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14. ABSTRACT
Most significant findings: 1)Responses to exertional heat stroke (EHS) are contrastingly different between male vs. female mice. Female mice withstand greater overall heat load and elevated exercise performance. 2)Males and females exhibit long term effects on the heart. Females appear particularly affected. At 9-14 days, post EHS, females exhibit signs of myocardial injury and inflammation.3) Mice exhibit epigenetic and phenotypic changes to their inflammatory cells and skeletal muscle at 30 days with reduced capacity to withstand a second EHS. These data demonstrate the potential for long term consequences of EHS exposure that may have been previously unrecognized. 4)Ingestion of high doses of ibuprofen over 48 hours prior to EHS result in increases in performance during heat exposure in male mice with no further damage seen to the intestines in either sex with ibuprofen. 5) The H2S-containing nonsteroidal anti-inflammatory drugs tested protected the intestines of both male and female mice during EHS, suggesting a potential GI application for military personnel.

15. SUBJECT TERMS
Sex differences, exertional heat stroke, multi-organ injury, heat stress, metabolic hormones, metabolomics, non-steroidal anti-inflammatory drugs.

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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 most recent Medical Military Surveillance Report, April, 2019 (8), there were 578 cases of heat stroke (largely EHS) and 2,214 cases of heat exhaustion reported among active service members. The rate of total heat illness in new military recruits is 12.55 per thousand. The rate in the US Army overall, is 3.67 per thousand. 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 in females remains lower than the incidence rate in males.

The Military needs solutions to 1) determine when warfighters are fit to return to duty without further risk of EHS or other complications and 2) to determine if there are long-term consequences of EHS that can be identified and prevented. 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. The aim of this work was 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 four years we have completed all of these aims and have begun to explore new avenues of research that emerged during this funding cycle that continue to address the aims.**

2. KEYWORDS

Sex differences, exertional heat stroke, multi-organ injury, metabolic hormones, NSAIDs, biomarkers, hydrogen sulfide, ibuprofen, diclofenac, naproxen, metabolomics, return-to-duty

3. ACCOMPLISHMENTS

What were the major goals of the project? (Stated from the original grant application)

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 addition, we have followed a new line of inquiry looking at epigenetic biomarkers of EHS exposure. Note: summary of the results of this objective are added to the next objective, as they go together)

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 analyses and initial reports of metabolomics and 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 A) (1)**. Male and female samples have been evaluated for metabolomics, metabolic hormone analysis and lipidomics (Appendix B), recently accepted for publication.**

KEY OUTCOMES:

1. Significant differences in performance in the heat were observed between male and female mice. These data were published in the Journal of Applied Physiology in 2018 by Garcia et al. that can be found in the appendix 1 (1).

Fig 1. Differences in exercise performance in the heat between young male and female mice From Garcia et al, 2018.

In brief, as shown in Fig. 1, females reached 1) ~80% greater running distances in the heat, 2) ~41% greater running speeds, 3) experienced ~50% greater heat loads, 4) ~56% higher levels of the stress hormone, corticosterone, and 5) lost 23% more body weight during the EHS exposure. However, both sexes terminated exercise at the nearly identical max core temperatures (Tc,max ~ =42.2°C. Marked plasma cytokine responses were seen immediately following heat stroke and were roughly

similar in both sexes with a predominance of IL-6, CXCL1 and some other chemokines. The responses of both males and females were mathematically modeled to be highly dependent upon sex, body surface area and exercise performance, as discussed in the next section.

The take home message for the military is that there were marked differences in male and female performance in the heat. These may reflect the species studied and the extent to which they carry over to humans is unknown at this time. Future studies should be directed towards understanding the underlying mechanisms for the sex-dependent response differences.

2. **Metabolomic and lipidomic responses in the blood and heart to EHS in male and female mice.**

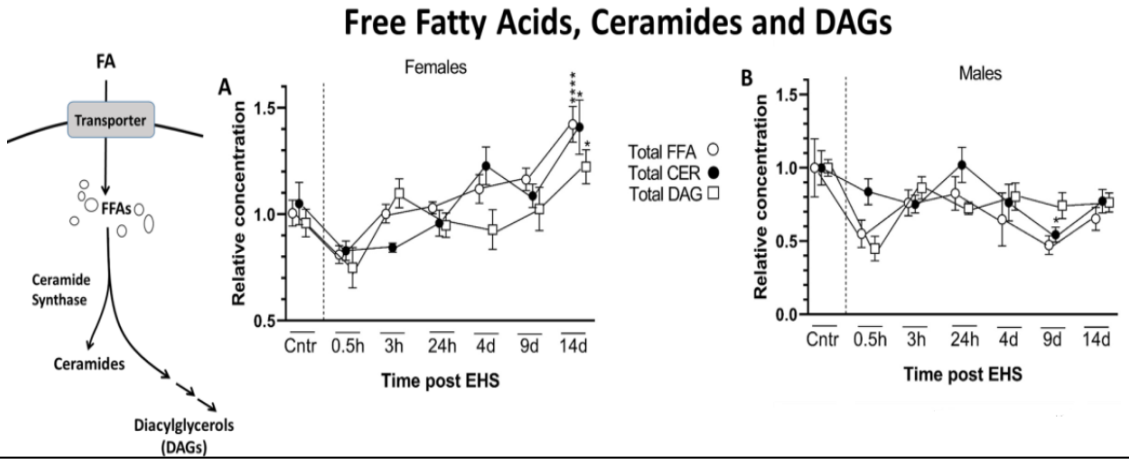

Fig 2. Fatty acid, ceramide and diaglycerol (DAG) accumulation in the myocardium of (A) female mice. (B). No changes were seen in male mice, from Laitano et al. 2019. FAA, Ceramide and DAG accumulation are characteristics of many types of myocardial disorders, including heart failure

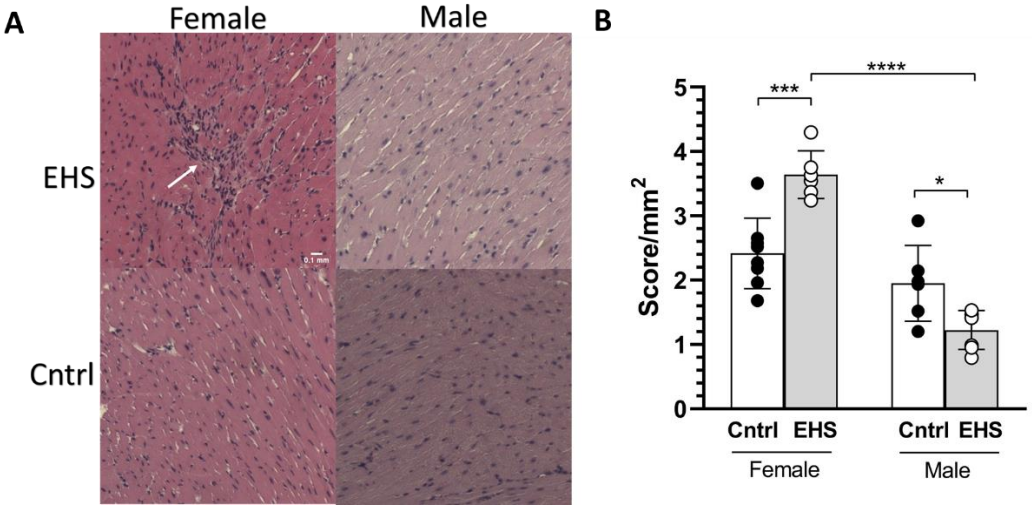

Fig 3. Accumulation of inflammatory cells in the myocardium of female mice but not male mice, 14 days after EHS. From Laitano et al. 2019. In B, entire cross sections of the ventricular myocardium in males and females were scored for incidence of localized inflammation. No significant inflammation was seen in EHS males.

The data obtained from the metabolomics component of this aim have been accepted with minor revision to the Journal of Physiology, (London) in a manuscript entitled, Laitano et al. "Delayed metabolic dysfunction in myocardium following exertional heat stroke in mice" (3) which can be found in Appendix B. Some of the more striking findings are shown below and are further developed later in the text.

For the results of the metabolomics study, in brief, in the first 24 hours, post EHS, both male and female blood exhibited what we have termed, an EHS-induced "energy crisis" characterized by reductions in blood glucose, reductions in plasma insulin, reductions in mobilized amino acids and delayed elevations in acylcarnitines indicating increased beta-oxidation of lipids. This pattern is characteristic of conditions of acute starvation in all animal models and in humans. By 24 hours these changes returned to near normal in both sexes. In the ventricular myocardium, similar changes were seen acutely in the intracellular compartment during this energy crisis period in both males and females. However, there was also evidence of oxidative stress during this period, particularly in females. In the female myocardium, starting at approximately day 9-14 of recovery, a progressive accumulation of fatty acids, ceramides and diacylglycerols was seen (See Fig 2A), all of which are considered evidence for development of a number of life-threatening myocardial disorders such as heart failure and coronary artery disease. Other abnormalities in female hearts during this late recovery time included disorders of glycolysis, bottlenecks within the tricarboxylic acid cycle in the mitochondria and disordered pyrimidine/ purine metabolism. Histologic evaluation of female hearts demonstrated increased incidence of inflammatory cell infiltration throughout the ventricular myocardium, as shown in Fig. 3. These data are discussed in further detail, later in the text and are in the manuscript in Appendix B.

The only changes observed in males were marked reductions in myocardial acylcarnitines and simultaneous elevations in acetylcarnitine. This response is more typical of elevations in beta oxidation of lipid substrates as might occur as a consequence of aerobic conditioning of some type of adaptation. The differences between males and females were striking.

These results raise some concern for the Military whether exposure to EHS may have longer term consequences in the heart, which may go unrecognized. This falls in line with epidemiological studies of victims of heat stroke over subsequent 14 years where the incidence of a cardiovascular incident is some 2 to 4 times higher than matched control populations (6, 7). It also may underlie the difficulty some Warfighters have in return to duty after only a few weeks of recovery from EHS, even when they appear to have fully recovered. The key to future work will be to develop non-invasive biomarkers that identify the underlying problem in at-risk Warfighters.

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.**

PROGRESS: All studies originally planned in males and females for the effects of Ibuprofen on EHS have been completed. A manuscript was submitted to Medicine Science in Sports and Exercise and is in revision for minor recommended revisions. See appendix C.

KEY OUTCOMES:

In brief, Ibuprofen (IBU) treatment, when given for 48 hours in chow prior to EHS exposure, resulted in an improved exercise performance in male mice (similar trends in females), resulting in a longer duration of exercise > 41°C ($P < 0.05$) and a greater peak core temperature, Tc,max ($P < 0.01$) , Fig 4 (2). We hypothesize that this outcome reflects the previously observed impact of NSAIDs (that can cross the BB barrier) on endurance exercise and heat accumulation during exercise in hot environments. This could have implications for the Military in terms of how NSAIDs are given to individuals at risk of EHS. There was little or no effect on females…but we attribute this largely to large PGE2 levels at rest and also the fact that the females reached Tc,max almost 2.0 h later in time. This is a point in time in which IBU effectiveness may be lost because of the limited half-life of the drug after ingestion.

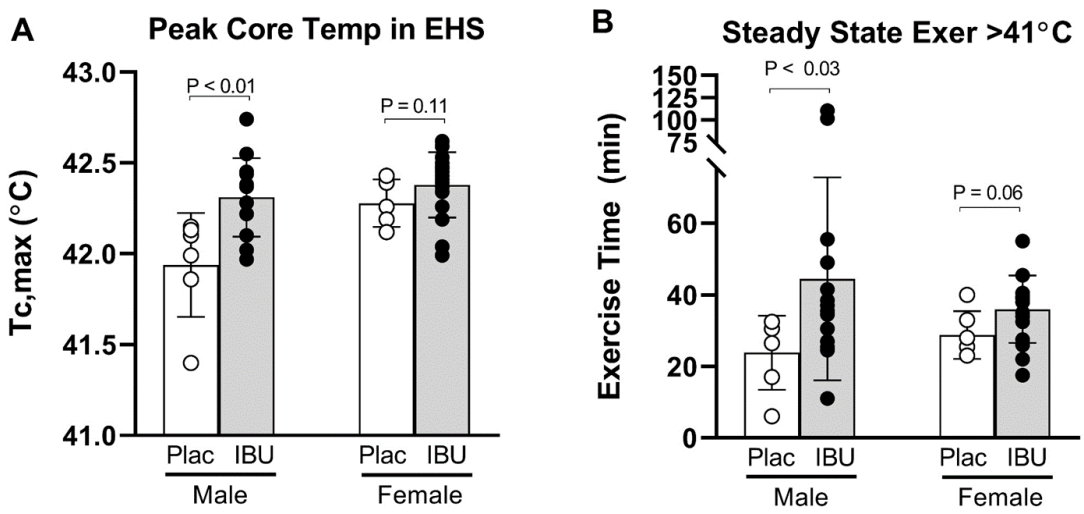

Fig. 4. Effects of ibuprofen (IBU) ingestion in male and female mice on a) max core temperature, Tc,max and b) the duration of steady state exercise in the heat. (*From Garcia et al. MSSE, under*

Though EHS elevated fatty acid binding protein-2 (FABP2) in the plasma (a biomarker for gut intestinal injury) IBU did not further elevate FABP2 in either sex suggesting no further intestinal injury. Though IBU alone caused apparent histological injury to the small intestine in both males and females (throughout all areas), for EHS exposed animals, there was no further injury, over and above the EHS in either sex (Fig. 5). In males, IBU treatment also reduced the loss of white blood cells due to exercise (seen in exercising controls) but this had no impact in EHS, where white blood cell counts plummeted in all groups. IBU treatment did reduce the % lymphocytes in the blood during EHS. However, there was no effect in females.

These data suggest that the ingestion of IBUPROFEN prior to EHS exposure is unlikely to complicate the progression toward heat stroke or the degree of organ injury. When taken with food, as recommended and as studied in this model, the consequences may not warrant great concern for the Military. How much this translates to the human Warfighter who often takes high doses of NSAIDs is something we cannot entirely predict at this time, but our data is consistent with little or no effect of IBUPROFEN on tissue injury during heat exposure.

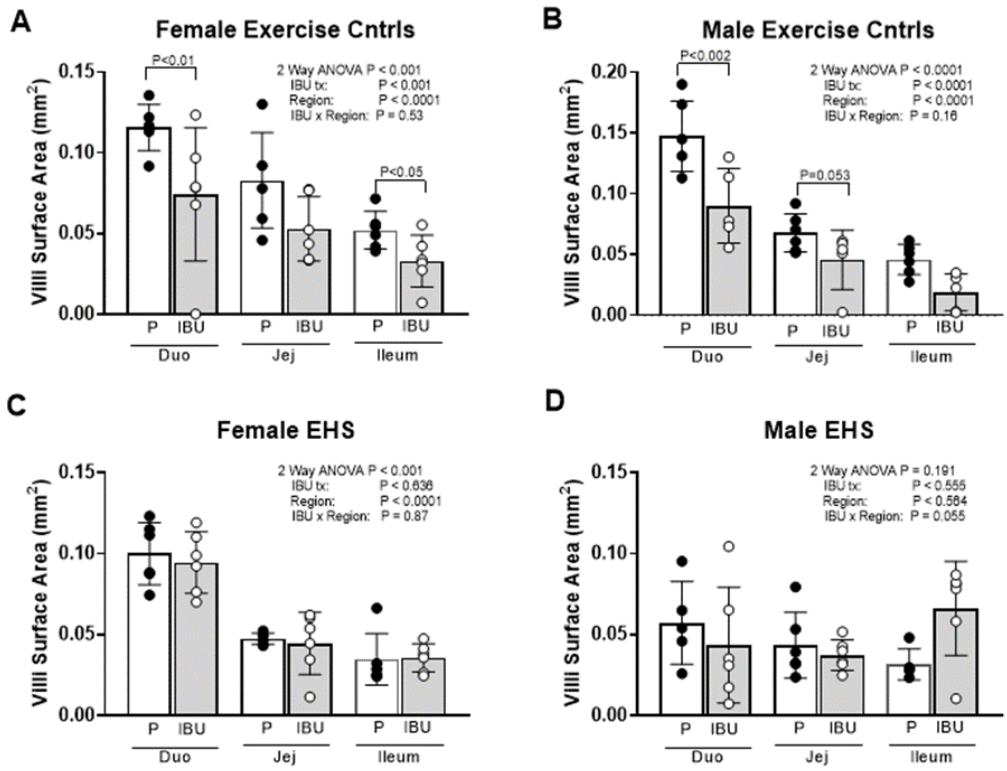

Fig. 5. Villi Surface area (an indicator of small intestinal injury) in response to EHS and exercise control, with or without Ibuprofen ingestion. Note, that IBUPROFEN in matched controls caused a reduction in villi surface area, and thus intestinal injury and hyperthermia caused a reduction in surface area, there was no interaction between IBUPROFEN ingestion and hypothermia on injury.

Year 3- 7 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**.

PROGRESS: All studies on ATB-337 and diclofenac that were originally planned in males have been completed. The results have been analyzed and are in preparation for publication with the data from the last series of experiments, as described below.

Year 3 Last 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.**

PROGRESS: All studies on ATB-346 and naproxen that were originally planned in males have been completed. The results have been largely analyzed and are in preparation for publication with the data from the preceding series of experiments on diclofenac.

KEY OUTCOMES:

Unlike Ibuprofen, discussed above, diclofenac, ATB-337 (diclofenac-H_2S derivative), naproxen, and ATB-346 (naproxen- H_2S derivative) had no significant effects on performance in the heat in male mice. They all ran for the same lengths of time and reached equivalent Tc,max temperatures. This may be due to the fact that none of these drugs appreciably crosses the BB-barrier, unlike Ibuprofen, studied in the last series,

Diclofenac, ATB-337, naproxen and ATB-346 had somewhat diverse effects on intestinal injury during EHS. Overall, neither diclofenac nor ATB346 greatly amplified intestinal damage cause by EHS. In fact damage scores in the duodenum (the location of the majority of injury in heat stroke, were significantly protected by ATB337. Drug treatment also significantly reduced crypt depth (Fig. 6A), which is an indicator of preservation of villi height (not shown). Interestingly, ATB337 (H_2S-donor) treatment resulted in a significantly elevated biomarkers for intestinal injury, i.e. FABP2, which is inconsistent with the histological measurements of injury measured in the same animals.

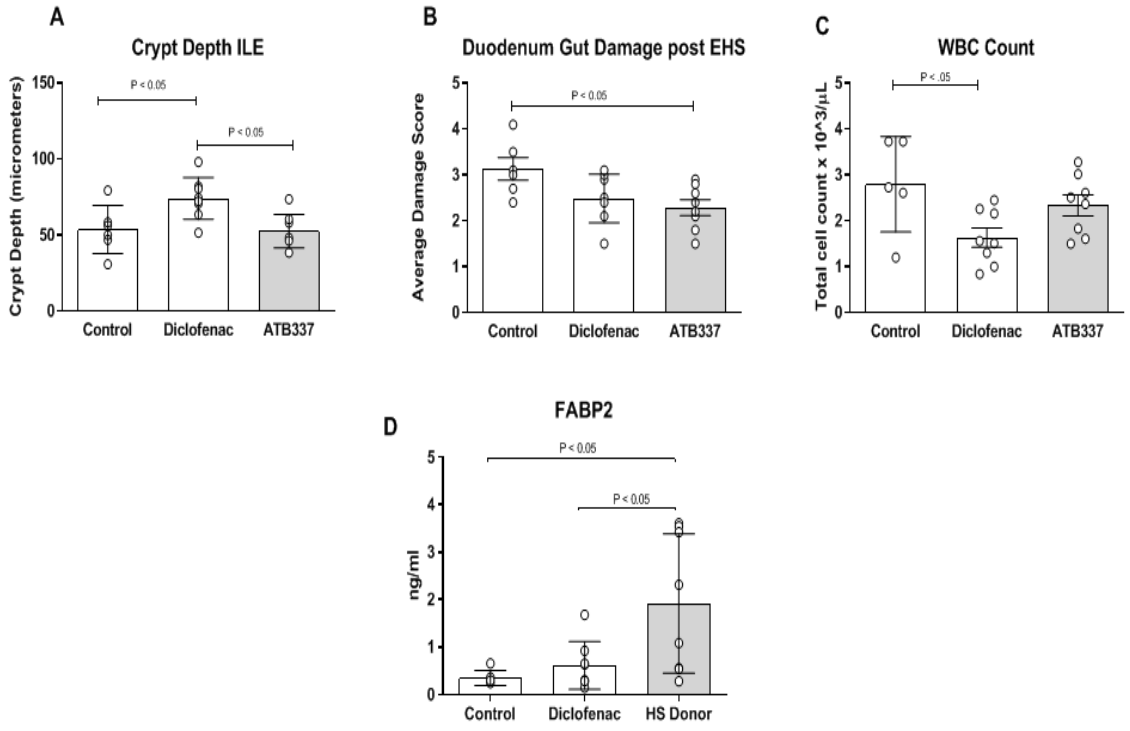

Figure 6. Some of the more interesting differences observed on the effects of diclofenac and the effects of the diclofenac derivative, ATB337. See text for details.

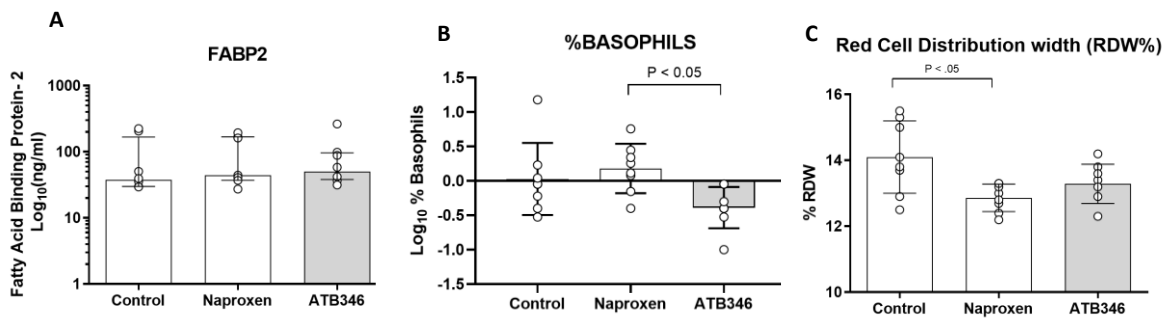

Fig. 7. Effects of naproxen and its H2S-containing analog, ATB346 on A) FABP2, a biomarker for intestinal injury, B) % Basophils and C) Red Cell Distribution width, which measures the O2 carrying capacity of the blood. B and C were the only differences in response to these drugs and we have interpreted this as evidence that the original over-the counter drugs are not harmful in heat stroke and that there is no particular benefit to the H_2S derivatives.

