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14. ABSTRACT **This report covers all experiments in 2016-2018, to date. The project is currently in 1 yr, no-cost extension. Over 128 male and female mice were used to evaluate the physiological responses to exertional heat stroke (EHS) and recovery (14 days). Females outperformed the males in distance run, mechanical work and time in the heat by 50-100%. Metabolomics measurements revealed striking differences between male and female mice with regard to myocardial lipid metabolism and hormonal responses. Males underwent a metabolic switch to beta-oxidation that was sustained for 14 days. We have recently studied 32 additional female mice to more closely compare these metabolomic responses in males. The results suggest that the performance advantage of females may relate to their alternative metabolic pathways. It is possible that this could translate to Warfighters' ability to perform in the heat if they converted to a more lipid-metabolic state. We also studied the influence of 48 hours of ibuprofen (IBU) ingestion on performance and intestinal injury in male and female mice. IBU increased the max core temperature prior to collapse in EHS. This was associated with increases in the exercise time (tolerance) during the last phases of the EHS protocol. We saw modest effects of IBU on intestinal injury, both histologically and through biomarkers. The primary source of injury was from EHS alone. Recent experiments involved the study of diclofenac, an alternative NSAID a diclofenac-H2S donor (ATB-337). Neither diclofenac nor ATB-337 had any impact on EHS performance. However, ATB337 significantly elevated biomarkers of intestinal injury (FABP2) compared to diclofenac alone. Interestingly, diclofenac reduced circulating white blood cells during EHS but this effect was attenuated by ATB337. Overall, the results suggest that the H2S-containing NSAIDs may not provide a protective role in EHS. We have made some major breakthroughs in additional work evaluating the epigenetic responses to EHS in this model and have identified relatively massive changes in DNA methylation in inflammatory cells and in skeletal muscle at 30 days post EHS. These are accompanied by changes in susceptibility to a second EHS exposure and to skeletal muscle abnormalities.**

15. SUBJECT TERMS
Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones, epigenetics, hydrogen sulfide.

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Table of Contents

Page

1. INTRODUCTION

Exertional heat stroke (EHS) is a serious medical problem in the U.S. Armed Forces, both during basic training and deployment operations. In the 2016 Medical Military Surveillance Report (1), there were 417 cases of heat stroke (largely EHS) and 2,350 cases of heat injury reported in the previous year. The rate of heat injury in active component members was 0.35/100 person years in males and 0.16/100 in females. The incidence rate of heat injury in males, however, were nearly identical. The reasons for these sex differences are not known.

The Military needs solutions to determine when warfighters are fit to return to duty without further risk of EHS or other complications and whether there are long-term consequences of EHS that can be identified and treated. Furthermore, more information is needed on risk differences between males and females as the demographics of active military personnel continues to be more distributed. We have developed the first preclinical EHS model in mice that resembles the condition in humans. It is our aim to utilize this model to solve a series of problems related to EHS, to identify biomarkers that will translate to the conditions experienced by Warfighters, to evaluate the influence of common drugs and agents that may amplify the deleterious effects of EHS, and to develop treatment and prevention strategies that are applicable to the needs of military medicine. Ultimately, our goal is to save lives and suffering of US Military personnel.

There are four basic purposes of this project 1) To identify relevant biomarkers that could be helpful to the US Military in identifying effective and complete recovery from exertional heat stroke and in identifying risk factors for long-term complications of EHS. 2) Determine if there are significant differences in the response to EHS between males and females. 3) To determine if non-steroidal anti-inflammatory drugs (NSAIDs) impose additional risk factors for complications of EHS, and 4) To evaluate a new line NSAIDs that may offer a safe line of protection from organ injury in EHS. In the past three years we have completed 90% of these aims and are performing experiments to prepare for further publication and to explore avenues of research brought up in the last years of the funding cycle.

2. KEYWORDS

Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones, non-steroidal anti-inflammatory drugs, biomarkers, epigenetics, hydrogen sulfide, ibuprofen, diclofenac, metabolomics

3. ACCOMPLISHMENTS

What were the major goals of the project?

Year 1: 2 Months: Complete approval of IACUC protocols, coordinate the data collection schedule between 3 centers, set up of new equipment and attain approval of Environmental Risk Assessment.

6 Months: Study EHS in male mice: surgical implantation of transmitters, recovery, exercise training and collection of data from 56 mice exposed to EHS or exercise control. Mice will be studied in groups of 8, implanted 2 weeks apart.

2 Months: Submission of samples and analytical and morphological tests of organ and tissue injury, submission of samples for immunological studies, metabolic hormone studies, metabolomics and proteomics analyses and integration of data from 3 centers.

PROGRESS: All of the original year one goals were completed and were published last year (manuscript in Appendix material. In additions, we have followed a new line of inquiry looking at epigenetic biomarkers of EHS exposure. This initial project is completed and is being prepared for publication.

Year 2: 6 Months: Study EHS in female mice: surgical implantation of transmitters, recovery, exercise training and collection of data from 56 female mice exposed to EHS or exercise control.
3 Months: Submission and analyses of samples for multiplex (Luminex) determination cytokines and metabolic hormones, development and testing of new assays for detection of targeted biomarkers from plasma and analyses of organ injury using histopathological analyses.

2 Months. Complete analysis and initial reports of metabolomics and proteomics, comparison of males and females and outcome of cytokine and metabolic hormone measurements.

PROGRESS: All studies originally planned in females have been completed and evaluated in a published manuscript (appendix). Male samples have been evaluated for metabolomics, metabolic hormone analysis and lipidomics. The metabolomic data has been analyzed and it was determined that we needed to repeat the metabolomics on female mice which is currently underway

Year 3: 4 Months: Completion of testing the impact of ibuprofen on organ injury in male and female mice during EHS in 48 mice. Submission of plasma samples for cytokine analyses and tissues for analysis of histopathological injury.

Months: Completion of testing for the impact of the predominant COX2 inhibitor, diclofenac vs. its H_2S-analog (ATB-337) on organ intestinal injury and damage to other organ systems following EHS in 32 male mice. Submission of plasma samples for cytokine analysis and multiple organ injury and for measurement of metabolomics and eicosanoid products.

3 Months: Completion of testing for the impact of the more predominant COX1 inhibitor, naproxen vs. its H_2S-analog (ATB-346) on organ intestinal injury and damage to other organ systems following EHS in 32 male mice. Submission of plasma samples for cytokine analysis and multiple organ injury and for measurement of metabolomics and eicosanoid products.

2 Months: Complete analysis of samples from mice, integrate data collection from the 3 laboratories and prepare final reports and manuscripts of experimental outcomes.

PROGRESS: All studies have been completed regarding the effect of ibuprofen on males and females. We have also completed the study of the effects of ATB-337 (diclofenac derivative) and diclofenac alone. Manuscripts are being prepared on these investigations. We have been currently staging the last ATB-346 studies after evaluating the results of the ATB-337. These last studies will be completed in coming months

What was accomplished under these goals? The following practical conclusions have been obtained that may affect the future translational development of preventative and treatment for Warfighters with EHS. Please note, that many of these will require further testing.

- Females appear to have a resistance to EHS that is markedly greater than males. Though these measurements in mice are unlikely to directly translate to humans, it suggests there is no fundamental biological impact of being in the female sex (or exposure to female hormones) that makes that females more susceptible to heat exposure.
- The metabolomics responses to both males and females suggest that an adaptive response, accentuating lipid metabolism may be of fundamental significance in acquiring resistance to EHS. From a translational viewpoint, would adjusting the diet to a predominantly fat and protein provide a performance advantage to Warfighters in hot environments? We hope to pursue this idea further in the coming year.
- There are long term consequences to EHS in our mouse model that result in marked epigenetic changes in at least 2 organ systems we have studied. These changes may be reversible, and might be useful in overcoming "return to duty" questions in Warfighters and also potentially understanding the significance of long term effects of EHS on health outcomes over a lifetime.
- In our hands, exposure to 48 hours of IBU in food, which we designed to mimic the conditions of the average Warfighter who is taking intermittent IBU with and without meals, did not greatly affect gastrointestinal damage in EHS. Furthermore, at least in one sense, IBU treatment resulted in a delay in the loss of consciousness at the end of the EHS exposure, resulting in animals achieving higher core temperatures prior to collapse. Although much additional work needs to be done to confirm this, these results challenge the idea that IBU and other NSAIDs are inherently dangerous to Warfighters who must perform at maximum in a hot environments. Again, more work needs to be done to confirm this finding and to ensure that that animals have a sufficient dose of the drug to make it relevant to Warfighters.
- In our hands diclofenac treatment also did not greatly elevate damage to the intestinal lining during EHS. Unexpectantly, the addition of the H_2S moiety to diclofenac appeared to worsen the intestinal damage and may have caused additional damage to the liver. This unexpected finding, which we will soon verify with other H_2S donors may result in an important precaution in using the next generation of NSAIDs in Warfighters.

Highlighted findings:

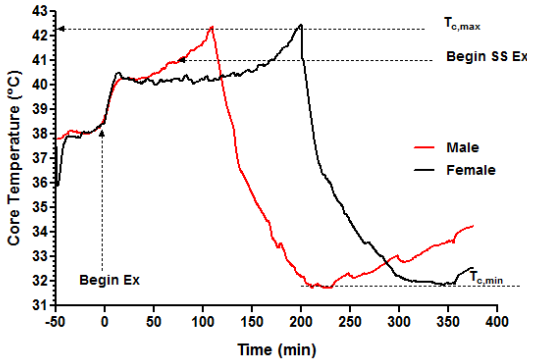

Figure 1. Typical core temperature profiles of male (red and female mice during EHS trials. (Garcia et al. 2018)

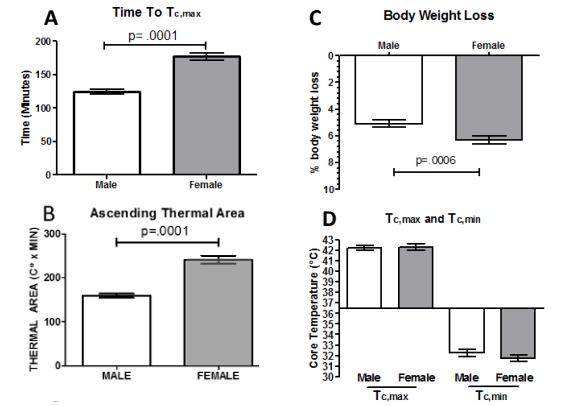

Fig. 2. Comparisons of ♂ & ♀mice of A) running time in the heat, B) thermal load, C) percent loss of body weight, and D) max and min core temperatures. Data are Means +/- SEM. (Garcia et al. 2018).

Temperature profile to EHS in ♂ & ♀ mice

Females exhibited a significant resistance to EHS compared to age-matched male mice. Figure 1 shows a typical example of this phenomenon in two representative mice. Note most of these results can be find in the Appendix material in a published manuscript (Garcia et al, 2018). The results are grouped in Fig 2. for all mice (N = 44 per group). As shown, female mice ran, on average, ~43% longer in the heat than male mice without losing consciousness. They were also exposed to a significantly higher overall heat loads because they ran longer in the heat (Fig. 2B, ascending thermal area). They exhibited a greater apparent fractional loss in body fluid, based on % body weight loss (Fig. 2C). Interestingly, both male and female mice collapsed, reaching the symptom limited end of EHS, at nearly identical peak core temperatures ($T_{c,max}$) and following EHS, their core temperatures dropped to nearly identical levels of hypothermia ($T_{c,min}$), Fig. 2D. This latter measurement is considered an index of severity of exposure to hyperthermic conditions (7). The specific results for the males in this study were very similar to results we have published previously (6).

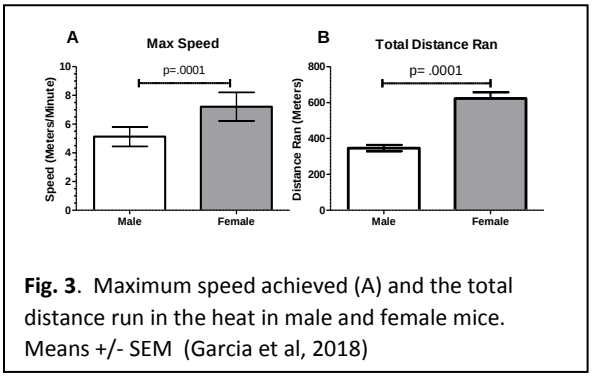

Fig. 3. Maximum speed achieved (A) and the total distance run in the heat in male and female mice. Means +/- SEM (Garcia et al, 2018)

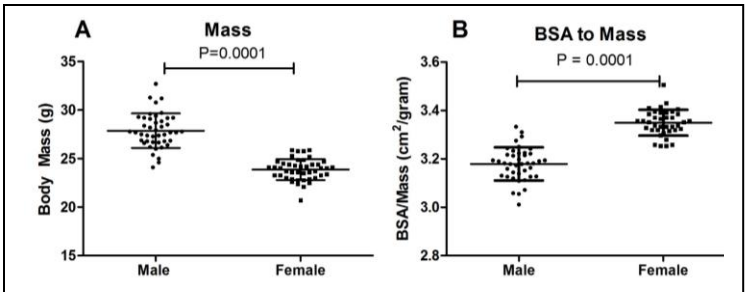

Fig. 4. Raw distributions showing **A)** The expected elevation in body mass of ♂ & ♀ mice, 20-24 weeks of age, and **B)** the calculated body surface area to mass ratio, showing that females had a greater surface area for heat dissipation.

Running Performance during EHS in ♂ & ♀ mice

Because the females could maintain their body temperature for longer periods during exercise in the heat, and the protocol called for incremental increases in running speed until a core temp. of 41°C was

achieved, they ran at much faster speed (~39%) by the end of the EHS protocol (Fig. 3A) resulting in a total distance in the heat ~80% further than the comparable males (Fig. 3B).

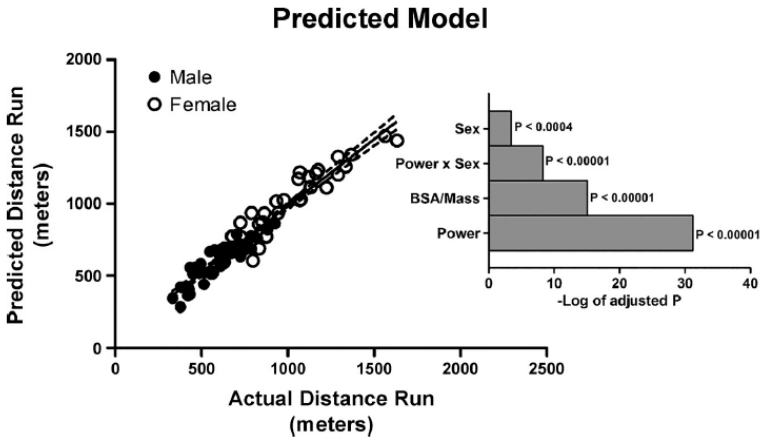

Fig. 5. Statistical modeling of physical determinants of differences in exercise performance in the heat between ♂s & ♀s. Both 'BSA/mass' ratio and 'Power' accounted for much of the variance in the distance run in the heat in both ♂s & ♀s. Mass is closely related to BSA/M ratio and demonstrates that mass appeared to be a significant limiting factor. The total model for multiple linear regression is shown in the figure using these variables. The bar graph on the right shows the relative significance of each variable in its contribution to the overall variance of the predictive model. Note that sex is a significant predictor of outcome, both as a direct factor and as a co-factor with power (Garcia et al, 2018).

Causative factors for differences in heat tolerance and running performance during EHS between ♂ & ♀ mice.

We considered several "physical" factors in trying to explain the origins of the better heat tolerance and performance of the females compared to males. Males had significantly higher body weights (~14%) but females had significantly higher body surface area/mass ratios (and body surface to mass ratios, on average ~5% higher (Fig. 4 A & B). Therefore, presumably, females had a physical advantage due to the greater relative surface area for heat dissipation. The females also had a mechanical advantage because at a given speed they would be performing less mechanical work and therefore producing less heat. The question we asked is whether we could account for the differences in performance between males and females in the heat, based solely only on differences in body surface area and power output. The statistical results are shown in Figure 5. Body BSA/mass and Power output were good predictors of performance in both sexes but there remained a powerful effect of sex, particularly when expressed as a crossed effect with Power that could not be accounted for by power and BSA/m alone (Figure 5).

We took from this analysis, as well as other analyses of rates of heat dissipation (not shown), that independent of physical laws governing heat dissipation and external work, there was an independent influence of sex on heat tolerance and work capacity.

Differences in Metabolic regulation in ♂ & ♀ mice

In both males and females, there were statistically significant reductions in blood glucose as measured at 30 min and 3 hours but there were no differences between males and females except at the 9d time point (we consider to be an anomaly). In general,

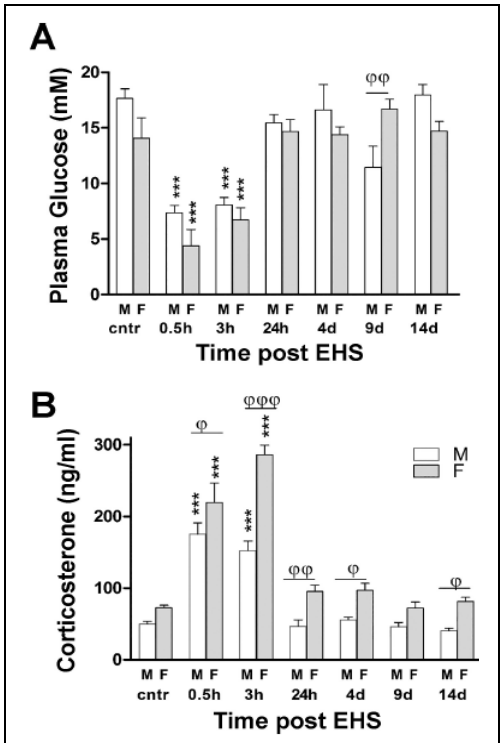

Fig. 6. Plasma levels of A) Glucose and B) corticosterone in sham control (cntrl) and during recovery from EHS in males and females * P < 0.05, ** <0.01 differences from cntrl. φ P <0.05, φφ P<0.01 between males and females at each time point.

glucose levels were higher than predicted in both groups between 1-14 days, as well as in the 4 d control animals (Fig 6A). *We conclude from this analysis that the mice go through a transient hypoglycemia that is resolved by 24 h post EHS. We do not believe that the hyperglycemia observed following EHS is due to EHS-induced pathology.*

We then measured metabolic hormone production in the plasma at each time point. The assays were run at USARIEM by Michelle King, Lisa Leon and colleagues. Corticosterone levels (the primary glucocorticoid produced in mice) were highly elevated at the 30 min and 3h time points (Fig 6B). Throughout recovery, females exhibited higher corticosterone levels than males. At the 3 the 3 hr time point the corticosterone was nearly double in females compared to males. These data demonstrate that females have a more robust glucocorticoid response to EHS.

Additional metabolic hormones were evaluated using Luminex multiplex technology (metabolic hormone panel). These results are displayed in figure 7 and 8.. In figure 7, metabolic hormones arising from the pancreas are shown. Insulin and c-peptide (a protein co-secreted with insulin from the β cells) were both significantly suppressed during the immediate recovery period following EHS (Fig 8 A & B). This is expected based on the lower plasma glucose. Interestingly, both in the exercise sham controls and throughout recovery, females had consistently higher levels of plasma insulin and c-peptide compared to males. In contrast, we could measure no elevations in plasma glucagon in male mice at any time point. In females, glucagon was present during the later recovery period. *These data suggest some abnormalities in glucose metabolism, particularly in male mice, during the recovery from EHS. Even though glucose levels were lower during 0.5-3 hr recovery, one would normally expect a robust glucagon response when insulin levels are low. The data has the appearance of revealing a deficient pancreatic enzyme response immediately post EHS. We speculate that this may reflect the reduction in blood flow to the pancreas during EHS due to splanchnic ischemia.*

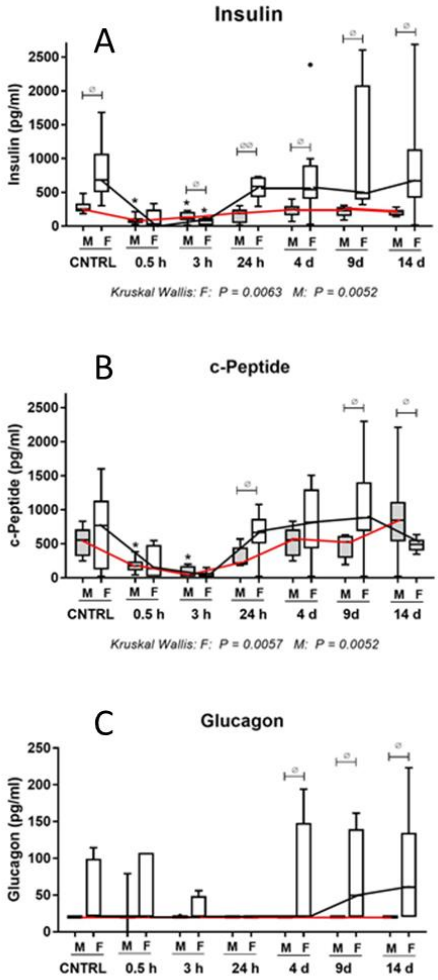

Figure 7. Plasma pancreatic enzyme responses during recovery from EHS. Red lines connect the medians in male, black lines are females. During 0.5-3 hr recovery all hormones were suppressed. Following EHS females demonstrated significantly higher values at most time points. θ = difference between male/female; * = difference from cntr.

