on the role HSP70 may play in other tissues/organs; it is also possible that HSP70 levels may correspond better to thermal profiles and heat stress in other organs such as skeletal muscle or the brain, neither of which were able to be examined in the current study. Taken together, HSP70 is a protein prominently involved in stress response in multiple organs with potential site-specific roles (e.g., immunoreactivity and protein folding for degradation).

The stress hormone corticosterone was elevated in all EHI groups at 30-min post-EHI. However, corticosterone was reduced in the EHI1 group at 3-hr post EHI exposure while it remained elevated for EHI0, EHI3, and EHI7 (Figure 3B). This time point is approximately the same time of hypothermic depth (T_{cmin}), such that the suppression of corticosterone may have attenuated hypothermia or vice versa. Corticosterone is a hormone typically associated with stress response and energy metabolism. Broiler chickens exposed to passive heat stress over several hours or several days showed increased corticosterone concentration, which is often associated with decreased body weight and food intake (64, 65). Prior data in mice also indicate that corticosterone is elevated following EHI (44) and this elevation reflects heat intolerance (30). Thus, the precipitous decline in corticosterone at 3-hr in EHI1 group indicates more rapid recovery and the mechanism mediating this response remains to be elucidated.

Intestinal damage, as evidenced by FABP2 (or I-FABP), was reduced in the EHI1 group but not in the EHI3 or EHI7 groups (Figure 4B). FABP2 is a protein found in enterocytes of the small intestine epithelium that is released into circulation upon intestinal damage (2). Previous studies of EHS in mouse models have indicated that intestinal damage is directly related to increased morbidity following an EHI event (5, 36). In humans, Yeh et al (78) have demonstrated that plasma claudin-3, another marker of intestinal permeability, and endotoxin

increases following exercise in a hot environment. Similar to our results, Yeh et al. (78) did not observe a significant increase in claudin-3 or endotoxin when participants were exercising in a cool environment. This indicates that exertional hyperthermia, and not exercise alone, is likely increasing intestinal permeability. The severity of the changes in intestinal permeability is directly related to the degree of hyperthermia (i.e., maximal core temperature) (18, 61). Intestinal damage during an EHI facilitates endotoxin translocation, which drives immune-system mediated SIRS. SIRS, which, is believed to exacerbate heat stroke and is strongly related to organ damage, sepsis and septic shock-like symptoms, and mortality (21, 46). Therefore, the reduction in FABP2 observed in the EHI1 group possibly reflects a critical protective mechanism against intestinal damage during the second heat stress event and may have facilitated greater thermal recovery. Exercise alone may even act as hormetic stressor that helps stimulate protective adaptations that help improve gut integrity (32). Numerous interventions – such as probiotics or amino acid supplementation – have been proposed as ways to improve intestinal barrier integrity (37). However, there is no definitive evidence that any proposed intervention is effective at preventing changes in intestinal permeability during an EHI event. Moreover, interventions to prevent changes in intestinal permeability may inhibit the positive adaptations that occur following exercise or hyperthermia (32).

Evidence of muscle damage, measured by CK, was highest in the EHI1 group. CK is a protein that is most abundant in skeletal muscles, and the elevations after an EHI are believed to originate primarily from damaged muscle tissue (15). In clinical practice, CK is often monitored in EHS patients as it is a strong prognostic indicator of rhabdomyolysis (29). It is uncertain what role CK played in this model of EHI. A prior study using a similar model found increased CK levels at 3 hours post-EHI (36), which is consistent with what we observed in EHI0 and EHI1 mice. However, we postulate that CK levels being highest in EHI1 is likely an indicator that they have sustained more muscle damage with two EHI bouts within 24 hours of each other rather than just a lack of clearance from the first EHI. This is reinforced by the fact

that 1) EHI0 mice had low CK concentration 1 day after their only EHI bout, and 2) EHI3 and EHI7 mice ran comparable distances/duration to EHI1 mice, but their CK levels were lower than the levels in EHI1 mice 3h after the 2nd EHI (Figure 4A). However, we cannot rule out that the 2nd EHI exposure caused additional renal stress that further limited the clearance of CK thereby causing the elevations of CK in the EHI1 mice at 3h.

Typically, increases in CK concentrations are associated with pathological conditions during heat stress (e.g., precursor to rhabdomyolysis) so it is noteworthy that we saw thermal protections in the EH1 mice despite such CK elevations. As such, the relationship between muscle damage and thermal adaptations may warrant further investigation. Interestingly, in the EHI3 and EHI7 groups there is a non-significant elevation in CK following the 2nd EHI exposure in spite of these groups running at faster speeds and for longer distances. It is possible that after 3-7 days of recovery from an EHI there may be a training effect and/or increased muscle resilience to contraction-induced damage. In humans an initial exercise bout with eccentric contractions can increase CK levels for up to 8 days following exercise, but subsequent exercise bouts 3 weeks and 5 weeks following the initial bout cause lower CK release than what was measured following just the first bout (54). This indicates some level of muscle protection or increased rate of CK clearance is established with a conditioning exercise session, and per the data from Newham et al. (54), this adaptation leading to lower circulating CK persists for at least 4 weeks following the initial bout. In rats, CK measurements are much higher in untrained rats vs. other groups of trained rats at 48 hours post-exercise (90 minutes of forced running at a decline on a treadmill (69). Another study in mice has indicated that following an initial exercise bout, there is reduced muscle injury [without CK measurements] and protection from damage after a 2nd exercise bout, and this protection from injury lasts ~21-84 days depending on the intensity of the exercise (67). Accordingly, the data from prior studies indicate that skeletal muscles are protected from damage during a second session of physical activity compared to the first, and this is consistent with what we observed in our double-hit EHI mice that

experienced a 2nd EHI at 3D and 7D after the 1st. However, the discrepancy is that rats and humans have chronically elevated CK following exercise for several days following the exercise (54, 69), but data from mice in groups EHI0-1D indicate that CK levels after activity return to control levels within 24 hours (Figure 4A).

AST, a marker of liver damage, was elevated in the EHI1 group compared to EHI3 and EHI7 (at least at 30-min and 3-hr post EHI; Figure 4C). This suggests that the liver was under greater stress in EHI1 mice compared to EHI3 or EHI7 mice. Previous evidence suggests that the liver may be the most sensitive and vulnerable organ to EHI (74), and this is likely caused by a combination of hypoxia and direct heat damage (28, 33). However, in all conditions, liver enzyme levels returned to normal values within 24 hours after any EHI exposure (Figure 4C). This suggests that the time course of recovery from an EHI may occur much faster in rodent models compared to what has previously been observed in humans (74). In addition, HSP70 in the liver was elevated above all other groups at 30-min and 3-hr post EHI. This is interesting because elevations in HSP70 are generally considered to have protective effects against heat stress (3), but were unable to prevent further increases in AST. However, while AST is often used as clinical biomarker of liver damage, it can be released from multiple tissues (45). Therefore, the elevations observed in this study may reflect a combination of liver and muscle damage, and would explain the nearly identical response of AST and CK (Figure 3A and 3C).

The inflammatory chemokine response was moderated by EHI and recovery time between the first and second EHI. Many inflammatory markers peaked around 30-min (just after T_{cmax}) to 3-hr post (approximately the same time ashypothermic depth; Figure 2), with the highest chemokine elevations in the EHI0 group and significant reductions in the EHI1 group (Figure 6A-D). Two chemokines that may play a role in heat stress response following EHI are MIP-1β and MIP-2. *In vivo*, MIP-1β is known to be a pyrogen in several animal models, and its levels are particularly elevated during sepsis in humans (56). Intravenous injections of lipopolysaccharide (LPS), a substance commonly observed in circulation with endotoxemia and

possibly heat stroke (10), induced a transient increase in plasma concentrations of MIP-1β (58) and therefore may be an indicator of EHI severity. MIP-2also mobilizes peripheral blood stem cells and hematopoietic stem and progenitor cells from bone marrow in response to severe stress. In conjunction with other chemokines (e.g., G-CSF and KC), MIP-2 stimulates the mobilization of neutrophils, and other polymorphonuclear cells to sites of inflammation where these cells act to initiate the repair processes at damaged tissues (11). Chemokine mobilization involving MIP-2 is often rapid with peak response occurring within minutes or hours following stress (59). Thirty minutes after T_{cmax} , the EHI1 group (the group with the longest time to collapse during a 2nd EHI bout) had the lowest levels of MIP-1β and MIP-2 compared to EHI3 and EHI7 at 30-min (Figure 6A & 6B) while the single exposure EHI0 group had the highest concentrations of MIP-1β and MIP-2 (Figure 6A & 6B). Accordingly, it is reasonable to conclude that decreased levels of MIP-1β and MIP-2 in the EHI1 mice at 30-min and 3h post-EHI are reflective of better heat resilience and an attenuated hypothermic depth, while the opposite would be true of drastically elevated levels of MIP-1β at 30-min and 3h. We do not know if MIP-1β have a causal effect on the severity of EHI, but increases in MIP-1β are least associated with increased mortality from during passive heat stroke in mice (17).