The direct comparison of naproxen with ATB 346 suggest some additional details. Effects of both naproxen and ATB-346 were almost undetectable compared to EHS controls. The only two measurements that came out significantly different were % basophils in the blood and % red blood cell distribution widths (Fig. 7), the meaning of which is uncertain. The measurements of the intestinal histology are still pending, but we do not expect major impacts of these drugs on the intestinal lining.

Overall, these results are consistent with little or no influence of either diclofenac or naproxen on gastrointestinal injury during or after exertional heat stroke. Furthermore there is little evidence for substantial improvements in outcome using the new NSAID-H_2S derivatives that are currently in phase III clinical trials in the civilian population. The data do not support further investment by the military I this alternative NSAID class of drugs if the only goal is to mitigate GI injury during EHS. However, they may be of some value when very high doses are administered where previous studies have demonstrated protection.

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

Overall Summary of what was accomplished under these goals?

The following practical conclusions are summarized below 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 within the female sex (or exposure to female hormones) that makes females more susceptible to exertional 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 but also developing a maladaptation to EHS. From a translational viewpoint, would adjusting the diet provide a performance or safety advantage to Warfighters in hot environments? We hope to pursue this idea further in the coming years.

- 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. This topic was developed in this grant cycle and is the main theme of a new grant cycle that was recently funded.

- 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 and naproxen treatment did not greatly elevate damage to the intestinal lining during EHS or to any other organ system. Unexpectantly, the addition of the H_2S moiety to diclofenac appeared to worsen the intestinal damage in the diclofenac derivative but not the naproxen derivative. In addition, there the H_2S-derivative may have caused additional damage to the liver. Though there were no findings in the naproxen-H2S studies that suggest that this is a general characteristic of this category of drugs, the overall findings thus far are not supportive of any specific advantage of the H2S-containing NASIDs for the Military.

ADDITIONAL Highlighted findings:

This section provides more detail and depth to the overall findings discussed above.

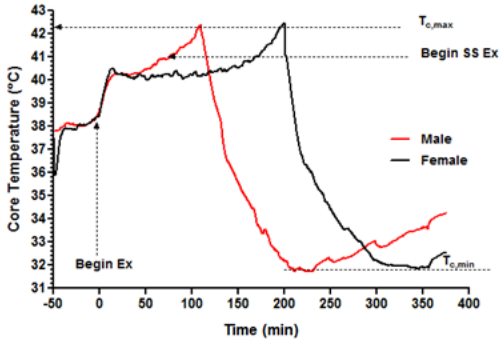

Figure 8. Typical core temperature profiles of male (red and female mice during EHS trials. (Garcia et al. 2018)

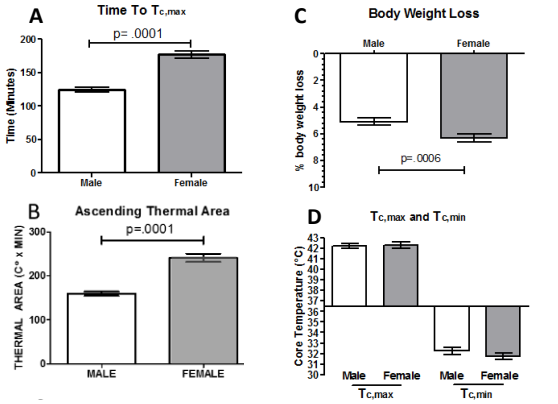

Fig. 9. Comparisons of ♂ & ♀mice of A) running time in the heat, B) thermal load, C) percent loss of body weight, and D) max and min core temperatures. Data are Means +/- SEM. (Garcia et al. 2018).

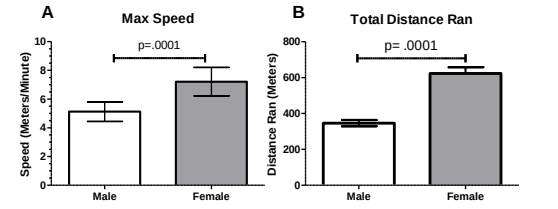

Fig. 10. Maximum speed achieved (A) and the total distance run in the heat in male and female mice. Means +/- SEM (Garcia et al, 2018)

Temperature profile to EHS in ♂ & ♀ mice

Females exhibited a significant resistance to EHS compared to age-matched male mice. Figure 8 shows a typical example of this phenomenon in two representative mice. Note most of these results can be find in the Appendix material in the published manuscript (1). The results are grouped in Fig 9. for all mice (n = 44 per sex). As shown, female mice ran, on average, ~43% longer in the heat than male mice, without losing consciousness. They were also exposed to significantly higher overall heat loads because they ran longer in the heat (Fig. 9B, ascending thermal area). They exhibited a greater apparent fractional loss in body fluid, based on % body weight loss (Fig. 9C). 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. 9D. 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) but this is the first time that females have been studied.

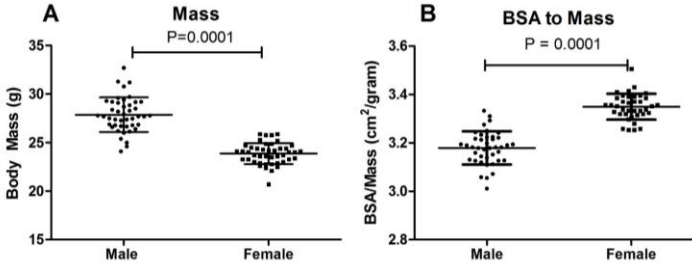

Fig. 11. Raw distributions showing **A)** The expected elevation in body mass of ♂ & ♀ mice, 20-24 weeks of age, and **B)** the calculated body surface area to mass ratio, showing that females had a greater surface area for heat dissipation.

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. 10A) resulting in a total distance in the heat ~80% further than the comparable males (Fig. 10B). This is supportive of a greater aerobic capacity in the females of this strain of mice, which was expected based on previous survey literature.

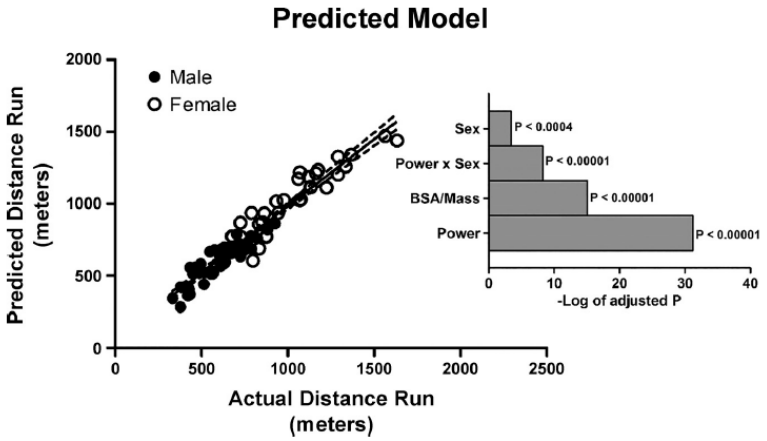

Fig 12. Statistical modeling of physical determinants of differences in exercise performance in the heat between ♂s & ♀s. Both 'BSA/mass' ratio and 'Power' accounted for much of the variance in the distance run in the heat in both ♂s & ♀s. Mass is closely related to BSA/M ratio and demonstrates that 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. 11 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 12. 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 either power and BSA/m alone (Figure 12).

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

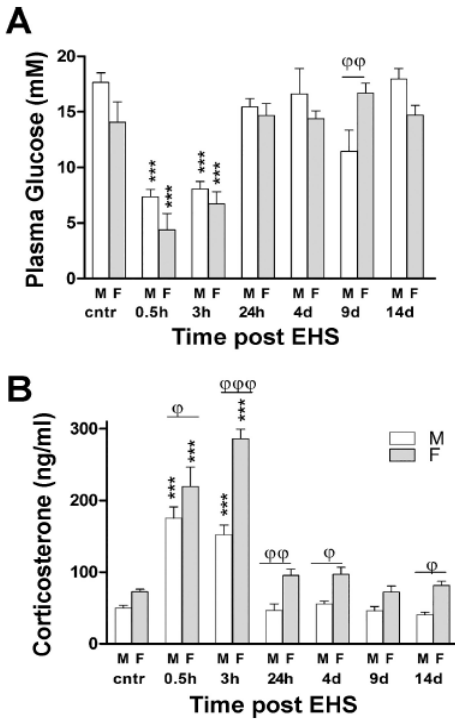

Fig. 13. Plasma levels of A) Glucose and B) corticosterone in sham control (cntrl) and during recovery from EHS in males and females * P < 0.05, ** <0.01 differences from cntrl. φ P <0.05, φφ P<0.01 between males and females at each time point.

independent influence of sex on heat tolerance and work capacity that favored the ability of females to withstand the heat.

Differences in regulation of metabolic hormones in ♂ & ♀ mice following EHS.

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, glucose levels were higher than predicted in both groups between 1-14 days, as well as in the 4 d control animals (Fig 13A). *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 form 0.5 h to 3 h time points (Fig 13B). Throughout recovery, females exhibited higher corticosterone levels than males. Between 3h to 24 hr, the corticosterone was nearly double in females compared to males. These data demonstrate that females have a more robust glucocorticoid response to EHS than males.

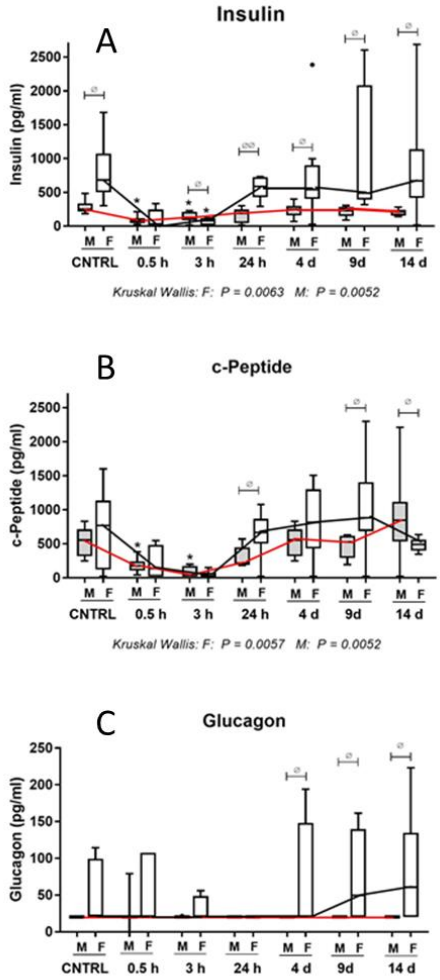

Figure 14. Plasma pancreatic enzyme responses during recovery from EHS. Red lines connect the medians in male, black lines are females. During 0.5-3 hr recovery all hormones were suppressed. Following EHS females demonstrated significantly higher values at most time points. θ = difference between male/female; * = difference from cntr.

Other metabolic hormones were evaluated using Luminex multiplex technology (metabolic hormone panel). These results are displayed in figure 1. Metabolic hormones arising from the pancreas, 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 14 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 15 illustrates the responses of two metabolic hormones generally attributed to secretion of 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 (Fig 15A & B). 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 or 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).

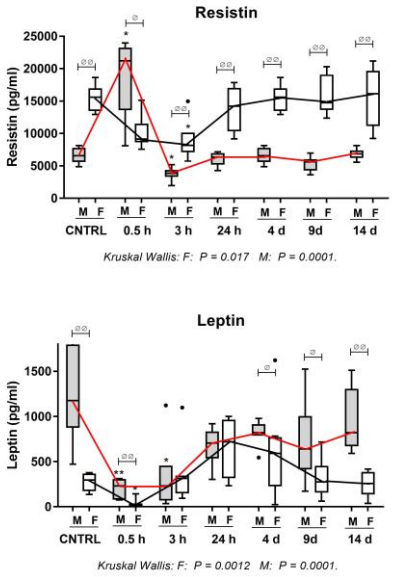

Figure 15. Adipose derived metabolic hormones, A) Resistin and B) Leptin during the recovery period from EHS.

Development of myocardial vascular abnormalities in female mice during late recovery from EHS: contrasting effects in males.

We performed extensive metabolomic studies of male and female hearts of the recovery period through collaboration with individuals at the USACHER, USARIEM and staff at Metabolon, Inc. This has resulted in a major publication, which is in 2nd review for the Journal of Physiology, Laitano et al. (3). Major disorders of metabolism were observed from Glycolysis (Fig. 16), alternative carbohydrate metabolic pathways (Fig. 17), the citric acid (TCA) cycle (Fig 18), beta-oxidation (Fig. 19), and pyrimidine and purine metabolism (Fig. 20 and 21), coupled with indicators of oxidative stress and myocardial injury (Fig. 22). ween 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).

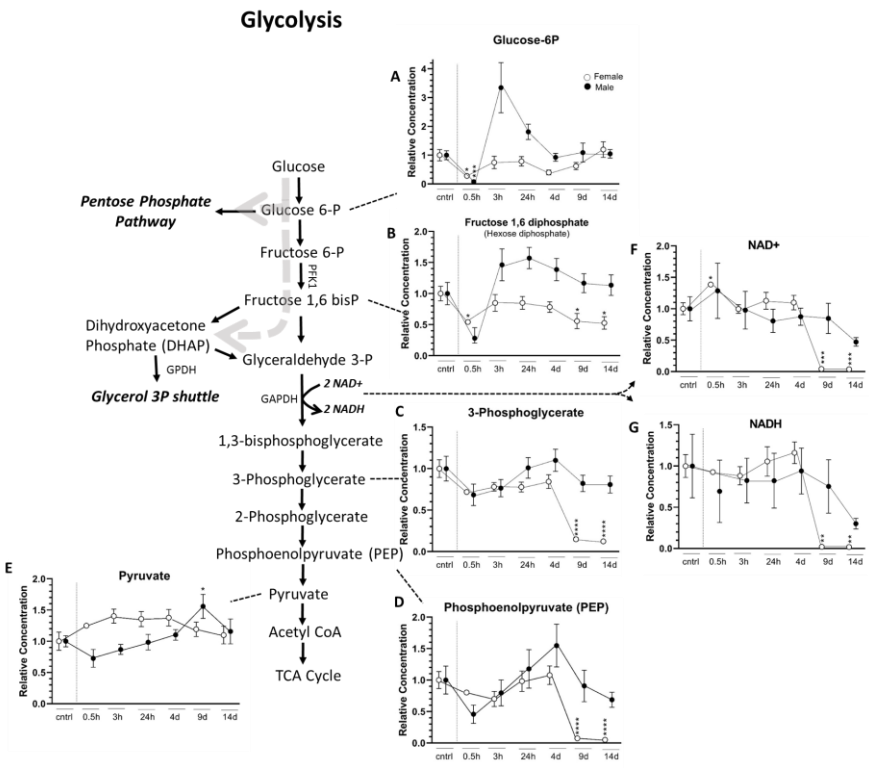

Figure 16. Changes in classic glycolysis metabolites in male and female mice during 14 days of recovery. By 9 days of recovery females exhibited a striking reduction in products downstream of glyceraldehyde 3-Phosphtae, including 3-Phosphoglycerate, phosphoenolypyruvate (PED) and NADH-NAD). Carbon substrates appear to be shuttling to alternative pathways (grey arrows), the Pentose Phosphate pathway and Glycerol 3-P shuttle, both described in the next figure.

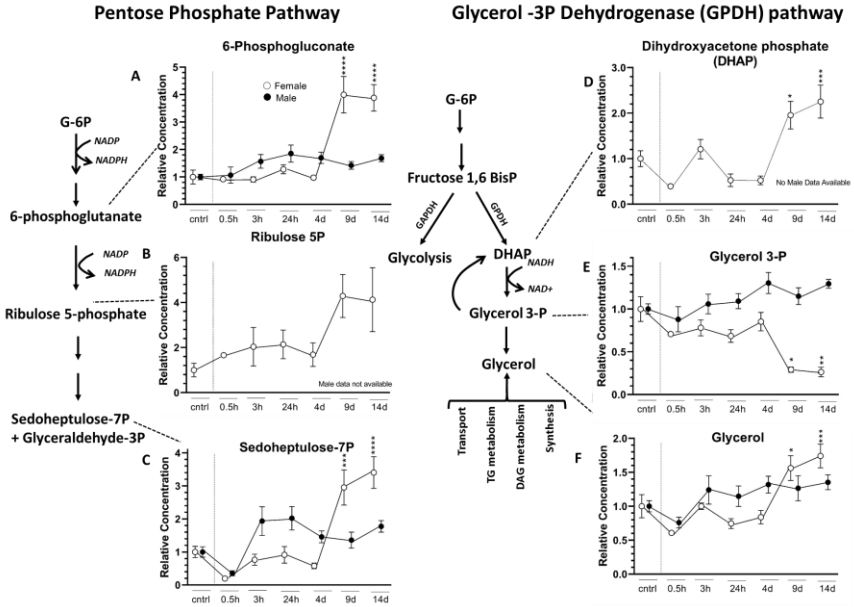

Figure 17. Changes in the alternative pathways of glucose metabolism in male and female hearts during recovery from EHS. The data demonstrates that females are shuttling carbon substrates from the classic glycolytic pathway to the Pentose pathway and the Glycerol=3P shuttle. Note that a bottleneck in the normal metabolic pathway occurs at dihydroacetate 3-phosphate. The probably cause of this is electron donation to the mitochondrial by DHAP, bypassing much of the TCA cycle.

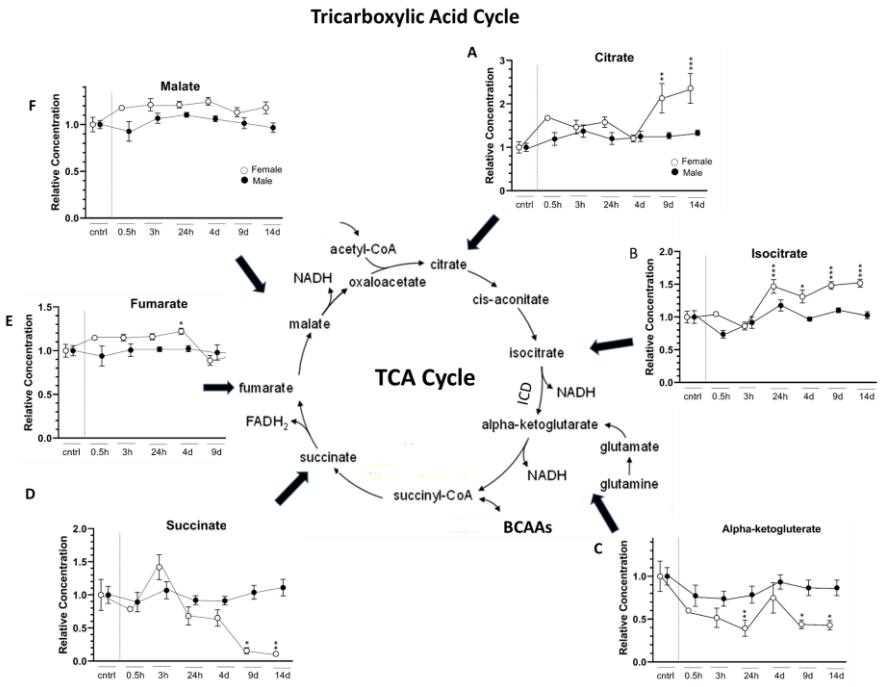

Figure 18. Changes in the TCA cycle (citric acid cycle) during recovery from EHS in male and female mice. Note the accumulation of citrate and isocitrate, the apparent bottleneck at isocitrate dehydrogenase, resulting in reductions in alpha-ketoglutarate and succinate. This suggests marked disorders in the flow of carbon substrates through TCA in female mice at 9-14 days.

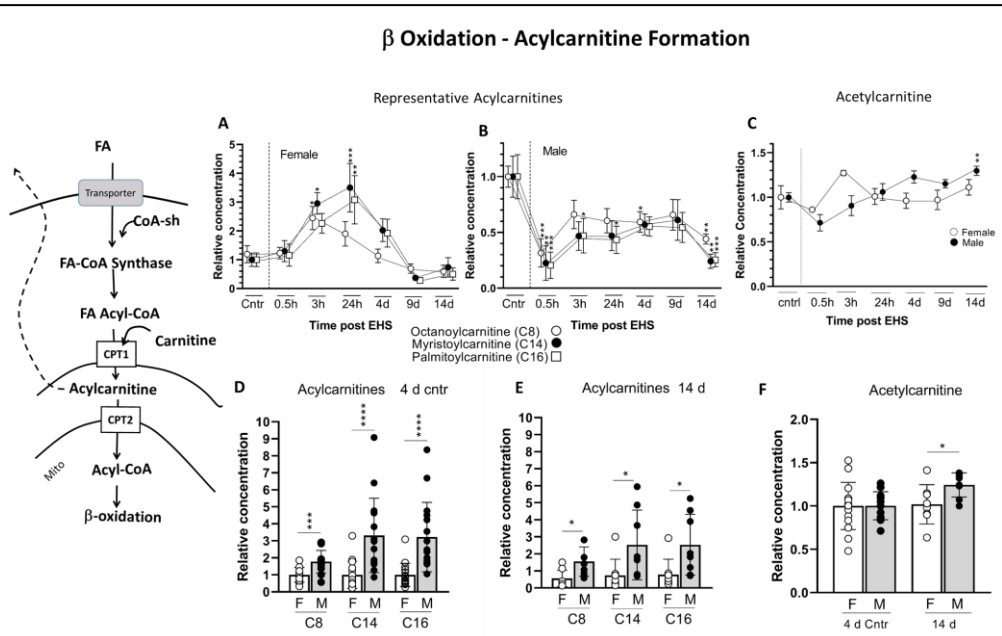

Figure 19. Changes myocardial beta oxidation in male and female mice. Elevations in acylcarnitines (A & B) suggest increased shuttling of fatty acids into cells or blockages in acylcarnitine metabolism by beta oxidation. In the transition to recovery males and females have striking differences in acylcarnitine accumulation, by 14 days, both sexes have reduced acylcarnitines, particularly significant in females. Despite these changes, males actually have greater total acylcarnitines at the 14 day time point.

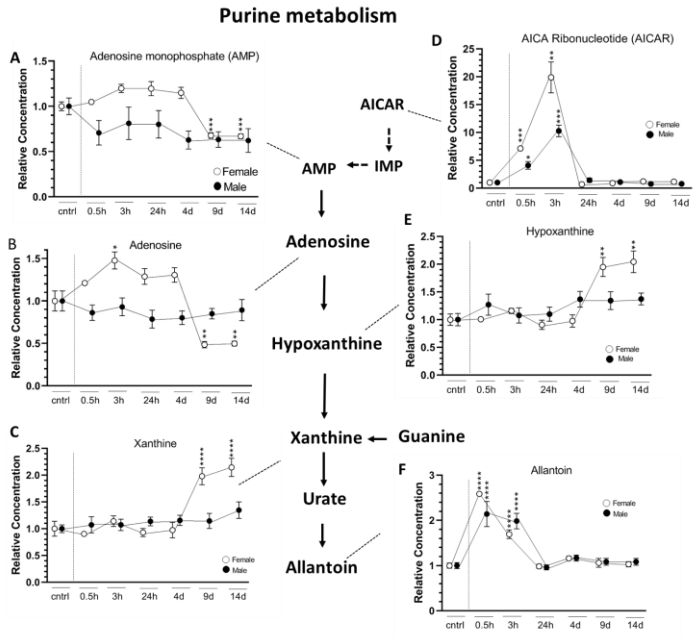

Figure 20. Changes in purine metabolism in male and female myocardial tissue during recovery from EHS. Note the general loss of adenosine metabolites and elevations in adenosine metabolic products. This loss of adenosine may be partially responsible for the overall loss of NAD metabolites shown in Fig. 16. Note the elevation in AICAR, a natural product that stimulates AMPK in muscle.