Figure 8 illustrates the responses of two metabolic hormones, generally attributed to adipose tissue (adipokines), leptin and resistin. Previous studies have demonstrated that resistin levels are much higher in control females but leptin levels are higher in males (2). Our results are consistent with these findings in the control mice, resistin (Fig. 9A) and leptin (Fig 9B). The response patterns were also significantly different between males and females and demonstrated strikingly different responses during EHS recovery. The best known function of resistin is that it reduces insulin sensitivity in tissues (9), though it also has recently been described as a secreted stress protein and chaperone, that may provide some protection to heat stress (8, 11). Resistin became acutely elevated at the 0.5 h recovery

period in males but showed little nor no change in females. From that point on resistin remained significantly lower in males than females. Leptin levels, were higher in males than females. This is not too unexpected since male C57/Bl6 mice have a higher percent fat content than females throughout life (3, 10). The females tend to rebound during the 1-4 days, post EHS, which corresponds to a period of weight gain in these mice (data not shown). The metabolic effects of leptin are largely associated with regulation of food intake, but recently it has been shown that leptin has the capacity to reduce thermal conductance (4).

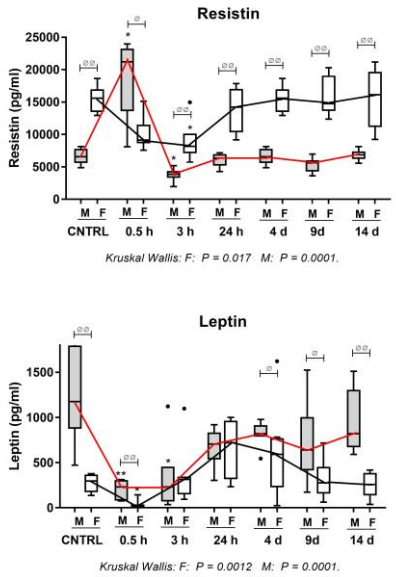

Figure 8. Adipose derived metabolic hormones, A) Resistin and B) Leptin during the recovery period from EHS.

We were also able to compare the metabolomics profiles between males and females (metabolomics data) at one time point following EHS, 3 hrs post vs. control. A more detailed metabolic profile follows below for the male population through the entire recovery period and in the next 2 months we should have a complete comparison of male and female EHS responses

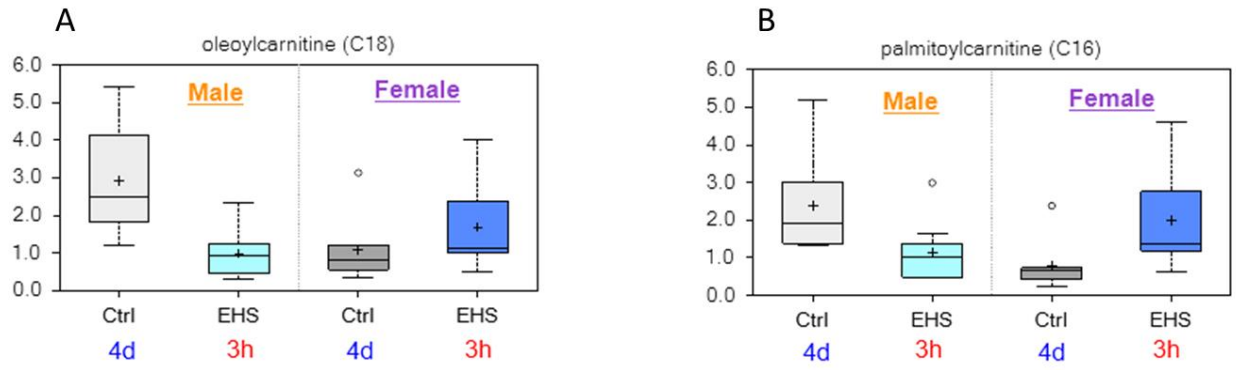

Figure 9. Changes in acylcarnitine abundance between male and female mice of two representative acylcarnitine forms. (Metabolon data). Only the 4d control land 3h post EHS data were studied between male and female mice. See text for details. These data illustrate striking contrasts between the metabolic responses of male and female mice to EHS.

over the 14 day recovery period. In most measurements, the 3 h and control time points were not different in terms of the metabolic profiles between males and females in the plasma or in heart. However, there was some striking and highly related findings. The acylcarnitine abundance (an indicator of ongoing fatty acid metabolism in the heart) was reduced in females compared to male mice in the sham controls (Figure 9). In males, these levels were higher at rest (control) but switched drastically lower after EHS suggesting a switch to mitochondrial fatty acid metabolism. In contrast in females, there was a low acylcarnitine abundance at rest but a modest trend toward an elevation at 3h post EHS. Since this was accompanied by what is considered control levels of acetylcarnitine supply, it suggests that females were actively metabolizing fatty acids in the heart at rest but males were metabolizing

relatively less. It also suggests that following EHS, males switched and relied more heavily on fatty acids (described in more detail below).

Differences in Cytokine Responses between male and female mice.

Plasma samples were evaluated for cytokine concentrations using an inflammation Luminex multiplex panel (27 cytokines). These analyses were performed at USARIEM. In general, the cytokines seen in the

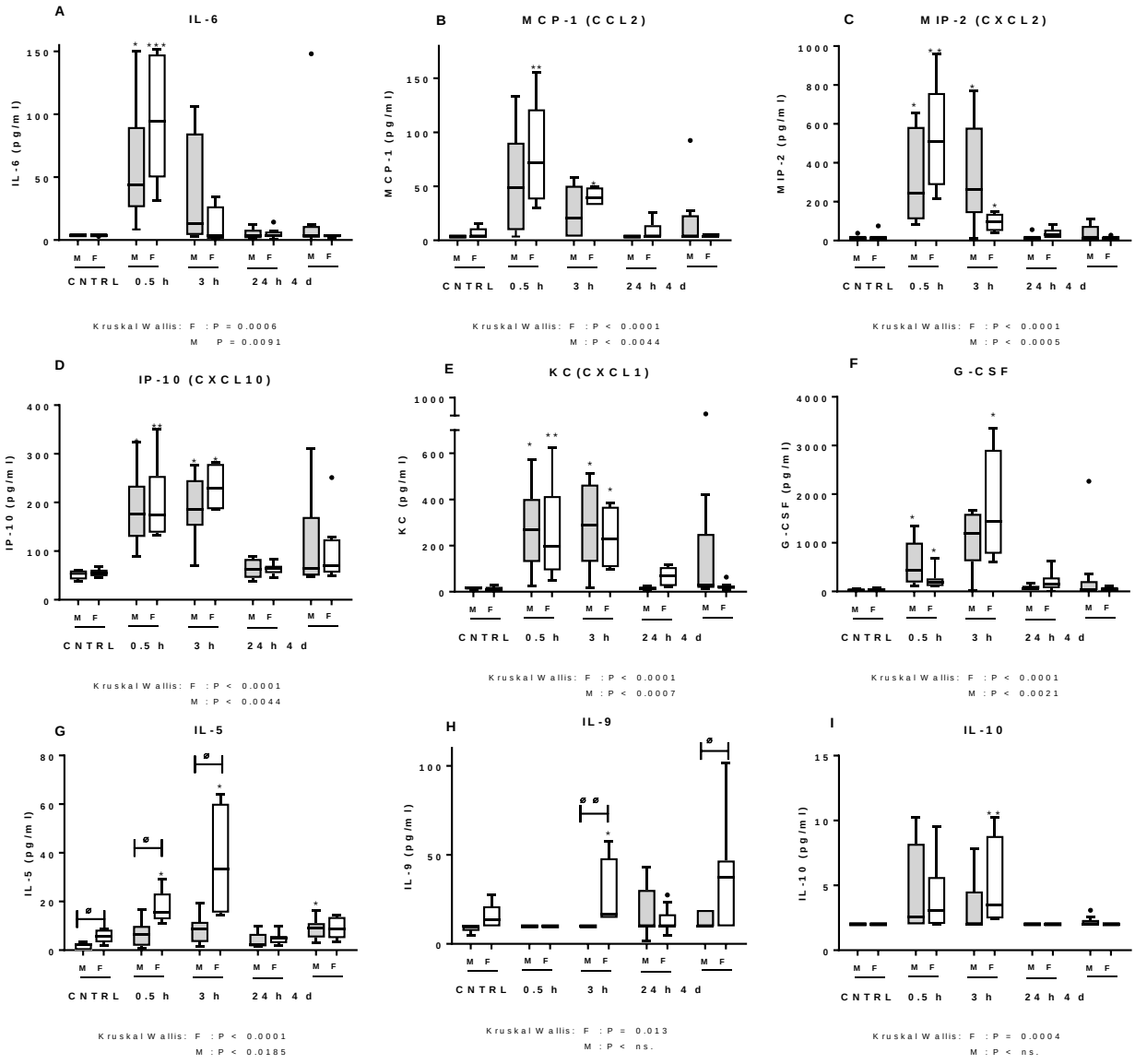

Fig. 10. Plasma cytokine measurements in male and female mice over the course of 0-4 d recovery from EHS. Most cytokines returned to normal or near normal by 4 days post. Data expressed as "whisker plots" because data was nonparametric. The lines represent the median values and the error bars are 95% Cis. Single dots are outliers (left in the analysis) * represent differences from sham 4 day control, θ = differences between males and females. (Garcia et al. 2018*)*

plasma during the recovery period had similar patterns between males and females (Fig. 10). Furthermore, the overall pattern in males was nearly identical to our previous publication in male mice (5). In general, in females the cytokine and chemokine responses appeared more robust and tended to be sustained longer, but most of these apparent changes did not reach statistical significance. Interestingly, IL-5 and IL-9 were expressed in plasma and higher levels in females than males. We have never before observed elevations in these two cytokines in animal models of heat stroke. However, differences in the cytokine responses in heat may contribute to the observation that the incidence of acute allergic reactions to exertion in the heat, though relatively rare, occurs at a rate of 15 fold higher in female humans compared to males (12) . We are trying to understand the implications of this. *The data suggests that the inflammatory responses of male and female mice to EHS are similar and therefore it appears to exclude the hypothesis that immunosuppression (perhaps from elevated glucocorticoid secretion, is responsible for better heat tolerance in female compared to male mice.*

Metabolomic Responses in male mice during recovery from Heat stroke.

From plasma and ventricular muscle samples submitted to Metabolon for analysis through Danielle Ippolito and our colleagues at USACEHR, over 1500 chemicals were identified in individual samples. The analyses is extremely complex and secondary analyses are ongoing. Based on summaries provided by Metabolon, the following chief conclusions are described Fig. 11. The early recovery period following

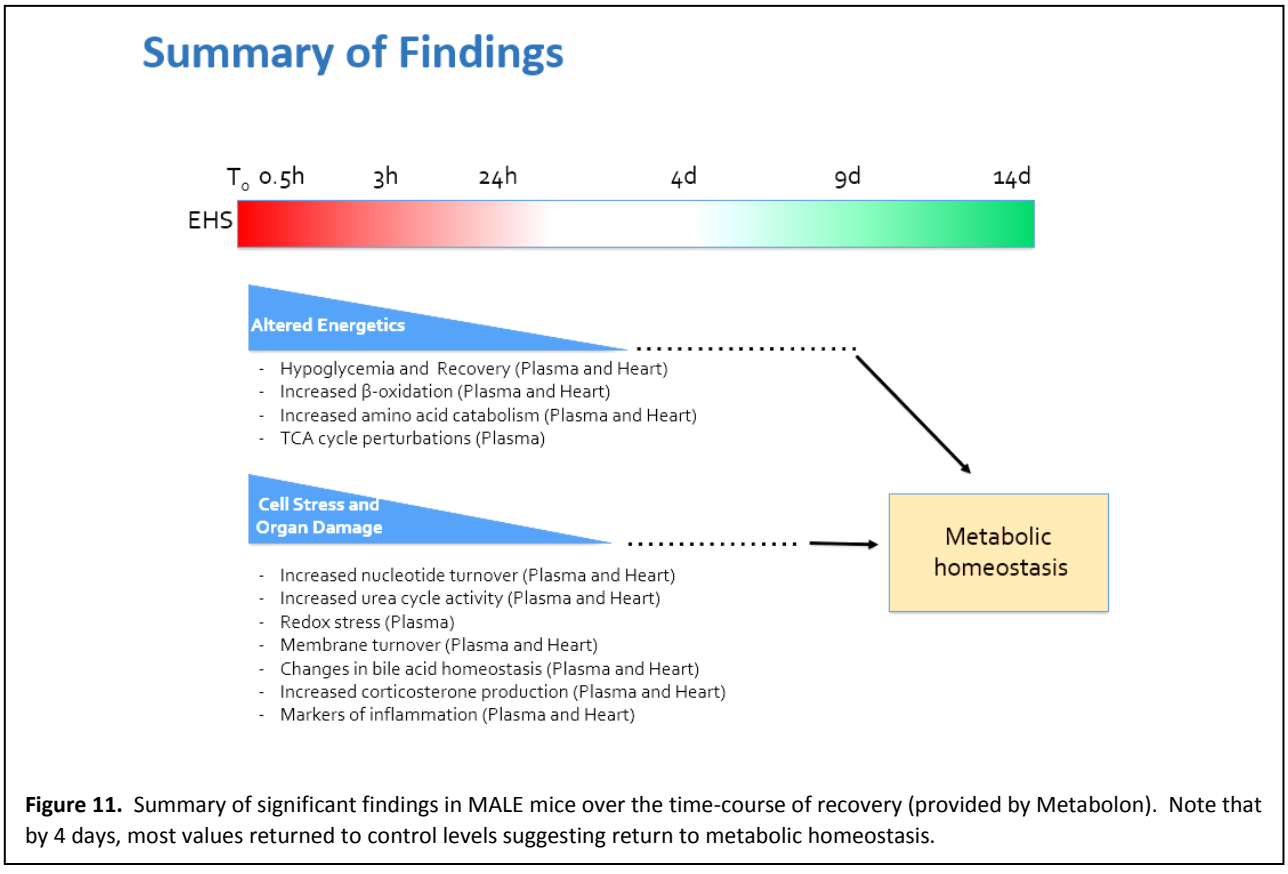

Figure 11. Summary of significant findings in MALE mice over the time-course of recovery (provided by Metabolon). Note that by 4 days, most values returned to control levels suggesting return to metabolic homeostasis.

EHS, was accompanied by significant changes in cellular energetics. The mice were initially hypoglycemic in the immediate post-EHS period, but appeared to compensate for this by increasing their rates of lipid oxidation and by utilizing amino acids as fuel sources. In parallel with these changes,

the mice also exhibited changes in a number of other catabolic processes (*e.g.,* the urea cycle and nucleotide degradation pathways). Markers of oxidative stress were also altered as was the stress hormone corticosterone (confirming results in fig. 6) and select inflammation-related metabolites. These latter responses, notably, are likely reflective of the cellular damage and/or organ dysfunction that accompanies EHS. Moreover, these responses appeared to be well conserved across the two genders. While some features persisted over several days, the majority of changes appeared to resolve at or before the 4 day recovery time point. Collectively, these findings provide a platform for further investigating how organisms respond to and recover from EHS."

One extremely interesting finding is illustrated in Figure 11. It is the only metabolic finding that separates EHS mice at the 14 day time-period from controls. In examining the heart of male mice

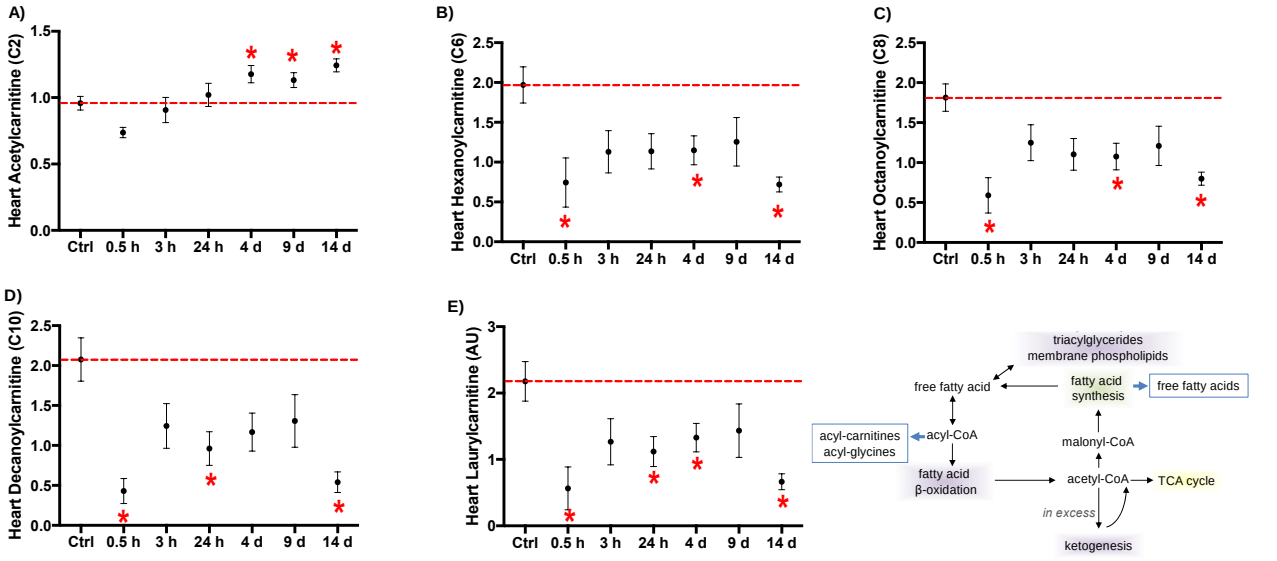

Figure. 11. Interesting metabolomics results showing a sustained elevation in β oxidation flux in the ventricles of mice that is sustained for over 15 days. The graphs on the left are the relative means and SDs of some representative acycarnitine and acetylcarnitine. The pathway analysis on the bottom right shows the specific elements affected over time. Laitano et al. *Manuscript in preparation.*

throughout recovery and at 14 days, they persisted in having a pronounced and significant change in the way they handled fatty acid oxidation in the ventricle. What you see graphically on the left upper panel is a gradually increasing heart acylcarnitine that is significantly elevated from 4-14 days above control. However, nearly every intermediate of β-oxidation product (B,C,D,E acylcarnitines) is significantly reduced compared to controls through 14 days. This means that the heart has undergone a shift in mitochondrial utilization of fatty acids such that this metabolic pathway is amplified and undergoing a large flux of carbon units. We feel this is the most significant finding of all of the metabolomics data we are working with. *It tells us that a single bout of EHS induces "switch" in energy metabolism towards more β-oxidation that lasts at least 14 days.* Is this maladaptive or adaptive? That is something we

hope to discover in future experiments. We also believe we can link epigenetic responses discussed below to these findings.

Results of Ibuprofen Effects on the GI tract during EHS.

We have completed the first round of the ibuprofen (IBU) studies in both males and females. The hypothesis was that IBU administration would make the mice more susceptible to heat stroke and they would have a greater amount of organ injury, particularly to the GI tract. Our data is analyzed for both males and females which were not significantly different in overall responses. Surprisingly, overall, mice ran longer in the final phase of the EHS trial when treated with IBU (P = 0.04), Fig. 12 A. They also had significantly higher core temperatures at Tc,max (i.e. the end point of EHS trial) , shown indirectly in Fig 12 B. Therefore under IBU, mice appeared to be somewhat more heat tolerant during exercise, a surprising finding.

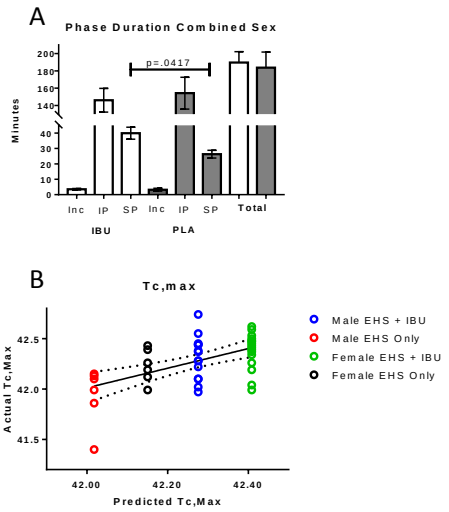

Fig. 12. Effects off 48 hours of ibuprofen administration on A) the exercise time in response to EHS. Note the elevation in the steady State phase (SP) of the EHS trial. B) Actual Tc,max in treated IBU-EHS and untreated EHS in males and females. Overall, in IBU treated animals there was a significantly higher Tc, max (Garcia et al. In preparation).

In IBU treated mice, the way we administered the IBU, there was little or no evidence of intestinal using a biomarkers for injury (FABP2, i.e. fatty acid binding protein 2) in the blood (Fig 13, B & C). However, with EHS, there was a strong elevation in FABP2. There was a greater elevation in FABP2 after EHS in the females vs. the males. We hypothesize that this was due to the much longer running times in the heat in the female population and therefore the greater heat load.

IBU in combination with EHS did appeared to accentuate the FABP2 responses in the female population compared to EHS alone, but we were not able to demonstrate a statistically significant interactive effect.

There is also very little effect of IBU treatment on the histological responses at rest or after EHS, as shown in Fig. 14.