Inflammatory cytokines (IL-6, IP-10, and IL-10) were also elevated following EHI and some of these responses were moderated by repeated EHI. At 3-hr after EHI, there was a reduction in IL-10 in the EHI1 group, which is important considering this may be related to heat stroke severity in humans (8) and rodent models (42, 75). Interestingly, the 2nd EHI had no significant effect on IL-6, which is unexpected since IL-6 is considered an important stimulus for IL-10 release (72) and is typically considered indicative of heat stroke severity (7). IP-10 was reduced in the EHI1 group suggesting some reduction in the stress response to EHI. Transient IP-10 elevations are typically associated with acute damage to some tissues or organs during a stressor event (e.g., neural tissue following cerebral ischemia) (14) and may also be associated with heat stress in hepatocytes (26). Upon release, IP-10 acts as a chemoattractant for

monocytes and T cells (22), thus initiating an *in vivo* immune response (17). Altogether, an increase in pro-inflammatory cytokines, which often occurs following a viral infection, may increase the vulnerability of the cells to hyperthermia (6, 71). During heat stroke, increased pro-inflammatory cytokines are associated with increased morbidity and mortality in both animal models (17) and humans (6). However, IL-6 may act as physiological stress hormone, and when mice are pretreated with IL-6 prior to extreme hyperthermia they exhibit reductions in organ damage and inflammation (60). MIP-1β on the other hand is increased following a viral infection and is associated with increased morbidity (17). Therefore, the reduction in MIP-1β with concurrent elevations in IL-6 in the EHI1 mice may indicate positive adaptation to the first EHI event. The stress of the EHI observed in our study may have acted as hormetic stressor and the reductions in some of these inflammatory cytokines may be indicative of positive adaptations to the original EHI stimulus. Together these chemokine and cytokine responses suggest a partially blunted inflammatory response on the 2nd EHI when it occurs within 24 hours of the first EHI.

Limitations

In this study we were able to assess fluctuations in cytokine/chemokine levels on a relatively compressed timescale to better elucidate their potential roles during key physiological events post-EHI (i.e., around T_{cmax} and T_{cmin}). However, we must note that this study did not take serial blood sampling from mice so it is possible that some fluctuations in cytokine/chemokine levels may have been missed. Additionally, we included a rodent model of EHI wherein almost all of the mice were able to survive the exposure to heat while exercising. Accordingly, although previous publications have indicated mice undergoing the current protocol mimic EHS (24, 25, 31, 35, 36, 38, 52), we have re-categorized the current model to be more representative of EHI since, even without active treatment, our mice were able to survive the event (no mortality) and showed increased heat tolerance during subsequent EHI events. Longer exposure to the heat following collapse in the mice may be necessary to increase the severity of the heat illness in order to be reflective of EHS (34). Additionally, the data from our

558 study should not be applied to more severe cases of heat illness where there is a high morbidity
559 and mortality risk. In cases of EHS, where organ damage can be much more severe, we still
560 speculate that rapid re-exposure to extreme heat stress could result in more organ damage and
561 less heat resilience.

562 **Conclusions**

563 Contrary to our expectations, an initial EHI induced heat resistance and/or increased
564 thermal tolerance during a second EHI exposure. The mice subjected to a 2nd EHI bout ran for a
565 longer duration and had higher T_{cmax} and blunted T_{cmin} compared to the 1st EHI exposure. This is
566 in contrast to human epidemiological data, which indicate a prior heat illness predicts
567 subsequent heat illness episodes (53, 55). Several key biomarkers related to heat tolerance and
568 immune or inflammatory responses were upregulated or downregulated in response to each
569 EHI event. An interesting feature of the study design was that sacrificing animals at various time
570 points after heat collapse enabled us to discern the timescale for cytokine and chemokine
571 responses in single exposures vs. 2-exposures separated by 1, 3, or 7 days of recovery.

572 Some level of prior heat exposure, even when a heat injury occurs, may increase
573 resilience to subsequent EHI conditions. Therefore, acclimation to the heat may occur in mild-to-
574 moderate cases of EHI and thus be beneficial during subsequent heat exposure. A major caveat
575 to our findings is that in order to more accurately recapitulate what occurs in humans during the
576 most severe heat illness, EHS, a mouse model with more severe heat illness symptoms and
577 higher mortality rates will likely be required for future studies. Studying the inflammation
578 pathways in specific heat-responsive target organs could inform more effective and appropriate
579 guidelines for recovery from EHI and EHS separately. Continued work in this area may provide
580 some direction for how and when patients recovering from an EHI should be reintroduced to
581 heat stress.

582

ADDITIONAL INFORMATION

Data Accessibility

All data and code to reproduce the analyses and figures included in this manuscript can be found on our Open Science Framework repository https://osf.io/n5ahf/?view_only=bca7ccb1b1554e1192ae776e6a7584d3

Disclosures

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. No conflicts of interest, financial or otherwise, are declared by the authors. Michelle King was with USARIEM at the time this study was conducted. She is currently employed by the Gatorade Sports Science Institute, a division of PepsiCo R&D.

Author Contributions

All authors meet the standards for authorship and all those who qualify are listed. All authors approved of the final version of this manuscript and agree to be accountable for all aspects of the work.

- Contributed to conception and design: MAK, TAM, MLP, JAW, SMW, LRL
- Contributed to acquisition of data: MAK, TAM, MLP, JAW, SMW
- Contributed to drafting and revising figures : TAM, MLP, JAW, SMW, LRL, ARC
- Contributed to analysis and interpretation of data: MAK, ARC, KO, TAM, MLP, JAW, SMW, LRL
- Drafted and/or revised the article: ARC, KO, JAW, SMW, LRL, MAK

FIGURE CAPTIONS

Figure 1. Experimental design. Following arrival and a 7 day quarantine period, mice were intraperitoneally implanted with temperature-sensitive radiotelemetry transmitters and allowed to recover ~7 days. Once recovered, running wheels were placed into individual cages for ad libitum running in addition to 4 days of 60-min forced exercise training sessions. Following a 2-day wash-out period, mice were exposed to one exertional heat injury (EHI0) or two EHI events separated by 1 day (EHI1), 3 days (EHI3) or 7 days (EHI7) of recovery. Samples were collected 30-min, 3-hr, 1 day, or 7 days after the first or second EHI exposure.

Figure 2. A) the average core temperature (T_c) and B) individual responses of C57BL/6J male mice exposed to the exercise control (EXC) condition, one exertional heat injury (EHI0) or two EHI events separated by 1 (EHI1), 3 (EHI3) or 7 (EHI7) days of recovery.

Figure 3. A) heat shock protein 70 (HSP70) and B) Corticosterone, log-transformed, data of male mice exposed to one exertional heat injury (EHI0) or two EHI events separated by 1 (EHI1), 3 (EHI3) or 7 (EHI7) days of recovery with 4 different times of sacrifice (30-min, 3-hr, 1 day, and 7 days). The exercise control (EXC) condition is represented by a horizontal black line (mean) and grey band (standard deviation). Summary data (dotand whiskers) represent the mean and standard deviation. Significant differences between EHI conditions are represented by letters (a,b,c,d) with a = difference from EHI0, b = difference from EHI1, c = difference from EHI3, d = difference from EHI7. Significant difference from control (EXC) condition denoted by an asterisk (*).