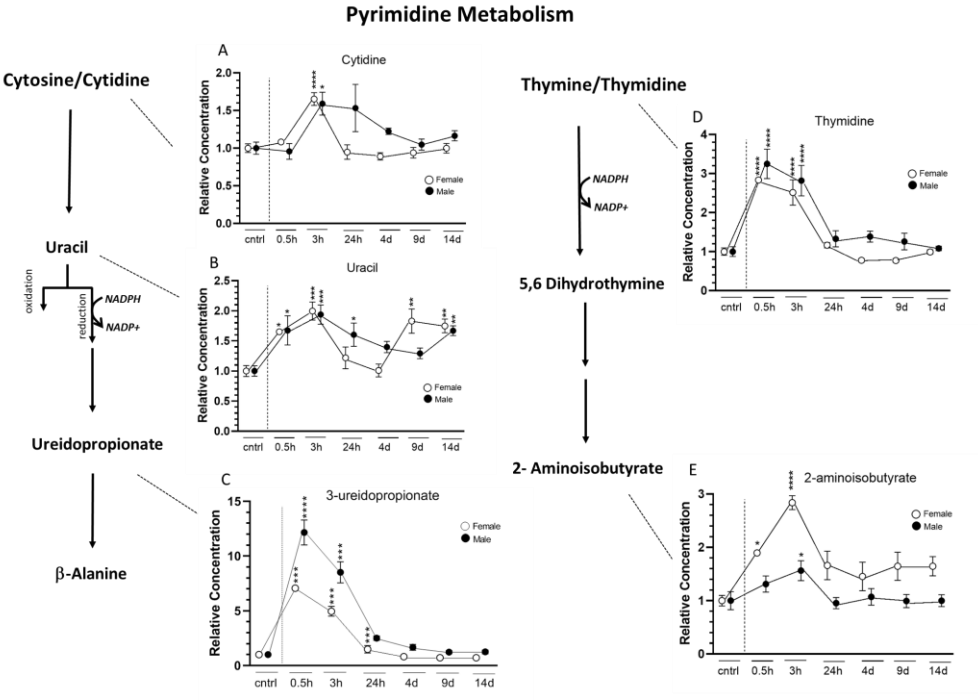

Figure 21. Changes in pyrimidine metabolism post EHS in male and female myocardium. Note the transient elevation in metabolites of cytosine and uracil over the first 24 hours post EHS.

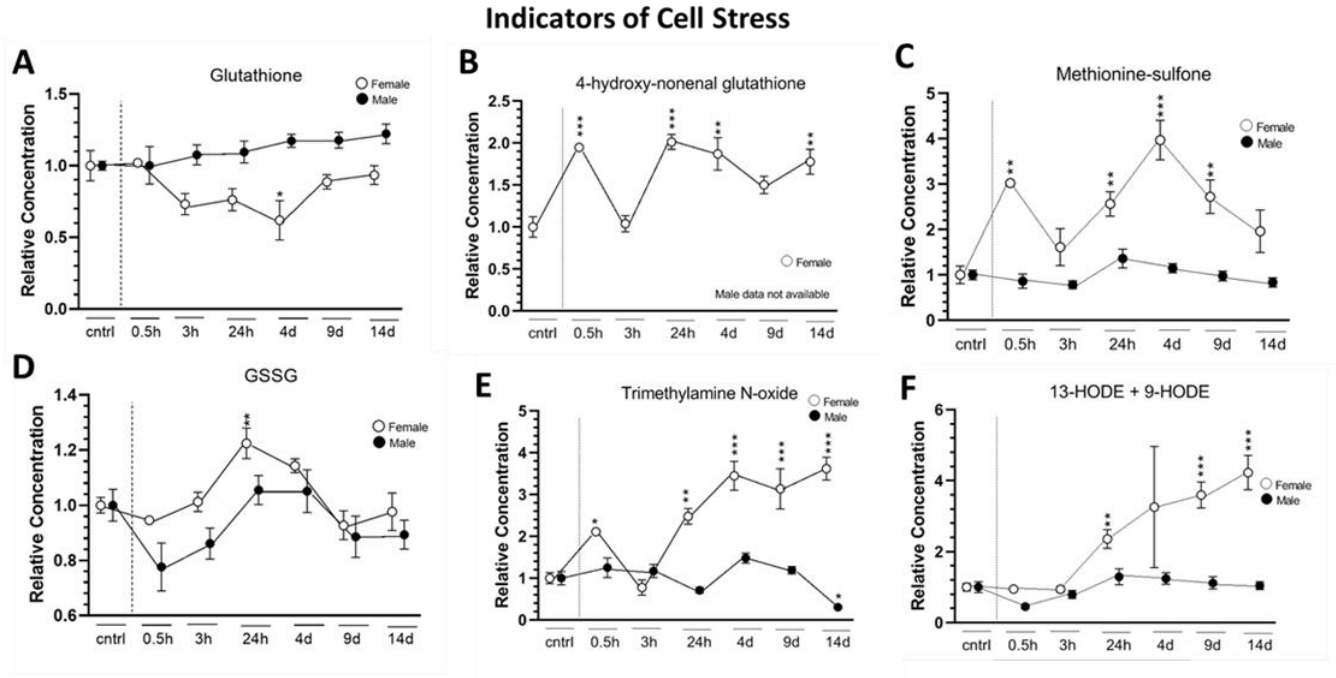

Figure 22 Changes in indicators of oxidative stress or damage in male and female myocardiums during 14 days of recovery from EHS. A. B, C,D and F are indicators of oxidative stress. E, Triethylamine N-oxide is a well known indicator of myocardial injury in heart failure.

In summary, these data demonstrate marked metabolic abnormalities in the myocardium in female hearts that fully develop only after 9-14 days of recovery. As was shown in Fig. 2, these changes are accompanied by large elevations in fatty acid, ceremide and diacylglycerol accumulation in the myocardium which is consistent with many forms of myopathy. We believe these data demonstrate that exposure to EHS can have a long term impact on cardiac metabolic function that may set up an individual for more susceptibility to heat stroke and greater potential for long term cardiovascular complications.

One of the big questions is why the males did not show a similar abnormality. We have two working hypotheses as to this sex difference. First, the females were exposed to a much higher heat load (temperature-time product) because they were able to sustain exercise for longer periods of time prior to collapse; therefore, it may be simply that the female hearts were under greater strain for a longer period of time. We are in the process of testing this hypothesis by developing new models in the males that mimic the heat load and exercise time of females. Our second hypothesis is that it may be related to hormonal differences between males and females. To test this hypothesis, we have experiments planned to study these responses in gonectomized males and females.

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

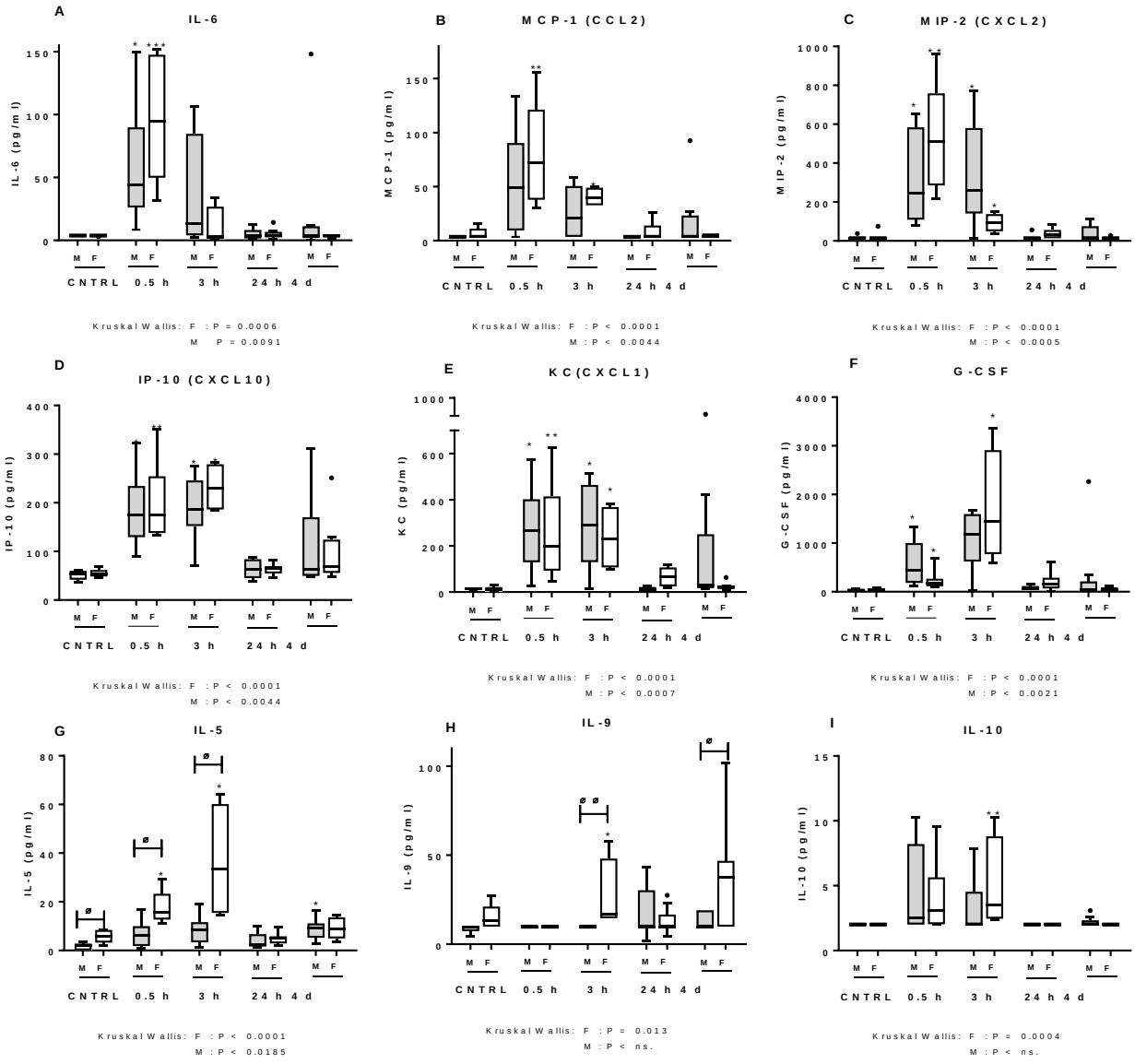

Fig. 22. Plasma cytokine measurements in male and female mice over the course of 0-4 d recovery from EHS. Most cytokines returned to normal or near normal by 4 days post. Data expressed as "whisker plots" because data was nonparametric. The lines represent the median values and the error bars are 95% 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. 22). 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.*

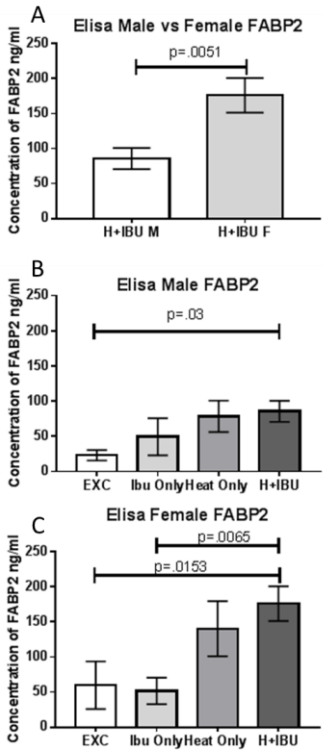

Fig. 23. FAB2 (biomarker for intestinal injury) was significantly increased by EHS and A) was larger in Females vs males. B and C: There were no significant effects of IBU alone in EX control mice, C nor was the response amplified by combined EHS and IBU treatment (Garcia et al. in preparation).

Effects of IBUPROFEN, NAPROXEN, DICLOFENAC and H2S derivatives of Naproxen and Diclofenac on the GI tract and performance during EHS.

We have completed the studies of the effects of ibuprofen (IBU) on EHS performance and gastrointestinal injury in both males and females. The primary data was summarized in Figures 4 and 5. Some additional data is shown in Figures 23 and 24.

In IBU treated mice, the way we administered the IBU, there was little or no evidence of additional damage caused by IBU to a biomarker of intestinal injury (FABP2, i.e. fatty acid binding protein 2) in the blood (Fig 23, 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 (Fig. 23 B and C). 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.

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 activities of IBU in this timeframe and as a result may repeat these experiments using a different delivery system.

As discussed above, there were only minor effects of diclofenac, naproxen and their derivatives on any measurements made during heat stroke. We do not believe there are significant interactions between these drugs and performance or tissue injury in EHS.

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. 24 and 25). 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.

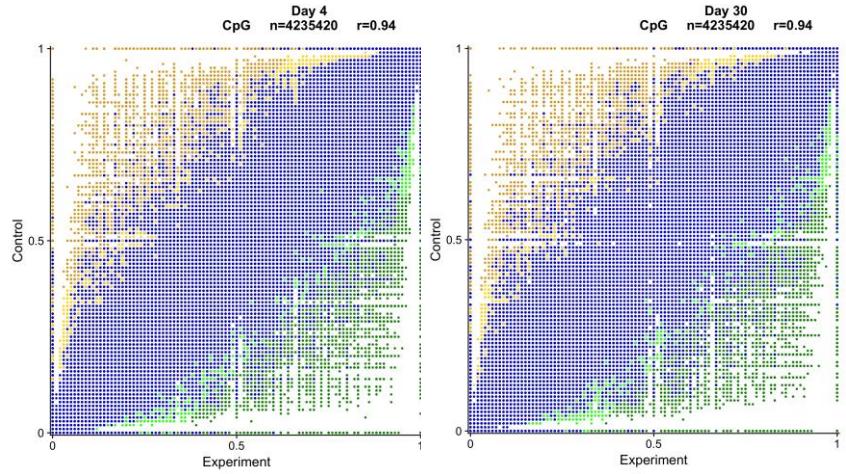

Fig. 24. Summary of statistically significant genes that were hypermethylated or hypomethylated , (LEFT) 4 days and (RIGHT) 30 days after EHS compared to matched exercise control mice at the same time points. The green data are significantly hypermethylated and the amber are significantly hypomethylated compared to controls.

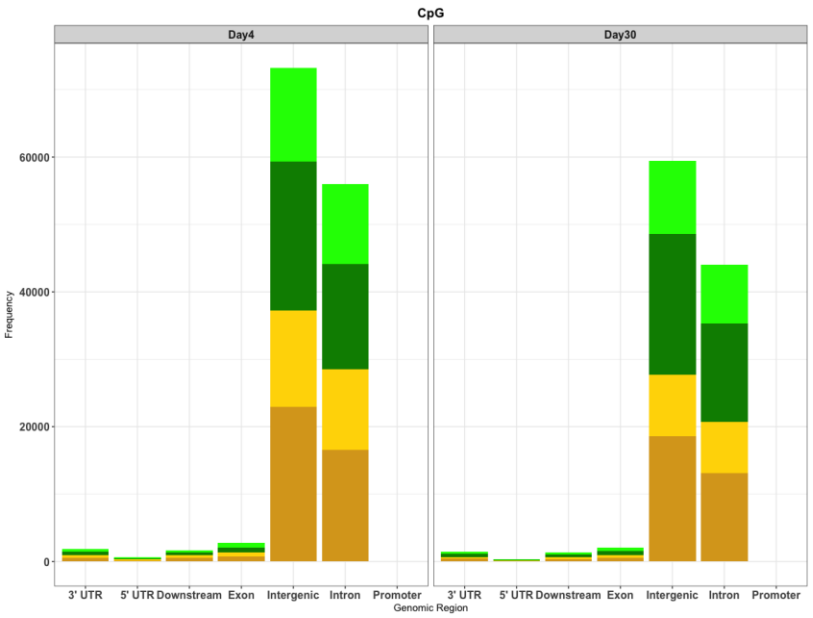

Fig. 25. The genomic topography of DNA methylation changes post EHS compared to matched controls at 4 days (Left) and 30 days (right). Both the 3' and 5'UTR responsible for transcriptional and translational regulation have significant changes. It is important to note that though many of the methylation 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. 26, 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.

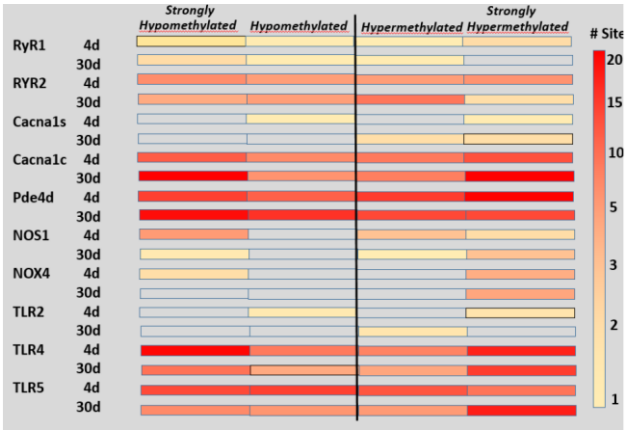

Fig 26. Targeted Hyper and hypomethylated genes of interest with multiple methylation sites (heat map on the right.

These experiments and results were used to apply for a new line of DOD funding, submitted in spring of 2018 and funded in October, 2019 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, 2018 and 2019. Finally, we provided training for our postdoc, Dr. Orlando Laitano who has since been promoted to Research Assistant Professor. He remains 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 has first authored our metabolomics work for this project.

We also have a number of undergraduate students who have been trained during this time, including Laila Sheikh (currently in Physical Therapy School), John Iwaniec (currently working in the laboratory and applying to med school), Lucas De Carvalho (currently enrolled in grad school), Reed Berlet (applying to med school; currently working in the medical field). Current undergrad students working in the lab who were involved in the tail end of these projects include Kevin Cusack and Taylor Doherty. We feel quite proud of all of our undergraduate and graduate trainees who are moving on in their careers to accomplish great things. Many of them have participated in both local and national meetings to present our work to the public. This would not have been possible without DoD support.

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 were 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, 2017, 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 presented the following work at the Experimental Biology Meeting:

  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_supplement 01 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 Ca^{2+} 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, 2018 we 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"**

- In spring of 2019 we presented the following abstracts to the Experimental Biology Meeting in Orlando Florida

Iwaniec, J., Robinson, G.P., Garcia, C.K., Murray, K. Laitano, O. Clanton, T.L. **Acute phase response to Exertional heat stroke in mice.** FASEB J 33(1), LB443 April, 2019

Garcia, KC, Sheikh, LH, Iwaniec, JD Robinson, GP, Berlet RA, Mattingly A, Laitano, O Murray, K and Clanton TL, **Ibuprofen effects on the response to exertional heat stroke in male and female mice** FASEB J 33(1), 842.6 April, 2019

Murray K, Sheikh, LHm Laitano, O, Iwaniec,J, Garcia,C, Robinson, R Hammamieh, R, Campbell, R, Yang ,R and Clanton, TL, **Epigenetic Memory and Phenotype Change Observed in Mouse Skeletal Muscle 30 Days after Exertional Heat Stroke** FASEB J 3Ne3(1): 842.5

Newly Submitted Publications

Garcia, CK. Sheikh, LH, Iwaniec, JD, Robinson GP, Berlet, RA, Mattingly, AJ, Murray, KO, Laitano, O., Clanton, TL **Ibuprofen impacts heat tolerance but not gut injury in exertional heat stroke in mice.** *Currently in 2nd revision for Medicine Science & Sports and Exercise.*

Laitano, O., Garcia, C.K., Mattingly, A.J, Robinson, G.P., Murray, K.O., King, M.A., Ingram, B.,, Ramamoorthy, S., Leon, L.R., Clanton, T.L. **Delayed metabolic dysfunction in myocardium following exertional heat stroke in mice.** *Currently in 3rd revision for Journal of Physiology (London).*

Publications in final stages of preparation.

Iwaniec, JD, Robinson, G.P., Garcia, C.K. Murray, K.O., deCarvalho, L, Clanton, TL, Laitano, O. **Acute phase response to exertional heat stroke in mice.** *In preparation for Experimental Physiology for a January submission.*

Publications in preparation

Robinson, G.P., Garcia, C.K. Murray, K.O., Laitano, O., Clanton, T.L. Effects of H_2S-containing NSAID on gastrointestinal injury during exertional heat stroke in male mice. *Target Journal. J Applied Physiology, target date: March 2020.*

Murray K, Sheikh, LHm Laitano, O, Iwaniec, J, Garcia, C, Robinson, R Hammamieh, R, Campbell, R, Yang,R and Clanton, TL, **Epigenetic Memory and Phenotype Change Observed in Mouse Skeletal Muscle 30 Days after Exertional Heat Stroke** *Target Journal: Journal of Physiology. Target date: March 2020.*

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

This is the final report for the grant. Nothing to report.

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 H_2S-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. However, we believe the danger of NSAID usage in subjects at risk of EHS may be very low. Finally, we believe that the metabolomic results shown in the female mice that only occur after 9-14 days following EHS is transformative research that may provide a window into previously

unrecognized long term impacts of EHS exposure. We now provide evidence that much like other disorders such as traumatic brain injury and concussion, there may be long term health impacts that require yeasts to develop that could sometimes be attributed back to an earlier EHS exposure. We will be continuing these studies in the next funded grant cycle to understand the implications of these findings.

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. The metabolic changes we observe in the heart in female mice, post EHS, resemble the phenomenon known as "stress-induced myocardial myopathy," more often referred to as "Takotsubo cardiomyopathy" (4, 5). This may be an experimental model for this disease and could have a long term impact in the field of cardiology and cardiovascular pathophysiology.

Impact on Technology Transfer: Nothing to report at this time. Although there is a possibility that biomarkers identified during this study, post EHS may emerge as a useful tool for the Military and other EHS-related populations, it is too early at this time to predict this outcome. We believe this will evolve in the next grant cycle.

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 in humans. 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.

It is also possible that the progress made in this study will direct research on treatment and prevention in the other populations susceptible to heat stroke, such as athletes and first responders.

We also believe that if we can substantiate long term consequences of EHS exposure we may be able to provide additional incentives through educational programs to prevent EHS in at risk populations. For example, the long term consequences of head injury or the health of athletes has made a major impact on the way games such as football, soccer and rugby are played. It is improving the precautionary measures that these sports can take to prevent long term bad outcomes.