We conclude the IBU's effect on intestinal damage in EHS is relatively mild if present at all, but that the IBU may have improved performance in the heat. These observations are strikingly different than we hypothesized. Current experiments we are proposing during the no cost extension (2018-19) will hopefully allow us to tease this out. We have concerns about the potency of the IBU in the animals over

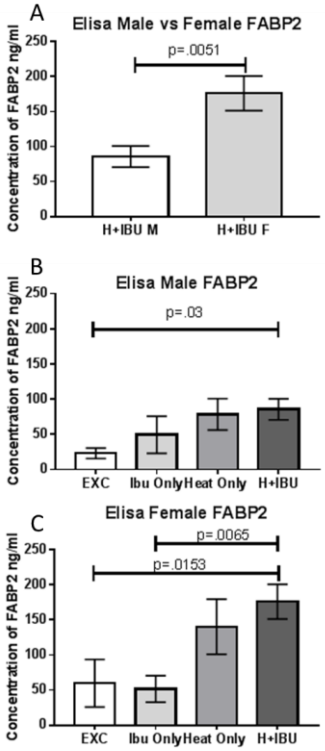

Fig. 13. FAB2 (biomarker for intestinal injury) was significantly increased by EHS and A) was larger in Females vs males. B and C: There were no significant effects of IBU alone in EX control mice, C nor was the response amplified by combined EHS and IBU treatment (Garcia et al. in preparation).

the course of the EHS protocol, which takes ~ 3-5 hours. The half-life of IBU is about 2 hours in the mouse. We are currently testing the anti-pain and anti-inflammatory activities of IBU in this timeframe and as a result may repeat these experiments using a different delivery system.

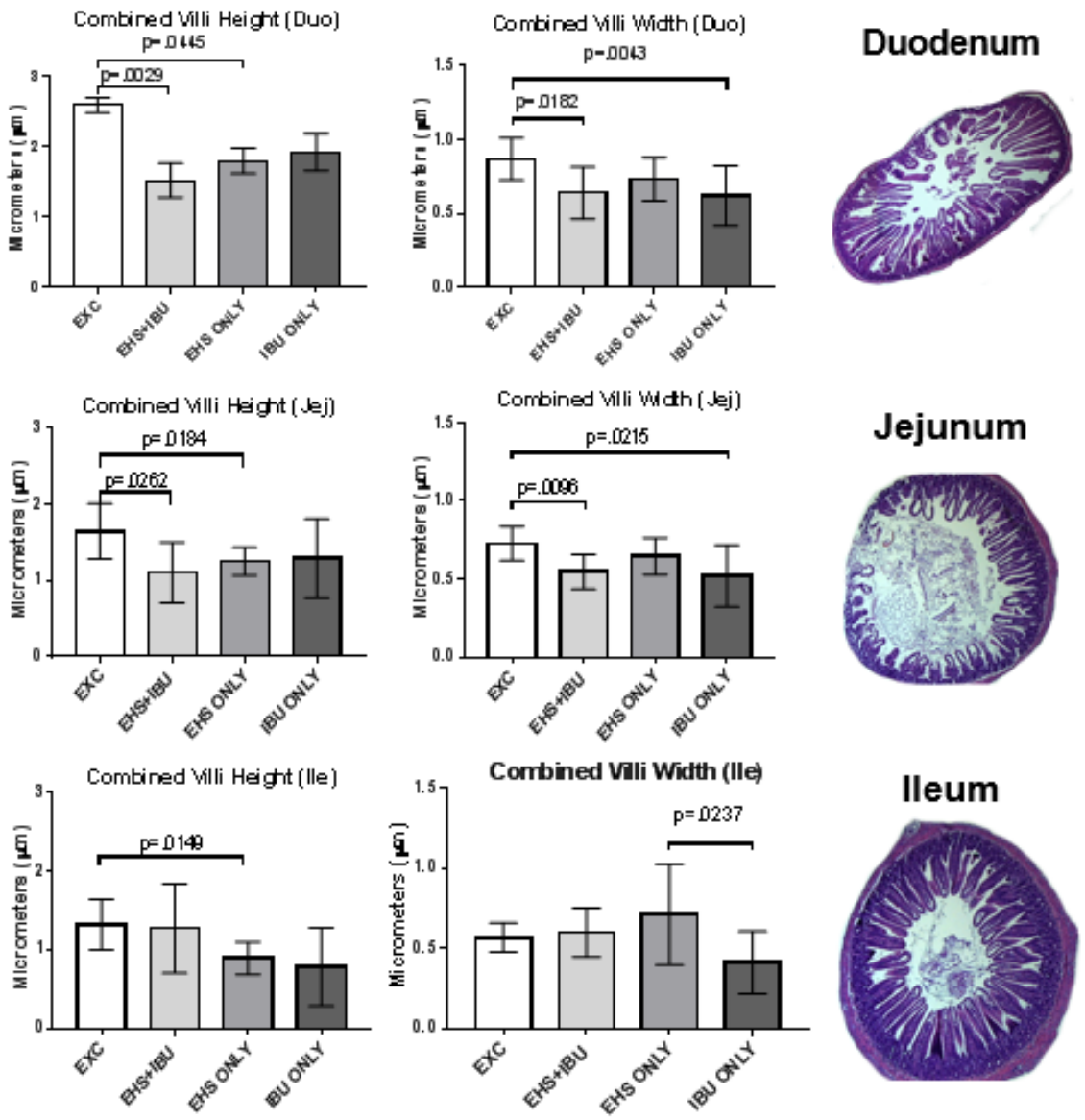

Fig 14. Histological responses to IBU treatment in the three primary regions of the small intestine. At this stage of recovery from heat stress we have previously shown that the primary significant response is a shortening of villi with an accompanying reduction in villi width. This is a process called "villi restitution" which represents the primary mechanism for recovery from injury. Injury was seen throughout the intestine during EHS. there was modest effects of IBU over and above the effects of EHS alone.

Effects of diclofenac and ATB-337 on performance and on intestinal permeability.

Groups of male animals were administered diclofenac, the diclofenac derivative ATB-337, and placebo for a 48 hour period prior to exposure to EHS. Unlike the IBU work discussed above, both diclofenac and ATB-337 had no apparent effect on peak core temperature (Tc,max) or on performance in the heat. However, as shown from Fig. 15, though there were no significant effects of diclofenac alone on any biomarker of organ injury, the HS donor version of diclofenac (ATB-337) had a striking impact on FABP2 in the presence of EHS and appeared to have an effect on liver injury. These data require further substantiation from histological measurements of intestines and livers. We also began in this series to measure the white blood cell differential counts in these animals. As shown in Fig. 16, Diclofenac administration significantly reduced the blood immune cell population in nearly all categories. However, administration of the ATB337 version of diclofenac attenuated this response.

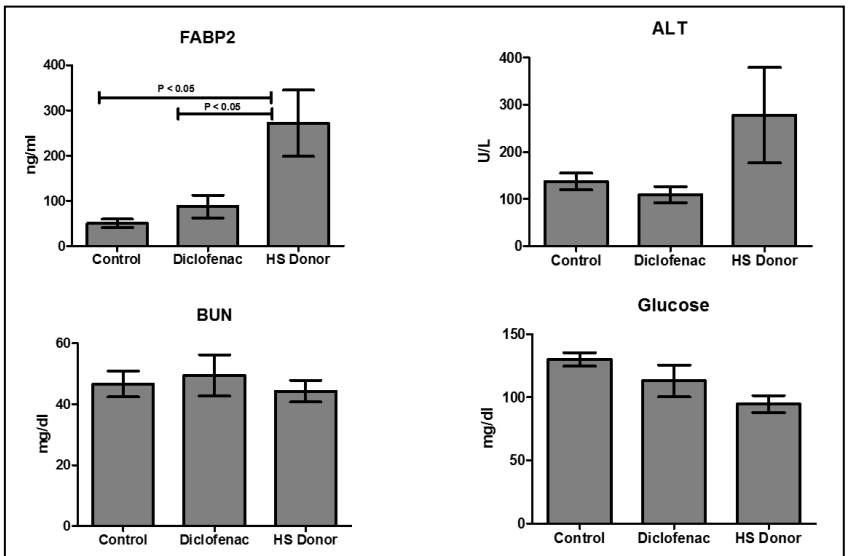

Fig. 15. Effects of diclofenac and ATB-337 (HS donor) on biomarkers of (upper left) intestinal injury, (upper right) liver injury, (lower left) kidney injury, and glucose metabolism

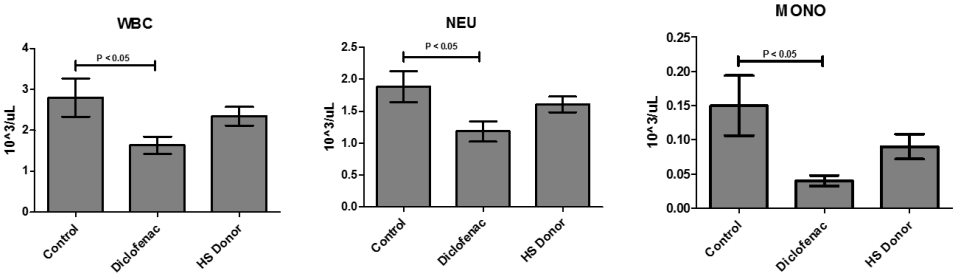

Fig. 16. Effects of diclofenac and ATB-337 treatment on blood white blood cell counts, neutrophil counts and monocyte counts.

Though verification with other assays is needed for this study. The results suggests that the H_2S donor has some unexpected side effects during heat exposure that have not been reported previously. This warrants caution in the translational application of these drugs to a military population.

Establishing reliable molecular biomarkers for the maladaptive responses to EHS

We continue to seek molecular markers that help identify responses to EHS that in individuals more susceptible to subsequent heat stroke and/or lead to long term complications of EHS. We submitted nuclear DNA for 4 EHS animals at 4 days and 30 days after heat stroke from both bone marrow derived monocytes and skeletal muscle and compared against sham exercise controls at each recovery time point. Extremely impressive differences in DNA methylation patterns were identified in the EHS

exposed animals. Much of the analysis of this work was done by the bioinformatics group at USACHER led by Rasha Hammamieh. Thousands of genes were either hyper- and hypo-methylated compared to controls (Fig. 15). Some genes of interest that were altered were involved with Ca^{+2} regulation, heat shock proteins, metabolic enzymes, and cytokine regulation. Secondary pathway analyses were performed by Rasha Hammamieh and colleagues at USACEHR and they have delivered those results to us. The results in skeletal muscle are even more striking and many of the specific methylation targets are reproduced in both skeletal muscle and monocyte. We are currently repeating these measurements at 30 days and coupling them to phenotype measurements consistent with heat stroke susceptibility.

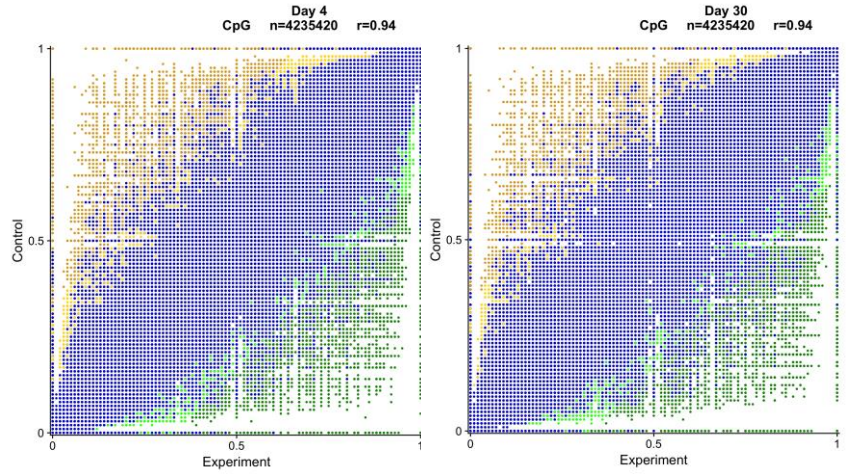

Fig. 15. Summary of statistically significant genes that were hypermethylated or hypomethylated , (LEFT) 4 days and (RIGHT) 30 days after EHS compared to matched exercise control mice at the same time points. The green data are significantly hypermethylated and the amber are significantly hypomethylated compared to controls.

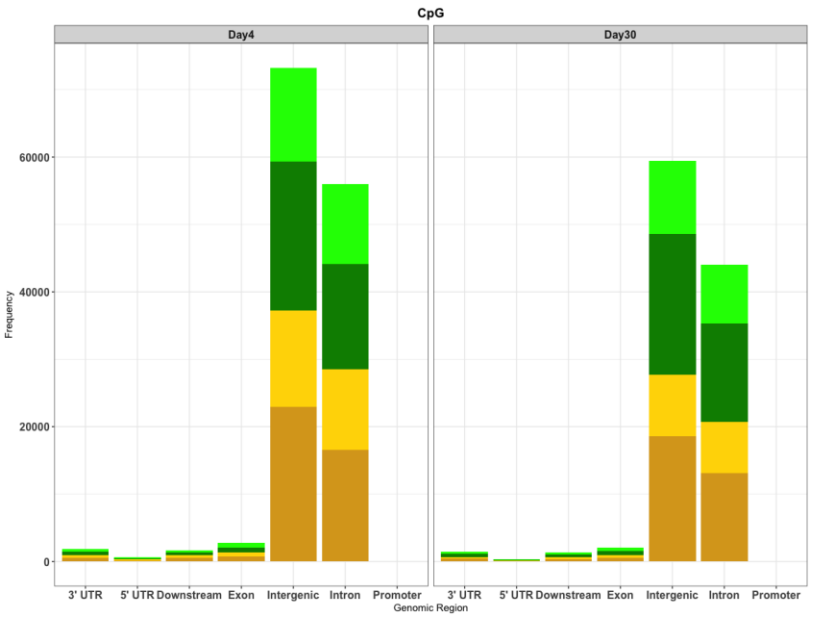

Fig. 16. The genomic topography of DNA methylation changes post EHS compared to matched controls at 4 days (Left) and 30 days (right). Both the 3' and 5'UTR responsible for transcriptional and translational regulation have significant changes. It is important to note that though many of the methylation changes occur in noncoding regions of the genome, only 1-1.5% of the total genome codes for actual protein (i.e. exons).

As shown in Fig. 17, specific targets of interest show a great deal of methylation changes in categories of genes involved with Calcium signaling (RYR1,RYR2, Cacna1, Cacna2, Pde4d, NOS1 and NOS4) and in immunological responsiveness (TLR 2,4,5) suggest that responses involving calcium signaling or immune responses are affected by long term methylation induced by EHS exposure. To test whether these responses change phenotype we have performed experiments on Ca^{+2} sensitivity to caffeine in isolated skeletal muscles and found a significant hypersensitivity to caffeine in muscle from animals previously (30 d) exposed to EHS (data not shown). We have also determined whether previous exposure to EHS results in an increased sensitivity to a second exposure to EHS. Indeed animals exposed to EHS after two weeks

show a marked reduction in the max temperature that they reach during EHS ($P < 0.05$) and significant reductions in measures of strength ($P < 0.05$), (data not shown)

We are now pursuing additional experiments to verify that the changes in epigenetic/DNA methylation profiles result in changes in mRNA and protein expression of particular proteins of interest.

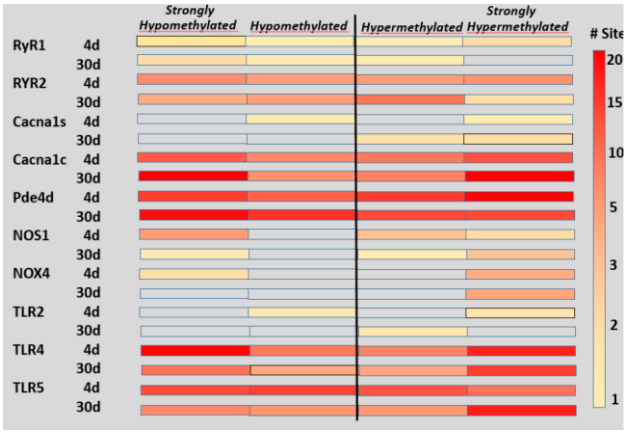

Fig 16. Targeted Hyper and hypomethylated genes of interest with multiple methylation sites (heat map on the right.

These experiments and results were used to apply for a new line of DOD funding, submitted in spring of 2018 entitled: Epigenetic markers for susceptibility and recovery from exertional heat stroke.

What opportunities for training and professional development did the project provide?

Because of this support, we were able to provide training opportunities for Alex Mattingly MS, who was supported for part of the year on the project. We were also able to use this support to employ two MS students in our Department, Christian Garcia and Gerard Robinson who have now converted to a full time Ph.D. program in spring of 2017. Both students are minority students. All three students have first author abstracts for the Experimental Biology Meeting in Chicago of 2017. Finally, we provided training for our postdoc, Dr. Orlando Laitano. He remains only been partially funded by this project but has not only provided the senior guidance in the lab but also developed a new line of research (funded by our endowment) which is looking at the molecular sources of rhabdomyolysis in heat, with and without coexisting hypertonic stress (relevant to heat stress and heat injury in the US Military). This is related to this study but not supported by this project. He is currently first authoring the metabolomics work for this project and has completed his draft of the paper while we await further experiments. Through our collaborations with USARIEM, some junior scientists on the team have played critical roles, most notably Michelle King and who will receive benefits from the interaction and the manuscripts that will emerge from this work in coming months.

How were the results disseminated to communities of interest?

- The P.I. presented the preliminary findings of these studies at Ft Detrick on October 19-20 in 2016 at the Extreme Environments Research in Progress Review.

- Two abstracts have been presented at the 2017 Experimental Biology Meetings in Chicago.

  Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton "**Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice." FASEB J.**

Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton "**Differences in tolerance to exertional hyperthermia between male and female mice" FASEB J**

- In January a new manuscript was published based on the preliminary work for this project with collaboration between USAIREM and UF. King, MA, Leon, LR, Morse, DA, Clanton, TL. "**Unique cytokine and chemokine responses to exertional heat stroke in mice."** J Appl Physiol, 1:122(2) 296-306, 2017

- In August of 2017 two abstracts were presented at the MHSRS Symposium in Orlando, Fl.

  Thomas L. Clanton, Michelle Kin, and Lisa Leon. **The intestinal epithelium is vulnerable to heat, exercise and NSAIDs.** MHSRS Symposium Orlando FL

  Orlando Laitano, Brian Ingram, Christian Garcia, Gerard Robinson, Alex Mattingly, Danielle Ippolito, Lisa Leon, Thomas L Clanton **Single exposure to exertional heat stroke results in a sustained metabolic switch to lipid oxidation in heart ventricular muscle of male mice.** MHSRS Symposium Orlando FL

- In April, 2018 we attended Experimental Biology and presented the following abstracts:

  Orlando Laitano, Christian K. Garcia, Brian Ingram, Gerard P. Robinson, Alex J. Mattingly, Danielle L. Ippolito, Lisa R. Leon, and Thomas L. Clanton **Heart Metabolic Responses to Exertional Heat Stroke Are Dependent Upon Sex** FASEB J Volume 32, Issue 1_supplement01 Apr 2018 NOTE: Won the Environmental and Exercise Physiology Award (Military Award)

  Christian Kyle Garcia, Jamal Alzahrani, Alex Mattingly, Orlando Laitano, Gerard Robinson, Kevin Murray, and Thomas Clanton **Ibuprofen increases resistance to exertional heat stroke in female mice** FASEB J Volume 32, Issue 1_supplement01 Apr 2018

  Kevin Murray, Orlando Laitano, Laila Sheikh, John Iwaniec, Gerard Robinson, Christian Garcia, Jamal Alzahrani, Rasha Hammamieh, Ross Campbell, Ruoting Yang, and Thomas Clanton **Epigenetic responses to exertional heat stroke in mice: a potential link to long term Ca2+ dysregulation in skeletal muscle** FASEBJ Volume 32, Issue 1_supplement01 Apr 2018

- In May we attended the American College of Sports Medicine meeting in San Diego and presented the following abstracts:

  Christian Kyle Garcia, Alex Mattingly, Gerard Patrick Robinson, Orlando Laitano, and Thomas Clanton **"Physical factors related to heat exchange in male and female mice during exertional heat stroke" ACSM May 29, 2018 Oral Presentation**

Orlando Laitano, Brian Ingram, Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Danielle L. Ippolito, Lisa R.Leon, Thomas L. Clanton. **Sustained metabolic switch to lipid oxidation In murine cardiac muscle after exertional heat stroke** FASEBJ Volume 32, Issue 1_supplement 01 Apr 2018 Oral Presentation

- In June, 2018we published a manuscript:

  Garcia CK, Mattingly AJ, Robinson GP, Laitano O, King MA, Dineen SM, Leon LR, Clanton TL. **"Sex-dependent responses to exertional heat stroke in mice."** J Appl Physiol (1985). 2018 Sep 1;125(3):841-849. doi: 10.1152/japplphysiol.00220.2018. Epub 2018 Jun 14. PubMed PMID: 29901435.

- In August of 2018 we presented abstracts at the MHSRS meeting in Orlando.