Figure 4. A) Creatine kinase (CK), B) fatty acid-binding protein 2 (FABP2), C) aspartate aminotransferase (AST), D) heat shock protein 70 (HSP70 Liver) content in the liver data (all log-transformed) of male mice exposed to one exertional heat injury (EHI0) or two EHI events separated by 1 (EHI1), 3 (EHI3) or 7 (EHI7) days of recovery with 4 different times of sacrifice (30-min, 3-hr, 1 day, and 7 days). The exercise control (EXC) condition is represented by a by a horizontal black line (mean) and grey band (standard deviation). Summary data (dot and whiskers) represent the mean and standard deviation. Significant differences between EHI conditions are represented by letters (a,b,c,d) with a = difference from EHI0, b = difference from EHI1, c = difference from EHI3, d = difference from EHI7. Significant difference from control (EXC) condition denoted by an asterisk (*).

Figure 5. A) IL-6, B) IL-10, and C) IP-10 data (all log-transformed) of male mice exposed to one exertional heat injury (EHI0) or two EHI events separated by 1 (EHI1), 3 (EHI3) or 7 (EHI7) days of recovery with 4 different times of sacrifice (30-min, 3-hr, 1 day, and 7 days). The exercise control (EXC) condition is represented by a by a horizontal black line (mean) and grey band (standard deviation). Summary data (dot and whiskers) represent the mean and standard deviation. Significant differences between EHI conditions are represented by letters (a,b,c,d) with a = difference from EHI0, b = difference from EHI1, c = difference from EHI3, d = difference from EHI7. Significant difference from control (EXC) condition denoted by an asterisk (*).

Figure 6. A) MIP-1β, B) MIP-2, C) G-CSF, and D) KC data (all log-transformed) of male mice exposed to one exertional heat injury (EHI0) or two EHI events separated by 1 (EHI1), 3 (EHI3) or 7 (EHI7) days of recovery with 4 different times of sacrifice (30-min, 3-hr, 1 day, and 7 days). The exercise control (EXC) condition is represented by a by a horizontal black line (mean) and grey band (standard deviation). Summary data (dot and whiskers) represent the mean and standard deviation. Significant differences between EHI conditions are represented

by letters (a,b,c,d) with a = difference from EHI0, b = difference from EHI1, c = difference from EHI3, d = difference from EHI7. Significant difference from control (EXC) condition denoted by an asterisk (*).

REFERENCES

1. Heat stress control and heat casualty management. *Technical Bulletin Medical* Department of Army and Air Force, 2003.
2. **Adriaanse MPM, Tack GJ, Passos VL, Damoiseaux JGMC, Schreurs MWJ, van Wijck K, Riedl RG, Masclee AAM, Buurman WA, Mulder CJJ, and Vreugdenhil ACE.** Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. *Alimentary Pharmacology & Therapeutics* 37: 482-490, 2013.
3. **Amorim FT, Fonseca IT, Machado-Moreira CA, and Magalhães FdC.** Insights into the role of heat shock protein 72 to whole-body heat acclimation in humans. *Temperature* 2: 499-505, 2015.
4. **Argaud L, Ferry T, Le QH, Marfisi A, Ciorba D, Achache P, Ducluzeau R, and Robert D.** Short- and long-term outcomes of heatstroke following the 2003 heat wave in Lyon, France. *Arch Intern Med* 167: 2177-2183, 2007.
5. **Audet GN, Dineen SM, Stewart DA, Plamper ML, Pathmasiri WW, McRitchie SL, Sumner SJ, and Leon LR.** Pretreatment with indomethacin results in increased heat stroke severity during recovery in a rodent model of heat stroke. *Journal of Applied Physiology* 123: 544-557, 2017.
6. **Bouchama A, Al-Sedairy S, Siddiqui S, Shail E, and Bezeig M.** Elevated pyrogenic cytokines in heatstroke. *Chest* 104: 1498-1502, 1993.
7. **Bouchama A, Al Mohanna F, El-Sayed R, Eldali A, Saussereau E, Chollet-Martin S, Ollivier V, de Prost D, and Roberts G.** Experimental heatstroke in baboon: analysis of the systemic inflammatory response. *Shock* 24: 332-335, 2005.
8. **Bouchama A, Hammami M, Al Shail E, and DeVol E.** Differential effects of in vitro and in vivo hyperthermia on the production of interleukin-10. *Intensive care medicine* 26: 1646-1651, 2000.
9. **Bouchama A and Knochel JP.** Heat stroke. *N Engl J Med* 346: 1978-1988, 2002.
10. **Bouchama A, Parhar RS, el-Yazigi A, Sheth K, and al-Sedairy S.** Endotoxemia and release of tumor necrosis factor and interleukin 1 alpha in acute heatstroke. *Journal of Applied Physiology* 70: 2640-2644, 1991.
11. **Butterfield TA, Best TM, and Merrick MA.** The dual roles of neutrophils and macrophages in inflammation: a critical balance between tissue damage and repair. *Journal of athletic training* 41: 457, 2006.
12. **Calderwood SK, Mambula SS, Gray PJ, Jr., and Theriault JR.** Extracellular heat shock proteins in cell signaling. *FEBS Lett* 581: 3689-3694, 2007.
13. **Calderwood SK, Theriault JR, and Gong J.** How is the immune response affected by hyperthermia and heat shock proteins? *Int J Hyperthermia* 21: 713-716, 2005.
14. **Chaitanya GV, Eeka P, Munker R, Alexander JS, and Babu PP.** Role of cytotoxic protease granzyme-b in neuronal degeneration during human stroke. *Brain Pathol* 21: 16-30, 2011.
15. **Clarkson PM and Ebbeling C.** Investigation of serum creatine kinase variability after muscle-damaging exercise. *Clin Sci (Lond)* 75: 257-261, 1988.
16. **Cole T.** Sympercents: symmetric percentage differences on the 100 loge scale simplify the presentation of log transformed data. *Statistics in medicine* 19: 3109-3125, 2000.
17. **Dineen SM, Ward JA, and Leon LR.** Prior viral illness increases heat stroke severity in mice. *Exp Physiol*, 2020.
18. **Dokladny K, Zuhl MN, and Moseley PL.** Intestinal epithelial barrier function and tight junction proteins with heat and exercise. *Journal of Applied Physiology* 120: 692-701, 2016.