5. CHANGES OR PROBLEMS

This is our final report. We have stayed within budget.

. **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

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

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

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**

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 MHSRS 2018, Orlando Florida**

- 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*.**

Yes. We have received funding for:

Department of Defense. US Army Medical Research Command Epigenetic markers for susceptibility and recovery from exertional heat stroke. E01 W81XWH1920050
P.I. Clanton Projected: 2019-2022. Start date Oct 1, 2019.

Kingdom of Saudi Arabia, Ministry of Higher Education. We have also received funding to support a new graduate student working on this project and on our new DoD project: Jamal Mohammed H. Alzahrani. He receives tuition and fees plus a stipend of $1886.17 per month ($22,634 per year) to take classes and perform his research in the P.I.s laboratory.

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 most recent contact was 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.

8. Special Reporting Requirements and APPENDIX MATERIAL

1. References
2. Quad Chart for FINAL REPORT
3. Publications

King et al. 2017,

Garcia et al, 2018

Laitano et al. 2019 accepted with minor revision

Garcia et al. 2019 accepted with minor revision

Iwaniec et al, 2020, in process.

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.
2. **Gui Y, Silha JV, and Murphy LJ**. Sexual dimorphism and regulation of resistin, adiponectin, and leptin expression in the mouse. *Obes Res* 12: 1481-1491, 2004.
3. **Hong J, Stubbins RE, Smith RR, Harvey AE, and Nunez NP**. Differential susceptibility to obesity between male, female and ovariectomized female mice. *Nutr J* 8: 11, 2009.
4. **Kaiyala KJ, Ogimoto K, Nelson JT, Muta K, and Morton GJ**. Physiological role for leptin in the control of thermal conductance. *Mol Metab* 5: 892-902, 2016.
5. **King MA, Leon LR, Morse DA, and Clanton TL**. Unique cytokine and chemokine responses to exertional heat stroke in mice. *J Appl Physiol (1985)* 122: 296-306, 2017.
6. **King MA, Leon LR, Mustico DL, Haines JM, and Clanton TL**. Biomarkers of multi-organ injury in a pre-clinical model of exertional heat stroke. *Journal of Applied Physiology accepted with minor revision*: 2015.
7. **Leon LR, DuBose DA, and Mason CW**. Heat stress induces a biphasic thermoregulatory response in mice. *Am J Physiol Regul Integr Comp Physiol* 288: R197-R204, 2005.
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9. **Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, and Rossetti L**. Role of resistin in diet-induced hepatic insulin resistance. *J Clin Invest* 114: 232-239, 2004.
10. **Reed DR, Bachmanov AA, and Tordoff MG**. Forty mouse strain survey of body composition. *Physiol Behav* 91: 593-600, 2007.
11. **Suragani M, Aadinarayana VD, Pinjari AB, Tanneeru K, Guruprasad L, Banerjee S, Pandey S, Chaudhuri TK, and Ehtesham NZ**. Human resistin, a proinflammatory cytokine, shows chaperone-like activity. *Proc Natl Acad Sci U S A* 110: 20467-20472, 2013.
12. **Vadas P, Sinilaite A, and Chaim M**. Cholinergic Urticaria with Anaphylaxis: An Underrecognized Clinical Entity. *J Allergy Clin Immunol Pract* 4: 284-291, 2016.

Prevention of Organ Injury in Exertional Heat Stroke: Preclinical evaluation of a new class of NSAIDs

Log Number: #14267001 FY 19 FINAL REPORT

W81XWH-15-2-0038 BAA Extramural Medical Research

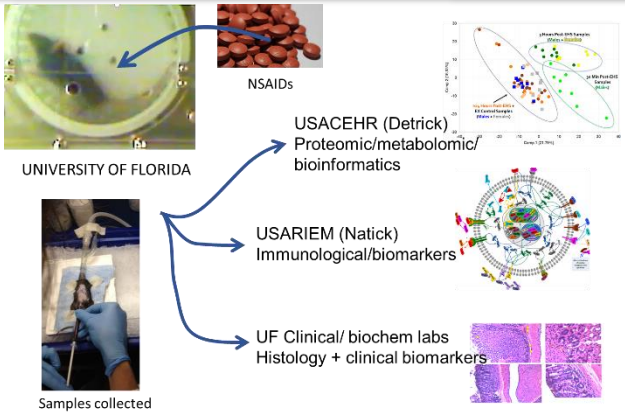
PI: Thomas L Clanton **Org: University of Florida** **Partners: USARIEM, USACEHR**

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: 12/23/2019 balance $0

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

- All experiments have been completed for the aims of the grant.
- We have completed most manuscripts reporting the results of this project. Are still completing the last manuscript on the NSAIDs.

Budget Expenditure to Date

Actual Expenditure: $545,155.

J Appl Physiol 122: 296–306, 2017.
First published December 1, 2016; doi:10.1152/japplphysiol.00667.2016.

RESEARCH ARTICLE

Unique cytokine and chemokine responses to exertional heat stroke in mice

Michelle A. King,[1] Lisa R. Leon,[2] Deborah A. Morse,[1] and Thomas L. Clanton[1]

[1]*Department of Applied Physiology and Kinesiology, College of Health and Human Performance, The University of Florida; and* [2]*Thermal and Mountain Medicine Division, United States Army Research Institute of Environmental Medicine, Natick, Massachusetts*

Submitted 25 July 2016; accepted in final form 28 November 2016

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).

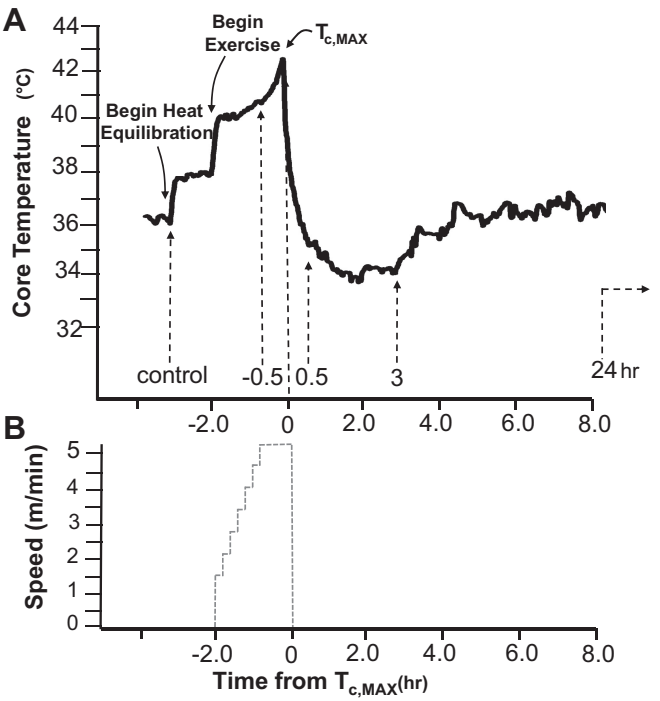

Fig. 1. *A*: typical core temperature (T_c) profile for the exertional heat stroke (EHS) protocol, showing the intervals of blood/tissue collections relative to peak core temp ($T_{c,max}$). *B*: average forced running wheel time course, starting at 2.5 m/min, with 0.3 m/min until 40.5°C and then held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

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

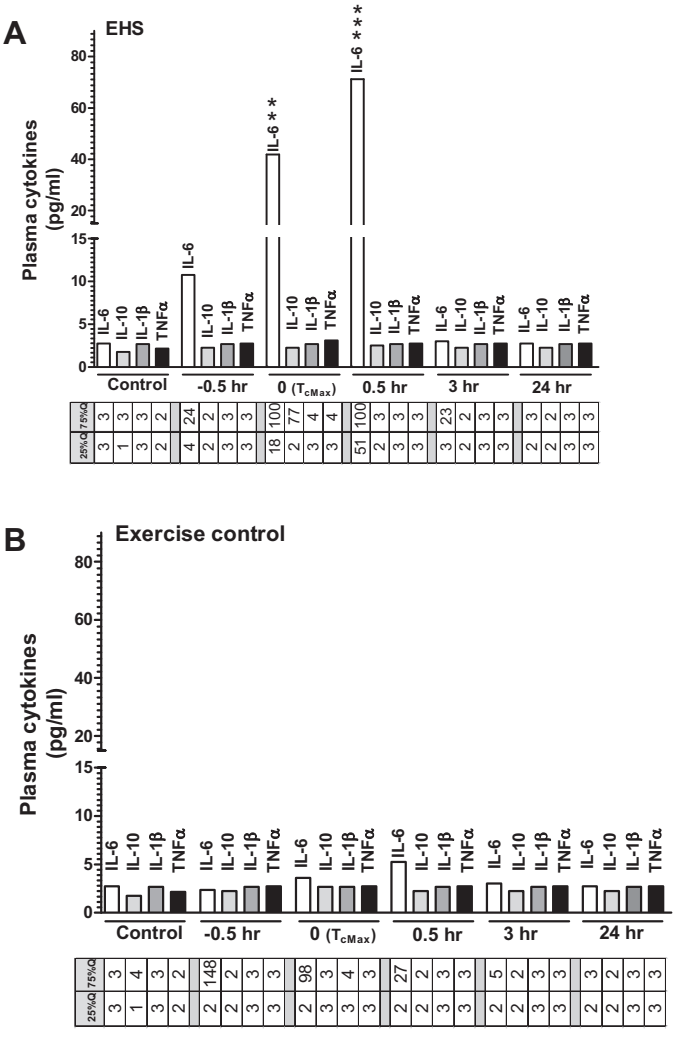

Fig. 2. Effects of EHS on common cytokines of innate immunity. *A*: responses of common innate immune cytokines to EHS. *B*: cytokine responses to sham exercise controls. Significance from naïve control: $P < 0.01$ (**) and 0.001 (**) (post hoc tests). MCP-1, monocyte chemoattractive factor-1; MIP, macrophage 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.

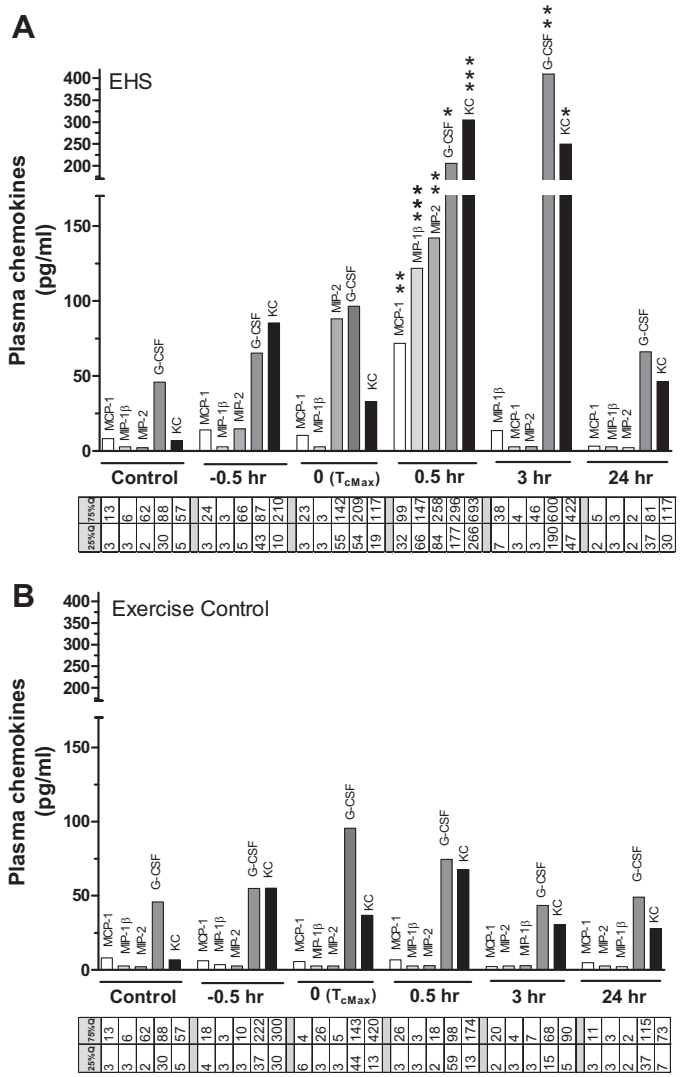

Fig. 3. Effects of EHS on chemokines and related cytokines. *A*: responses during and following EHS. *B*: responses to sham exercise controls. Post hoc significance from naïve control: $P < 0.05$ (*), 0.01 (**), and 0.001 (***) (post hoc tests). Benjamini-Hochberg procedure for multiple ANOVAs = FDR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

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-

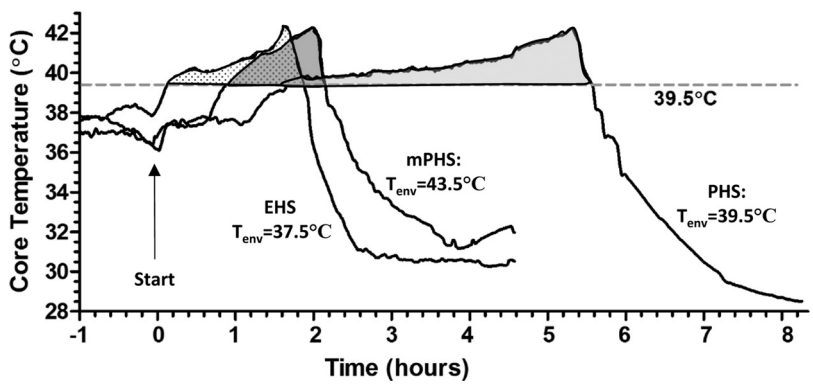

Fig. 4. Typical T_c profiles for EHS, passive heat stroke (PHS), and PHS at thermal area matched to EHS (PHS_m). Shaded areas represent the thermal areas (time·temperature >39.5°C). T_{env}, environmental temperature.

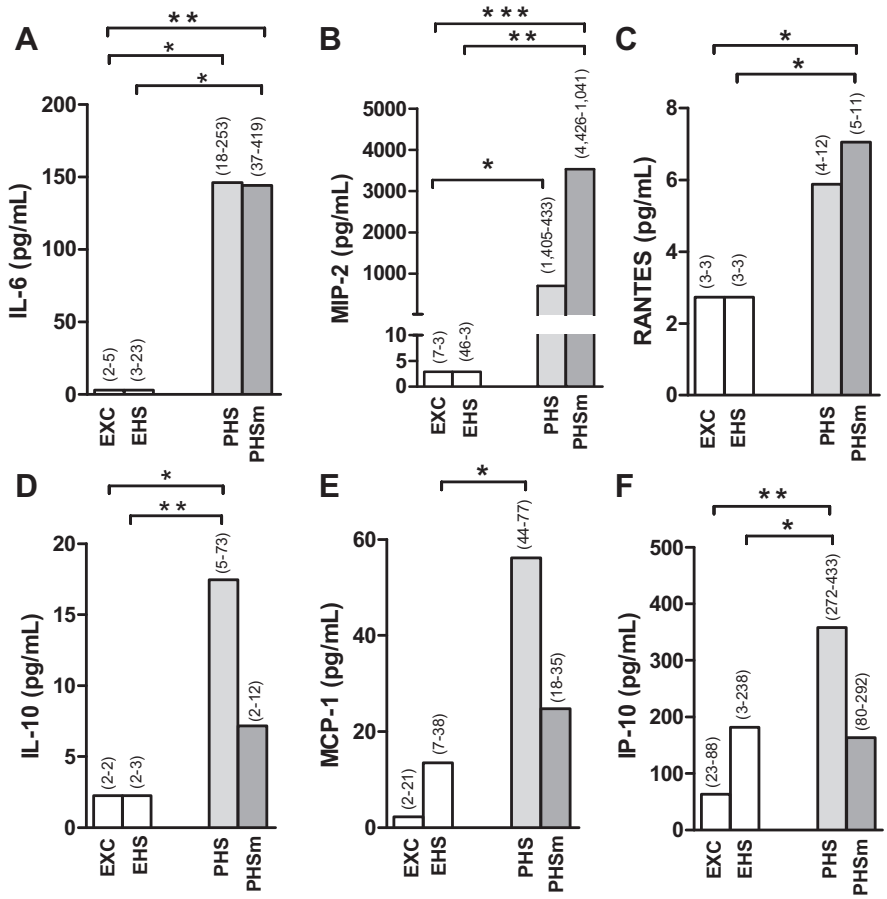

Fig. 5. Comparison of cytokines and chemokines significantly different at 3 h between EHS and models of PHS. EXC, sham controls. $P < 0.05$ (*), 0.01 (**), and 0.001 (***), Kruskall Wallis, Dunn's post hoc comparisons. Bars = median with 25–75% quartiles. Benjamini-Hochberg procedure for multiple ANOVAs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

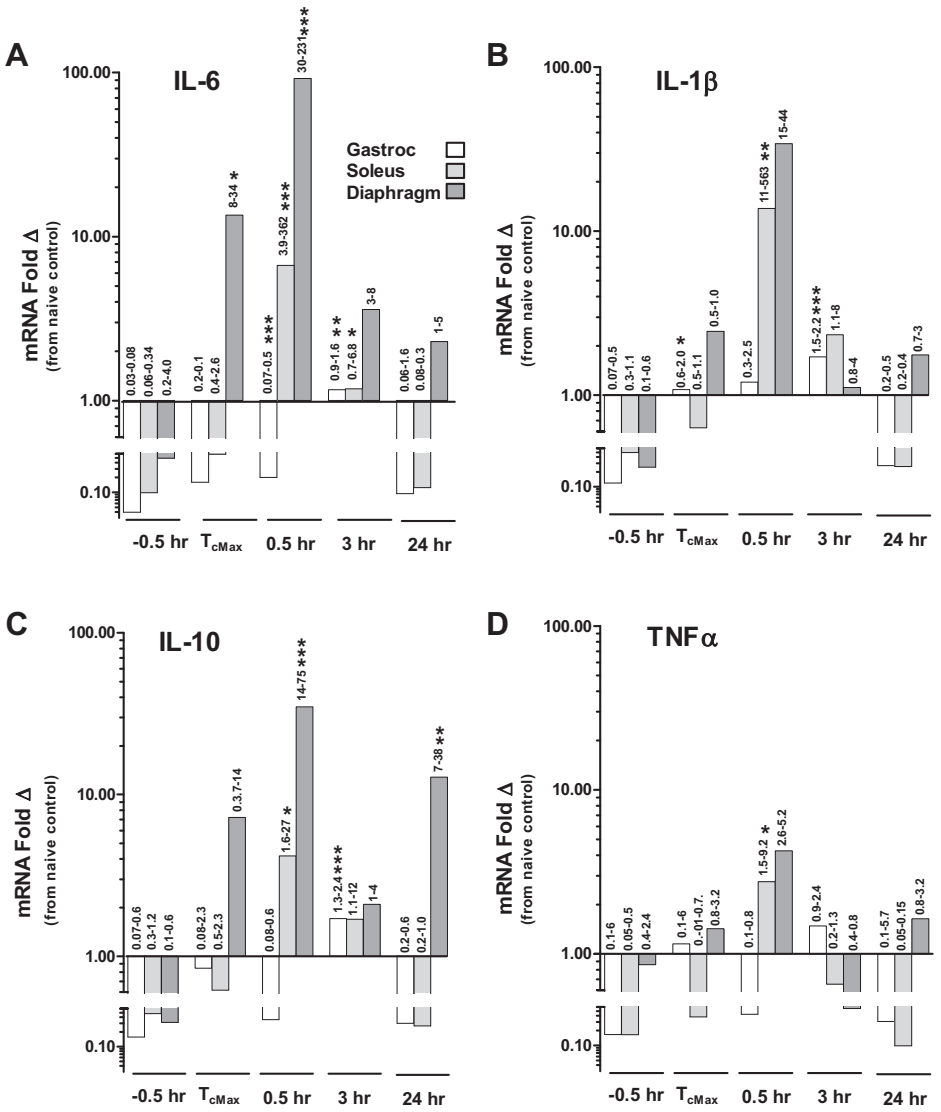

Fig. 6. Fold changes in innate immune cytokine mRNA in EHS gastrocnemius (gastroc), soleus, and diaphragm muscle. All changes reported relative to naïve control mouse muscle. Kruskal-Wallis ANOVA, Dunns post hoc: $P < 0.05$ (*), 0.01 (**), and 0.001 (***). Medians ± 25–75% quartiles. Benjamini-Hochberg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

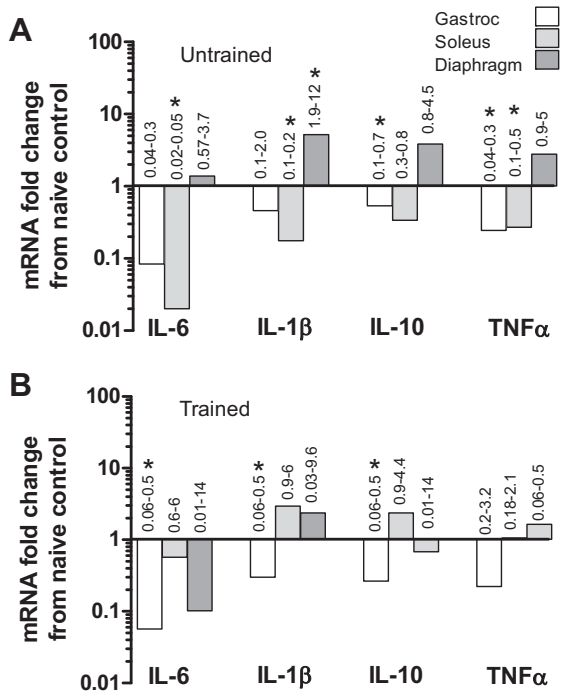

Fig. 7. Effects of a single bout of exercise (matched to EHS) on innate immune cytokine gene expression in muscle. Samples collected at 0.5 h post-$T_{c,max}$. *A*: untrained mice without cage running wheels or exercise training. *B*: response of EXC mice. Medians ± 25–75% quartiles. FDR = 0.15 using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

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

The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. No conflicts of interest, financial or otherwise, are declared by the authors.

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.

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.