  Clanton, TL, Murray, K, Laitano, O. "**DNA methylation as a historical epigenetic record of environmental exposure: applications to exertional heat stroke" Oral presentation**

  Orlando Laitano, Laila H. Sheikh, Alex J. Mattingly, Kevin O. Murray, Leonardo F. Ferreira, and Thomas L. Clanton **Hyperthermia and extracellular hyperosmolality affect resting isometric tension, sarcolemma damage and protein oxidation in mammalian skeletal muscle in vitro**

- In Sept 2018 we presented one abstract at the APS/ACSM meeting on the Integrative Physiology of Exercise

  Kevin O. Murray, Orlando Laitano, Laila Sheikh, John Iwaniec, Gerard P. Robinson, Christian K. Garcia, Jamal Alzahrani, Rasha Hammamieh, Ross Campbell, Ruoting Yang, Thomas L. Clanton. **"DNA methylome in monocytes for up to 30 days of exertional heat stroke in mice"**

What do you plan to do during the next reporting period to accomplish the goals and objectives:

We are currently beginning the ATB-346 experiments and submitting new protocols to ACURO and IACCUC to study a simple H_2S donor that is not a derivative of a NSAID. We are also completing experiments to verify that the dose of NSAIDs that we have given is still effective at the time the animals are exposed to EHS. These latter experiments are necessary before publication.

We are continuing to develop the preliminary data for molecular biomarkers, most notably the epigenetic biomarkers. We have since submitted a series of muscle samples for RNAseq analysis to back up the DNA methylation studies. We plan on submitting this manuscript within two month of this report.

We are awaiting analysis of female metabolomics of the heart in additional groups of animals. A draft of the manuscript for the metabolomics data has been completed but we wish to make a more powerful and compelling paper by adding this additional information.

We have completed all of the experiments for the ibuprofen study and are now writing up the manuscripts for submission.

Our biggest challenge is to complete the manuscripts of the studies we have experimentally finished. It is a slow process as most of these are being written by graduate students who have little previous experience in writing. We will get it done, however.

4. IMPACT

Impact on the Field. This model has become extremely refined and predictable and we believe it will stand the test of time as the first go-to model for preclinical studies in EHS research. We continue to be surprised by new findings that are not expected from other models such as passive heat stroke. The NSAID studies provide some insights we did not expect, particularly the ability of mice to withstand a greater exertional heat load. We feel this could impact the way the Armed Services approaches guidelines for NSAID usage. We also feel work done on the H2S-containing NSAID during EHS may encourage military medicine to proceed to utilization of some of these drugs which are now finishing Phase II clinical trials.

Impact on other Disciplines: We have confidence that biomarkers we can identify may be applicable across other fields, particularly with respect to studies underway on epigenetic markers. We also are of the opinion that our work identifies a unique "stress induced immune response" which can be separated from classic innate immunity. This may ultimately impact the field of immunology.

Impact on technology transfer: Nothing to report

Impact on Society beyond science and technology: It is possible that our work will impact the evaluation and treatment of exertional heat stroke patients. However, at this time, it is premature to predict how this will be manifest itself. We have found the differences between male and female mice, from a metabolic and hormonal aspect to be remarkable. These striking differences may help to understand health related questions between the sexes that are not related to EHS but involve the same integrative physiological systems.

For impact on the Military, please read the summary statement at the beginning of this document under the heading: ***What was accomplished under these goals.***

5. CHANGES OR PROBLEMS

Our expenditures are in line with expectations and we should be able to complete the studies over the next year without interruption. We currently have ~$60K in direct costs available to complete the remaining objectives.

We are making some changes and will be adding additional animals to firm up our conclusions. The primary change is the timing and administration of the NSAIDs which we may give much sooner before starting the EHS protocol. We are currently testing the effectiveness of the way we gave the oral dose.

We wish to add some additional experiments to further explore the male and female differences using castration and ovariectomy to diminish the impact of sex hormones on the responses. These experiments are already approved at our IACUC and we will submit to ACURO to pursue these further.

We wish to further pursue the effects of a single EHS exposure on susceptibility to EHS, later in time. This protocol has been submitted to our IACUC and will be submitted to ACURO shortly.

Overall, there have been few problems with completing this work. We continue to develop new questions which is driving us to dig deeper into these problems.

. **6. PRODUCTS:**

- Abstracts:

  Gerard P. Robinson, Michelle A. King, Alex J. Mattingly, Christian K. Garcia, Orlando Laitano, David Van Steenbergen, Lisa R. Leon, Thomas L. Clanton "**Major Metabolic Hormone Responses to Exertional Heat Stroke in Mice." FASEB J. 31(1) suppl 1085.a**

  Christian K. Garcia, Gerard P. Robinson, Alex J. Mattingly, Orlando Laitano, David Van Steenbergen, Michelle A. King, Lisa R. Leon, Thomas L. Clanton "**Differences in tolerance to exertional hyperthermia between male and female mice" FASEB J 31(1) suppl 1018.10**

  Thomas L. Clanton, Michelle Kin, and Lisa Leon. **The intestinal epithelium is vulnerable to heat, exercise and NSAIDs.** MHSRS Symposium Orlando FL

  Orlando Laitano, Brian Ingram, Christian Garcia, Gerard Robinson, Alex Mattingly, Danielle Ippolito, Lisa Leon, Thomas L Clanton **Single exposure to exertional heat stroke results in a sustained metabolic switch to lipid oxidation in heart ventricular muscle of male mice.** MHSRS Symposium Orlando FL

  Orlando Laitano, Christian K. Garcia, Brian Ingram, Gerard P. Robinson, Alex J. Mattingly, Danielle L. Ippolito, Lisa R. Leon, and Thomas L. Clanton **Heart Metabolic Responses to Exertional Heat Stroke Are Dependent Upon Sex** FASEB J Volume 32, Issue 1_supplement01 Apr 2018 NOTE: Won the Environmental and Exercise Physiology Award (Military Award)

  Christian Kyle Garcia, Jamal Alzahrani, Alex Mattingly, Orlando Laitano, Gerard Robinson, Kevin Murray, and Thomas Clanton **Ibuprofen increases resistance to exertional heat stroke in female mice** FASEB J Volume 32, Issue 1_supplement01 Apr 2018

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Christian Kyle Garcia, Alex Mattingly, Gerard Patrick Robinson, Orlando Laitano, and Thomas Clanton **"Physical factors related to heat exchange in male and female mice during exertional heat stroke" ACSM May 29, 2018 Oral Presentation**

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Clanton, TL, Murray, K, Laitano, O. "**DNA methylation as a historical epigenetic record of environmental exposure: applications to exertional heat stroke" Oral presentation MHSRS 2018, Orlando Florida**

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- Manuscripts

King, MA, Leon, LR, Morse, DA, Clanton, TL. **Unique cytokine and chemokine responses to exertional heat stroke in mice.** J Appl Physiol, 1:122(2) 296-306, 2017

Garcia CK, Mattingly AJ, Robinson GP, Laitano O, King MA, Dineen SM, Leon LR, Clanton TL. **"Sex-dependent responses to exertional heat stroke in mice."** J Appl Physiol (1985). 2018 Sep 1;125(3):841-849.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project.

Personnel at UF.

Name: Thomas Clanton Ph.D.
Project Role: P.I.
Researcher Identifier Orchid: 0000-0003-0600-7150
Nearest person-month worked 3.5 Person Months
Contribution to project : All aspects of the project.
Funding support: Univ of Florida

Name: Orlando Laitano, Ph.D.
Project Role: Postdoctoral fellow
Researcher Identifier
Nearest person-month worked: 2 Person Months
Contribution: Data collection, planning design of experiments, directing other lab personnel.
Funding support: Rest of support from the National Institutes of Health

Name: Alex Mattingly, MS
Project Role Senior Graduate Student/Research Assistant
Researcher Identifier
Nearest person-month worked 3 Person Months
Contribution: Oversees surgeries, data collection and managing activities and training of other personnel.
Funding support: Univ of Florida Research Assistantship.

Name: Christian Garcia
Project Role: Graduate student research assistant
Researcher Identifier
Nearest person-month worked 8 Person Months
Contribution: Ran most of the training and EHS experiments, collected specimens, animal care, histology
Funding support: Entirely from this award.

Name: Gerard Robinson
Project Role: Graduate student research assistant
Researcher Identifier
Nearest person-month worked: 6 Person Months
Contribution: Ran training and EHS experiments, collected specimens, animal care, histology
Funding support: Entirely from this award.

Name: John Iwanaiec
Project role: Masters student paid in summer to monitor animals in the lab.
Nearest person-month worked: 1.5 person months.

Name: Kevin Murray
Project role: Ph.D. student paid in summer to run the epigenetics experiments
Nearest person-month worked: 2.4 months

Name: Laila Sheikh
Project role: Undergraduate student worked in summer to monitor animals and collect data.
Nearest person months: 2 months.

***Has there been a change in the active other support of the PI since the last reporting period*.**

None:

What other organizations were involved as partners:

Organization Name: USARIEM (Lisa Leon, primary contact, Michelle King)
Location of Organization: Natick MA.
Contribution to the Project:

Financial Support: USARIEM receives separate financial support for their part of the project. I have never received a report of the amounts distributed for this purpose.
The role of USARIEM is to evaluate samples for metabolic hormones and cytokine expression. The also collaborate and plan experiments, help with writing manuscripts and data analysis.

Organization Name: USACEHR (Danielle Ippolito, primary contact, currently left the program. Our current contact is Valerie T. Divito)
Location of the Organization: Frederick MD
Contribution to the Projects:

Financial Support: USARIEM receives separate financial support for their part of the project. I have never received a report of the amounts distributed for this purpose.
UASCEHR helps evaluate samples for metabolomic, lipidomic and proteomic markers. They help with bioinformatics and interpretation of results and writing of manuscripts.

APPENDIX MATERIAL

1. References
2. Quad Chart for 4th Quarter 2017-2018
3. Publication King et al. 2017, Garcia et al, 2018

APPENDIX 1: References:

1. **Armed Forces Health Surveillance B**. Update: Heat injuries, active component, U.S. Army, Navy, Air Force, and Marine Corps, 2015. *MSMR* 23: 16-19, 2016.
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Prevention of Organ Injury in Exertional Heat Stroke: Preclinical evaluation of a new class of NSAIDs

Log Number: #14267001 FY 18

W81XWH-15-2-0038 BAA Extramural Medical Research

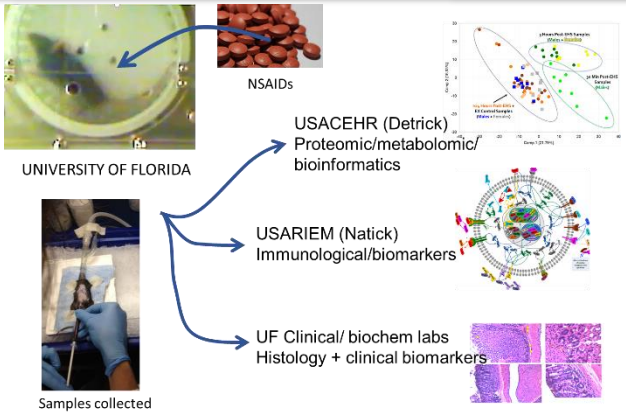
PI: Thomas L Clanton **Org: University of Florida** **Partners: USARIEM, USACEHR** **Total Award Amount: $265 (3rd yr)**

Study/Product Aim(s)

- to define the time course of multi-organ injury, repair and recovery of metabolic control in exertional heat stroke (EHS)
- to determine sex differences in susceptibility to EHS in mice
- to identify metabolomic and proteomic biomarkers that define underlying disorder in EHS
- to test the impact of commonly used NSAIDs on susceptibility to organ injury in EHS
- to test the effectiveness of new H_2S-containing NSAIDs on reducing intestine and organ damage in EHS

Approach

Instrumented and exercise-trained mice (♂ &♀) run on a running wheel within an incubator (37.5°C) until symptom limited (neurological). Samples of blood and various organ systems are taken at intervals up to 14 days and prepared for proteomic, metabolomic and genomic analysis. In upcoming experiments, the animals will be given different varieties of NSAID to determine susceptibility to organ injury.



Accomplishments: Completed EHS studies on 112 (♂ &♀) mice. Completed the initial metabolomics and lipidomics analyses in collaboration with USACEHR and USARIEM. Completed physiological analyses of the response to heat in ♂ &♀ . Completed first series of ♂ &♀ mice exposed to ibuprofen and EHS. Completed experimental work on ATB-337. Paper accepted to Journal of Applied Physiology. Three other manuscripts at various levels of completion

Timeline and Cost

Activities CY 19	15	16	17	18-
Collection of tissues from EHS studies in male and female mice				
Proteomic/metabolomic/and immunological analysis of samples				
Test effects of common NSAIDs on organ injury in EHS				
Effects of new generations of H2S containing NSAIDs in EHS				
Estimated Budget ($K)		**$325K**	**$265K**	**$268K**

Updated: 10/29/2018

Goals/Milestones

CY15 Goal – ☑ purchase equipment, train personnel begin EHS

CY16 Goals – ☑ Complete male & Female EHS and control experiments☑ male/female samples to USACEHR for metabolomics

CY17 Goal –☑ Begin studies of effects of predominant NSAIDs on organ injury. ☑ Completed metabolomics and lipidomics analyses. ☑ Writing manuscripts on male-female difference and metabolomics responses to EHS.

CY18-19 Goal –☑ Complete NSAID-H_2S studies, expanded ibuprofen studies. and analyze and write up data □ Begin studies of NSAID-H_2S drug studies. Complete manuscript preparation and submission

Comments/Challenges/Issues/Concerns

- Experimental model working well, there are no major problems.
- Have completed data collection for years 1 and 2 and most of yr 3

Budget Expenditure to Date

Projected Expenditure $545,155 (by end of year 3)

Actual Expenditure: $496,043. (09/18/2018) **Remaining balance = $59,100**

FY3 (direct) Currently in NO COST EXTENSION FOR 12 months

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RESEARCH ARTICLE

Unique cytokine and chemokine responses to exertional heat stroke in mice

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King MA, Leon LR, Morse DA, Clanton TL. Unique cytokine and chemokine responses to exertional heat stroke in mice. *J Appl Physiol* 122: 296–306, 2017. First published December 1, 2016; doi:10.1152/japplphysiol.00667.2016.—In heat stroke, cytokines are believed to play important roles in multiorgan dysfunction and recovery of damaged tissue. The time course of the cytokine response is well defined in passive heat stroke (PHS), but little is known about exertional heat stroke (EHS). In this study we used a recently developed mouse EHS model to measure the responses of circulating cytokines/chemokines and cytokine gene expression in muscle. A very rapid increase in circulating IL-6 was observed at maximum core temperature ($T_{c,max}$) that peaked at 0.5 h of recovery and disappeared by 3 h. IL-10 was not elevated at any time. This contrasts with PHS where both IL-6 and IL-10 peak at 3 h of recovery. Keratinocyte chemoattractant (KC), granulocyte-colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-2, MIP-1β, and monocyte chemoattractive factor-1 also demonstrated near peak responses at 0.5 h. Only G-CSF and KC remained elevated at 3 h. Muscle mRNA for innate immune cytokines (IL-6, IL-10, IL-1β, but not TNF-α) were greatly increased in diaphragm and soleus compared with similar measurements in PHS. We hypothesized that these altered cytokine responses in EHS may be due to a lower $T_{c,max}$ achieved in EHS or a lower overall heat load. However, when these variables were controlled for, they could not account for the differences between EHS and PHS. We conclude that moderate exercise, superimposed on heat exposure, alters the pattern of circulating cytokine and chemokine production and muscle cytokine expression in EHS. This response may comprise an endocrine reflex to exercise in heat that initiates survival pathways and early onset tissue repair mechanisms.

NEW & NOTEWORTHY Immune modulators called cytokines are released following extreme hyperthermia leading to heat stroke. It is not known whether exercise in hyperthermia, leading to EHS, influences this response. Using a mouse model of EHS, we discovered a rapid accumulation of interleukin-6 and other cytokines involved in immune cell trafficking. This response may comprise a protective mechanism for early induction of cell survival and tissue repair pathways needed for recovery from thermal injury.

interleukin-6; CXCL1; granulocyte-colony-stimulating factor; exercise; hyperthermia

EXERTIONAL HEAT STROKE (EHS) is a life-threatening condition where the body is no longer able to dissipate the heat load produced during physical exertion. This can lead to extreme elevations in core temperature (T_c), central nervous system dysfunction, and subsequent multiorgan damage (7). This condition affects seemingly healthy individuals, such as military personnel, occupational workers, and athletes, making this illness even more enigmatic. While EHS is distinct from passive heat stroke (PHS) (35), the etiologies of both conditions are still poorly understood, and although multiorgan dysfunction is common in both (35, 38, 39, 53), the extent to which they share underlying mechanisms is not known. Despite efforts to prevent multiorgan damage via rapid cooling, many individuals still succumb to multiorgan failure. Furthermore, for those individuals who survive the initial heat injury, 40% are more likely to die earlier in life than their matched counterparts (62). To develop clinical interventions and prevent long-term organ damage, it is important to understand the underlying causes responsible for multiorgan injury.

The multiorgan dysfunction that occurs as a consequence of heat stress has been suggested to be the result of excessive inflammatory processes, where cytokines serve as important mediators (38, 56). The local response to tissue damage involves the production of cytokines at the injury site, which, with the help of chemokines, function in attracting lymphocytes, neutrophils, and monocytes to aide in the healing process (69). PHS models, as well as hyperthermia itself, display an acute rise in cytokines with dominant elevations in interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-1β (IL-1β) and a lesser rise in tumor necrosis factor-α (TNF-α) (12, 30, 39). Importantly, the circulating cytokine pattern following PHS is unique from that seen following exposure to endotoxin or acute exercise (39, 49, 64, 67). However, the circulating cytokine pattern following EHS has yet to be determined.

One of the distinct differences between PHS and EHS is the role of the exercising muscle. Exercising muscle is not only the main contributor to increases in T_c during physical activity but also has the ability to act as an endocrine organ, contributing cytokines, particularly IL-6, to the circulation (49, 58). Furthermore, skeletal muscle has been shown to be responsive to heat stress following PHS (64). However, the role of the skeletal muscle in contributing to the circulating cytokine profile is not known in EHS.

To understand the acute cytokine responses to EHS, our objective was to determine the pattern of cytokines and chemokines expressed in the circulation and the expression of select cytokines in skeletal muscle throughout the course of EHS and recovery. Because there may be a cumulative effect of hyperthermia, exercise, and other potential factors such as endotoxemia or release of catecholamines, we hypothesized that the stress-induced cytokine response to EHS would be greater in magnitude but follow a similar time course as that observed in

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PHS. We predicted that the additional stress of exercise would exacerbate the associated cytokine and chemokine profile.

METHODS

Animal care. All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee. 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. Ninety-five mice were used for data collection in this study. A subset of these mice had been used previously to determine multiorgan dysfunction in EHS (35). All were C57BL/6J males (Jackson Laboratories, Bar Harbor, ME) weighing an average of 29.1 ± 3 (SD) g, approximately 4 mo of age. Mice were housed in groups until they were implanted with telemetry devices, after which they were individually housed in 7.25 in. wide × 11.75 in. deep × 5 in. high cages lined with Harlan corn cobb bedding and maintained on a 12:12-h light-dark cycle at 20–22°C/30–60% relative humidity (RH). A standard chow diet (LM-485m Envigo; Teklad, Madison, WI) and water were provided ad libitum until the EHS protocol. Experiments were performed in the morning of the light cycle (~0700–1000).

Animal preparation and training. As described previously (35), under isoflurane anesthesia, mice were implanted with temperature telemetry transmitters (TA-E-Mitter; Starr Life Sciences, Oakmont, PA) for monitoring T_c. The mice were allowed to recover with subcutaneous buprenorphine injections every 12 h for 48 h and then recovered undisturbed for >2 wk. Following this recovery period, exercise wheels and enrichment huts (Silent Spinner and Small Animal Igloo Hideaways; PETCO, San Diego, CA) were introduced in the cages for 3 wk. During this period, mice had ad libitum access to the running wheel throughout the day and night. On the 3rd wk, additional exercise training/acclimation 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 forced running wheel system (model 80840; Lafayette Instrument, Lafayette, IN). The first exercise session in the chamber consisted of 15 min of freewheeling, where the mouse was free to run and explore their surroundings. This was followed by a short recovery period (<5 min). Next, mice were started at an initial speed of 2.5 m/min and then 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 for 60 min. At the fourth and final session, the same protocol was used, but exercise time and incremental speed were elevated until the animals exhibited fatigue. Fatigue was defined as refusal to run or walk on the wheel for >5 s. No shock or any other manual stimuli were used to maintain running speed.

EHS. Following the last training session, mice were given 2 days of rest with free access to the running wheel in their cages. The evening before or the morning of the EHS test, mice were brought to the laboratory in their own cage. T_c was monitored with a data acquisition system, averaged over 30-s intervals (VitalView; Starr Life Sciences). After at least 2 h of resting data in the environmental chamber, each mouse was monitored until T_c dropped to <37.5°C for >15 min. At this time, the environmental temperature (T_{env}) and RH were increased to 37.5°C, 50% RH; water, food, and the cage lid were removed leaving only the wire rack exposed. This T_{env} was based on previous work where we studied EHS at three different T_{env} (between 37.5 and 39.5) and RH values (35–90%) (35). At this temperature, the animals' exertional heat production had the greatest contribution to overall heat load and therefore had the greatest potential for distinguishing differences from PHS. As soon as the environmental chamber equilibrated to the target T_{env} (~1 h), the chamber was opened, and the animal was quickly placed in the running wheel. The forced running wheel protocol was then initiated. The mouse's behavior was monitored continuously in real time with a video camera. Running speed began at 2.5 m/min and increased 0.3 m/min every 10 min until the mouse reached a T_c of 41°C, which served as threshold beyond which the running speed was kept constant (Fig. 1, *A* and *B*). The end point of the EHS test was "symptom limited," since nearly all mice (≈98%) displayed a sudden loss of consciousness and collapse. However, reaching a T_c of 42.5°C was also considered a humane end point but was a rare occurrence. At the end of the protocol, T_{env} was adjusted back to room temperature, the chamber door was opened, and the mouse was carefully watched until it regained consciousness. At this time, it was weighed and returned to its home cage. T_c continued to be monitored for a 24-h recovery or until death at an earlier time point (described below). The 12-h light-dark cycle was maintained in the environment during the recovery period.