19. **DuBose DA, Wenger CB, Flinn SD, Judy TA, Dubovtsev AI, and Morehouse DH.** Distribution and mitogen response of peripheral blood lymphocytes after exertional heat injury. *J Appl Physiol (1985)* 95: 2381-2389, 2003.
20. **Epstein Y and Roberts WO.** The pathopysiology of heat stroke: an integrative view of the final common pathway. *Scandinavian journal of medicine & science in sports* 21: 742-748, 2011.
21. **Epstein Y, Roberts WO, Golan R, Heled Y, Sorkine P, and Halpern P.** Sepsis, septic shock, and fatal exertional heat stroke. *Curr Sports Med Rep* 14: 64-69, 2015.
22. **Farber JM.** Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol* 61: 246-257, 1997.
23. **Flanagan SW, Ryan AJ, Gisolfi CV, and Moseley PL.** Tissue-specific HSP70 response in animals undergoing heat stress. *Am J Physiol* 268: R28-32, 1995.
24. **Garcia CK, Mattingly AJ, Robinson GP, Laitano O, King MA, Dineen SM, Leon LR, and Clanton TL.** Sex-dependent responses to exertional heat stroke in mice. *J Appl Physiol (1985)* 125: 841-849, 2018.
25. **Garcia CK, Sheikh LH, Iwaniec JD, Robinson GP, Berlet RA, Mattingly AJ, Murray KO, Laitano O, and Clanton TL.** Effects of Ibuprofen during Exertional Heat Stroke in Mice. *Medicine and science in sports and exercise* 52: 1870-1878, 2020.
26. **Hassanshahi G, Patel SS, Jafarzadeh AA, and Dickson AJ.** Expression of CXC chemokine IP-10/Mob-1 by primary hepatocytes following heat shock. *Saudi Med J* 28: 514-518, 2007.
27. **Horowitz M.** Epigenetics and cytoprotection with heat acclimation. *Journal of Applied Physiology* 120: 702-710, 2016.
28. **Hubbard RW, Criss RE, Elliott LP, Kelly C, Matthew WT, Bowers WD, Leav I, and Mager M.** Diagnostic significance of selected serum enzymes in a rat heatstroke model. *Journal of Applied Physiology* 46: 334-339, 1979.
29. **Huerta-Alardín AL, Varon J, and Marik PE.** Bench-to-bedside review: Rhabdomyolysis – an overview for clinicians. *Critical Care* 9: 158, 2004.
30. **Islam A, Abraham P, Hapner CD, Andrews-Shigaki B, Deuster P, and Chen Y.** Heat exposure induces tissue stress in heat-intolerant, but not heat-tolerant, mice. *Stress* 16: 244-253, 2013.
31. **Iwaniec J, Robinson GP, Garcia CK, Murray KO, de Carvalho L, Clanton TL, and Laitano O.** Acute phase response to exertional heat stroke in mice. *Experimental physiology* 106: 222-232, 2021.
32. **Keirns BH, Koemel NA, Sciarrillo CM, Anderson KL, and Emerson SR.** Exercise and intestinal permeability: another form of exercise-induced hormesis? *American Journal of Physiology-Gastrointestinal and Liver Physiology* 319: G512-G518, 2020.
33. **Kew M, Bersohn I, Seftel H, and Kent G.** Liver damage in heatstroke. *The American Journal of Medicine* 49: 192-202, 1970.
34. **King MA, Alzahrani JM, Clanton TL, and Laitano O.** A Preclinical Model of Exertional Heat Stroke in Mice. *Journal of visualized experiments: JoVE*, 2021.
35. **King MA, Leon LR, Morse DA, and Clanton TL.** Unique cytokine and chemokine responses to exertional heat stroke in mice. *Journal of Applied Physiology* 122: 296-306, 2017.
36. **King MA, Leon LR, Mustico DL, Haines JM, and Clanton TL.** Biomarkers of multiorgan injury in a preclinical model of exertional heat stroke. *J Appl Physiol (1985)* 118: 1207-1220, 2015.
37. **King MA, Rollo I, and Baker LB.** Nutritional Considerations to Counteract Gastrointestinal Permeability during Exertional Heat Stress. *Journal of Applied Physiology*, 2021.
38. **Laitano O, Garcia CK, Mattingly AJ, Robinson GP, Murray KO, King MA, Ingram B, Ramamoorthy S, Leon LR, and Clanton TL.** Delayed metabolic dysfunction in

myocardium following exertional heat stroke in mice. *The Journal of Physiology* 598: 967-985, 2020.
39. **Laitano O, Leon LR, Roberts WO, and Sawka MN.** Controversies in exertional heat stroke diagnosis, prevention, and treatment. *J Appl Physiol (1985)* 127: 1338-1348, 2019.
40. **Lambert GP, Gisolfi CV, Berg DJ, Moseley PL, Oberley LW, and Kregel KC.** Selected contribution: Hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. *J Appl Physiol* 92: 1750-1761, 2002.
41. **Lee WC, Wen HC, Chang CP, Chen MY, and Lin MT.** Heat shock protein 72 overexpression protects against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke. *J Appl Physiol (1985)* 100: 2073-2082, 2006.
42. **Leon LR, Blaha MD, and DuBose DA.** Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. *Journal of Applied Physiology* 100: 1400-1409, 2006.
43. **Leon LR, Dineen S, Blaha MD, Rodriguez-Fernandez M, and Clarke DC.** Attenuated thermoregulatory, metabolic, and liver acute phase protein response to heat stroke in TNF receptor knockout mice. *Am J Physiol Regul Integr Comp Physiol* 305: R1421-1432, 2013.
44. **Leon LR, DuBose DA, and Mason CW.** Heat stress induces a biphasic thermoregulatory response in mice. *Am J Physiol Regul Integr Comp Physiol* 288: R197-204, 2005.
45. **Leon LR and Helwig BG.** Heat stroke: role of the systemic inflammatory response. *Journal of applied physiology* 109: 1980-1988, 2010.
46. **Leon LR and Helwig BG.** Role of endotoxin and cytokines in the systemic inflammatory response to heat injury. *Front Biosci (Schol Ed)* 2: 916-938, 2010.
47. **Leon LR, Walker LD, DuBose DA, and Stephenson LA.** Biotelemetry transmitter implantation in rodents: impact on growth and circadian rhythms. *Am J Physiol Regul Integr Comp Physiol* 286: R967-974, 2004.
48. **Li GC, Li L, Liu RY, Rehman M, and Lee WM.** Heat shock protein hsp70 protects cells from thermal stress even after deletion of its ATP-binding domain. *Proc Natl Acad Sci U S A* 89: 2036-2040, 1992.
49. **Maloyan A, Palmon A, and Horowitz M.** Heat acclimation increases the basal HSP72 level and alters its production dynamics during heat stress. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 276: R1506-R1515, 1999.
50. **Mayer MP and Bukau B.** Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* 62: 670-684, 2005.
51. **Mitchell KM, Cheuvront SN, King MA, Mayer TA, Leon LR, and Kenefick RW.** Use of the heat tolerance test to assess recovery from exertional heat stroke. *Temperature* 6: 106-119, 2019.
52. **Murray KO, Brant JO, Iwaniec JD, Sheikh LH, de Carvalho L, Garcia CK, Robinson GP, Alzahrani JM, Riva A, Laitano O, Kladde MP, and Clanton TL.** Exertional heat stroke leads to concurrent long-term epigenetic memory, immunosuppression and altered heat shock response in female mice. *The Journal of Physiology* 599: 119-141, 2021.
53. **Nelson DA, Deuster PA, O'Connor FG, and Kurina LM.** Timing and Predictors of Mild and Severe Heat Illness among New Military Enlistees. *Med Sci Sports Exerc* 50: 1603-1612, 2018.
54. **Newham D, Jones D, and Clarkson P.** Repeated high-force eccentric exercise: effects on muscle pain and damage. *Journal of applied physiology* 63: 1381-1386, 1987.
55. **O'Connor FG, Heled Y, and Deuster PA.** Exertional Heat Stroke, the Return to Play Decision, and the Role of Heat Tolerance Testing: A Clinician's Dilemma. *Curr Sports Med Rep* 17: 244-248, 2018.