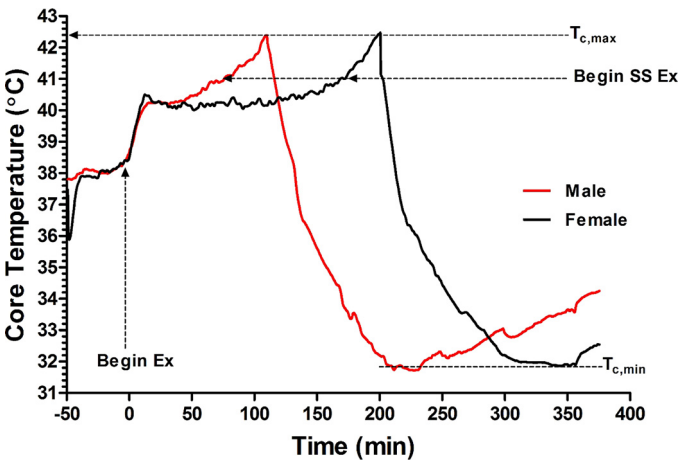

Fig. 1. Typical core temperature profiles of male (red line) and female mice (black line) during exertional heat stroke (EHS) trials. The time scale is set so that 0 = the beginning of exercise in the heat. For ~60 min before the beginning of exercise, the mice were equilibrated to the environmental 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

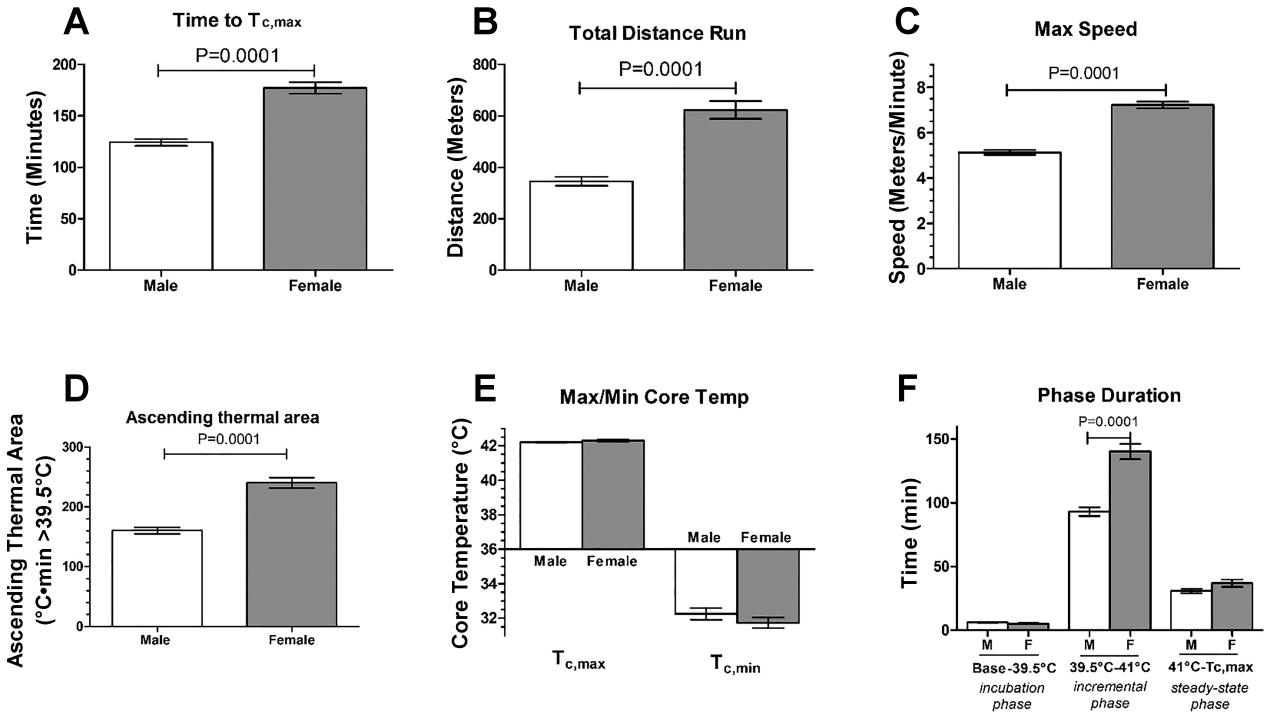

Fig. 2. *A–F*: performance differences between male and female mice during exertional heat stroke (EHS). (Values are means ± SE; 2-sample *t*-tests, $n = 43–44$ for both groups). See text for details.

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

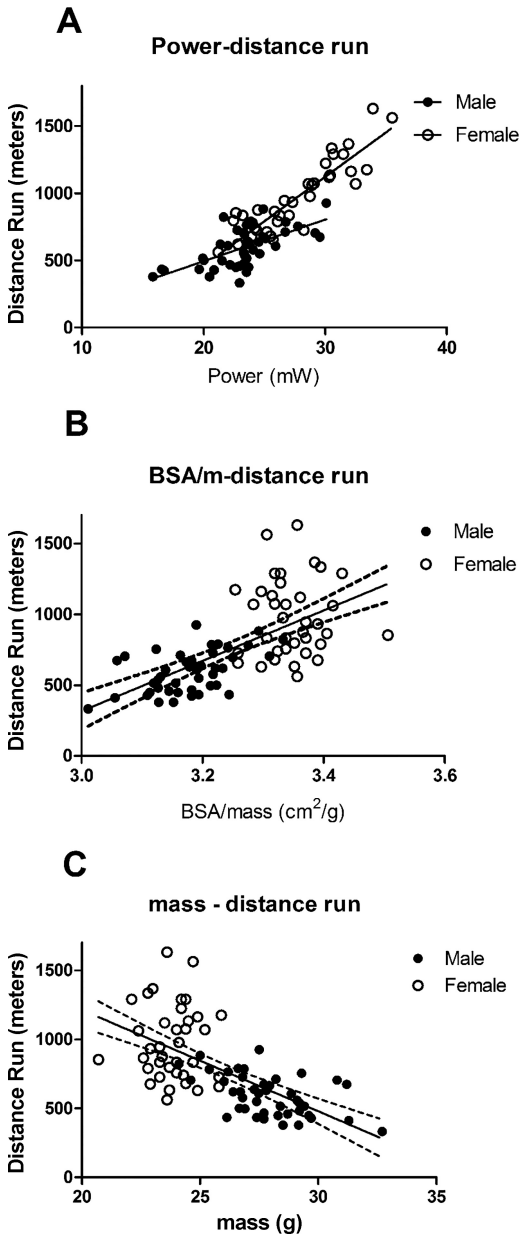

Fig. 3. *A*: relationship between power output and distance run. The linear fit for this relationship was significantly different between males and females ($P < 0.001$). *B*: relationship between BSA/mass and distance run in males and females. There was no statistical difference in the relationship 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).

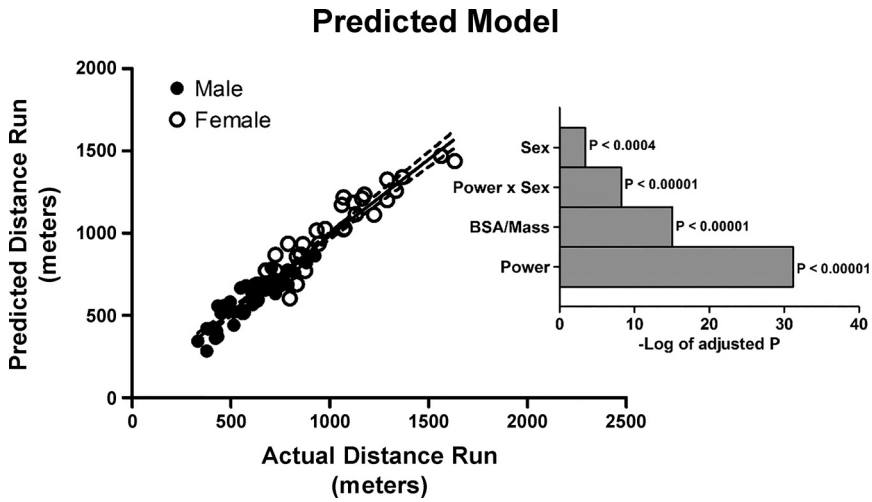

Fig. 4. Results of the ANCOVA statistical model showing the actual distance run by the mice vs. the distance predicted by the statistical model. The dashed lines are the 95% CI for the regression slope. The *inset* shows the −log of the P values, an index of relative contribution to the model. Note that 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.

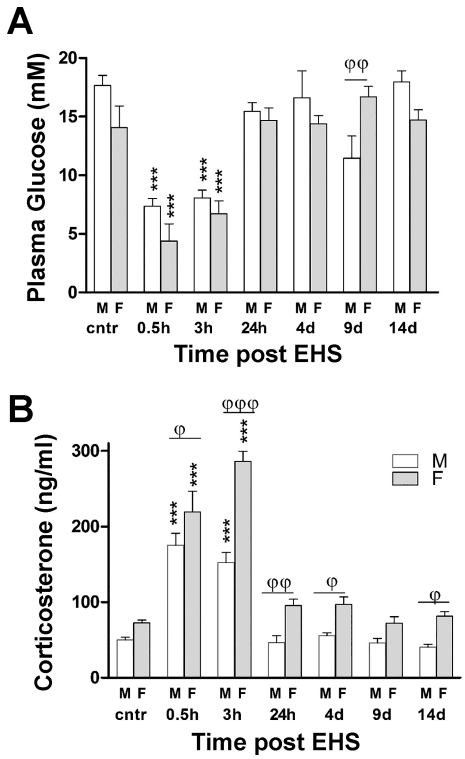

Fig. 5. *A*: measures of plasma corticosterone in males vs. females over the course of 14 day recovery. ANOVA Effects: sex <0.0001, time <0.001, time × sex <0.001. *B*: plasma glucose, non-fasting, measured at the time of sacrifice during post EHS recovery and under isoflurane anesthesia. ANOVA = $P < 0.05$. 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

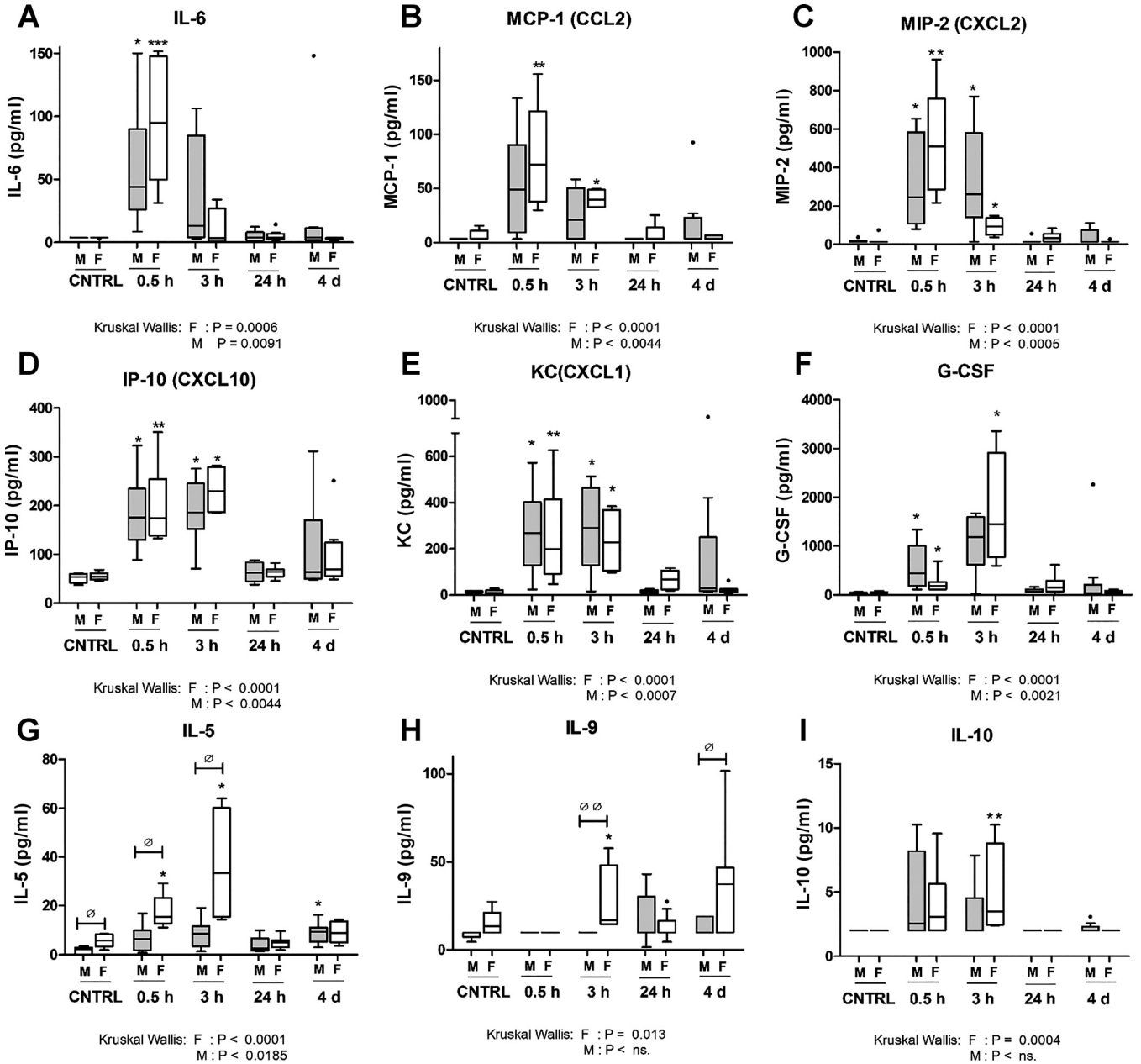

Fig. 6. *A–I*: plasma cytokines measured over 4 days of recovery post-EHS. Kruskal Wallis ANOVA *P* values under each relationship, post hoc between groups: Steel-Dwass, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ from control (CNTRL). $\theta\theta P < 0.01$, $\theta P < 0.05$ from time-matched male-female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

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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Delayed metabolic dysfunction in myocardium following exertional heat stroke in mice

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Key Points Summary

- Exposure to exertional heat stroke (EHS) is associated with increased risk of long-term cardiovascular disorders in humans.

- We demonstrate that in female mice, severe EHS results in metabolic changes in the myocardium, emerging only after 9-14 days. This was not observed in males that were symptom-limited at much lower exercise levels and heat loads compared to females.

- At 14 day recovery in females, there were marked elevations in myocardial free fatty acids, ceramides and diacylglycerols, consistent with development of underlying cardiac abnormalities.

- Glycolysis shifted towards the pentose phosphate and glycerol-3P dehydrogenase pathways. There was evidence for oxidative stress, tissue injury and microscopic interstitial inflammation. The tricarboxylic acid cycle and nucleic acid metabolism pathways were also negatively affected.

- We conclude that exposure to EHS in female mice has the capacity to cause delayed metabolic disorders in the heart that could influence long term health.

ABSTRACT

Exposure to exertional heat stroke (EHS) is associated with a higher risk of long term cardiovascular disease in humans. Whether this is a cause-and-effect relationship remains unknown. We studied the potential of EHS to contribute to development of a "silent" form of cardiovascular disease using a preclinical mouse model of EHS. Plasma and ventricular myocardial samples were collected over 14 d of recovery. Male and female C57bl/6J mice underwent forced wheel running for 1.5-3 h in a 37.5°C/40% relative humidity until symptom limitation, characterized by CNS dysfunction. They reached peak core temperatures of 42.2±0.3°C. Females ran ~40% longer, reaching ~51% greater heat load. Myocardial and plasma samples (n = 8/group) were obtained between 30 m-14 d recovery, analyzed using metabolomics/lipidomics platforms and compared to exercise controls. The immediate recovery period revealed an acute energy substrate crisis from which both sexes recovered within 24 h. However, at 9-14 d, the myocardium of female mice developed marked elevations in free fatty acids, ceramides, and diacylglycerols. Glycolytic and tricarboxylic acid cycle metabolites revealed bottlenecks in substrate flow, with build-up of intermediate metabolites consistent with oxidative stress and damage. Males exhibited only late stage reductions in acylcarnitines and elevations in acetylcarnitine. Histopathology at 14 d showed interstitial inflammation in the female hearts only. The results demonstrate that myocardium of female mice is vulnerable to a slowly emerging metabolic disorder following EHS that may harbinger long-term cardiovascular complications. Lack of similar findings in males may reflect their lower heat exposure.

KEY WORDS: metabolomics, lipotoxicity, myocardium, sex differences, hyperthermia

INTRODUCTION

Successful short-term recovery from exertional heat stroke (EHS) may not be the end of problems for victims. For instance, a 14-year epidemiological study of military personnel hospitalized for severe heat illness reported a ~2.2 times greater risk of dying of ischemic heart disease and a ~1.7 greater risk of dying of other forms of cardiovascular disease (Wallace *et al.*, 2007). Another 14-year follow-up study of heat stroke victims reported a ~3.9 times higher incidence of major cardiovascular events, a ~5.5 times greater incidence of ischemic stroke and a ~15 times greater incidence of a diagnosis of atrial fibrillation (Wang *et al.*, 2019). These findings suggest that heat stroke and severe heat injury are associated with long-term cardiovascular complications that may not be evident for many years. However, exposure to EHS has never been shown to cause any long-term cardiovascular disorders in any kind of controlled experiment.

A second medical concern that may be related involves the uncertainties in the time required for recovery and safe "return to duty" for the military or "return to physical activity" for athletes following EHS. In response to acute cooling, fluid replacement and intensive medical care, most patients with EHS appear to quickly recover overall health in a few days. However, this apparent recovery may be misleading. For example, in the French military, 15.4% of the patients experiencing EHS reported a previous EHS episode (Abriat *et al.*, 2014). More recently, another study reported an incidence of 4.1% of recurrent exertional heat illnesses episodes in a cohort of 145 patients (Schermann *et al.*, 2018). Likewise, US active military personnel, who experienced EHS in the previous year, had 7.3 times greater chance of getting a second EHS episode (King et al., unpublished

observations). Despite the apparent return to homeostasis, underlying repair or regenerative processes may be ongoing after EHS that are relatively invisible to normal clinical testing and could ultimately impose an increased risk. This is particularly true if such repair mechanisms are ongoing in the heart, because cardiovascular collapse is the hallmark of the final stages of heat stroke in animals (Quinn *et al.*, 2015) and in severe heat stressed humans (Crandall & González-Alonso, 2010). At a cellular/molecular level, exposure to acute hyperthermia can result in long term effects on cellular function and epigenetics (Weyrich *et al.*, 2016) that may remain hidden until exposure to a secondary stress or in response to long-term processes associated with aging (Velichko *et al.*, 2015).

The problem with the epidemiological/retrospective data is distinguishing whether EHS increases susceptibility to cardiovascular disease or whether individuals with underlying propensities toward cardiovascular disease are more prone to EHS. There is evidence that individuals with a history of heart disease are more susceptible to heat stroke (Semenza *et al.*, 1996) and indirect evidence that middle-aged patients with chronic heart failure (CHF) have compromised thermoregulation in heat (Cui *et al.*, 2005; Cui & Sinoway, 2014). Thus, there seems little doubt that cardiovascular disease is a risk factor for heat stroke. However, we hypothesize the reverse, that is, that the exposure to exertional heat stroke can lead to the emergence of cardiovascular diseases later in life. To test this, we employed an established preclinical model of exertional heat stroke (EHS) in an inbred mouse strain that has little genetic variance and identical environmental history between animals. Metabolomic profiling of cardiac ventricular muscle was followed over 14 days of recovery. This time period was chosen because, by all behavioral appearances, mice have

fully recovered at this time and it is a common time window for return to physical activity in the military or sports. We studied metabolic biomarkers of changes in cardiac metabolites that could indicate long-term consequences. We also compared metabolomic responses between male and female mice because they have strikingly different exercise responses in the heat (Garcia *et al.*, 2018) and exhibit marked differences in their predominant metabolic strategies for energy storage and utilization (Blaak, 2001; Lyons *et al.*, 2013; Mauvais-Jarvis, 2015).

METHODS

One hundred and forty-four male and female C57bl/6J mice (Jackson Laboratories, Bar Harbor, ME), 3-4 months old, were used in this study. Animals were housed at University of Florida Animal Care Facility under a 12:12 dark/light cycle, where lights were switched on at 7AM and off at 7PM. The ambient room temperature and relative humidity range were 20-25˚C and 30-60% respectively. Animals had *ad libitum* access to food (2918 Envigo Chow) and water through an automatic tap system. All procedures included in the study protocol were approved by the University of Florida Institutional Animal Care and Use Committee. We 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. Reported information about the procedures conformed to the Animal Research Reporting of *in vivo* Experiments – ARRIVE guidelines (Kilkenny *et al.*, 2010).

Animal preparation and exercise training. One week after arriving at the University's animal housing facility, we surgically implanted a radio telemetric sensor for real time core temperature (T_c) monitoring (TA-E-Mitter; Starr Life Sciences, Oakmont, PA). A small incision was made at the peritoneal cavity while animals were kept under isoflurane anesthesia and the telemetric device was placed on the lower right quadrant. Muscle and skin layers were sutured, and animals were allowed to recover from surgery for 2 weeks. To reduce pain and discomfort, mice were single-housed and given injections of buprenorphine every 12 h for 48 h after surgery. Two weeks after surgery, a running wheel (Columbus Instruments, 0297-0521) and an enrichment hut were placed into the cages so that animals could freely exercise and learn to voluntarily run on the running wheels. Three weeks after placement of the running wheels the mice were trained on forced running wheels (Lafayette Inst, 80840). The first exercise training session consisted of 15 min of freewheeling, where the mouse was free to run and explore its surroundings. This was followed by a short recovery period (~5 min) and a second exercise period with initial speeds of 2.5 – 3.1 m/min, increased by 0.3 m/min every 10 min for 60 min. Training sessions on the next three consecutive days consisted of only the incremental protocol for 60 min. Electric shock or other manual stimuli were not utilized to maintain running speed. After the last training session, animals rested for 2 days prior to undergoing the EHS trial.

Exertional Heat Stroke. The pre-clinical model of EHS employed in the current study was previously described in detail (King *et al.*, 2015, 2017; Garcia *et al.*, 2018). Briefly, mice were taken to the lab from the animal facility 24 h prior to the experiment but kept on the same light cycle and in their home cage. We monitored T_c with a data

158 acquisition system (VitalView; Starr Life Sciences) averaged over 30-s intervals. All
159 experiments were performed at the same time, beginning early in the daylight cycle of the
160 next day. Mice rested in the environmental chamber for at least 1 hour, while they were
161 monitored until T_c was maintained <37.5°C for >15 min. Thereafter, we increased the
162 environmental temperature (T_{env}) in the chamber to 37.5°C and relative humidity (RH) to
163 40%, which required 45-60 min to be achieved. Once the chamber temperature was stable
164 the animal was quickly placed in the running wheel, which was encased in ventilation
165 plexiglass chamber. The animal's behavior during the EHS protocol was monitored
166 continuously in real time through an infrared video camera display system. Mice initially
167 ran at a speed of 2.5 m/min and increased 0.3 m/min every 10 min until a T_c of 41°C was
168 attained, which served as threshold beyond which the running speed was kept constant (Fig.
169 1). The end point of the EHS test was "symptom limited," since all mice displayed a sudden
170 loss of consciousness and collapse near peak core temperature ($T_{c,max}$). We calculated "heat
171 load" based on the area under the curve for a core temperature >39.5 ˚C over time. This
172 method was described previously for passive heat stroke conditions in mice (Leon *et al.*,
173 2005). At the end of the protocol, we immediately placed the animals back in their original
174 cages. We carefully watched each mouse until it regained consciousness and then recorded
175 its body mass. T_c continued to be monitored for a 10-12-hours post EHS or until a planned
176 death point at an earlier time (described below). Only one animal died spontaneously
177 following EHS exposure. All other animals were euthanized at the time of sample
178 collection.