EHS experiments. Five groups of mice were studied (n = 6–9/group) to determine the time course of cytokine expression. Mice were euthanized at 80 min into the protocol (which was set to be ≈0.5 h before $T_{c,max}$) at $T_{c,max}$ and 0.5, 3, and 24 h post-$T_{c,max}$. At each time point, blood and tissue samples were collected. Five other groups of sham controls (EXC) were treated identically without heat exposure, and tissues were sampled at the same times. These mice were exercised at the average time and intensity of the EHS mice (maximum speed: 5.2 m/min, duration: 113 min) but with the environmental chamber maintained at 25°C and 50% RH (35).

For sample collection, the mice were placed under isoflurane anesthesia, and blood samples were obtained by transthoracic cardiac stick. Soleus, gastrocnemius, and diaphragm were removed for later biochemical or histological analyses. Thoracotomy and heart removal were performed under deep anesthesia.

Tissue and blood samples were obtained from another group of naïve control mice (NC) that did not undergo surgery, any exercise training, any specific enrichment, or any exercise or heat interventions (n = 6).

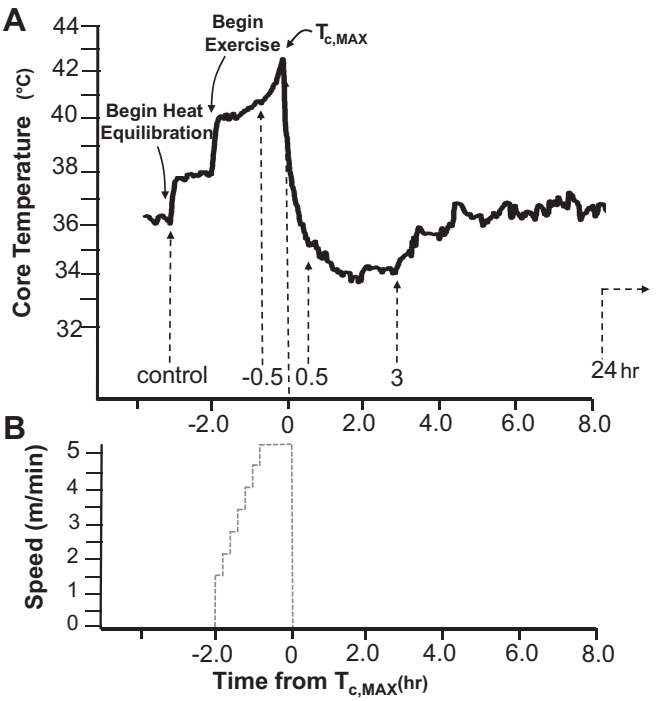

Fig. 1. *A*: typical core temperature (T_c) profile for the exertional heat stroke (EHS) protocol, showing the intervals of blood/tissue collections relative to peak core temp ($T_{c,max}$). *B*: average forced running wheel time course, starting at 2.5 m/min, with 0.3 m/min until 40.5°C and then held at steady-state exercise until $T_{c,max}$.

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PHS experiments. Two more groups of animals were exposed to a PHS protocol. One set ($n = 6$) was exposed to 39.5°C at 30% RH, identical to previous approaches described by Leon and colleagues (40) except that the end point for these PHS mice was T_c of 42.1°C, rather than 42.4–42.7°, which was used for previous studies (40, 64). This end point temperature was used because it was the average $T_{c,max}$ acquired by the EHS mice in this study. This was done to determine if differences in response of EHS could be attributed to the lower peak T_c reached. We only took samples at the 3-h time point in these mice because this corresponds to a time when there is marked cytokine expression in PHS but a time when there is almost no circulating cytokine expression in EHS.

Another set of mice [matched PHS (PHS_m)] ($n = 6$) underwent a passive heating protocol designed to mimic the shortened thermal area (heat load) experienced in EHS groups. Thermal area was calculated as defined by Leon et al. (40), adapted from Hubbard et al. (32). Mathematically this equals approximately the area under the curve of the temperature profile for all points at which T_c was >39.5°C (units = °C·min). To obtain a very similar thermal area in PHS_m, the environmental temperature was elevated to 43.5°C/50% RH, determined by trial and error in a group of test mice. These mice were also studied at the single time point of 3 h post-$T_{c,max}$ for the same reasons identified in PHS mice.

Plasma cytokine measurements. Blood was collected, using heparin as the anticoagulant, and spun at 2,000 relative centrifugal force, and plasma (250 μl) was pulled off the buffy coat, separated into aliquots, and stored at −80°C. Plasma cytokines and chemokines were determined using a Luminex system, employing MILLIPLEX MAP Mouse cytokine/chemokine-premixed 25 plex assay kits, which include the antibodies for the following analytes: granulocyte-colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, interferon-γ-induced protein-10, keratinocyte chemoattractant (KC), monocyte chemoattractive factor-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, regulated on activation, normal T cell expressed and secreted (RANTES), and TNF-α. The test was performed according to the manufacturer's protocols, as described elsewhere (67).

RNA isolation, reverse transcription, and real-time PCR. To determine innate immune cytokine expression in skeletal muscle, the soleus, diaphragm, and gastrocnemius muscles were dissected and flash-frozen at the −0.5 h, $T_{c,max}$, 0.5-, 3-, and 24-h time points. As previously described (67) RNA was separated from DNA by bromochloropropane and precipitation in isopropanol. After a 75% ethanol wash and resuspension in DEPC water, purity of RNA was quantified by spectrophotometry. Total mRNA was reverse transcribed using a Verso cDNA Synthesis Kit. Preformulated Taqman Gene Expression assays were used for IL-1β, IL-6, IL-10, and TNF-α. Relative quantitative real-time reverse transcription-polymerase chain reaction was done using TaqMan Gene Expression Master Mix on a StepOnePlus. Hypoxanthine phosphoribosyltransferase was used as a housekeeping gene based on previous studies in which we observed the gene to be stable in hyperthermic myofibers and tissues (67). Changes in target gene expression were independent of changes in the level of mRNA for hypoxanthine phosphoribosyltransferase. Relative quantitation was calculated using the $\triangle\triangle C_T$ method as described previously (31).

Statistical analyses. Statistical analyses were performed using SAS JMP (Cary, NC) and Graphpad Prism (La Jolla, CA). The large majority of cytokine and mRNA data was nonparametric, and, therefore, Kruskal-Wallis was used for all ANOVAs. Post hoc tests were done with Dunn's multiple-comparison test for nonparametric comparisons. Central tendency and variance of data were expressed as medians ± 25–75% quartiles because of the nonparametric nature of the datasets. To determine the probability of type 1 error due to multiple comparisons, the Benjamini-Hochberg procedure for estimating false discovery rate was applied (6), using a cutoff of 0.15 as an acceptable false discovery rate.

RESULTS

Plasma cytokine and chemokine responses to EHS. We sampled plasma cytokines and chemokines at time intervals denoted on a typical EHS T_c profile in Fig. 1*A*. Cytokines such as IL-1β, IL-6, IL-10, and TNF-α, which are classically involved in the innate immunity, are elevated following heat stroke (10, 11, 39, 64). However, in this model of EHS, only IL-6 was significantly elevated at any time point over the course of EHS, reaching a peak at +0.5 h into recovery (Fig. 2*A*). This response was suppressed by 3 h and remained undetectable at 24 h. Sham exercise controls displayed no significant changes in IL-6 nor any of the cytokines measured in this study, at any time (Fig. 2*B*).

As shown in Fig. 3*A*, plasma chemokines, MCP-1, MIP-1β, and MIP-2 followed a similar trajectory seen for IL-6, where peak concentrations occurred at 0.5 h of recovery, disappearing

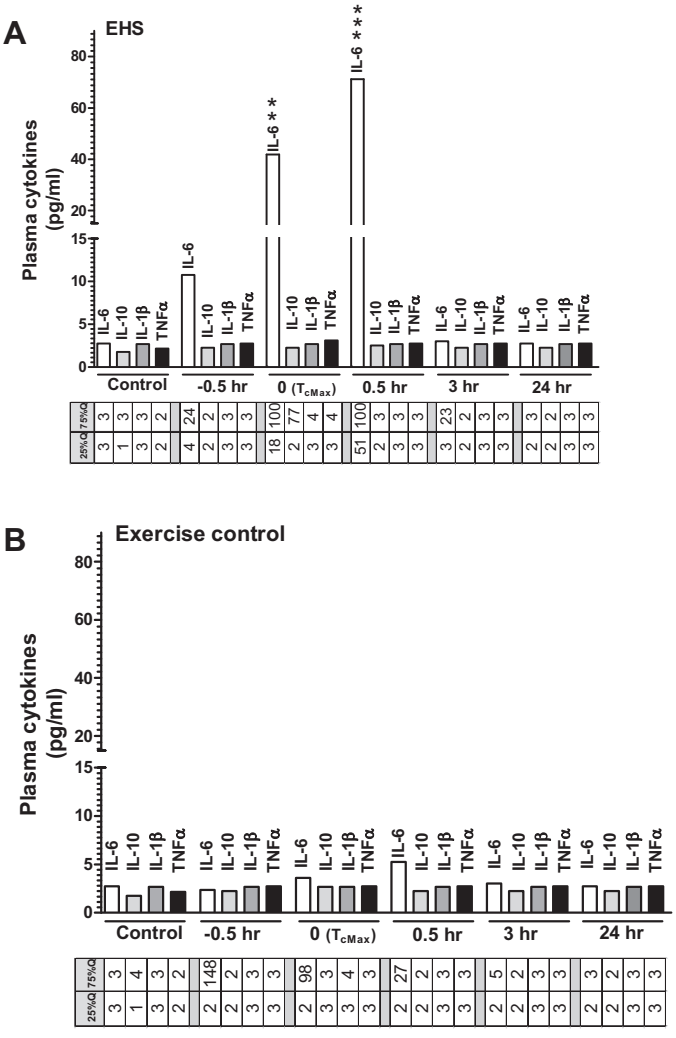

Fig. 2. Effects of EHS on common cytokines of innate immunity. *A*: responses of common innate immune cytokines to EHS. *B*: cytokine responses to sham exercise controls. Significance from naïve control: $P < 0.01$ (**) and 0.001 (**) (post hoc tests). MCP-1, monocyte chemoattractive factor-1; MIP, macrophage inflammatory protein; G-CSF, granulocyte-colony-stimulating factor; KC, keratinocyte chemoattractant. Benjamini-Hochberg procedure for multiple ANOVAs = false discovery rate (FDR) <15%. Bars = medians; tables below = 25–75% quartiles.

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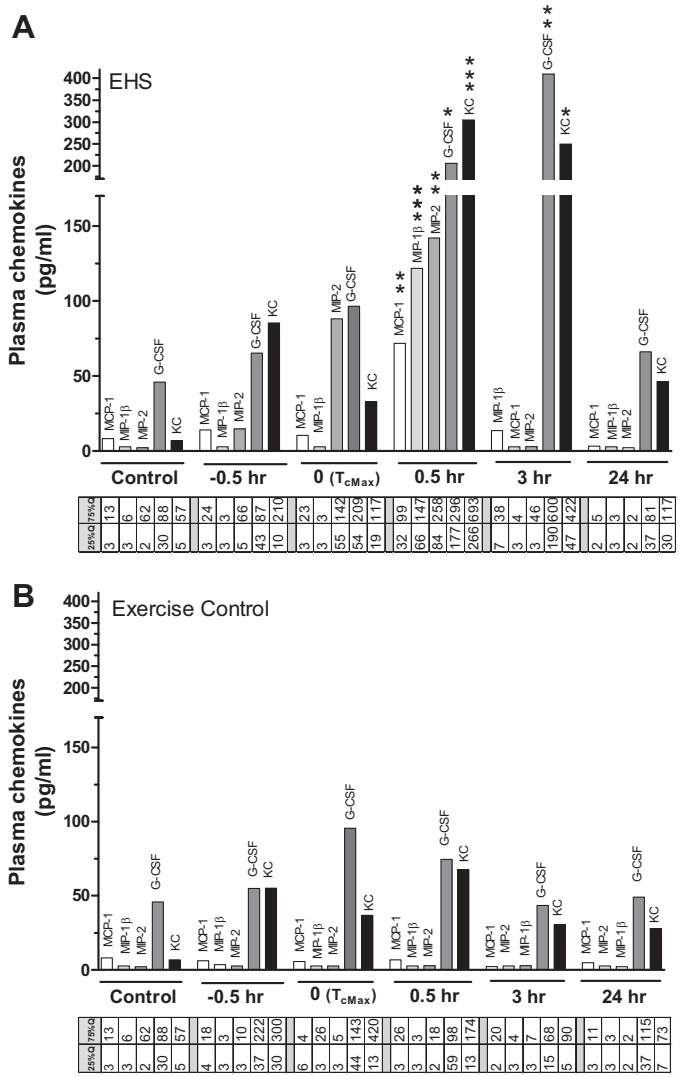

Fig. 3. Effects of EHS on chemokines and related cytokines. *A*: responses during and following EHS. *B*: responses to sham exercise controls. Post hoc significance from naïve control: $P < 0.05$ (*), 0.01 (**), and 0.001 (***) (post hoc tests). Benjamini-Hochberg procedure for multiple ANOVAs = FDR <10%. Bars = medians; tables below = 25–75% quartiles.

by 3 h (Fig. 2*A*). G-CSF and KC were also significantly elevated at 0.5 h but showed sustained or increasing levels at 3 h. G-CSF is not structurally classified as a chemokine but works synergistically with many other chemokines like KC to mobilize immune cells (68). All chemokines returned to control values by 24 h. There were no significant elevations in these chemokines in sham exercise controls (Fig. 3*B*). All other cytokines and chemokines tested with the multiplex array showed no significant elevation during EHS (data not shown). Refer to Table 1 for functional and structural classifications of responsive chemokines observed in this study.

PHS experiments. Previous PHS studies have shown that circulating IL-6 and IL-10 reach a peak response at 3 h of recovery (39, 64), with little or no response at $T_{c,max}$ and only modest responses at ≈0.5 h of recovery (64). To understand the origins of this delay in the PHS cytokine profile compared with the EHS profile, we tested several possible experimental mechanisms related to heat exposure.

First, because our EHS animals achieved an average symptom-limited $T_{c,max}$ of only 42.1°C [−0.3 to −0.6°C lower than the $T_{c,max}$ in studies by Leon and colleagues (39) and Welc et al. (64)], we repeated the standard PHS experiment in mice but stopped exposure when T_c reached 42.1°C. A typical temperature profile for this group (PHS) compared with EHS is shown in Fig. 4. Second, the PHS protocol resulted in an increased thermal area compared with EHS, averaging 409 ± 71°C·min in this series compared with 146 ± 30 (SD) °C·min in EHS. Therefore, we hypothesized that the altered cytokine response to EHS might reflect differences in the overall thermal load between PHS and EHS. To test this, we studied a second group of PHS animals (PHS_m) in which the thermal area was matched, using an elevated T_{env} in the chamber (43.5°C). This resulted in an average thermal area = 148 ± 20 (SD) °C·min (not significant from EHS). A typical thermal profile for PHS_m experiments is also shown in Fig. 4. We tested only the 3-h time point in these experiments because it represented a time when EHS cytokine responses were nearly absent in EHS but reached peak concentrations in PHS.

Comparisons of cytokines and chemokines between sham EXC, EHS, PHS, and PHS_m animals at the 3-h recovery point are shown in Fig. 5. In Fig. 5, *A–C*, are cytokine/chemokine responses to PHS that showed no response in EHS or EXC but were significantly elevated in PHS and PHS_m (i.e., IL-6, MIP-2, and RANTES). In Fig. 5, *D–F*, are cytokines/chemokines for which there were no responses in EHS, EXC, or PHS_m, but there were significant elevations in PHS. Both G-CSF and KC (data not shown) were significantly elevated in PHS and/or PHS_m and were not significantly different from EHS (data not shown). Elevations during EHS in these two chemokines are shown in Fig. 3.

Skeletal muscle innate immune cytokine gene expression. Skeletal muscle mRNA expression of IL-6, IL-10, IL-1β, and TNF-α was evaluated over the course of the EHS and EXC protocol through 24 h of recovery. The primary rationale was that significant muscle injury is associated with EHS but not PHS, based on plasma creatine kinase measurements (35) and unpublished observations of hindlimb motor dysfunction during recovery. In addition, in a previous study, the same approach was used in PHS at the similar time points, making comparison possible (64). Therefore, measuring the mRNA expression of important inflammatory cytokines in muscle can provide an indication of the timing of ongoing damage and repair processes in the muscle.

The results are summarized in Fig. 6 using samples from the whole gastrocnemius, soleus, and diaphragm. Results are expressed as fold change compared with samples taken from "naïve controls" that did not undergo surgery or acute exercise and were not exercise trained or exposed to heat. Note the tendency in early time points (−0.5 h to $T_{c,max}$) for cytokine mRNA to be suppressed before reaching $T_{c,max}$ (discussed below). There was very little mRNA response at any time point in gastrocnemius; however, in soleus and diaphragm, elevations in cytokine gene expression (IL-6, IL-1β, and IL-10) peaked at 0.5 h after $T_{c,max}$. IL-6 mRNA was also evident in diaphragm at $T_{c,max}$. These elevations in mRNA are 3–10 times higher than seen in comparable conditions and times during PHS (64). Note that TNF-α mRNA was not significantly elevated at any time point. Furthermore, in exercise controls, exercised to match EHS, and trained identically, there were no

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Table 1. *Functional-structural classes of chemokines/related cytokines observed in heat stroke*

Common Abbreviations	Name	Structure Name	Human Homolog	Observed in	Primary Functions
MCP-1	Monocyte chemoattractive factor-1	CCL2	Human MCP-1	EHS/PHS	Induces migration of monocytes and other immune cells
MIP-1β	Macrophage inflammatory protein-1β	CCL4	Human MIP-1β	EHS/PHS	Induces migration of monocytes and other immune cells
RANTES	Regulated on activation, normal T cell expressed and secreted	CCL5	Human RANTES	PHS	Stimulates T cells, basophils, and eosinophils
IP-10	Interferon-γ induced protein-10	CXCL10	Human IP-10	PHS	Induces migration of neutrophils, macrophages, and other immune cells.
MIP-2	Macrophage inflammatory protein-2	CXCL2	Human MIP-2 (90% IL-8 homolog)	EHS/PHS	Induces migration of neutrophils, macrophages, and other immune cells.
KC	Keratinocyte chemoattractant	CXCL1	IL-8 (similar to MIP2)	EHS/PHS	Stimulates hematopoietic and other stem cells and migration, similar to MIP-2
G-CSF	Granulocyte-colony-stimulating factor	CXC synergist	Human G-CSF	EHS/PHS	Not a chemokine but synergistic with CXCL1 and CXCL2; stimulating hematopoietic and stem cell release

PHS, passive heat stroke; EHS, exertional heat stroke.

significant elevations in muscle cytokine gene expression at any time point.

Based on the plasma cytokine results, we hypothesized that moderate acute exercise or the exercise training protocol itself may be responsible for suppression of cytokines. To test this, we compared our EXC group (which received enrichment and training sessions as previously described) with mice that were exposed to a single bout of moderate exercise, matched in timing and intensity to the EHS experiments. This experimental bout was preceded by only a familiarization trial the day prior, identical to the 60-min incremental training session that EXC mice received. We then measured inflammatory cytokine gene expression at 0.5 h of recovery because this time point displayed the greatest cytokine response in plasma. As shown in Fig. 7*A*, exercise suppressed IL-6, IL-1β, and IL-10 mRNA in the gastrocnemius and soleus but not in the diaphragm. Comparable trends were seen in the EXC (i.e., trained) animals, but fewer time points were statistically significant (Fig. 7*B*). The data are consistent with acute moderate exercise inducing an acute inhibition of inflammatory cytokine gene expression in skeletal muscle.

DISCUSSION

We have demonstrated that EHS results in cytokine/chemokine responses in plasma and skeletal muscle that are uniquely different in the timing, magnitude, and/or species compared with passive models of heat stroke. Contrary to our original hypothesis where we proposed the combined effects of exercise and hyperthermia would amplify the IL-6-induced response, circulating IL-6 emerges rapidly, reaching a peak level at 0.5 h of recovery and disappearing by 3 h, a point in time when the magnitude of circulating IL-6 is highest in PHS. Similar responses were seen for MIP-1β, MCP-1, and MIP-2, whereas G-CSF and KC increased rapidly but remained elevated at 3 h of recovery. At that time point, they were not different in magnitude from PHS. There was no evidence for elevations in circulating IL-10 at any time during recovery from EHS, whereas this is routinely elevated during recovery from PHS (Refs. 39 and 64 and Fig. 4).