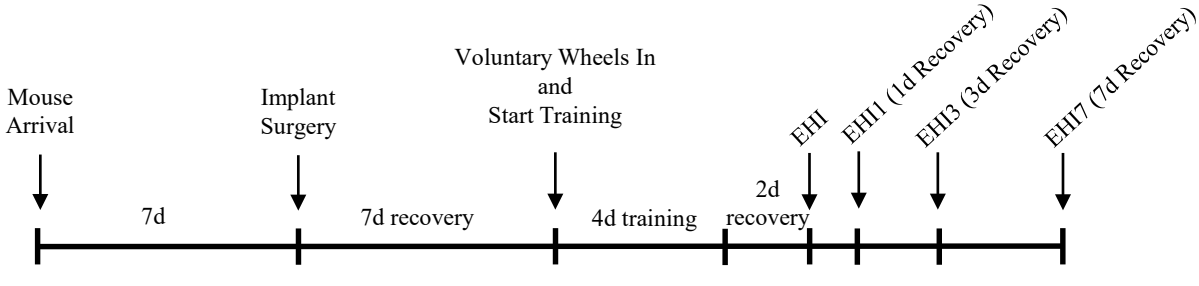
56. **O'Grady NP, Tropea M, Preas HL, 2nd, Reda D, Vandivier RW, Banks SM, and Suffredini AF.** Detection of macrophage inflammatory protein (MIP)-1alpha and MIP-1beta during experimental endotoxemia and human sepsis. *J Infect Dis* 179: 136-141, 1999.
57. **Oishi Y, Taniguchi K, Matsumoto H, Ishihara A, Ohira Y, and Roy RR.** Differential responses of HSPs to heat stress in slow and fast regions of rat gastrocnemius muscle. *Muscle Nerve* 28: 587-594, 2003.
58. **Olszyna DP, Pajkrt D, Lauw FN, van Deventer SJ, and van Der Poll T.** Interleukin 10 inhibits the release of CC chemokines during human endotoxemia. *J Infect Dis* 181: 613-620, 2000.
59. **Pelus LM and Fukuda S.** Peripheral blood stem cell mobilization: The CXCR2 ligand GROβ rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Experimental Hematology* 34: 1010-1020, 2006.
60. **Phillips NA, Welc SS, Wallet SM, King MA, and Clanton TL.** Protection of intestinal injury during heat stroke in mice by interleukin-6 pretreatment. *The Journal of physiology* 593: 739-753, 2015.
61. **Pires W, Veneroso CE, Wanner SP, Pacheco DA, Vaz GC, Amorim FT, Tonoli C, Soares DD, and Coimbra CC.** Association between exercise-induced hyperthermia and intestinal permeability: a systematic review. *Sports Medicine* 47: 1389-1403, 2017.
62. **Pockley AG.** Heat shock proteins as regulators of the immune response. *Lancet* 362: 469-476, 2003.
63. **Pockley AG, Shepherd J, and Corton JM.** Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. *Immunol Invest* 27: 367-377, 1998.
64. **Quinteiro-Filho WM, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Sakai M, Sa LR, Ferreira AJ, and Palermo-Neto J.** Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poult Sci* 89: 1905-1914, 2010.
65. **Quinteiro-Filho WM, Rodrigues MV, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Sa LR, Ferreira AJ, and Palermo-Neto J.** Acute heat stress impairs performance parameters and induces mild intestinal enteritis in broiler chickens: role of acute hypothalamic-pituitary-adrenal axis activation. *J Anim Sci* 90: 1986-1994, 2012.
66. **Rav-Acha M, Hadad E, Heled Y, Moran DS, and Epstein Y.** Fatal exertional heat stroke: a case series. *The American journal of the medical sciences* 328: 84-87, 2004.
67. **Sacco P and Jones D.** The protective effect of damaging eccentric exercise against repeated bouts of exercise in the mouse tibialis anterior muscle. *Experimental Physiology: Translation and Integration* 77: 757-760, 1992.
68. **Sawka MN, Leon LR, Montain SJ, and Sonna LA.** Integrated physiological mechanisms of exercise performance, adaptation, and maladaptation to heat stress. *Compr Physiol* 1: 1883-1928, 2011.
69. **Schwane J and Armstrong R.** Effect of training on skeletal muscle injury from downhill running in rats. *Journal of Applied Physiology* 55: 969-975, 1983.
70. **Skidmore R, Gutierrez JA, Guerriero V, Jr., and Kregel KC.** HSP70 induction during exercise and heat stress in rats: role of internal temperature. *Am J Physiol* 268: R92-97, 1995.
71. **Sonna LA, Wenger CB, Flinn S, Sheldon HK, Sawka MN, and Lilly CM.** Exertional heat injury and gene expression changes: a DNA microarray analysis study. *Journal of applied physiology* 96: 1943-1953, 2004.
72. **Steensberg A, Fischer CP, Keller C, Møller K, and Pedersen BK.** IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *American Journal of Physiology-Endocrinology And Metabolism* 285: E433-E437, 2003.

73. **Wallace RF, Kriebel D, Punnett L, Wegman DH, and Amoroso PJ.** Prior heat illness hospitalization and risk of early death. *Environ Res* 104: 290-295, 2007.
74. **Ward MD, King MA, Gabrial C, Kenefick RW, and Leon LR.** Biochemical recovery from exertional heat stroke follows a 16-day time course. *PLOS ONE* 15: e0229616, 2020.
75. **Welc SS, Clanton TL, Dineen SM, and Leon LR.** Heat stroke activates a stress-induced cytokine response in skeletal muscle. *Journal of Applied Physiology* 115: 1126-1137, 2013.
76. **Weshler Z, Kapp DS, Lord PF, and Hayes T.** Development and decay of systemic thermotolerance in rats. *Cancer research* 44: 1347-1351, 1984.
77. **Wissing D and Jaattela M.** HSP27 and HSP70 increase the survival of WEHI-S cells exposed to hyperthermia. *Int J Hyperthermia* 12: 125-138, 1996.
78. **Yeh YJ, Law LYL, and Lim CL.** Gastrointestinal response and endotoxemia during intense exercise in hot and cool environments. *European journal of applied physiology* 113: 1575-1583, 2013.

Table 1. Responses of mice during heat exposure and recovery

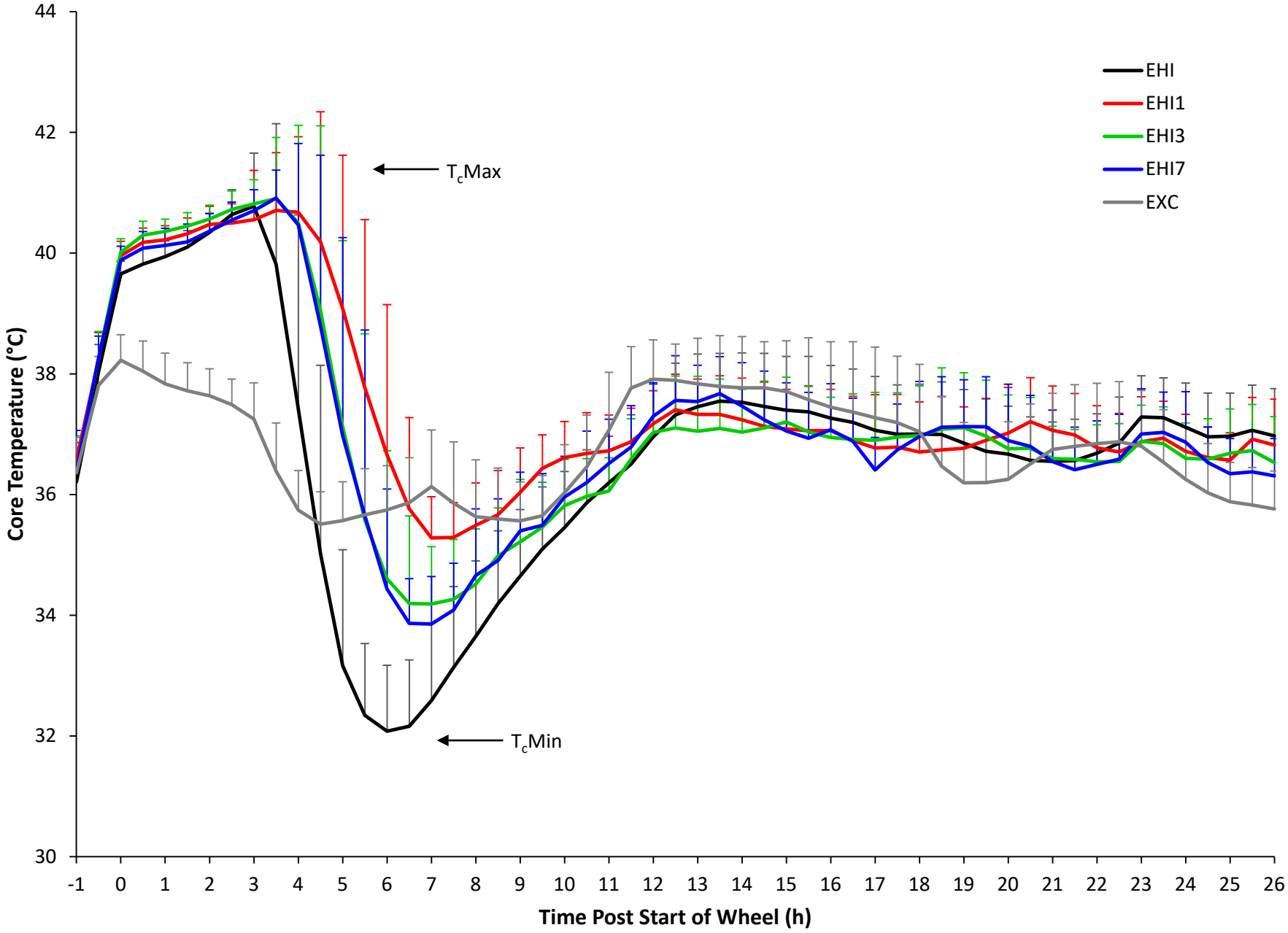
	EHI0 (*n* = 148)	EHI1 (*n* = 40)	EHI3 (*n* = 33)	EHI7 (*n* = 36)
Heat Exposure				
T_{cmax} (°C)	42.17 (0.2) **b,c**	42.48 (0.2) **a,d**	42.38 (0.2) **a,d**	42.20 (0.2) **b,c**
Time to T_{cmax} (min)	273.67 (35.2) **b,c,d**	343.60 (49.9) **a**	322.09 (45.1) **a**	321.64 (39.8) **a**
Thermal Load (°C·min)	727.88 (94.4) **b,c,d**	1028.66 (158.6) **a,d**	978.09 (161.3) **a**	926.59 (135.9) **a,b**
Running Performance				
Running Time (min)	239.14 (35.1) **b,c,d**	309.43 (50.2)**a**	287.12 (45.5) **a**	287.33 (39.6) **a**
Distance Run (m)	1399.75 (340.1) **b,c,d**	1999.04 (525.2) **a**	1778.28 (445.1) **a**	1826.45 (437.0) **a**
Dehydration (%)	12.32 (1.7) **b,c,d**	14.08 (2.2) **a**	14.54 (1.9) **a**	13.95 (1.6) **a**
Recovery				
Hypothermic Depth (T_{cmin}; °C)	31.59 (0.9) **b,c,d**	34.71 (0.4) **a,c,d**	33.28 (0.8) **a,b**	33.33 (0.6) **a,b**
Hypothermia Duration (min)	259.32 (62.1) **b,c,d**	16.00 (30.0) **a,c,d**	141.18 (79.4) **a,b**	154.63 (49.4) **a,b**