Tissue collection. We studied 7 groups of male and 7 groups of female mice (n = 8/group) to determine the time course of metabolite expression in plasma and heart ventricular tissue. Tissues were collected at 0.5 h, 3 h, 24 h, 4 d, 9 d, 14 d after EHS. Four other groups of 8 male and 8 female animals were collected to obtain matched data for pairing certain time points for metabolomic analyses in the same run of the mass spectrometry.

At sample collection, the animals were placed under deep isoflurane anesthesia. Blood was first rapidly collected using a transthoracic cardiac stick from below the xiphoid process using a 1 ml Luer Lock syringe, preloaded with EDTA. Thereafter, the chest was opened, and the heart rapidly removed and placed in ice-cold phosphate buffer saline (PBS). The entire process of blood and heart collection took ~3-4 min. The cooled heart was rapidly cut horizontally midway from the atria to the apex. The apical right and left ventricular chambers were combined and immediately flash frozen in liquid nitrogen. All myocardial samples consisted of combined left and right ventricles in the apex of the heart. The remaining top section of the ventricles and atria were then fixed in 10% formalin for later histological analyses and hematoxylin/eosin (H&E) staining. Whole blood was centrifuged in 4°C at 2000 rcf for 10 min and the plasma pipetted off, immediately snap frozen in liquid nitrogen and stored at -80°C.

A group of sham exercise controls (cntrl), identically treated, but without heat exposure, were sampled at the 4-day time point of recovery from exercise. These mice were exercised at the average time and intensity of the EHS mice for the given sex, but the environmental chamber was maintained at 22-23°C and 50% RH. The 4 d time of sample

collections for the cntrls was designed to be an approximate midway point of the entire recovery period for all of the animals and one in which the animals should have recovered completely from their exercise bout.

Metabolomics analyses. Metabolomics analysis was conducted by Metabolon (Durham, NC), through collaboration with the US Army Center for Environmental Health Research. In short, plasma samples (100 µl) and of heart ventricular tissue were thawed, and extracts were prepared according to Metabolon's standard protocols, which are designed to remove protein, dislodge small molecules bound to protein or physically trapped in the precipitated protein matrix, resulting in recovery of a wide range of chemically diverse metabolites. Plasma samples for the complete 14-day recovery groups were analyzed for males; plasma samples for females were only studied for the 3 h time point and for cntrl mice. Heart samples were studied for all males and females, at all time points. The samples were extracted and split into equal parts for analysis on the gas chromatography mass spectrometer and liquid chromatography mass spectrometer platforms (HD4 and Lipidomics platforms). Proprietary software was used to match ions to an in-house library of standards for metabolite identification and for metabolite quantitation by peak area integration. All experimental groups had an n = 8 except for the female hearts at 3 h post EHS (n=6) and the male 0.5 h post EHS (n=7). The number was reduced because of insufficient sample at the time of analyses.

Histological measurements. After seeing metabolic abnormalities in female hearts at the 14 d recovery period compared to their respective cntrls, the remaining apical components of these ventricles, which had been fixed in 10% formalin, were thin sectioned

(4 um), stained for H&E and evaluated for lesions in both males and females. Images were divided into equal areas and scored for a) inflammation, b) vacuolization and c) hemorrhage, based on the pathologic descriptions and examples provided by the National Toxicology Program (Johnson & Nyska, 2014). We identified little or no consistent changes in either hemorrhage or vacuolization across samples and so concentrated on scoring inflammation. 0 = no significant clustered accumulation of nuclei; 1= slight accumulation (1 cluster of nuclei)/small area; 2= moderate accumulation (2+ clusters of nuclei)/moderate area, and 3= very high accumulation (3+ clusters)/area. The hearts were evaluated by blinded evaluators (CKG, GPR) and the results averaged for each heart for each investigator prior to statistical testing.

Statistical treatment. Statistical analyses were performed using SAS JMP® and GraphPad Prism. Time-dependent data were tested with one-way ANOVA. Most populations of time data followed a normal distribution, with similar variances; however, when one or more groups within an ANOVA exhibited widely variant distributions, the Welch's ANOVA correction was utilized (McDonald, J.H., 2014). For extremely non-parametric data, Kruskal-Wallis was utilized instead of ANOVA. Post-hoc analyses were performed using Dunnett's test, against the cntrl sample for standard or Welch's ANOVA or Tukey'. For populations requiring Kruskal-Wallis, a nonparametric post hoc test was used against cntrl, i.e. Steel (SAS JMP®). For visual clarity of the data within a time series, results for each time point are expressed as a fraction of the mean 4-day exercise control, and as mean ± SEM for parametric data or median ± 25-75% quartiles for nonparametric groups. Other single variable data is expressed as mean ± SD for parametric

data or median ± 25-75% quartiles for nonparametric data, with individual data points shown. Single unpaired parametric measurements were made with using two sample T test.

RESULTS

Exercise Performance during EHS: Fig. 1 illustrates core temperature profiles during the EHS protocol in a typical female (panel A) and male (panel B) mouse. All mice were symptom-limited near $T_{c,max}$, characterized by loss of apparent consciousness and unresponsiveness to external physical stimuli. Within a few minutes of rest, after being unconscious, they returned to normal resting behavior. The composite performance data for these male and female mice were reported previously (Garcia *et al.*, 2018), but the individual groups at each time point are included here in Table 1 and 2 for interpretation of metabolomic results among groups. As shown in Table 2, female mice ran ~39% longer in the heat, covered nearly twice the distance, and reached speeds ~40% higher than their male counterparts. Both groups lost a significant amount of body weight during the EHS trial. Based on previous analyses this is largely accounted for by water loss. In general, body weights returned to near baseline values within 24 hours. The greater exercise time in the heat for females resulted in a significant elevation in the total exertional heat load as quantified by the 'temperature • time product' when $T_c > 39.5°$ C, as described for passive heat stroke conditions by Leon et al. (Leon *et al.*, 2005).

Plasma evidence of an energy crisis during early EHS recovery period:

Male plasma samples were available for all time points over 14 days and were used to establish a time profile of metabolic substrate availability in the blood during recovery from EHS. Female plasma samples were available at 3 h and are compared to males (Figs

2D,E and F). As shown in Fig. 2A and D, plasma glucose fell precipitously and remained decreased through the 3 h of recovery. Lactate and fructose also remained significantly reduced between 0.5-3 hours. Nearly identical responses were seen in females at the 3 h time point (Fig. 2D). By 3 hours, plasma total free fatty acids were mobilized, and representative acylcarnitines, ketones and acetylcarnitine were elevated in the plasma (Fig. 2B and E). Such elevations in plasma acylcarnitines are considered to reflect the effectiveness of mitochondrial fatty acid β-oxidation and have been shown to be elevated in heart failure patients with reduced ejection fraction (Ruiz *et al.*, 2017). Branch chain amino acids like valine increased rapidly but other amino acid substrates like glycine and alanine (Fig. 2C), leucine and serine (Fig. 2F) were diminished by 3 h, a pattern reflective of early stages of starvation (Felig *et al.*, 1969). Acetylated forms of amino acids, generally indicative of the protein degradation processes (Alamdari *et al.*, 2013), were uniformly and significantly increased in plasma by 0.5h in males, but these returned to control levels by 3h. Essentially all other metabolomic measurements from the plasma in males returned to control within 24 h. Overall, this pattern of metabolic substrate availability in the blood fits the classic energy depletion model proposed earlier as a major factor in the pathophysiology of EHS in a rat model (Hubbard *et al.*, 1987).

Carbohydrate metabolism in the ventricular myocardium

At 0.5 h of recovery, glucose-6P (Fig. 3A) and glucose (not shown) were markedly reduced in the ventricular tissue, parallel to carbohydrate measurements in plasma, but rebounded to normal levels by the 3 to 24 h time points. Similar outcomes were observed at subsequent steps of glycolysis (Fig. 3B), including formation of fructose 1,6 bisphosphate.

289 However, by day 9 and 14, females showed an unexpected fall in these metabolites,
290 suggesting an impairment of glycolytic flux. Downstream of glyceraldehyde phosphate
291 dehydrogenase (GAPDH), there was also a drastic reduction in metabolite concentrations of
292 3-phosphoglycerate (Fig. 3C) and phosphoenolpyruvate (PEP, Fig 3D) in females but not
293 males at the 9 and 14-day recovery periods. In addition, the nicotinamide adenine
294 dinucleotide (NAD^+) and NADH concentrations, relative to controls, were markedly
295 diminished. This may reflect reductions in flux through GAPDH, the primary cytosolic
296 source of NAD^+ reduction or by disorders in nucleotide metabolism discussed later in the
297 text. Pyruvate concentrations showed minimal changes over the recovery period (Fig. 3E).

298 Figure 4 illustrates the changes in metabolites that reflect alternative pathways for
299 glucose metabolism (see arrows in Fig 3), the pentose phosphate pathway (PPP, left) and
300 the glycerol-3P dehydrogenase (GPDH) pathway (right). For PPP, a marked elevation in
301 the product of-glucose 6P metabolism (i.e. 6-phosphogutonate, Fig. 4A) was seen in the
302 female population at day 9 and 14. Products of this multi-step pathway, such as ribulose-5P
303 (Fig. 4B, NS) and sedoheptulose-7P (Fig 4C) either appeared elevated or reached
304 significance by days 9 and 14.

305 The GPDH pathway also appeared to receive considerable flux of glucose
306 metabolites. The product of GPDH metabolism, dihydroacetone phosphate (DHAP, Fig.
307 4D) was greatly elevated at days 9 and 14. However, the product resulting from DHAP
308 reduction by NADH, glycerol-3P, was greatly decreased at these time points (Fig. 4D).
309 These observations may reflect the conversion of glycerol-3-P to DHAP from the glycerol-
310 3P shuttle or shunting of substrate for glycerol synthesis (Fig. 4F). In summary, these data

311 are consistent with marked reductions in glycolytic flux, decreases in NAD+ and a shunting
312 of carbon substrates into the PPP and GPDH pathways late in the recovery period.

313 ***Tricarboxylic acid (TCA) cycle in the ventricular myocardium.***

314 Metabolites of the TCA cycle were also greatly impaired in the female mice at days
315 9 and 14. Marked elevations in citrate were observed at day 9 and 14 (Fig 5A). Isocitrate
316 was elevated from the first 24 hours of recovery (Fig. 5B). A striking reduction in alpha-
317 ketogluterate was also observed beyond 24-hour time point (Fig 5C) and succinate
318 concentrations plummeted, particularly at 9 and 14 d (Fig. 5D). Neither fumarate (Fig 5E)
319 nor malate (Fig. 5F) were affected over the course of recovery. These data are consistent
320 with a substantial decrease in carbon flux within the TCA cycle beyond the level of
321 isocitrate dehydrogenase (ICD). This may have contributed to low NADH in the
322 mitochondria, as this is a major source of mitochondrial NAD^+ reduction. (Fig. 3G).

323 ***Indicators of β oxidation in the myocardium: acylcarnitine formation***

324 Strikingly different patterns of fatty acylcarnitine levels were observed in
325 myocardial tissue between males and females. In females, over the first 24 hours there was
326 a large buildup of essentially all acylcarnitines that were measured. Three very typical
327 responses of common acylcarnitine species are shown in Fig 6A. In contrast, males
328 exhibited a marked reduction in acylcarnitines in the early time points that never fully
329 returned to the level of controls and then dropped substantially again at day 14 (Fig 6B).
330 Acetylcarnitine levels did not change in females but were significantly elevated at 14 d of
331 recovery in males (Fig 6C).

By comparing groups of animals in the same metabolomics analysis run, we were able to compare relative acylcarnitine and acetylcarnitine concentrations in the myocardium between males and females at two time points, in cntrl and at the 14-day recovery. As shown, in figures 6D and E, male mice had significantly higher levels of acylcarnitines at both time periods compared to females. The levels of acetylcarnitine were not different between males and female cntrl groups. However, after 14 days had elapsed, acetylcarnitine in the males was significantly higher than both females and each respective cntrl group. In summary, these data are consistent with a period of time that extends from 3h and 4d, when females have either suppressed flux of lipid metabolites into β-oxidation and the TCA cycle or a marked elevation in fatty acid uptake that could not be metabolized by the mitochondria. In the male population, the data are consistent with a greater flux of lipid metabolites into β-oxidation following EHS that appear sustained or even increasing by 14 d.

Fatty acids in the myocardium

As shown in Fig. 7A females exhibited a marked accumulation of total free fatty acids (FFA) and their reactive metabolic products, ceramides and diaglycerols (DAGs) that appeared to gradually accumulate throughout the recovery period, compared to cntrls, reaching statistical significance at 14 d. In comparison, these products did not increase in males and, in fact, total ceramides were significantly decreased at day 9 (Fig. 7B). In female hearts, when comparing the cntrl vs. the 14-day recovery, it is clear that a wide variety of subtypes of these FFA products are increasing following exposure to EHS in females (Fig. 7 C and D and E).

354 ***Pyrimidine and Purine metabolism in the myocardium***

355 There was strong evidence for considerable purine turnover in female mice,
356 particularly during the 9-14 d recovery periods. A marked reduction in both AMP and
357 adenosine was evident (Fig. 8A and B). This is consistent with the loss of the
358 NAD+/NADPH pool seen in Fig. 3. In the same time period, there were also elevations in
359 their degradation products, hypoxanthine and xanthine (Fig. 8C and E), which come from
360 redox reactions of xanthine oxidase. One of the more stable down-stream end products of
361 adenosine metabolism, allantoin (Fig. 8F), was elevated in both males and females in the
362 first 3 h of recovery, suggesting that this period is also an active interval of purine turnover.
363 A similar pattern was seen for urate, a degradation product of adenine and well established
364 marker of antioxidant response (not shown). We also observed a 10-20-fold increase in
365 5'aminoimidazole-4-carboxamid ribonucleotide (AICAR), which is an intermediate in
366 synthesis of inosine monophosphate.

367 Most of the pyrimidine metabolism intermediates were elevated at the 0.5-3 h, in
368 both males and females, as shown in Fig 9, with little change over the rest of the 14-day
369 recovery period. Only uracil (Fig 9D) showed a second wave of elevations in the 9-14-day
370 recovery period, seen in both males and females.

371 ***Indicators of cell stress in the myocardium***

372 A number of indicators of oxidative stress were evident in the myocardium during
373 recovery from EHS. Reduced glutathione levels showed a significant decrease by day 4
374 and appeared to recover at later time points in females (Fig 10A). Oxidized glutathione

(GSSG) peaked in females at 24 h and then returned to control levels (Fig. 10B). The glutathione conjugate of hydroxy-nonenal is a biomarker of lipid peroxidation and hydroxy-nonenal activity (Völkel *et al.*, 2005) and is strongly elevated in females, both at the 0.5 recovery time and beyond 24 h (Fig 10C). Methionine sulfone (Fig 10C) is a rare oxidation product of methionine and is thought to reflect very strong oxidant exposure because it is only created when methionine sulfoxide undergoes a second oxidation step to the sulfone (Hoshi & Heinemann, 2001). It too exhibited a biphasic response at 0.5 hours and beyond 24 hours. The 9- and 13-hydroxyoxtadecadienoic (HODE) acid products (Fig 10F) were elevated throughout most of the recovery period and are often cited as a another highly stable product of lipid peroxidation reactions and of eicosanoid enzyme activity (Yoshida *et al.*, 2005). Trimethylamine N-oxide (TMAO) (Fig 10E) also showed a biphasic response, continuing to elevate over 14 days in females. This metabolite, when found in the blood, is a known risk factor for acute cardiac events (Li *et al.*, 2019) and has also been associated with possible cardiac illness-induced changes in the gut microbiome (Chioncel & Ambrosy, 2019).

Histological changes in the female myocardium

We submitted both male and female 14 d hearts and their respective 4 d cntrls for histopathological injury scoring (Fig 11). The frequency of hemorrhage and vacuolization were not significantly different between controls and EHS (data not shown) but the frequency and intensity of areas of inflammation were consistently elevated in the female EHS hearts when compared to either controls or to male EHS hearts

DISCUSSION

Collectively, these results demonstrate that female mice develop a large array of metabolic disorders and stress responses in the myocardium following EHS collapse that worsen over the last few days of a 14 d recovery period. Although males and females undergo a severe and largely similar nutrient depletion in both plasma and myocardium during the first 24 h of recovery, male mice appear to largely recover metabolic function beyond this point. The delayed metabolic disorders in the female mice consist of, 1) accumulation of a wide variety of free fatty acids and their signaling metabolites, 2) alterations in glycolytic flux towards the PPP and GPDH pathways, 3) loss of NAD^+-NADH content and homeostasis, 4) inhibition of the TCA cycle beyond isocitrate dehydrogenase, 5) pronounced elevations in the turnover of nucleic acids with a loss of adenine nucleotides, 6) an apparent increase in oxidative stress and tissue injury throughout the recovery period and 7) histological evidence of extensive myocardial inflammation. In the male mice, the most unique finding was a marked reduction in myocardial acylcarnitines that remain suppressed throughout the recovery period, whereas myocardial acetylcarnitine was significantly increased at 14 d of recovery.

Delayed metabolic responses in the female heart

The accumulation of free fatty acids and their metabolites in the female hearts is consistent with a number of known heart diseases and disorders (Kurisu *et al.*, 2003; Sharma *et al.*, 2004; Lopaschuk *et al.*, 2010; Schulze *et al.*, 2016; Wu *et al.*, 2017). Based largely on studies done in myocardial complications with high fat diet or diabetes, intracellular elevation of FFAs, DAGs and ceramides have been shown to stimulate

excessive oxidant production (Boudina *et al.*, 2007; Law *et al.*, 2018), induce Ca^{+2} dysregulation (Egnatchik *et al.*, 2014; Arruda & Hotamisligil, 2015), alter mitochondrial function (Hsu *et al.*, 2016) and suppress insulin signaling (Holland *et al.*, 2007). Our data cannot distinguish the causes of this imbalance in lipid supply and demand or apparent lipotoxicity, because similar outcomes have been observed from either disorder (Abdurrachim *et al.*, 2015). However, the fact that both glycolytic and TCA pathways show parallel disturbances in metabolite profiles in the later stages of recovery in females, suggest that it is the global processes that limit the metabolic flexibility of the heart to sustain energy production. Strong candidates for a mechanism include oxidative stress, which is apparent throughout the recovery period, generalized mitochondrial dysfunction and unbalanced mitophagy.

Differences between males and females

It was completely unexpected that females demonstrated such profound metabolic disturbances compared to males during the late stages of recovery. They greatly outperformed the males in terms of their exercise performance and resistance to hyperthermia, which we previously reported (Garcia *et al.*, 2018), and there were no outward indications of underlying cardiovascular difficulties or health issues during this period. Several potential alternative mechanisms are worthy of consideration. First, the females were exposed to a much greater overall heat load over time, though they reached nearly identical $T_{c,max}$ values as the males. Perhaps the effects we observed are therefore due to the greater heat load over time and greater exercise exposure. It follows then that the males were exposed to a stimulus that may have been insufficiently long or intense enough

to result in long-term metabolic alterations. Though the females also were exposed to a greater exercise load than the males, it is unlikely, by itself, to cause the myocardial disorder, since no such changes were observed in the cntrl female mice that ran for equivalent speeds and distances as the EHS mice.

A second set of possibilities involves the distinct differences in preferred metabolic pathways between males and females. Both male and female sex steroids have profound effects on specific metabolic pathways and on channels involved in Ca^{+2} homeostasis (Wittnich *et al.*, 2013). For example, female humans and rodents have an overall greater propensity toward fat uptake, utilization and storage than males, reviewed in (Mauvais-Jarvis, 2015). During exercise, male humans rely on a greater proportion of carbohydrate metabolism, whereas females rely to a greater extent on lipid metabolism (Horton *et al.*, 1998). Although both male and female hearts generally rely on 60-90% fatty acids as metabolic substrates, females rely on fatty acids to a greater extent than males (Wittnich *et al.*, 2013). When faced with a stressful environmental exposure or cardiac disease, all hearts tend to shift to a greater reliance on carbohydrates, which are more efficient at producing ATP (Wittnich *et al.*, 2013). However, if carbohydrate metabolism and overall mitochondrial metabolism are compromised, as it appears to be in the female mice at 9-14 days of recovery, fatty acid metabolite concentrations would presumably accumulate, perhaps ultimately leading to lipotoxicity. This might be amplified in females since they are programmed for lipid uptake and metabolism.

An interesting and possibly related clinical condition is Takotsubo cardiomyopathy, a transient cardiomyopathy occurring predominantly in women (>90%) (Said *et al.*, 2016).

It is clinically characterized by “left ventricular apical ballooning in the presence of normal coronary arteries” and is histologically characterized by the presence of increased inflammatory cells in the myocardium (Scally *et al.*, 2019). It is sometimes called the “broken heart syndrome” as it occurs following extreme exposures to emotional or physical stress and is also referred to a “stress induced myopathy”(Scally *et al.*, 2019). In rodents it can be induced with serial injections of high dose isoproterenol and is characterized not only by ventricular dilation but also accumulation of myocardial lipids (Sachdeva *et al.*, 2014). The predominant occurrence in postmenopausal females illustrates the possibility that when exposed to some forms of extreme levels of stress, female hearts may be more prone to metabolic disturbances compared to males. Whether this applies to this experimental model is unclear but the experimental data fit this syndrome.

The only notable long-term effects observed in the males within the myocardium were a reduction in essentially all species of myocardial acylcarnitines and an elevation in acetylcarnitine. Interestingly, fatty acylcarnitines remained 2.0-16.7-fold lower than cntrl through 14 d in males, suggesting a major and sustained switch toward increased β-oxidation in the hearts, post-EHS, which is a completely different pattern than observed in the females. In animal models exposed to endurance exercise training, this is generally considered a reflection of metabolic reprogramming towards greater and more effective fatty acid oxidation (Lai *et al.*, 2014). The significant increase in acetylcarnitine at 14 d recovery in males is also of interest. In cardiac muscle, endogenous acetylcarnitine is a marker and buffer for acetyl-CoA, derived from pyruvate (glycolysis), amino acids, and β-oxidation of fatty acids. It is reversibly converted to acetylcarnitine and CoA in the

presence of carnitine by the enzyme, carnitine acetyltransferase. The equilibrium between acetylcarnitine, carnitine and CoA is key for proper mitochondrial metabolism (Schroeder *et al.*, 2012) and thus, the content of endogenous acetylcarnitine is indicative of aerobic metabolism of acetyl-CoA. Together with the observed reduction in acylcarnitines at day 14, this suggests a positive adaptation to stress that would presumably enhance the ability of the male mouse's cardiovascular system to cope with subsequent heat or exercise exposures. Perhaps the absolute lower intensity of exercise and lower total heat load in males provided enough stimulus to produce a conditioning effect, but not enough to induce a maladaptive metabolic dysregulation in the myocardium that was seen in females.