Exploration of possible environmental variables related to the timing and magnitude of heat exposure failed to provide a suitable explanation for these phenomena. Therefore, the data suggest that the predominant experimental factor driving the rapid and unique cytokine/chemokine responsiveness of EHS is related to the influence of moderate forced exercise performed during hyperthermia. Neither matched exercise alone nor matched heat exposure alone could reproduce this pattern.

Possible origins of the cytokine/chemokine response pattern in EHS. There are several underlying stimuli that are thought to interact to produce the pattern of cytokine production seen in heat stroke that may be differentially affected by exercise in heat. One frequently mentioned stimulus is endotoxin or other pathogen-associated molecular patterns (PAMPs) released in the circulation from a leaky intestinal barrier (29, 56). How-

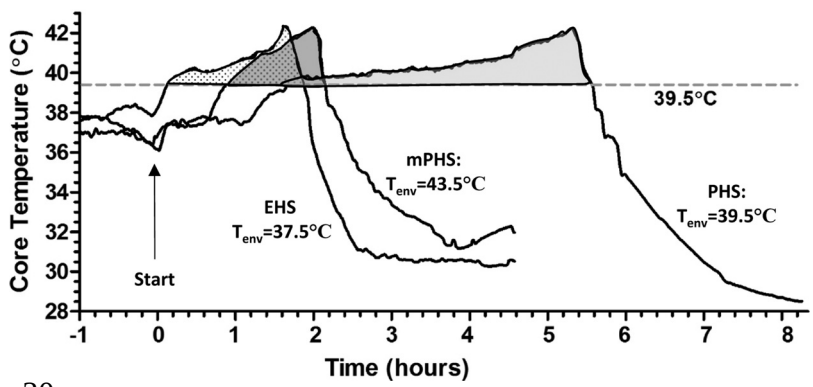

Fig. 4. Typical T_c profiles for EHS, passive heat stroke (PHS), and PHS at thermal area matched to EHS (PHS_m). Shaded areas represent the thermal areas (time·temperature >39.5°C). T_{env}, environmental temperature.

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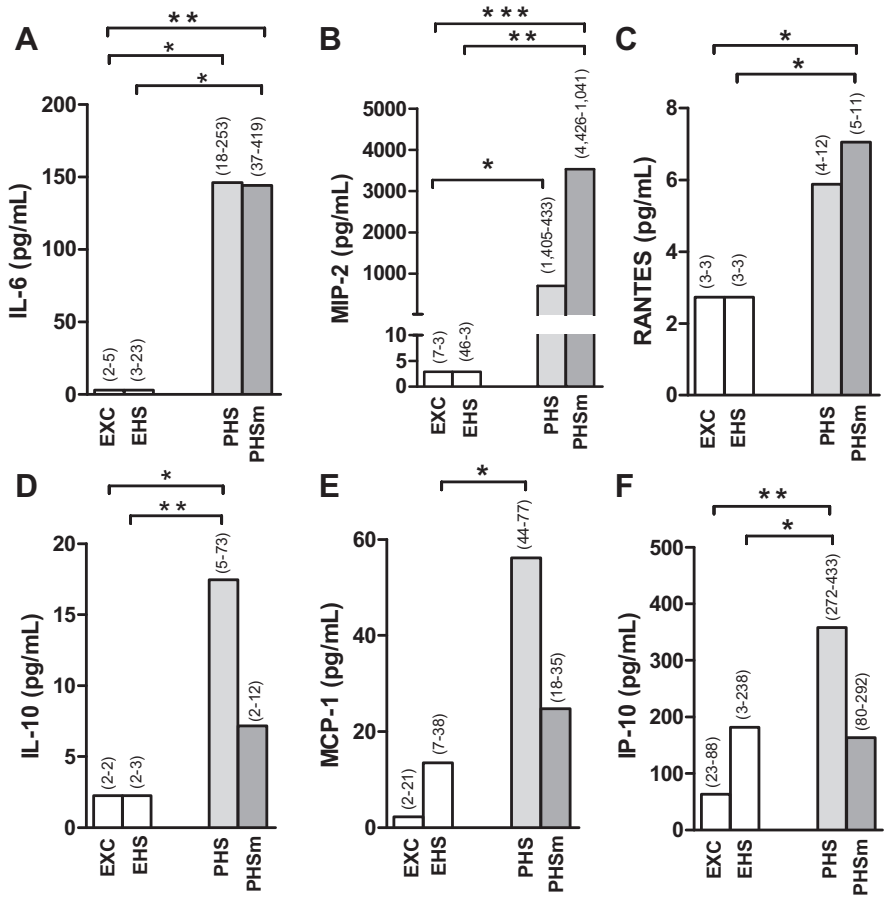

Fig. 5. Comparison of cytokines and chemokines significantly different at 3 h between EHS and models of PHS. EXC, sham controls. $P < 0.05$ (*), 0.01 (**), and 0.001 (***), Kruskall Wallis, Dunn's post hoc comparisons. Bars = median with 25–75% quartiles. Benjamini-Hochberg procedure for multiple ANOVAs = FDR <15%.

ever, the pattern of cytokines seen in the plasma during EHS is not typical of known cytokine responses to PAMPs, e.g., there is an absence of circulating TNF-α, IL-1β, or IL-12, at any time point. It appears more likely that the response is driven by a "stress-induced cytokine response" in which IL-6 is a predominant element. We have previously described this concept in the context of PHS in mice (64) where we observed altered expression of cytokine genes and Toll-like receptor isoforms that are uniquely different from the responses seen in the classic innate immune response. Its theoretical origins are based on observations of the response of isolated skeletal muscles to a variety of forms of cellular and systemic stress mediators (63, 65, 66).

Other possible influences that may contribute to the uniqueness of the PHS response include effects of intense endurance exercise alone, which produce rapid elevations in IL-6 and a variety of other cytokines and chemokines (47, 48). However, in paired exercise controls, there were no significant elevations in cytokines or chemokines. This may have been due to the moderate intensity of exercise. It is possible that hyperthermia amplified the exercise-induced IL-6 (52) as it does with other stimuli (66), but the exercise alone cannot account for the response.

Muscle injury is another potential factor. Local cytokines and chemokines produced following injury play important roles in tissue regeneration and repair (24, 59). Muscle injury was likely present in this model since elevations in plasma creatine kinase are present in this model of EHS but not PHS (35). In addition, Fig. 5 suggests ongoing inflammatory gene expression in both limb and diaphragm muscle during the recovery period that exceed by many fold what is seen in PHS (64). The responses appear to be local because mRNA for cytokines such as IL-1β and IL-10 are greatly upregulated in muscle, but these do not appear elevated in blood during the course of recovery. In addition, previous reports of the timing and magnitude of the circulating cytokine responses in the blood following muscle injury appear to be too small and slow to account for observations seen in EHS (59, 61).

Because the EHS animals received training sessions and had access to running wheels before EHS, this may have modified the cytokine responses during heat stroke. Previous studies have shown that endurance exercise training alters or dampens immune responsiveness (25, 45). It takes only 2 wk of voluntary wheel running in C57BL/6J mice to induce significant increases in heart-to-body mass ratio and percentage of oxidative fibers (1), suggesting that endurance training was likely in the mice provided running wheels. Resolving this variable will require a different approach, since mice unaccustomed to wheel running have more difficulty completing the EHS protocol and likely would experience much higher levels of psychological stress.

One important difference in the cytokine profile in EHS compared with PHS was the absence of circulating IL-10, at any time point. This was unexpected, since increases in circulating IL-10 are one of the most predictive circulating cytokines seen in human patients in heat stroke (9) and in animal

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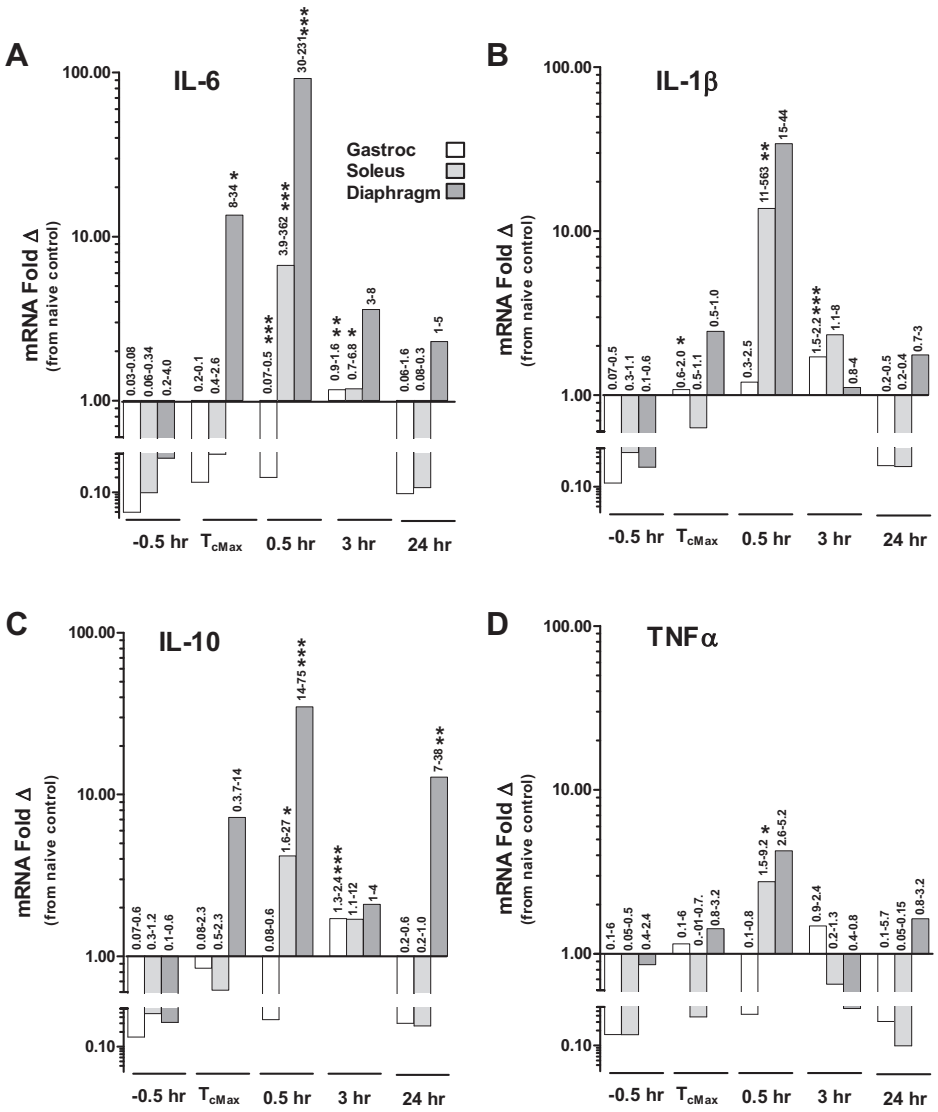

Fig. 6. Fold changes in innate immune cytokine mRNA in EHS gastrocnemius (gastroc), soleus, and diaphragm muscle. All changes reported relative to naïve control mouse muscle. Kruskal-Wallis ANOVA, Dunns post hoc: $P < 0.05$ (*), 0.01 (**), and 0.001 (***). Medians ± 25–75% quartiles. Benjamini-Hochberg procedure for multiple ANOVAs = FDR <15%.

models in PHS (10, 39, 64). Furthermore, IL-6 has been shown to be an important stimulus for IL-10 production (57), and intense exercise alone stimulates IL-10 (46). One possible explanation may reflect the effects of "forced" exercise on immune modulators such as corticosterone. In mice, during forced swimming exercise, corticosterone levels exceed 800 ng/ml within 5 min, approximately one-half of the value seen in parallel experiments in mice exposed only to passive heat (42°C) (26). In the mouse model for PHS, corticosterone has been shown to exceed 400 ng/ml, but this value is reached after ≈3 h of recovery (39). Although we did not measure plasma glucocorticoids in this setting, it is possible that forced running resulted in an early stress-induced surge in glucocorticoids that may have suppressed global cytokine gene expression. This could also explain the apparent suppression of muscle cytokine mRNA seen immediately after forced running (Fig. 6, *A* and *B*). Almost all cytokines and chemokines are suppressed by glucocorticoids, including IL-10 (19). Interestingly, one cytokine not affected appreciably by glucocorticoids is G-CSF (13), which turned out to be one of the most profoundly expressed plasma cytokines in EHS, rising rapidly in the circulation but continuing to rise up to 3 h.

A second important and unexpected finding was the very rapid emergence of IL-6, which was elevated in the plasma, at or shortly before $T_{c,max}$ (Fig. 1). This would seem to be too fast to reflect de novo protein synthesis, particularly when there appears to be simultaneous suppression of IL-6 mRNA (at least in muscle, Fig. 6). Most of the circulating chemokines also emerged during this time frame (Fig. 2). One possible mechanism is that these cytokines/chemokines were prestored in microvesicles or endosomes and were then released early in EHS. In mouse limb muscle, IL-6 is stored in such microvesicles and then released within 25 min from the beginning of an exercise protocol (37). Microvesicle or exosome release has also been shown in some systems to be facilitated by heat stress or by costimulation with other cytokines like IL-1β (18, 72). For example, in tumors, heat stress is a powerful stimulus for release of exosomes that contain many of the same CCL- and CXC-chemokine species we describe here (18). In theory, triggered release of prestored cytokines in this manner could supersede opposing immunosuppressive influences of glucocorticoids produced in the stress of exercise in the heat. This could be a kind of fail-safe acute endocrine stress response

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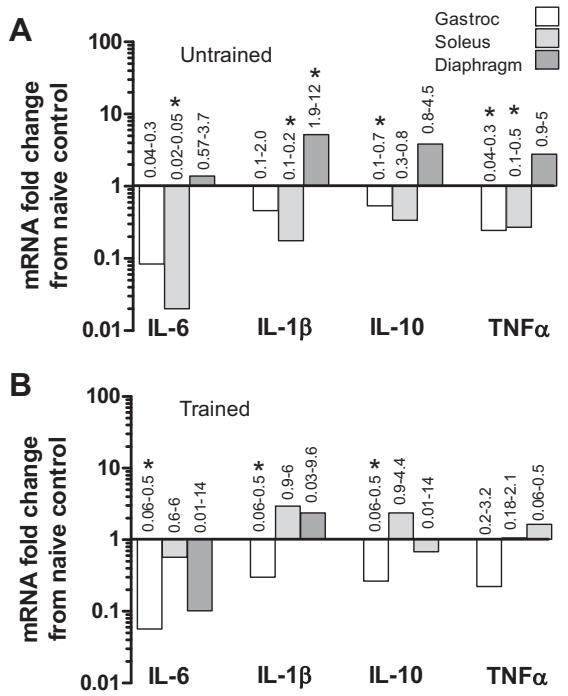

Fig. 7. Effects of a single bout of exercise (matched to EHS) on innate immune cytokine gene expression in muscle. Samples collected at 0.5 h post-$T_{c,max}$. *A*: untrained mice without cage running wheels or exercise training. *B*: response of EXC mice. Medians ± 25–75% quartiles. FDR = 0.15 using Benjamini-Hochberg procedure. *$P < 0.05$.

from tissues that could be important in recovery from acute illness.

Because of the large role muscle plays in exercise, we have focused on it as a source of circulating cytokines in EHS. However, it is highly plausible that other organs make significant contributions to the cytokine profile seen in EHS. Tissue damage resulting from heat stress may impart damage to the liver, kidney, heart, spleen, lung, small intestine, and brain as well as the skeletal muscle (8, 10, 23, 27, 35, 43). When these organs are damaged, they may release cytokines, or resident macrophages, dendritic cells, endothelial cells, or astrocytes may participate in the inflammatory response to injury. Therefore, although we did not directly measure other organs as potential sources of circulating cytokines, it is likely that they contribute to the cytokine profile seen in plasma.

Functional significance of the pattern of cytokine/chemokine production in EHS. In this model all experimental animals survived up to two weeks or to the point of sample collection. After a few hours of recovery, they show a remarkable ability to return to near-normal behavior, despite evidence of underlying organ damage (35). One of the primary functions of both cytokines and chemokines, besides defending against pathogens, is to participate in the process of wound healing and damage repair (69). This occurs, in part, through recruitment of peripheral blood mononuclear cells (PBMCs) and other immune cells in damaged tissue (59) but also by stimulation, recruitment, and mobilization of stem cell or progenitor cell populations in the bone marrow or other tissues (5, 42, 50).

In a previous study (51) we demonstrated that, in PHS, early injection of low levels of recombinant IL-6 enabled anesthetized mice to withstand hyperthermic temperatures for longer periods of time, to have protection from intestinal injury, and to demonstrate suppression of proinflammatory cytokines in the circulation. The protective influence of IL-6 in similar acute life-threatening conditions, or the loss of protection in knockout studies, has now been well established in a number of models, including hemorrhagic shock (2), sepsis (4, 41), acute pancreatitis (21), ischemic heart injury (22), and liver failure (20). Several mechanisms have been proposed but include pre- or postconditioning through Janus kinase/signal transducer and activator of transcription 3 signaling, promoting cell survival (22, 44, 55), upregulation of manganese superoxide dismutase in critical organs such as liver (14), activation of acute-phase response in liver (15), and stimulation of anti-inflammatory cytokines and cytokine receptors (60). We hypothesize that the early secretion of IL-6 and possibly chemokines in this model of EHS may have played an overall protective role in supporting survival and protection from multiorgan injury.

The specific sets of chemokines secreted may also have contributed to recovery from heat injury. There are two broad categories, as shown in Fig. 2 and Table 1: the CCL-chemokines (i.e., MCP-1|CCL2 and MIP-1β|CCL4) and CXCL-chemokines (i.e., MIP-2|CXCL2 and KC|CXCL1). The CCL-chemokines are important for stimulating chemotaxis of monocytes out of the bone marrow and in injured tissues to begin the process of repair (28), and CCL4 has an additional role in stimulating migration of natural killer (NK) lymphocytes (28), which are important in surveillance and ultimate clearing of heavily damaged cells (16, 33). CXCL-chemokines primarily trigger release of neutrophils and other immune cells from bone marrow and also function as a chemotactic stimulus for movement of neutrophils in damaged tissues (28). The cytokine G-CSF stimulates granulopoiesis in the bone marrow and works in synergy with MIP-2 and KC to increase several types of circulating leukocytes (68). As importantly in this setting, G-CSF is a critical stimulus for mobilization of adult stem cells from the bone marrow (5). Although IL-6, in combination with its soluble receptor, has been shown to contribute to promotion of progenitor cells (50), its role in this process is not as clearly understood. Some of the chemokines seen in EHS may act like IL-6 and may also have direct protective effects of tissues exposed to stressful conditions, e.g., CXCL1 (3) and G-CSF (36). IL-6 does have extensive effects on immune cell trafficking that include transition from innate to acquired immunity (34) and stimulation of lymphocyte movement across the endothelium and in tissues (17).

The marked elevation in circulating G-CSF is consistent with human data during short-term hyperthermia (41.8°C) where circulating G-CSF rapidly increases in the circulation (54). It is also very modestly increased during exercise in some studies (71) or not at all in others (70), although there may be a closer association with muscle damage than there is with exercise (70). The source of G-CSF in this setting is not known, but muscle fibers have been shown to be capable of secreting G-CSF following lipopolysaccharide exposure (70).

In summary, we have demonstrated that EHS displays a unique pattern of circulating cytokines and cytokine gene expression in muscle that is unlike that seen in PHS, sepsis, or intense exercise. This response is characterized by the greatest elevations in IL-6, and several chemokines, at the beginning of the recovery period. We verified that this pattern of expression is not simply a result of exposure to lower peak T_c or exposure

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to decreased thermal loads but, by elimination, appears to be an effect arising from acute exercise superimposed on heat.

Clinical and Integrative Perspectives

It is apparent from these data that exercise, whether acute or chronic, can play a unique role in the overall immune responsiveness to severe hyperthermia exposure. The data are consistent with the existence of an exercise- and hyperthermia-induced rapid physiological response system that is geared toward initiating survival pathways and recruitment of immune cells involved in rapid wound healing and repair from thermal injury. One would expect that different exercise intensities, levels of exercise training, and the timing of exposure of exertion vs. hyperthermia would likely impact the background immune responsiveness and clinical outcomes in conditions in which EHS can occur.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

M.A.K. and D.A.M. performed experiments; M.A.K. and T.L.C. analyzed data; M.A.K., L.R.L., and T.L.C. interpreted results of experiments; M.A.K. and T.L.C. prepared figures; M.A.K. drafted manuscript; M.A.K., L.R.L., D.A.M., and T.L.C. edited and revised manuscript; M.A.K., L.R.L., D.A.M., and T.L.C. approved final version of manuscript.