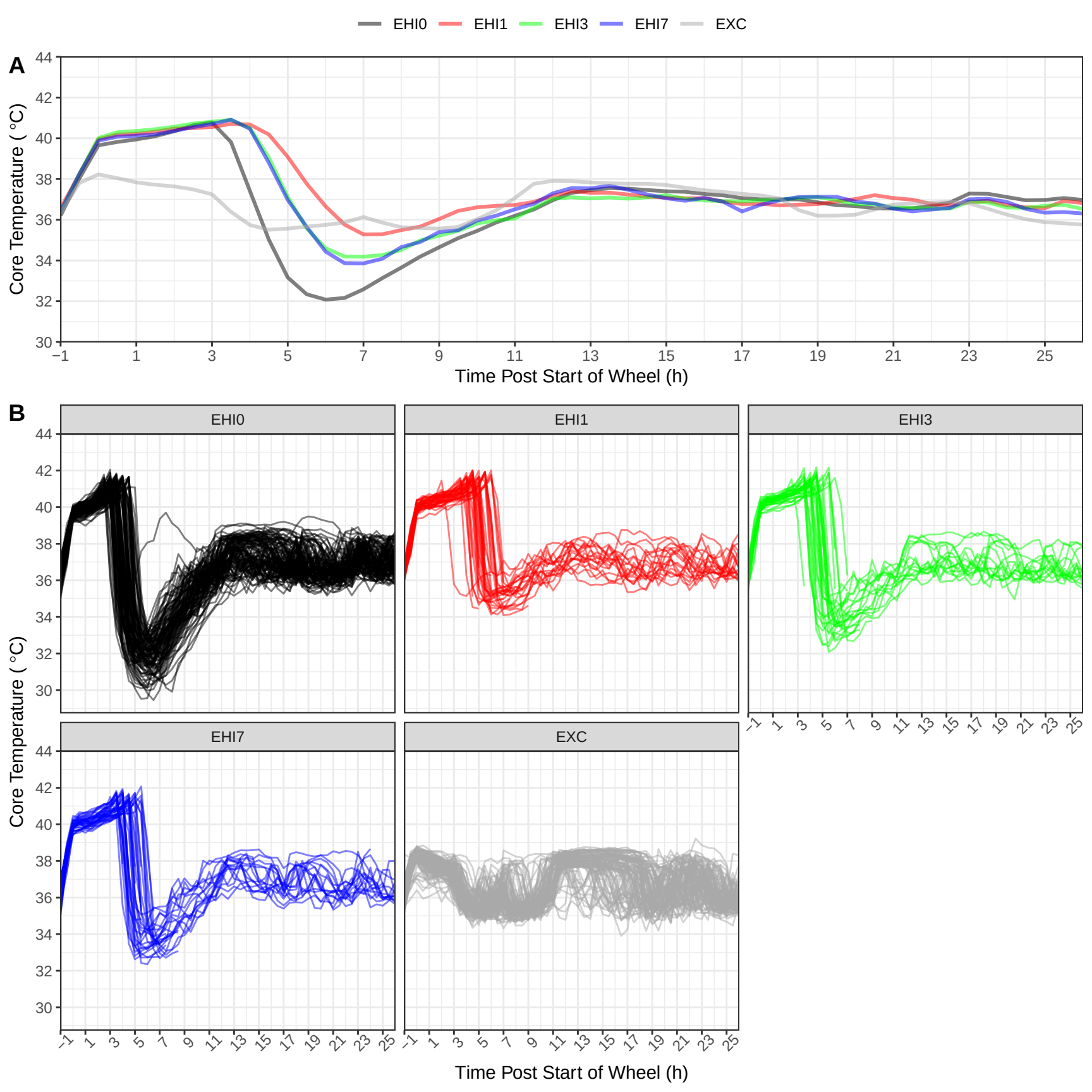
Values are mean (SD). Heat exposure data represent all mice with animal numbers indicated in parentheses. Recovery data represent mice that were sacrificed at 1 or 7 days post-heat. T_{cmax}*, maximum core temperature during heat exposure. Hypothermic Depth (*T_{cmin}*), minimum core temperature during recovery. Significant differences between EHI conditions are represented by letters (a,b,c,d) with a = difference from EHI0, b = difference from EHI1, c = difference from EHI3, d = difference from EHI7. Significance was determined by Welch's One Way ANOVA with Games-Howell's Post-Hoc Test.*