Another interesting and unexpected finding was the marked early (0.5-3 h post EHS) elevation (10-20 fold) in AICAR in both male and female hearts. AICAR is an intermediate in the synthesis of inosine monophosphate (IMP), reviewed in (Daignan-Fornier & Pinson, 2012). However, it is more well known as a drug that is given to activate AMP kinase, but was recently shown to be endogenously produced by intense endurance exercise (Ezagouri *et al.*, 2019).

Potential roles of increased oxidants

Metabolomic evidence of redox changes in the myocardium reflecting lipid and protein oxidation in the late stages of recovery was substantial in the female mice but not in the males (Fig. 10). It is possible that the oxidizing environment contributed to a variety of deteriorating pathways seen throughout the metabolic network. For example, within the glycolytic pathway, both phosphofructokinase-1 (PFK1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are highly sensitive to inhibition by oxidative modification

508 (Mullarky & Cantley, 2015). This event has the net effect of lowering fructose 1,6 bisP
509 (PFK1) and lowering the movement of glucose metabolites converted to PEP and
510 ultimately to pyruvate via GAPDH. Both of these outcomes were observed in the female
511 data presented in Fig 3. This could have a secondary effect of decreasing the level of total
512 nicotinic adenine dinucelotides (NAD^+ and NADH; also observed) and increasing the
513 movement of glucose substrates towards PPP. This would elevate NADPH reducing
514 equivalents for reduction of oxidized sulfhydryls such as GSSG and oxidized thioredoxin.
515 This shift toward PPP has been proposed as a targeted strategy that cells use to defend
516 against severe oxidative stress (Mullarky & Cantley, 2015).

517 In the TCA cycle there appeared to be a bottleneck in the flow of metabolites
518 between isocitrate and alpha-ketoglutarate (Fig. 5). The enzyme responsible for this
519 reaction is isocitrate dehydrogenase (ICD). One of the critical mitochondrial isoforms of
520 this enzyme is $NADP^+$-dependent ICD. In the myocardium, this enzyme is particularly
521 sensitive to oxidation by 4-hydroxy-nonenal (Benderdour *et al.*, 2003). We observed an
522 elevated glutathione-adduct of 4 hydroxy-nonenal during the late recovery period in
523 females, suggesting a possible source of inhibition of this enzyme (Fig. 10). Finally, a loss
524 of total nicotine adenine dinucleotides observed at day 9 and 14 in females is consistent
525 with very high levels of oxidant exposure that have been shown to deplete NAD(H) stores
526 in some conditions (Schraufstatter *et al.*, 1986). Importantly, in the female heart, most
527 metabolites associated with oxidative stress (e.g. 4-hydroxy-nonenal glutathione,
528 methionine-sulfone and amine and trimethylamine N-oxide) displayed a biphasic response
529 over time, characterized by earlier (0.5 h post-EHS) and later (24 h post-EHS onwards)

elevations interspersed by a return to baseline levels at 3h. These biphasic responses presumably reflect the time-course of the impact of altered metabolic status in the myocardium during recovery from EHS. Overall, these findings are consistent with an increase in oxidative state, at multiple locations within the metabolic network, that could conceivably account for many of the changes in metabolic profile observed in females in this study.

Implications for long-term effects of exertional heat stroke

It is important to emphasize that this study does not suggest that female humans or females of any species are more susceptible to long-term effects of EHS. Simply put, females in this context were operating like 'super athletes,' able to sustain higher intensities of exercise during severe thermal stress. However, it does help to frame the epidemiological studies in humans by showing the possibility that increased risk of cardiovascular disorders may be induced by a previous EHS or severe heat illness exposure in some patients. Therefore, the epidemiological projection of long term effects of heat illness on cardiovascular health may very well be appropriate and not just coincidental (Wallace *et al.*, 2007; Wang *et al.*, 2019). At least over this relatively short recovery period of 14 days, we report that an emerging myocardial pathology can develop in some conditions that would likely increase susceptibility to further cardiovascular problems later in life. This parallels our emerging understanding of the long-term effects of severe head injuries on the health of the central nervous system, which do not manifest themselves for decades after the initial insult.

COMPETING INTERESTS

The authors have no conflicts of interest or competing interests with this work.

AUTHOR CONTRIBUTIONS

All authors read and approved the final version of the manuscript, agree to share authorship and are accountable for the integrity of the work.

OL: Wrote original draft of the manuscript, assisted in data acquisition and interpretation of the data and contributed to intellectual content.

CKG: Performed animal experiments, collected and interpreted data, assisted in drafting and revising manuscript.

AJM: Assisted in performing animal experiments, collection of data and revising manuscript.

GPR: Performed animal experiments, collected and interpreted data and assisted in drafting and revising experiment.

KOM: Assisted in performing animal experiments, collecting specimens and revising manuscripts.

MAK: Assisting in data collection and interpretation, revising manuscript.

BI: Assisting in study design and interpretation of metabolomic data. Assisted in revising manuscript

SR: Assisted in data interpretation of metabolomic data and in interpretation and revision of manuscript.

LRL: Shared in original design of experimental plan, data interpretation and revision of manuscript.

TLC: Designed original study, provided leadership in all experimental components of the study, assisted in drafting the manuscript, interpretation of data and revision.

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FIGURE LEGENDS

Figure 1. Typical core temperature profiles in representative female (panel A) and male (panel B) mice during the EHS protocol. Note the much longer exposure to high temperatures > 39.5°C (heat load) in the female mice. Also note that both males and females reached symptom limitation at similar Tc,max values.

Figure 2. Plasma measurements of metabolic substrates over the first 24 hours of recovery from heat stroke. Panels: A,B and C, Plasma glucose, beta-oxidation substrates and represetative plasma amino acids in males. Statistical comparisons are between matched 4 d exercise controls and each time point, means ± SE. D,E and F are comparisons of plasma values of cntrl vs EHS mice for both male and female mice at the 3 h time point, post EHS, D plasma carbohydrates, E) plasma components of fatty acid oxidation, and F) plasma amino acids. Data are normalized to average of all cntrl values for each group (i.e. Cntrl average = 1.0). (*P* designations*:* * < 0.05 ** <0.01, *** < 0.001, ****< 0.0001; n = 8 per group, except where noted in methods). In A-C means ± SEM, D-F, means ± SD.

Figure 3. Changes in representative metabolites of the glycolytic pathway in the myocardium. Statistical comparisons are against matched 4 d exercise controls (cntrls) at each time point. (*<0.05 **, <0.01, *** 0.001, ****<0.0001; n=8 per group, except where noted in methods, mean ± SEM). The grey arrow emphasizes the partitioning of the glycolytic pathway away from pyruvate formation in the EHS females, shown in detail in Fig. 4.

Figure 4. Metabolites in the myocardium from alternative glycolysis pathways. Statistical comparisons are between matched 4 d exercise controls (cntrl) at each time point. (* $P<$ 0.05, ** $P<$ 0.01, *** $P<$ 0.001, **** $P<$0.0001, n=8 per group, except where noted in Methods; mean ± SEM)

Figure 5. Metabolites associated with the tricarboxylic Acid (TCA) cycle in the myocardium. Statistical comparisons are between matched 4 d exercise controls (cntrl) at each time point. (* $P<$ 0.05 ** $P<$ 0.01, *** $P<$ 0.001; n =8 per group except where noted in methods; means ± SD)

Figure 6. Myocardial fatty acylcarnitine formation in the myocardium in A) females and B) males showing three highly concentrated metabolites. C) Acetyl carnitine changes in both males and females during recovery, D) comparisons between male and female species of the acylcarnitines shown in A, normalized to the mean female control values in the same metabolomic run. E) Comparisons of acylcarnitines in males and females at 14 days of recovery. F) Comparisons of acetyl carnitines between males and females from the same metabolomic run; significance is between male and female at 14 days. (* $P<$ 0.05, ** $P<$0.01, $P<$ 0.001, $P<$0.0001, n=8 per group, means ± SEM).

Figure 7. Total free fatty acids (FFA) and their derivatives, ceramides, and diacylglycerols (DAGs) in the myocardium of (A) females and (B) males. C, D& E) Female data showing the large number of species significantly elevated from the exercise control at 14 d post EHS. Data from C, D, E, from the lipidomics platform (* $P<$ 0.05 ** $P<$ 0.01, $P<$ 0.001, $P<$ 0.0001, N=8 per group except where noted in methods; A-B = means ±SE ,in C,D and E, means ± SD).

Figure 8. Changes in purine metabolism in the myocardium. Degradation of guanine is through xanthine. Solid lines are degradation pathways, broken lines are synthesis pathways (e.g. AICAR) (* $P< 0.05$, $P< 0.01$, *** $P< 0.001$, **** $P<0.0001$, n=8 per group except as noted in methods, means ± SEM.)

Figure 9. Changes in pyrimidine metabolism in the myocardium. Cytosine is metabolized to uracil. (*$P< 0.05$, ** $P< 0.01$, $P<$ *** 0.001, $P<$ ****0.0001, n=8 per group except where noted in methods, means ± SEM).

Figure 10. Indicators of oxidative stress and other forms of stress or injury in myocardium. A,B,C,E and F are indicators of oxidative stress. D, trimethylamine N-oxide is a biomarker for acute cardiac disease. (*$P< 0.05$, $P<$ ** 0.01, $P<$ *** 0.001, **** $P<$ 0.0001, n=8 per group except as noted in methods; means ± SEM).

Figure 11. A) Examples of H & E staining of ventricular tissue from female and male EHS vs Cntrl mice. Arrow: example of an area of localized inflammation. B) Results of scoring for inflammation in all cross sectional areas of the ventricles in EHS vs cntrl in males and females. ANOVA followed by Tukey's post hoc (**** P < 0.0001, ***P < 0.001, *<0.05. For females N = 8 Cntr; 6 EHS; for males N = 6 cntrl, 7 EHS.

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810

Table 1. Body mass measured before and immediately after EHS followed by body mass immediately before tissue collection for each time point. Pre EHS and Post EHS are mean ± SD for all groups combined. *p<0.05 in comparison to males.

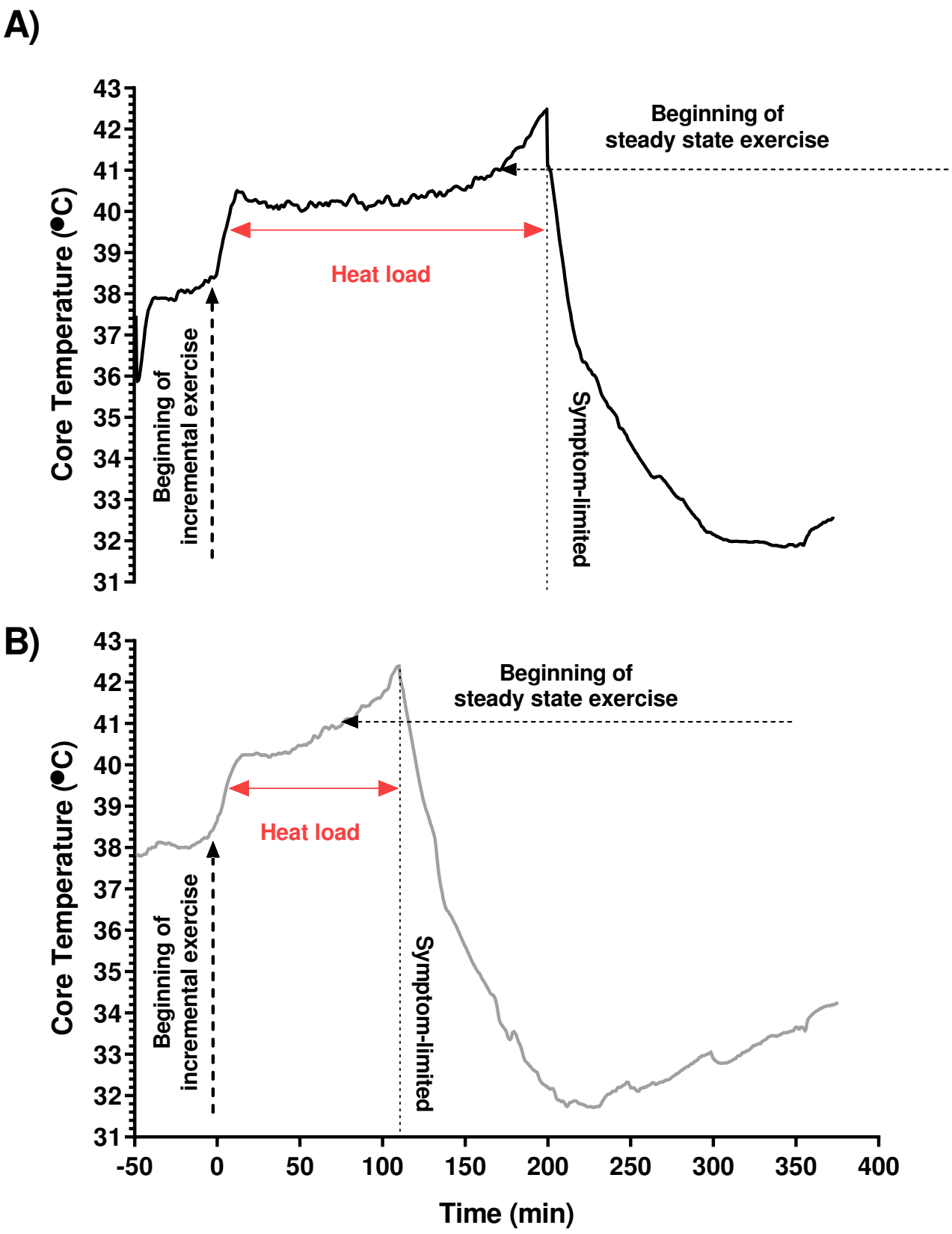
	Sex	Pre EHS	Post EHS	0.5 h post	3 h post	24 h post	4 days post	9 days post	14 days post
Body mass (g)	**M**	27.8 ± 1.7	25.7 ± 2.0	25.5 ± 1.6	25.0 ± 1.0	28.4 ± 2.3	26.2 ± 1.5	26.5 ± 1.3	28.6 ± 1.2
	F	23.9 ± 1.0*	21.7 ± 1.0*	21.7 ± 1.6	22.7 ± 5.3	23.0 ± 0.7	23.9 ± 1.3	23.0 ± 0.7	23.4 ± 0.6

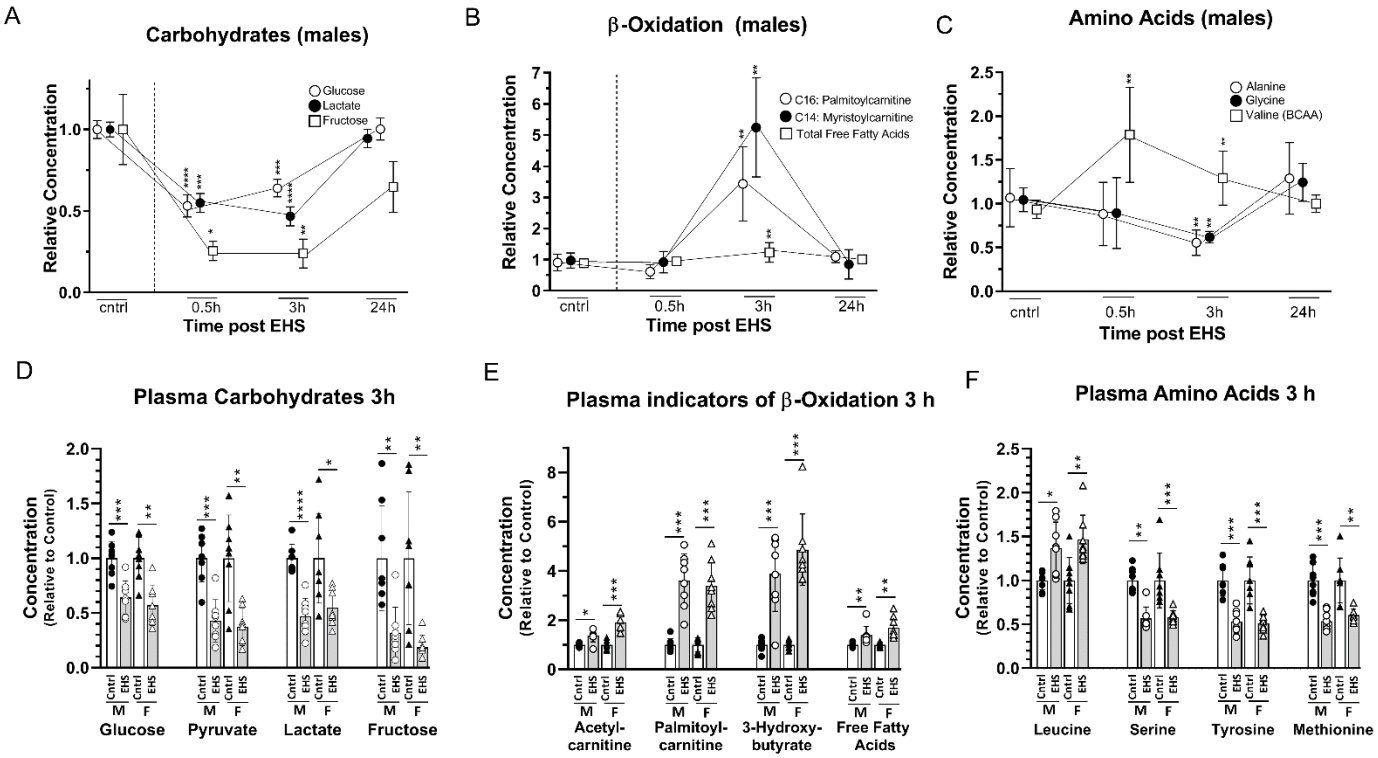
Table 2. Thermoregulatory and performance indexes during or immediately following EHS for male and female mice.

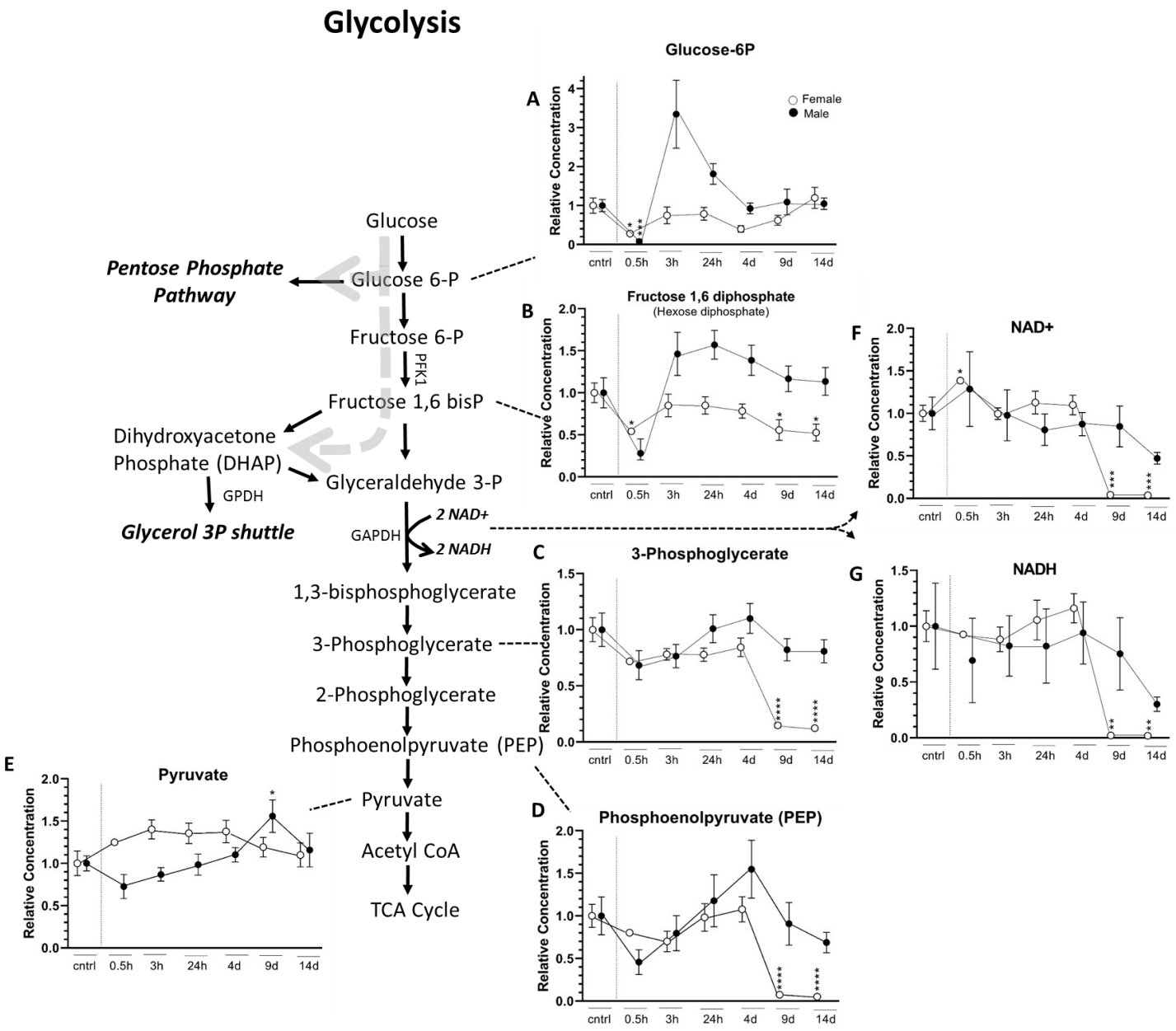
	Dehydration after EHS (%)		**Tc,max (˚C)**		**Distance Ran (m)**		**Time to EHS (min)**		**Heat load (˚C.min>39.5˚C)**	
	Male	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*	*Male*	*Female*
All groups	7.5± 2.1	9.2 ± 2.3*****	42.2 ± 0.2	42.2 ± 0.3	595 ± 147	987 ± 281*****	124 ± 23	178 ± 34*****	160.2 ± 35.7	241.3 ± 57.8*****
0.5h group	7.0 ± 2.1	9.0 ± 2.4	42.2 ± 0.1	42.4 ± 0.1	604 ± 191	1017 ± 402	125 ± 29	180 ± 50	164 ± 39	260 ± 72
3 h group	7.9 ± 2.4	8.5 ± 2.0	42.1 ± 0.1	42.1 ± 0.2	576 ± 144	1082 ± 278	121 ± 22	187 ± 33	151 ± 40	243 ± 47
24 h group	8.0 ± 1.1	9.1 ± 1.6	42.3 ± 0.1	42.2 ± 0.3	623 ± 106	885 ± 262	129 ± 16	164 ± 33	173 ± 23	221 ± 66
4 days group	5.8 ± 3.1	9.9 ± 2.1	42.2 ± 0.1	42.4 ± 0.2	566 ± 129	1173 ± 315	119 ± 19	200 ± 36	156 ± 43	289 ± 78
9 days group	8.1 ± 1.8	10.0 ± 1.2	42.0 ± 0.4	42.4 ± 0.3	667 ± 173	957 ± 222	135 ± 25	174 ± 27	167 ± 43	253 ± 21
14 days group	7.9 ± 1.6	8.1 ± 3.8	42.1 ± 0.1	42.0 ± 0.3	534 ± 103	898 ± 230	115 ± 16	166 ± 28	146 ± 26	203 ± 46