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RESEARCH ARTICLE

Sex-dependent responses to exertional heat stroke in mice

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Garcia CK, Mattingly AJ, Robinson GP, Laitano O, King MA, Dineen SM, Leon LR, Clanton TL. Sex-dependent responses to exertional heat stroke in mice. *J Appl Physiol* 125: 841–849, 2018. First published June 14, 2018; doi:10.1152/japplphysiol.00220.2018.—With increasing participation of females in endurance athletics and active military service, it is important to determine if there are inherent sex-dependent susceptibilities to exertional heat injury or heat stroke. In this study we compared responses of male and female adult mice to exertional heat stroke (EHS). All mice were instrumented for telemetry core temperature measurements and were exercise-trained for 3 wk before EHS. During EHS, environmental temperature was 37.5°C (35% RH) while the mice ran on a forced running wheel, using incremental increases in speed. The symptom-limited endpoint was loss of consciousness, occurring at ~42.2°C core temperature. Females ran greater distances (623 vs. 346 m, $P < 0.0001$), reached faster running speeds (7.2 vs. 5.1 m/min, $P < 0.0001$), exercised for longer times (177 vs. 124 min, $P < 0.0001$), and were exposed to greater internal heat loads (240 vs.160°C·min; $P < 0.0001$). Minimum Tc during hypothermic recovery was ~32.0°C in both sexes. Females lost 9.2% body weight vs. 7.5% in males ($P < 0.001$). Females demonstrated higher circulating corticosterone (286 vs 183 ng/ml, $P = 0.001$, at 3 h), but most plasma cytokines were not different. A component of performance in females could be attributed to greater body surface area/mass and greater external power performance. However, there were significant and independent effects of sex alone and a crossed effect of "sex × power" on performance. These results demonstrate that female mice have greater resistance to EHS during exercise in hyperthermia and that these effects cannot be attributed solely to body size.

NEW & NOTEWORTHY Female mice are surprisingly more resistant to exertional heat stroke than male mice. They run faster and longer and can withstand greater internal heat loads. These changes cannot be fully accounted for by increased body surface/mass ratio in females or on differences in aerobic performance. Although the stress-immune response in males and females was similar, females exhibited markedly higher plasma corticosteroid levels, which were sustained over 14 days of recovery.

cytokines; exercise; hyperthermia; stress; thermoregulation

INTRODUCTION

With the increasing participation and higher levels of performance of women in endurance athletics (30) and with higher expectations of women serving in active military service (41), there have been concerns regarding the susceptibility of women to exertional heat illness (EHI) and exertional heat stroke (EHS) (19, 29, 31). In military medicine, heat stroke is a subset of heat illness and in most cases it involves exertion in the heat. It is defined as "a severe heat stress injury to the central nervous system, characterized by central nervous system dysfunction and often accompanied by heat injury to other organs and tissues" (4a), whereas heat injuries other than heat stroke "include moderate to severe heat injuries associated with strenuous exercise and environmental heat stress…that require medical intervention and loss of duty time" (4a). In the civilian population, specifically defined EHI is seen more frequently in emergency room and hospital admissions in male populations in the U.S. by a factor of >2-fold (15, 45). In the active military, a 2005 review concluded that the risk of heat illness in women is ~1.21 fold higher than in men (11). However, more recent military surveillance data puts the rate of EHI at nearly the same level in both men and women, but the incidence of EHS is lower in women over the past several years (4). The validity of this kind of data is challenged by differing behaviors and participation rates of men and women, amount of exposure to heat, exercise intensities, differences in inherent aerobic fitness, and body composition. Nevertheless, whether women and men have different susceptibilities to EHI is poorly understood.

Specific sex-based differences in the way humans and other mammals thermoregulate during heat exposure have been identified (e.g., 6, 24, 28, 33, 52), but it is unclear whether these differences in biology provide any specific advantage to one sex or another during EHS. Although it is highly feasible to measure differences in thermoregulatory response mechanisms in men and women, it is not feasible to determine if these differences translate into a greater or lesser susceptibility to EHS, because of the severity of the stimulus in humans, and because other variables such as cellular stress responses, vulnerability to multiorgan injury, immune responses to pathogens, and blood flow distribution disturbances may only emerge in severe exposure and may be equally important in the progression to heat stroke (50).

Our primary objective in this study was to determine if there is a biological basis for sex differences in susceptibility to EHS in an established preclinical model of EHS in mice (35). We hypothesized that male and female mice would be equally susceptible to EHS, when variables such as body size, body weight, and external work rate were accounted for. A secondary objective was to identify other immunological and/or hormonal factors that could account for sex differences that go beyond simple physical characteristics.

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METHODS

This study was approved by the University of Florida's Institutional Animal Care and Use Committee and by the Animal Care and Use Committee of the US Army Medical Research and Material Command. All mice were C57BL/6J (Jackson Laboratories, Bar Harbor, ME) of both sexes. Mice were housed in 19.4-cm-wide × 29.8-cm-diameter × 12.7-cm-high cages on a 12:12-h light/dark cycle at 20–22°C and 30 – 60% relative humidity (RH). Standard chow (LM-485m Envigo; Teklad, Madison, WI) and water were provided ad libitum. All mice were 4–6 mo of age. The light cycle was "lights on" during daytime hours (6 AM–6 PM) and "lights off" between 6 PM and 6 AM. No specific considerations were given to the estrus phase of the female mice.

Animal surgery and exercise training. Surgery for implantation of telemetry devices was conducted under isoflurane anesthesia and performed under sterile conditions. Briefly, a small incision was made to allow placement of temperature telemetry emitters into the abdominal cavity (G2 E-Mitter; Starr Life Sciences, Oakmont, PA) for measurements of core temperature (Tc). Following surgical closure of the abdomen, the mice were singly housed throughout the rest of the experiment. They were monitored postoperatively for 48 h, and subcutaneous injections of buprenorphine given every 12 h. Mice were allowed 2 wk to fully heal from emitter implantation. After 2 wk they were given in-cage running wheels (model 0297–0521, Columbus Instruments, Columbus, OH) to allow for voluntary exercise training for 3 wk. During the third week, animals were brought to the laboratory, placed inside of an environmental chamber, and exercised on a forced running wheel (Lafayette, model 80840, Lafayette, IN) powered by a DC power supply, as described previously (35). Once training was completed, mice were given two full days of rest to recover from the forced wheel training, before the EHS trial. Their in-cage running wheels were available during this 2-day period.

EHS protocol. The mice were brought to the laboratory the afternoon before EHS. Tc was monitored overnight in 30-s intervals to ensure normal temperature profiles before EHS. The 12:12-h light/dark cycle was maintained. The EHS procedure was run in the early-mid morning. Mice remained in their cages with Tc being monitored during equilibration of the environmental chamber (Thermo Forma, 3940, Thermo-Fisher, Waltham, MA) to a set point of 37.5°C, and 30–40% relative humidity (RH). Chamber temperature and humidity were measured, recorded, and controlled at the location of the running wheel. Once the temperature equilibrated in 30–45 min, the mice were placed in the enclosed running wheel. Mice were given ~5 min to recover from the stress of being moved and then, once Tc stabilized at 36–37.5°C, the running wheel was started on an incremental preprogrammed protocol. Speed began at 2.5 m/min and increased 0.3 m/min every 10 min until the mouse reached a Tc of 41°C. Once Tc reached 41°C, the speed was maintained until mice reached the symptom-limited end point. The EHS end point was defined by loss of consciousness, specifically, three consecutive revolutions of the wheel with no physical response by the mouse. Mice remained immobile for 5–10 min following EHS; during that time they were returned to their original cage for recovery at room temperature.

Mice recovered for >0.5 h at 23–25°C with full access to food and water while Tc was being continuously monitored. The mice recovered for varying time points, before euthanasia, i.e., 0.5 h, 3 h, 24 h, 4 days, 7 days, 9 days, and 14 days, with six males and six females at each time point. One set of male and female sham mice were designated "exercise control mice" and were treated identically to the EHS mice except there was no elevation of temperature within the chamber. The exercise time and speed of the control mice was matched to the mean value of the EHS male or female mice as previously described (35). The control mice were euthanized 4 days after exercise. This time was chosen for controls throughout the study to compare one set of data to all time points. These mice reflected the effects of training and conditioning, single housing, and recovery from acute exercise. Furthermore, in previous studies we found little difference in exercise control effects between naive baseline controls at this time point (35).

At the time of euthanasia, mice were anesthetized with isoflurane. Immediately after induction and steady-state anesthesia, heparinized blood was collected by transcardiac stick, using a caudal approach, just left of the xiphoid process. Whole blood was immediately tested for glucose using a portable glucose meter (One Touch, Lifescan). The remaining blood was centrifuged, plasma removed, aliquoted, and stored at −80°C. Plasma samples were then shipped on dry ice to the U.S. Army Research Institute of Environmental Medicine (USARIEM) for corticosterone and cytokine analyses.

Plasma cytokines and chemokines were determined using a Luminex system, (MILLIPLEX MAP Mouse cytokine/chemokine-premixed 25 plex assay kits) and analyzed for granulocyte-colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor (GMCSF), interferon gamma (IFN-γ), interleukin 1 alpha (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, keratinocyte chemoattractant (KC), monocyte chemoattractive factor-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and tumor necrosis factor-alpha (TNFα). The test was performed according to the manufacturer's protocols. Corticosterone was measured in duplicate using a commercial ELISA kit for mouse corticosterone (Assay Designs, Ann Arbor, MI).

Calculations and statistical approach. The physical relationship between mass and surface area across a sphere is referred to as Meeh's equation, i.e., $BSA = k \times mass^{2/3}$ (43). The relationship has been shown to be predictive of BSA from mass across all species of animals, with the k constant changing with different species and even between different strains of mice. Cheung et al. (14) reported $k = 9.822 \pm 0.09$, applicable to both male and female, nonobese, C57bl/6 mice, and this was the approach taken in this experiment. External mechanical power output was calculated in milliwatts (mW) by the formula: [mass (kg) × 9.806 m/s^2 × running speed (m/s) × 1,000]. Ascending thermal area was used as an index of thermal load and calculated as described previously (37).

All statistical analyses were performed by SAS JMP (SAS; Cary NC). Single male vs. female differences were tested for normality and when normally distributed were tested using an unpaired t-test. Nonparametric distributions were tested using the Wilcoxon for unpaired samples. For multiple groups (time course during recovery), normally distributed samples were tested using ANOVA, followed by post hoc t-tests (orthogonal comparisons). For nonparametric distributions (cytokines) ANOVA was performed using Kruskal-Wallis, and post hoc analysis of specific pairs was performed by nonparametric Steel-Dwass. Performance data were submitted to multiway ANCOVA to establish a model for explaining the variance between males and females. Covariates were removed from the model if they did not reach a statistical significance of $P < 0.05$.

RESULTS

Physical characteristics. Table 1 compares physical characteristics of the male and female mice. As expected, for this C57BL/6J strain (14), male mice had significantly higher mass and body surface area (BSA) compared with females, but females had a greater ratio of BSA/mass. Although the male and female mice lost approximately the same absolute weight during EHS exposure, females lost a greater fraction of the total body weight (~1.2%) during EHS.

Exercise performance in the heat. Figure 1 illustrates typical core temperature profiles during EHS in a male and a female mouse. Female mice demonstrated a higher capacity to sustain exercise for longer times in the heat compared with males.

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Table 1. *Physical characteristics of the male and female mice*

Sex	Body Wt[a], g	Body Wt (Post-EHS), g	ΔBody Wt, %	BSA[a,b], cm^2	BSA/Mass[a], cm^2/g
Male	27.88 ± 1.77	25.80 ± 2.05	7.52 ± 2.22	88.47 ± 3.75	3.18 ± 0.01
Female	23.87 ± 1.09	21.67 ± 1.07	9.23 ± 2.33	79.80 ± 2.43	3.34 ± 0.01
P value[c]	$P < 0.0001$	$P < 0.001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

Values are means ± SE. [a]Based on measurements immediately before exertional heat stroke (EHS). [b]Body surface area (BSA) based on Cheung et al. (14) for C57/bl/6 strain. [c]Two-sample *t*-test ($n = 43$ or 44 per group).

Grouped data are shown in Fig. 2*A*. The females achieved longer distances in the heat (Fig. 2*B*) and operated at higher maximum speeds during the final phase of steady-state exercise (Fig. 2*C*). Because of the greater exercise time in the heat, females were exposed to a greater overall heat load, as estimated by ascending thermal area in Fig. 2*D*. However, maximum core temperatures (Tc,max) attained before symptom limitation and minimum core temperatures during post-EHS hypothermia (Tc,min) were nearly identical between males and females (Fig. 2*E*).

To further explore the differences in the EHS response between males and females, the temperature profiles were further subdivided into an "incubation phase," from baseline to 39.5°C, an "incremental phase" where the speed of the running wheel was increased incrementally (from 39.5°-41°C), and a "steady state" exercise phase, when exercise intensity was maintained constant until symptom limitation. As shown in Fig. 2*F*, the durations of both the incubation phase and steady-state phase were not different between males and females, but the duration of the incremental phase was significantly higher in females. This means that the females resisted elevations in Tc while their running speed and mechanical power output were increasing, which allowed them to reach higher running speeds and achieve greater overall distances over the course of the experiment.

Physical factors influencing performance. Sex differences in body mass and surface area (Table 1) can impact heat dissipation and heat storage whereas body weight and running speed, determinants of power output, affect the rate of heat generation and the accumulated distance run. Therefore, we wished to determine if these physical factors could fully account for differences in performance between males and females. As an indicator of performance in the heat, we chose to use the total distance run in the heat before symptom limitation, because it reflects both the duration of exercise tolerance in the heat and the elevations in running speed over the course of the trial.

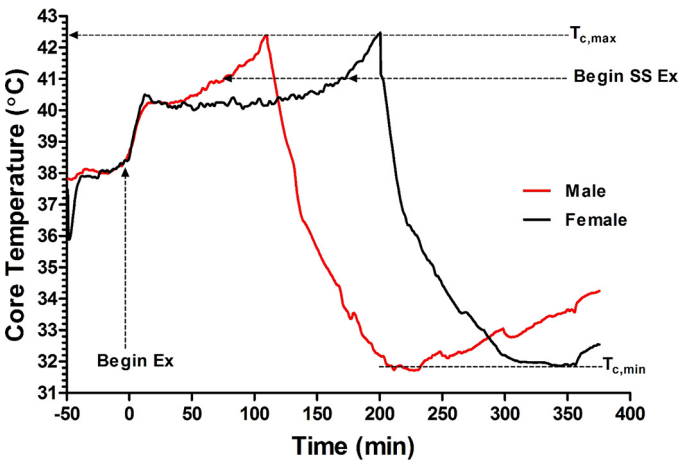

Fig. 1. Typical core temperature profiles of male (red line) and female mice (black line) during exertional heat stroke (EHS) trials. The time scale is set so that 0 = the beginning of exercise in the heat. For ~60 min before the beginning of exercise, the mice were equilibrated to the environmental chamber at 37.5°C. When mice attained a core temperature of 41°C the forced running wheel was kept at a constant speed throughout the rest of the EHS trial and thus steady-state exercise began at that point (SS Ex). Both males and females reached similar maximum core temperatures (Tc,max) and minimum core temperatures (Tc,min).

Figure 3*A* expresses the distance run as a function of the maximum power achieved in the final steady-state exercise phase. The total distance run by both male and female mice was strongly dependent upon the maximum power achieved in this phase. Female mice exhibited higher capacities to generate power, even though they had lower body mass, demonstrating that they attained these values by running at faster velocities. The linear relationships between power output achieved and distance run were statistically different for males and females (ANCOVA).

We also expressed distance run as a function of BSA/mass (BSA/m) ratio in Fig. 3*B*. Animals with higher BSA/m should be able to dissipate heat more effectively. In Fig. 3*C*, we also plotted distance against body mass alone, since heavier mice would presumably accumulate heat faster at a given speed. Since BSA in the mouse is strain-dependent but predicted based on mass (14), Fig. 3, *B* and *C*, are mirror transformations of each other. In essence, mice with greater BSA/m ratios and lower body mass attained greater distances and were more resistant to exercise in the heat, regardless of sex. There was no statistical effect of sex on the linear relationship between BSA/m and distance, or mass and distance.

Using these relationships we constructed a statistical model to predict the probability that the variance in exercise performance (distance run) in the heat between sexes could be accounted for by physical size and BSA rather than inherent sex differences. We reasoned that the rate of heat accumulation would be a function of the mechanical power exhibited during exercise, whereas the rate of heat dissipation and heat storage should be a function of BSA/m. To this end, we used the following multiway ANCOVA model:

$$\text{Performance} = A + B(\text{Power}) + C\left(\frac{\text{BSA}}{\text{m}}\right) + D(\text{Sex}) + E(\text{Sex} \times \text{Power}) + F\left(\text{Sex} \times \frac{\text{BSA}}{\text{m}}\right) + \text{Error} \quad (1)$$

The results are illustrated in Fig. 4, which is a unity plot of the actual distance run vs. the predicted distance run. The residual error represents only 0.4% of the total variance of the populations. The significance of each factor in contributing to the variance in the population response is illustrated using

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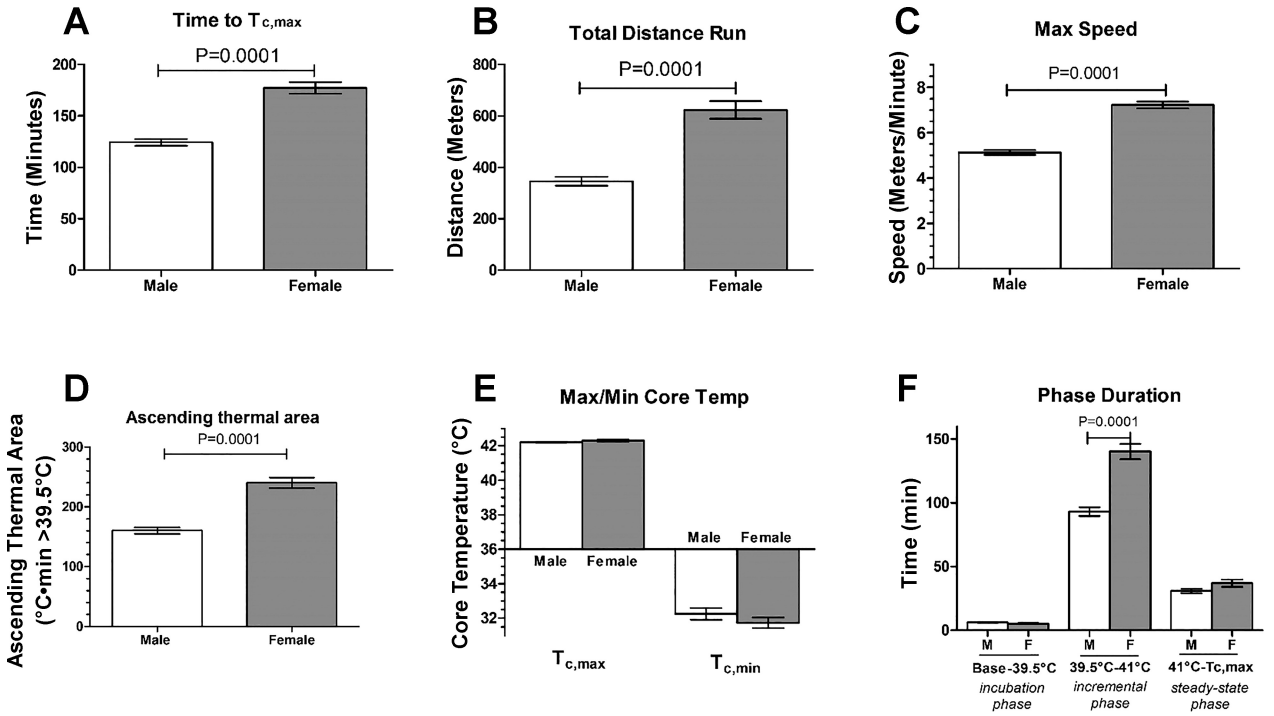

Fig. 2. *A–F*: performance differences between male and female mice during exertional heat stroke (EHS). (Values are means ± SE; 2-sample *t*-tests, $n = 43–44$ for both groups). See text for details.

negative log plot of *P* values (logworth, SAS JMP). These *P* values were adjusted for False Discovery Rate (FDR), based on Benjamini-Hochberg (7).

The factors of greatest significance were the maximum power exerted while running on the wheel and the BSA/m, independent of sex. There was no significant crossed effect of sex × BSA/m, so this factor was eliminated from the model (factor *F* in *Eq. 1*) and graphically handled as a univariate regression in Fig. 3*B*. Sex had two important impacts. There was a small but independent effect of sex alone ($P < 0.0004$) and there was a strong crossed effect of sex × power ($P < 0.00001$). This latter relationship can be seen in Fig. 3*A* by the differing slopes of the power-distance plots in males and females. In summary, these results are consistent with a unique property of female mice, independent of body mass or BSA, that allow them to perform at higher power outputs and for longer periods of time in the heat. The final equation of the line from the ANCOVA model was:

$$\text{Performance} = -5,659.0 + 1,505.6\left(\frac{\text{BSA}}{\text{m}}\right) + 54.4(\text{Power}) - 17.7\left[\text{Sex} \times (\text{Power} - 25.2)\right] - 62.4(\text{Sex}) \quad (2)$$

where Sex = [1 (male); −1 (female)], Performance is in meters, and maximum Power is in milliwatts.

Immunological and hormonal responses of males and females. We hypothesized that male and female mice may exhibit different immunological responses to exercise hyperthermia, which could influence development of and recovery from EHS (50). Corticosterone, the primary stress-glucocorticoid expressed in mice (22), was significantly elevated in female mice compared with males throughout the recovery period (Fig. 5*A*; ANOVA crossed effects: Time × Sex, $P < 0.001$). Interestingly, this elevation remained significant through 14 days of recovery. Since glucocorticoids play important roles in regulation of glucose homeostasis in stress (36), we also evaluated differences in plasma glucose between male and female mice throughout recovery. As shown in Fig. 5*B*, at all but one time point (9 days), there were no significant differences in plasma glucose. Overall, the plasma glucose values measured in controls and throughout recovery beyond 24 h were higher than predicted values in isoflurane-anesthetized, nonfasting mice (17).