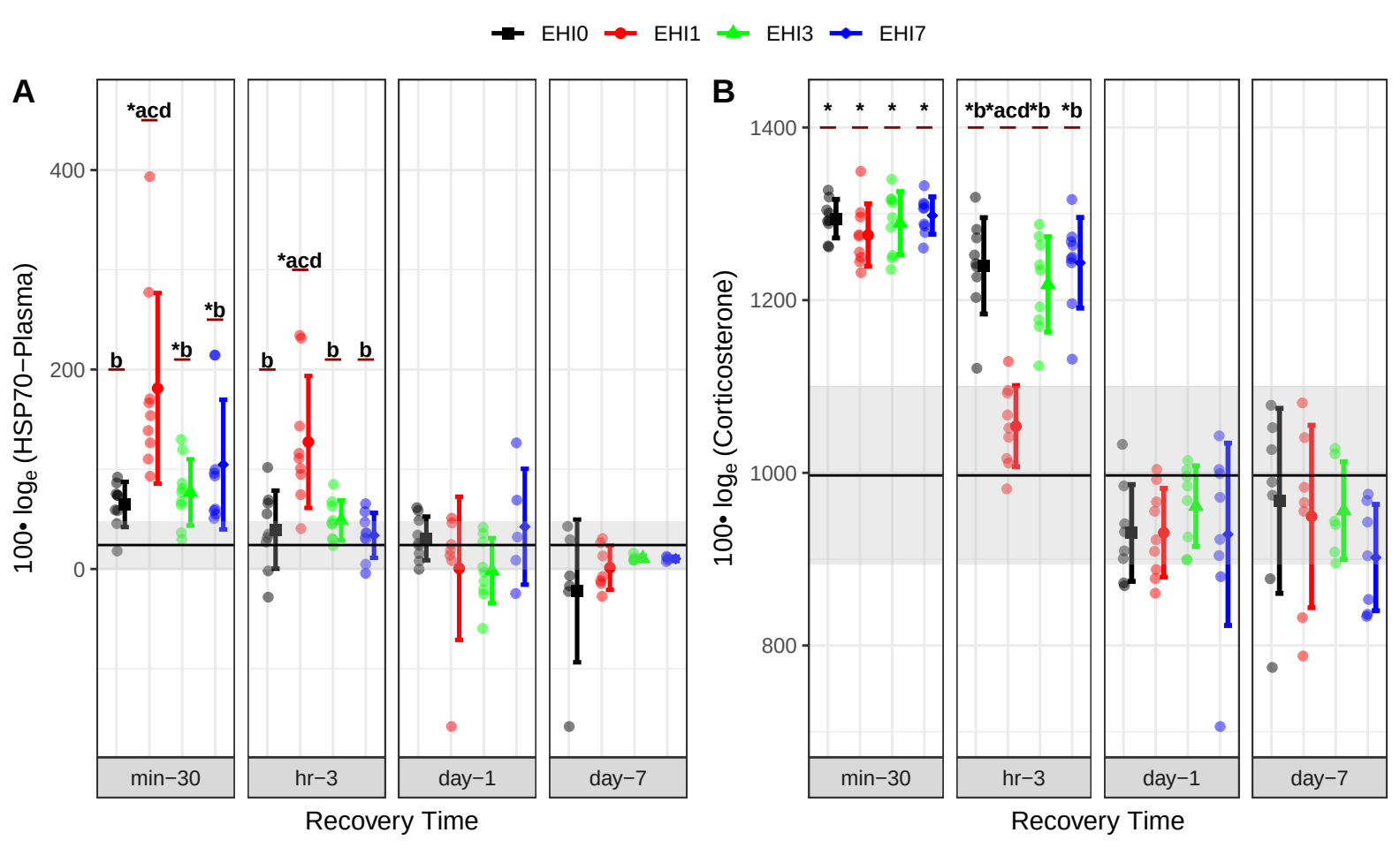

Mouse Arrival
Implant Surgery
Voluntary Wheels In and Start Training
EHI
EHI1 (1d Recovery)
EHI3 (3d Recovery)
EHI7 (7d Recovery)
7d
7d recovery
4d training
2d recovery