Another important function of corticosterone is suppression of inflammatory cytokines and inflammatory cells involved with innate and stress-induced immunity. We therefore compared the plasma cytokine and chemokine responses in male and female mice over 4 days of recovery. As shown in Fig. 6 the time course of the EHS-induced cytokine responses were similar between males and females and were largely back to baseline by 24 h of recovery. Only cytokines that reached the $P < 0.05$ level of significance in any comparison are shown. This time course was similar to previous reports in male mice undergoing EHS (34). The only differences observed between males and females were significant elevations in interleukin-5 (IL-5), interleukin-9 (IL-9), and interleukin-10 (IL-10) in females only, observations previously not associated with cytokine response to EHS (34).

DISCUSSION

These results demonstrate that female C57BL/6J mice exhibit a significantly elevated tolerance to exercise exertion in the heat, and resistance to EHS, compared with male mice. During the interval in which mechanical power output is increasing (i.e., the incremental phase as shown in Fig. 1 and

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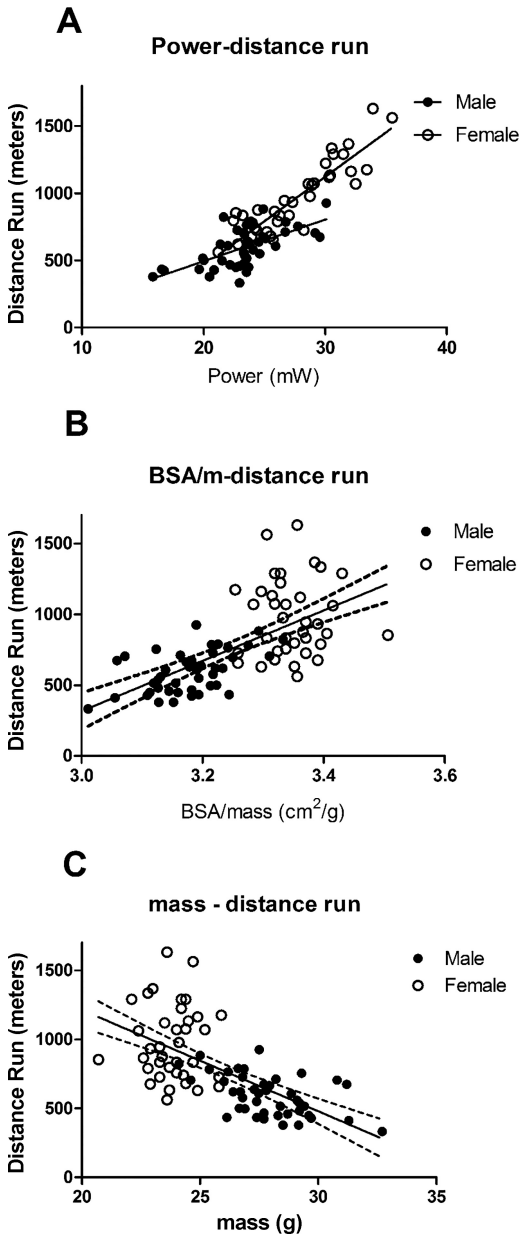

Fig. 3. *A*: relationship between power output and distance run. The linear fit for this relationship was significantly different between males and females ($P < 0.001$). *B*: relationship between BSA/mass and distance run in males and females. There was no statistical difference in the relationship between males and females, but females had predominantly higher BSA/mass values. *C*: relationship of body mass to distance run. These two linear relationships in males and females were not statistically different. Dashed line is 95% confidence of the regression slope.

Fig. 2*F*) females are better able to defend their Tc without entering into the progressive hyperthermia leading to EHS. Interestingly, once males and females enter this final phase, their accelerated hyperthermia follows a similar time course, ending at a near identical Tc at collapse (Tc,max). This suggests that the underlying pathophysiology converges similarly in both sexes. However, of note, the females enter this final phase at a much higher aerobic output, with greater metabolic requirements and presumably greater requirements for dissipation of heat.

No doubt, a number of underlying mechanisms account for the different responses in female and male mice. Based on the statistical model, the predominant factor may simply be that the female mice had a greater capacity for aerobic work than males. The method utilized requires 3 wk of in-cage training on a voluntary running wheel, with 4 days of forced running wheel training. Three weeks of voluntary wheel running has previously been shown to induce greater aerobic performance in this strain of female mice and may reflect a known capacity for greater aerobic training in females (32) and a greater propensity to utilize in-cage running wheels at faster velocities in females (38, 42). This behavioral difference is, in part, strain dependent, as some inbred strains and also wild deer mice show no such differences in voluntary exercise behaviors between the sexes (13, 38). One consideration in comparing males and females is that male C57BL/6J mice (and most strains) are heavier than females and, therefore, for a given running speed, males must perform more mechanical work. Therefore, they may be training at similar relative rates even though their absolute speeds are lower. However, when total external work is estimated based on body mass and velocity during the EHS run, it appears that females in this study performed at much greater work rates and could presumably dissipate heat more effectively while running at greater velocities (Fig. 2*A*). Male and female C57BL/6J mice have nearly identical proportions of body fat (47); therefore, this would not likely be a factor contributing to differences in performance.

Another potential factor that may have been an advantage for female mice is that they may inherently express a more aerobic muscle phenotype, and therefore would have a greater energy efficiency and presumably a lower heat production during exercise (24). The evidence for this phenomenon in the hindlimbs of C57BL/6J mice is slim, but in general, a fiber-type sexual dimorphism has been demonstrated in mice, humans, and other mammals with slower, more oxidative fibers being more predominant in females (24).

There are several important aspects to the statistical model that are important to consider. First, the biomechanics of quadrupeds running on forced running wheels is more complex than simple calculations of raw external power. Mice are not smooth runners on running wheels, particularly forced running wheels, and take on start-and-stop behaviors (1), frequent braking, and occasional performance of widely varying angular motions within the caged wheel. During spontaneous running on wheels, predominant amounts of force are developed in the hindlimbs (more so than in flat field running), and peak forces exceed the forces required to overcome gravity (48). There are also additional forces required by the forelimbs and core musculature to extend toward the forward angular surface of the wheel, which is dependent on the circumference of the wheel and would be reduced in flat running. These additional mechanical factors are significant, and our calculations no doubt underestimate the total external power output. However, this would apply similarly across male and female mice, although the somewhat shorter stature of females could provide a very small mechanical advantage.

The estimates of the BSA available for heat exchange is another potential source of error. The actual BSA calculation for C57BL/6J mice, based on weight, is highly predictive of actual surface area and shown to not be affected by sex, but greatly affected by mouse strain and body composition (14).

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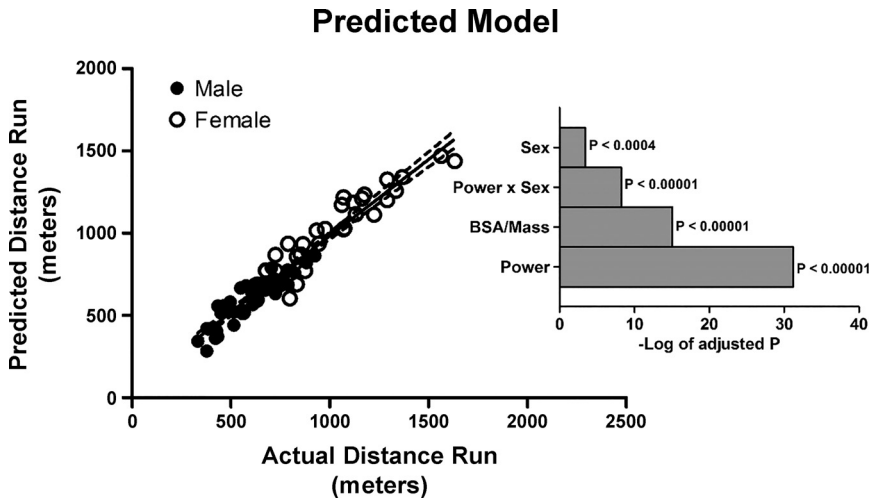

Fig. 4. Results of the ANCOVA statistical model showing the actual distance run by the mice vs. the distance predicted by the statistical model. The dashed lines are the 95% CI for the regression slope. The *inset* shows the −log of the P values, an index of relative contribution to the model. Note that males and females sat on different regions of the curve. See text for details.

However, in terms of heat dissipation, the area available for radiative heat exchange in the mouse is not uniformly distributed between furred and nonfurred areas of the skin. In the deer mouse, in an environment of 34°C, ~32% of the heat is dissipated from the unfurred appendages (ears/tail/feet) and the remainder from the body torso (16). The fraction of radiative heat loss from the facial region where saliva spreading is used for evaporative heat loss is not well documented. Nevertheless, in C57BL/6J mice, appendages, such as tail length, increase proportionately with body weight over time (20), and therefore, it is likely that BSA in most regions of the body is changing roughly proportionately with the body mass.

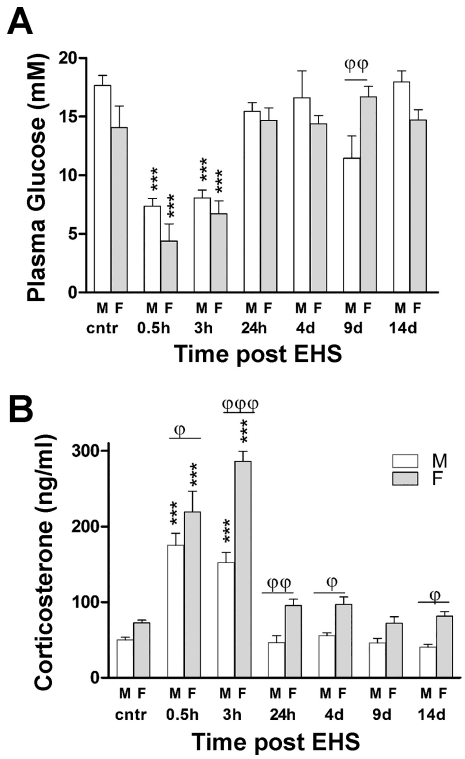

Fig. 5. *A*: measures of plasma corticosterone in males vs. females over the course of 14 day recovery. ANOVA Effects: sex <0.0001, time <0.001, time × sex <0.001. *B*: plasma glucose, non-fasting, measured at the time of sacrifice during post EHS recovery and under isoflurane anesthesia. ANOVA = $P < 0.05$. No crossed effect of sex × time could be identified. $N = 6$ in each group means ± SE, post hoc comparisons from sex-matched controls: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Post hoc differences between males and females at each time point: φ$P < 0.05$, φφ$P < 0.01$, φφφ$P < 0.001$.

One of the more striking differences between male and female mice was the difference in plasma corticosterone seen immediately after EHS and throughout the recovery phase (Fig. 5*A*). This may represent a greater responsiveness of the stress-induced pituitary-adrenal axis, as has been described for some female mammals during exposure to different kinds of stress conditions (1a, 40). Alternatively, it could reflect the greater overall heat exposure and level and duration of exercise in the female mice. The persistence over many days is somewhat surprising and illustrates that recovery from EHS exposure is a long process and possibly sex dependent.

The possible therapeutic impact of glucocorticoid supplementation on prevention and recovery from heat stroke has been explored, but there is no consensus on whether it improves outcomes. In a model of passive heat stroke in anesthetized rats, administration of dexamethasone, a common pharmaceutical corticosteroid, has been shown to be effective in increasing survival, and reducing arterial hypotension, cerebral ischemia, and organ damage (39). However, in the baboon model of passive heat stroke, administration of dexamethasone during recovery had no influence on survival and resulted in the presence of significantly elevated and prolonged plasma biomarkers of organ injury during the recovery phase (8).

The biological effects of elevations in glucocorticoids in conditions of stress are many and encompass metabolic, immunological, and cellular effects. In terms of glucose metabolism, through differing mechanisms in skeletal muscle and liver, glucocorticoids function to preserve plasma glucose in times of stress or substrate depletion to maintain glucose-dependent brain function (36). Glucocorticoids are also involved with fatty acid mobilization in endurance exercise and may contribute to endurance performance (2, 12). Interestingly, females may be more effective, in general, at recruiting lipid metabolic pathways during endurance exercise compared with males (18). The third influence of glucocorticoids is their inhibitory effects on inflammatory signaling pathways and

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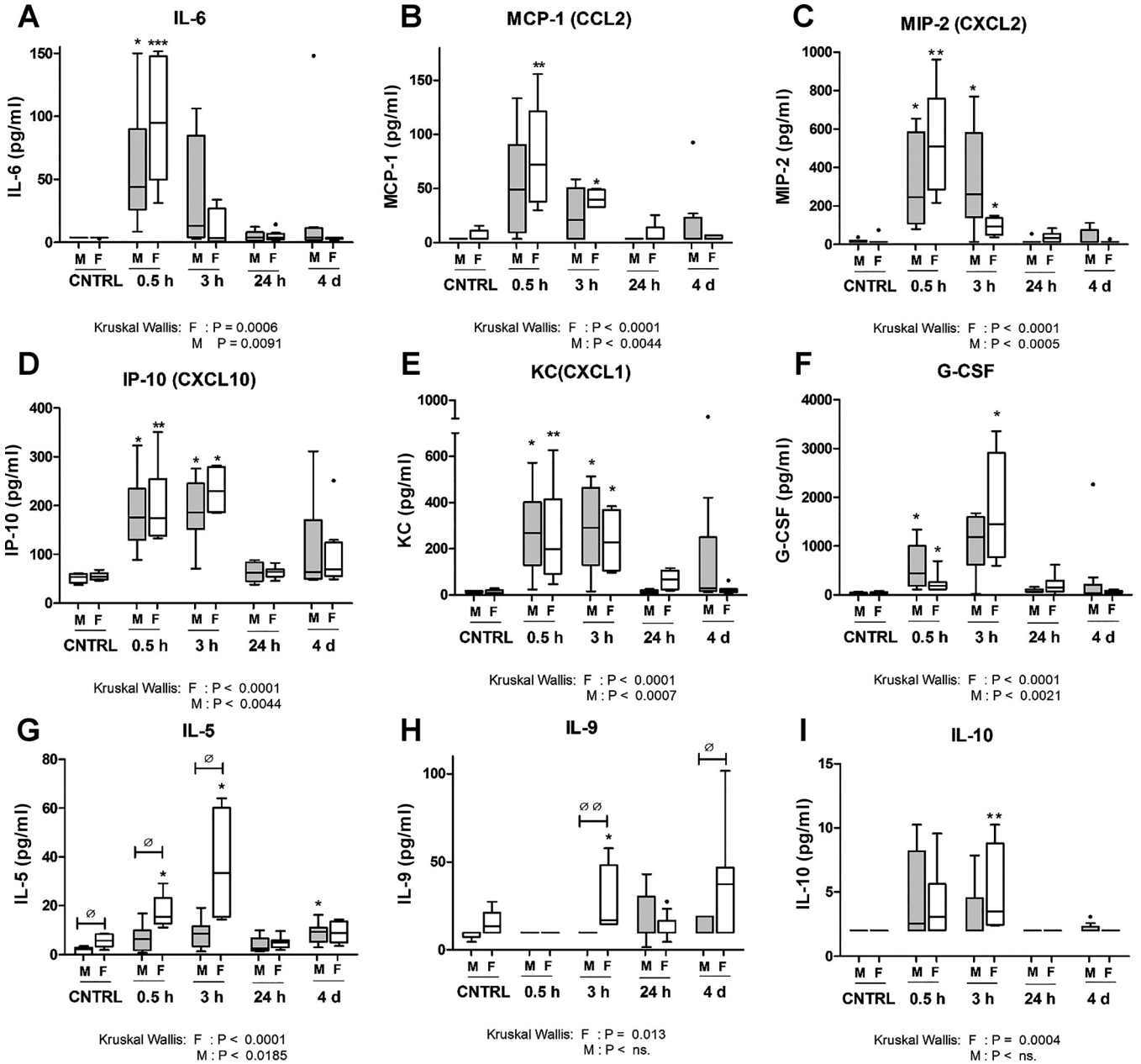

Fig. 6. *A–I*: plasma cytokines measured over 4 days of recovery post-EHS. Kruskal Wallis ANOVA *P* values under each relationship, post hoc between groups: Steel-Dwass, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ from control (CNTRL). $\theta\theta P < 0.01$, $\theta P < 0.05$ from time-matched male-female differences.

specifically on IL-6 expression. Most cytokines and chemokines involved with innate and stress-induced immunity are directly or indirectly inhibited by glucocorticoid receptor activation (27). IL-6 needs particular consideration because it has important metabolic effects that support lipolysis in adipose tissue and glucose homeostasis during exercise, some of these effects are general effects (21) and some sex specific (44). Exogenous IL-6 administration improves thermal tolerance and tissue protection from heat stroke (46). In general, however, there were no striking differences between most plasma cytokines between males and females.

Interestingly, three cytokines that were upregulated in females have not previously been seen in this model, namely, IL-5, IL-9, and IL-10. We do not have a mechanistic reason that these would be uniquely increased in females during EHS recovery. IL-5 is produced by a large number of organ systems in the mouse (49) and is involved with eosinophilic activity and inflammatory cell survival in conditions such as asthma (26), whereas IL-9 is largely produced by innate lymphoid cells and plays a poorly understood anti-apoptotic role in immune cells and many other cell populations (54). As an anecdote, both IL-5 and IL-9 are involved in promotion of anaphylaxis, and there is a rare condition called "exercise-induced anaphylaxis" or "physical urticaria" that occurs following exertion in hot environments. It is seen in females at a threefold higher rate than males (53).

One of the limitations of the study was that we did not standardize the estrus stage of the female animals during the

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EHS trial. The stage of estrus cycle could influence both Tc and the thermoregulatory responses to heat (33). However, because of the rigid timing inherent in the study design, trying to standardize the estrus cycle became infeasible. The timing of each stage of the experiment was scheduled >3 wk before each animal's EHS trial and included events occurring up to 2 wk after the trial. Although mice can cycle through the estrus cycle within 4 days, this is the minimum, and there is a wide variation in the average mouse (10). Alternatively, we could have measured estradiol levels in the mice, but again, they were euthanized for tissue collection between 3 h and 14 days after EHS, so it would be impossible to extrapolate the estradiol levels back to the EHS exposure. We also could have estimated the estrus cycle using visual clues at the time of EHS and used this as a covariate in the analyses, but the predictability of the responses in females without using this factor (Fig. 4) makes it unlikely that it would have substantially improved the power of our statistical model.

Translational implications. In this controlled preclinical study, some unknown variables that are independent of physical factors such as BSA/m ratios, power, or fitness appear to provide a biological advantage to female mice for enduring exertion in the heat, before experiencing EHS. The same holds true in the absence of exertion, as female mice have been shown to exhibit greater resistance to sustained passive heat exposure compared with males (37°C for 5 days) (9). Can we translate this to humans? It is very difficult because marked differences exist in the thermoregulatory mechanisms used by rodents vs. humans in overcoming hyperthermia (23). However, as women in the military and in athletics begin to approach levels of fitness of their male counterparts, concerns that women are inherently more susceptible than men to EHS (19, 29, 31) may be premature. For example, the most recent two years of military surveillance data in the US have demonstrated that although the proportion of men and women in active service who are diagnosed with exertional heat injury are approximately equal, the incidence of heat stroke in both 2015 and 2016 were 1.75- to 2.22-fold higher in men (3, 4). Therefore, the question remains unresolved as to whether women are more or less susceptible to EHS. We can say that female mice clearly have some advantage, but this cannot be extrapolated yet to women. Furthermore, although women represent fewer cases of EHS, based on the epidemiological literature, there are several other differences, in addition to biological sex, that may be mediating this effect, such as differences in behavior, risk taking, or frequency of exposure.

There are numerous examples of sexual dimorphism between males and females with regard to responses to heat exposure and thermoregulation. For example, in studies in men and women with similar fitness levels, similar acclimation states and similar body sizes, men and women display very similar thermoregulatory responses to heat stress but women demonstrate some advantages in hot humid environments but not dry environments, particularly before acclimatization (5, 33, 51). Women have different body distributions of sweat production (52) and a greater density of heat-activated sweat glands (6), but overall lower rates of sweat production (reviewed in 33). Women use different signaling pathways for the cutaneous vascular responses to local warming than males (28). These observations illustrate that there is much room for continued exploration of sex differences in thermoregulation and heat tolerance and that conclusions based only on responses to transient exercise in mild hyperthermia such as the standardized heat tolerance test are unlikely to provide a complete picture.

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DISCLAIMERS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.K.G., A.J.M., G.P.R., O.L., M.A.K., and S.M.D. performed experiments; C.K.G., A.J.M., G.P.R., M.A.K., and T.L.C. analyzed data; C.K.G. and T.L.C. interpreted results of experiments; C.K.G. and T.L.C. prepared figures; C.K.G. drafted manuscript; C.K.G., G.P.R., O.L., M.A.K., S.M.D., L.R.L., and T.L.C. approved final version of manuscript; A.J.M., G.P.R., O.L., M.A.K., S.M.D., L.R.L., and T.L.C. edited and revised manuscript; L.R.L. and T.L.C. conceived and designed research.

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