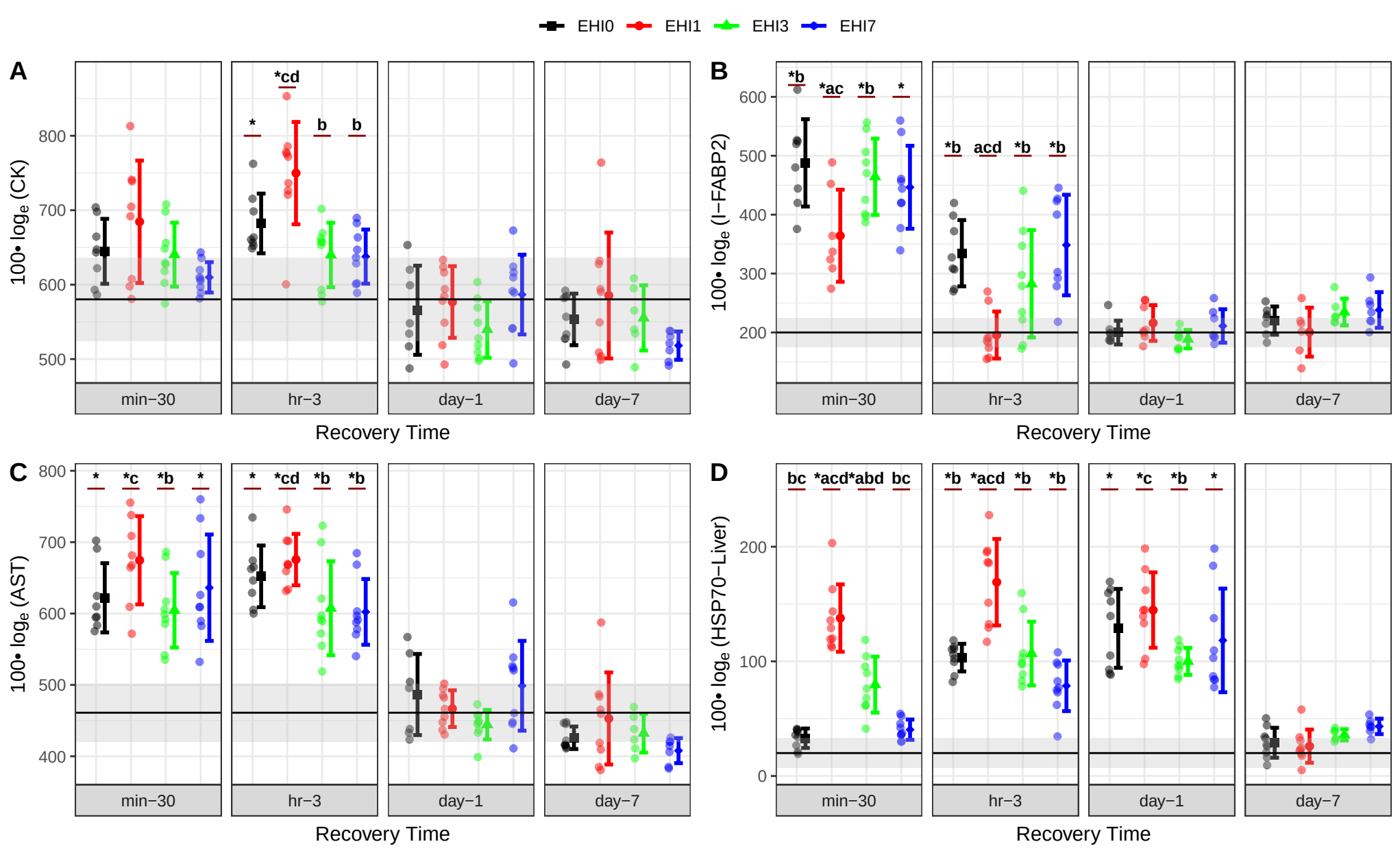
Leon et al. Figure 2

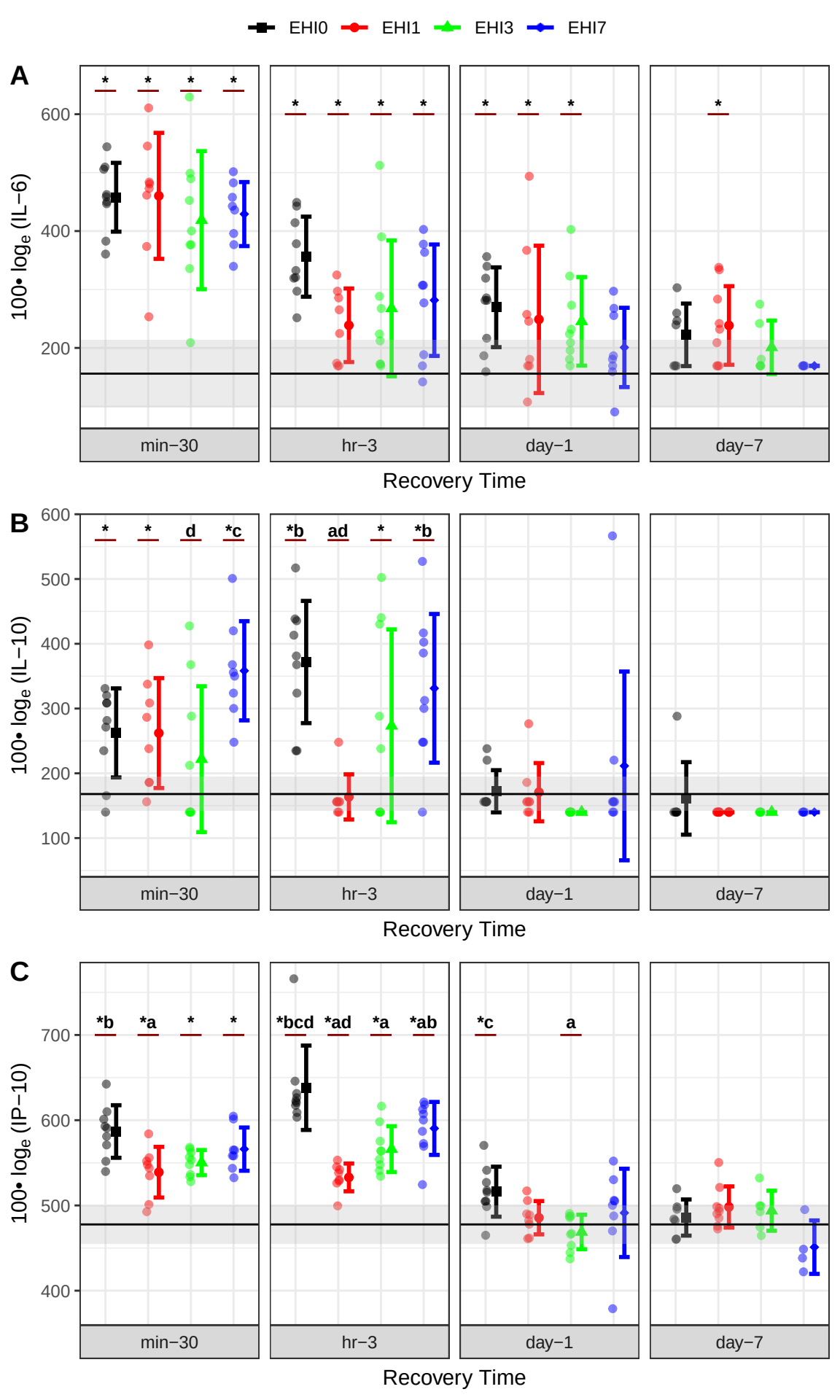
*SD

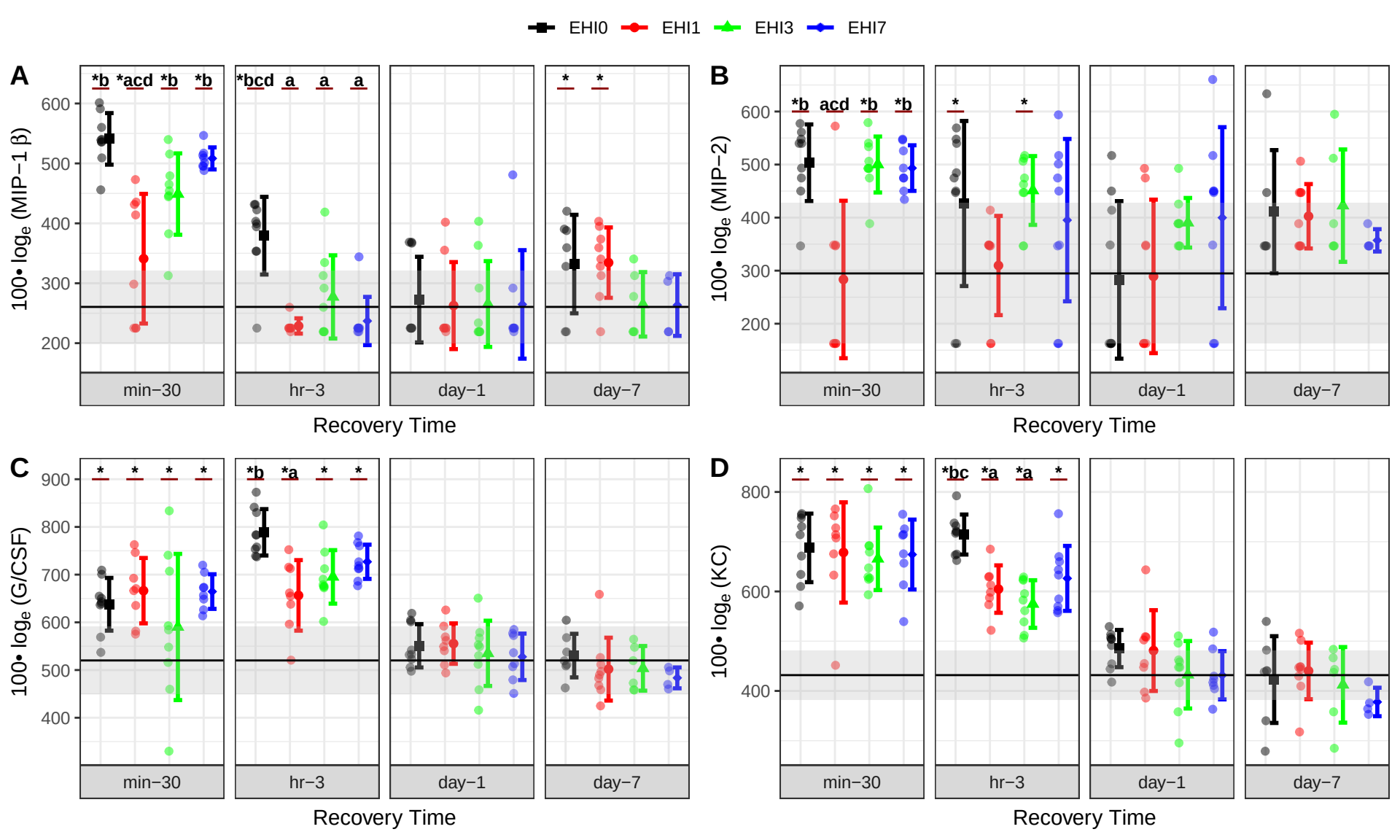



EHI0
EHI1
EHI3
EHI7
EXC
A
Core Temperature (°C)
Time Post Start of Wheel (h)
B
EHI0
EHI1
EHI3
EHI7
EXC
Core Temperature (°C)
Time Post Start of Wheel (h)


EHI0
EHI1
EHI3
EHI7
A
100• log$_e$ (HSP70−Plasma)
400
200
0
*acd
*acd
*b
*b
b
b
b
b
min−30
hr−3
day−1
day−7
Recovery Time
B
100• log$_e$ (Corticosterone)
1400
1200
1000
800
* * * *
*b*acd*b *b
min−30
hr−3
day−1
day−7
Recovery Time


EHI0
EHI1
EHI3
EHI7
A
100• log$_e$ (CK)
*cd
*
b
b
min−30
hr−3
day−1
day−7
Recovery Time
B
100• log$_e$ (I−FABP2)
*b
*ac
*b
*
*b
acd
*b
*b
C
100• log$_e$ (AST)
*
*c
*b
*
*cd
D
100• log$_e$ (HSP70−Liver)
bc
*acd
*abd
bc
*acd


EHI0
EHI1
EHI3
EHI7
A
100• log$_e$ (IL−6)
600
400
200
*
min−30
hr−3
day−1
day−7
Recovery Time
B
100• log$_e$ (IL−10)
600
500
400
300
200
100
*
d
*c
*b
ad
min−30
hr−3
day−1
day−7
Recovery Time
C
100• log$_e$ (IP−10)
700
600
500
400
*b
*a
*bcd
*ad
*ab
*c
a
min−30
hr−3
day−1
day−7
Recovery Time


EHI0
EHI1
EHI3
EHI7
A
100• log$_e$ (MIP−1 β)
*b *acd *b *b
*bcd a a a
* *
min−30
hr−3
day−1
day−7
Recovery Time
B
100• log$_e$ (MIP−2)
*b acd *b *b
* *
Recovery Time
C
100• log$_e$ (G/CSF)
* * * *
*b *a * *
Recovery Time
D
100• log$_e$ (KC)
* * * *
*bc *a *a *
Recovery Time