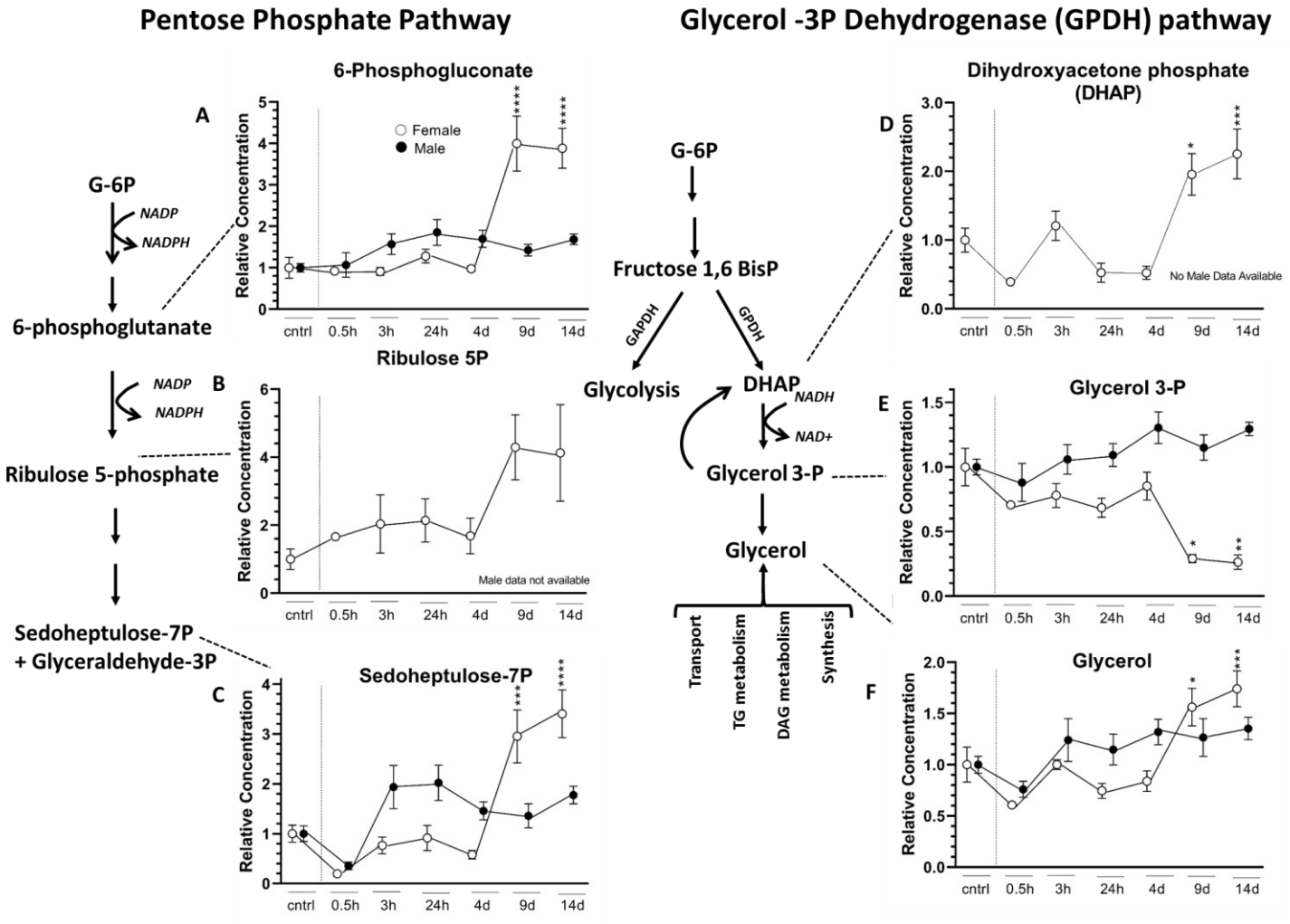
n=8/group, total n = 48 per sex group.

mean ± SD, *P < 0.05 for comparisons of males and females in entire group.

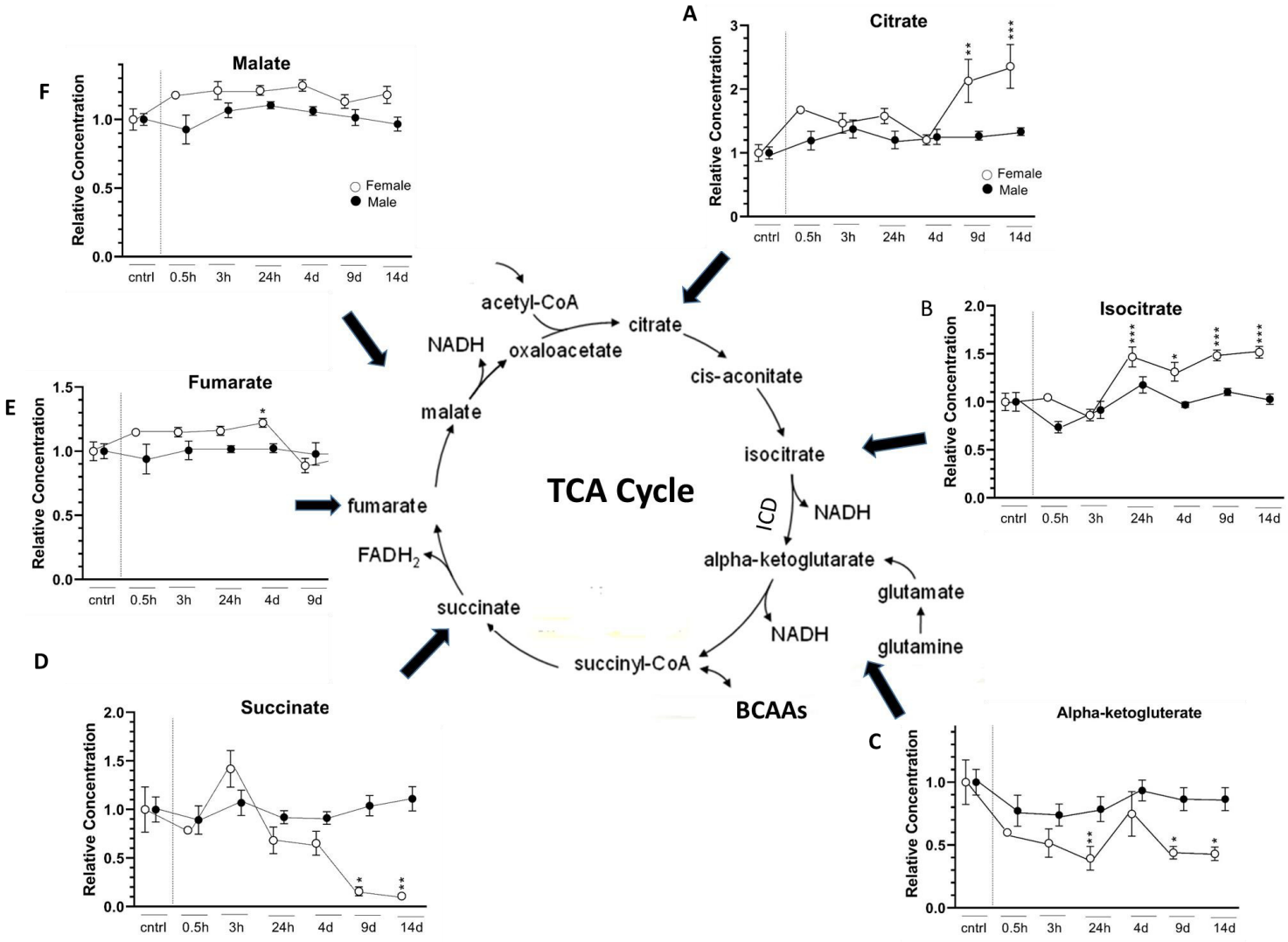

A)
Core Temperature (•C)
43
42
41
40
39
38
37
36
35
34
33
32
31
Beginning of steady state exercise
Heat load
Beginning of incremental exercise
Symptom-limited
B)
Core Temperature (•C)
43
42
41
40
39
38
37
36
35
34
33
32
31
Beginning of steady state exercise
Heat load
Beginning of incremental exercise
Symptom-limited
-50
0
50
100
150
200
250
300
350
400
Time (min)


A
Carbohydrates (males)
Glucose
Lactate
Fructose
Relative Concentration
cntrl
0.5h
3h
24h
Time post EHS
B
β-Oxidation (males)
C16: Palmitoylcarnitine
C14: Myristoylcarnitine
Total Free Fatty Acids
Relative Concentration
Time post EHS
C
Amino Acids (males)
Alanine
Glycine
Valine (BCAA)
Relative Concentration
Time post EHS
D
Plasma Carbohydrates 3h
Concentration
(Relative to Control)
Cntrl
EHS
M
F
Glucose
Pyruvate
Lactate
Fructose
E
Plasma indicators of β-Oxidation 3 h
Concentration
(Relative to Control)
Acetyl-carnitine
Palmitoyl-carnitine
3-Hydroxy-butyrate
Free Fatty Acids
F
Plasma Amino Acids 3 h
Concentration
(Relative to Control)
Leucine
Serine
Tyrosine
Methionine

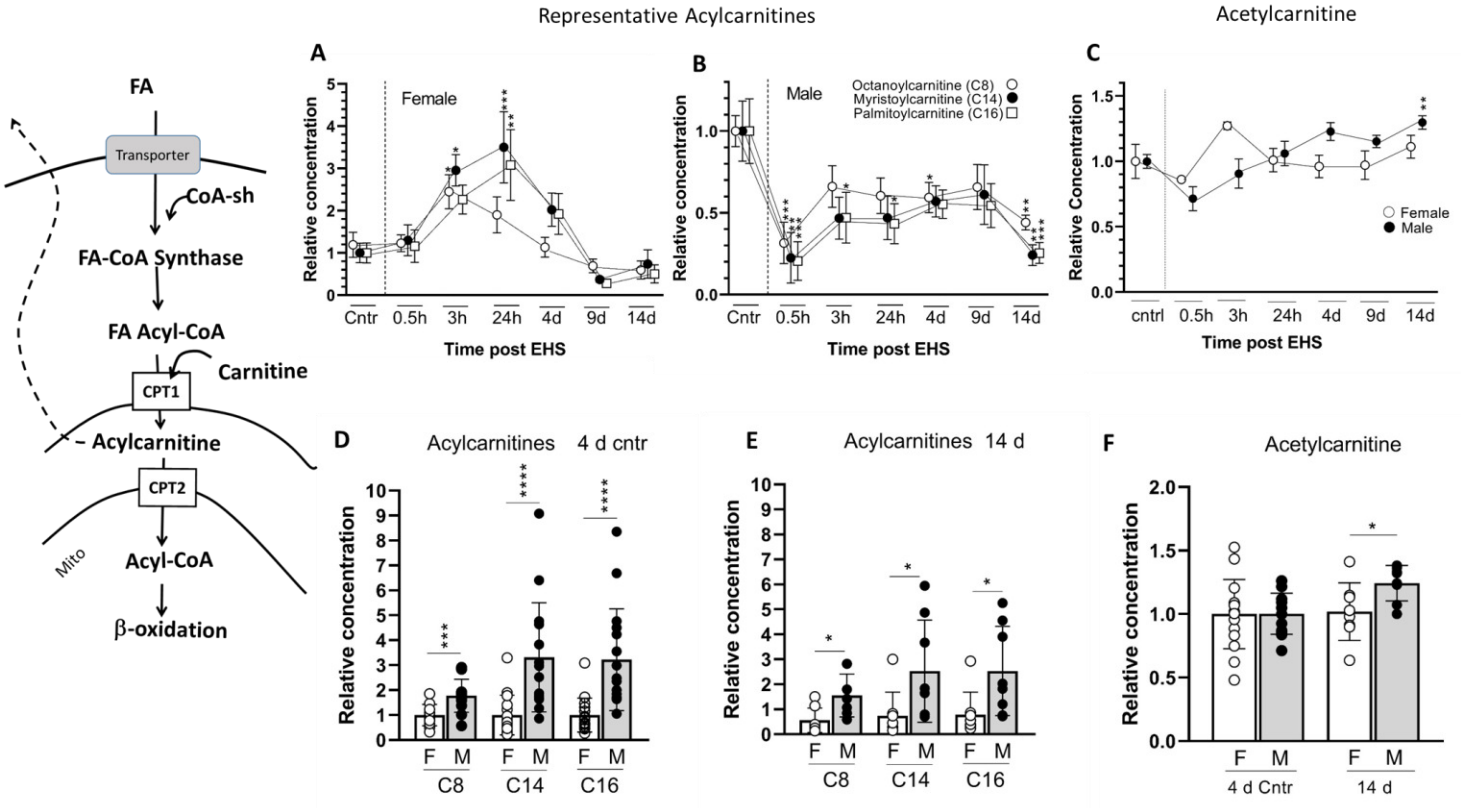

Glycolysis
Glucose-6P
A
Female
Male
Relative Concentration
cntrl 0.5h 3h 24h 4d 9d 14d
Glucose
Pentose Phosphate Pathway
Glucose 6-P
Fructose 6-P
PFK1
Fructose 1,6 bisP
Dihydroxyacetone Phosphate (DHAP)
GPDH
Glycerol 3P shuttle
Glyceraldehyde 3-P
GAPDH
2 NAD+
2 NADH
1,3-bisphosphoglycerate
3-Phosphoglycerate
2-Phosphoglycerate
Phosphoenolpyruvate (PEP)
Pyruvate
Acetyl CoA
TCA Cycle
B
Fructose 1,6 diphosphate
(Hexose diphosphate)
C
3-Phosphoglycerate
D
Phosphoenolpyruvate (PEP)
E
Pyruvate
F
NAD+
G
NADH


Pentose Phosphate Pathway
Glycerol -3P Dehydrogenase (GPDH) pathway
G-6P
NADP
NADPH
6-phosphoglutanate
NADP
NADPH
Ribulose 5-phosphate
Sedoheptulose-7P
+ Glyceraldehyde-3P
A
6-Phosphogluconate
Female
Male
Relative Concentration
cntrl 0.5h 3h 24h 4d 9d 14d
B
Ribulose 5P
Male data not available
C
Sedoheptulose-7P
G-6P
Fructose 1,6 BisP
GAPDH
GPDH
Glycolysis
DHAP
NADH
NAD+
Glycerol 3-P
Glycerol
Transport
TG metabolism
DAG metabolism
Synthesis
D
Dihydroxyacetone phosphate
(DHAP)
No Male Data Available
E
Glycerol 3-P
F
Glycerol

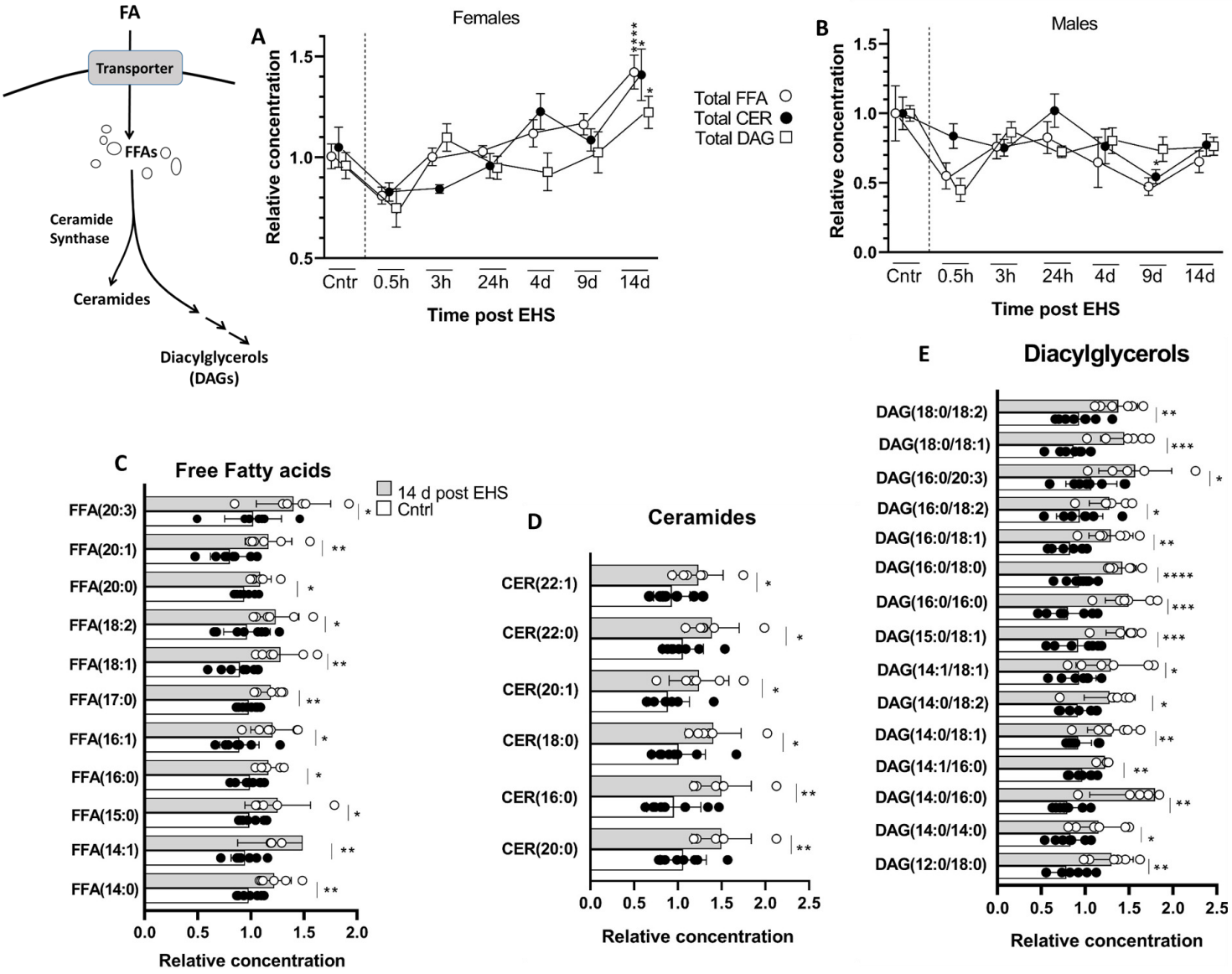
Tricarboxylic Acid Cycle

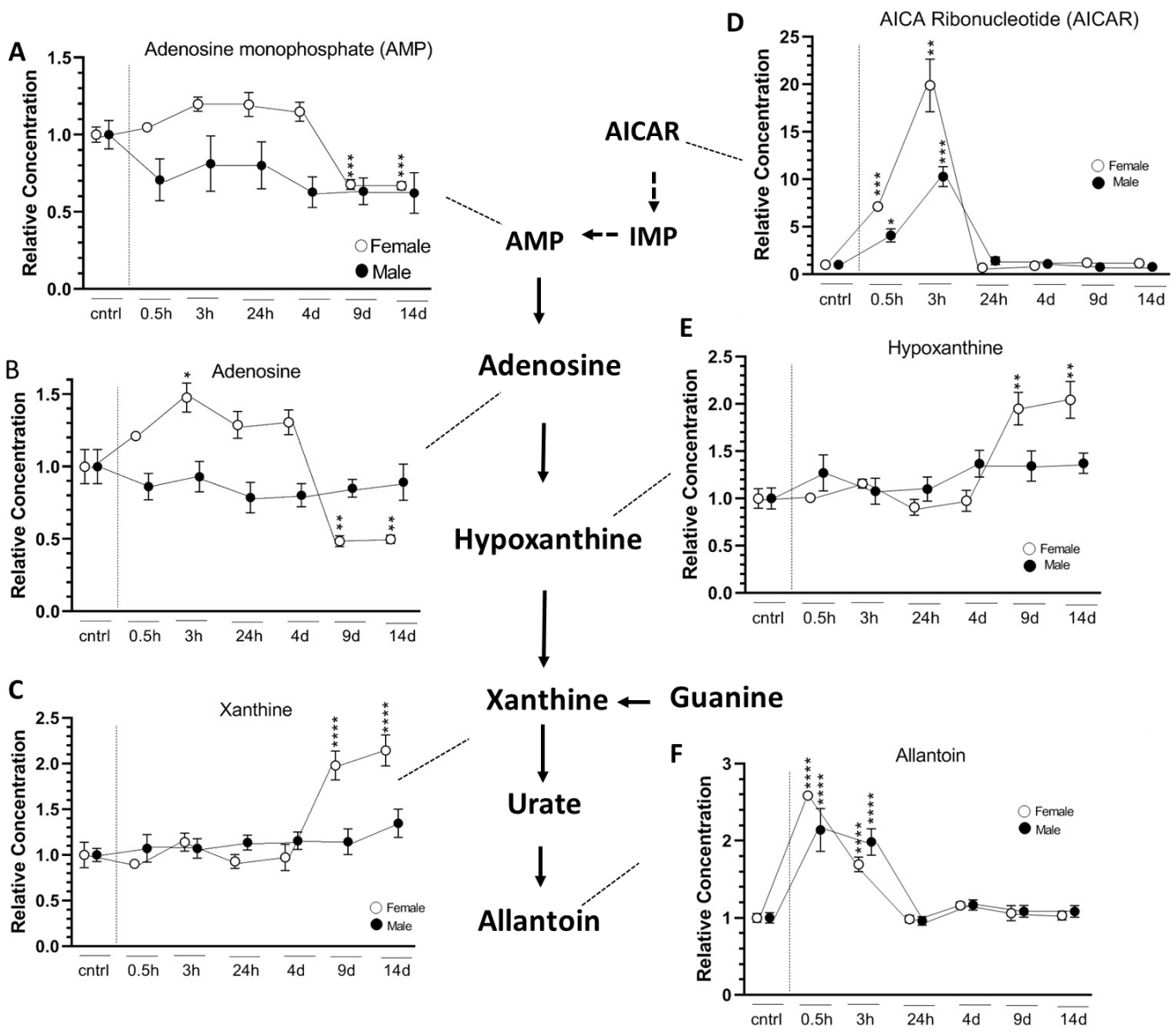


β Oxidation - Acylcarnitine Formation

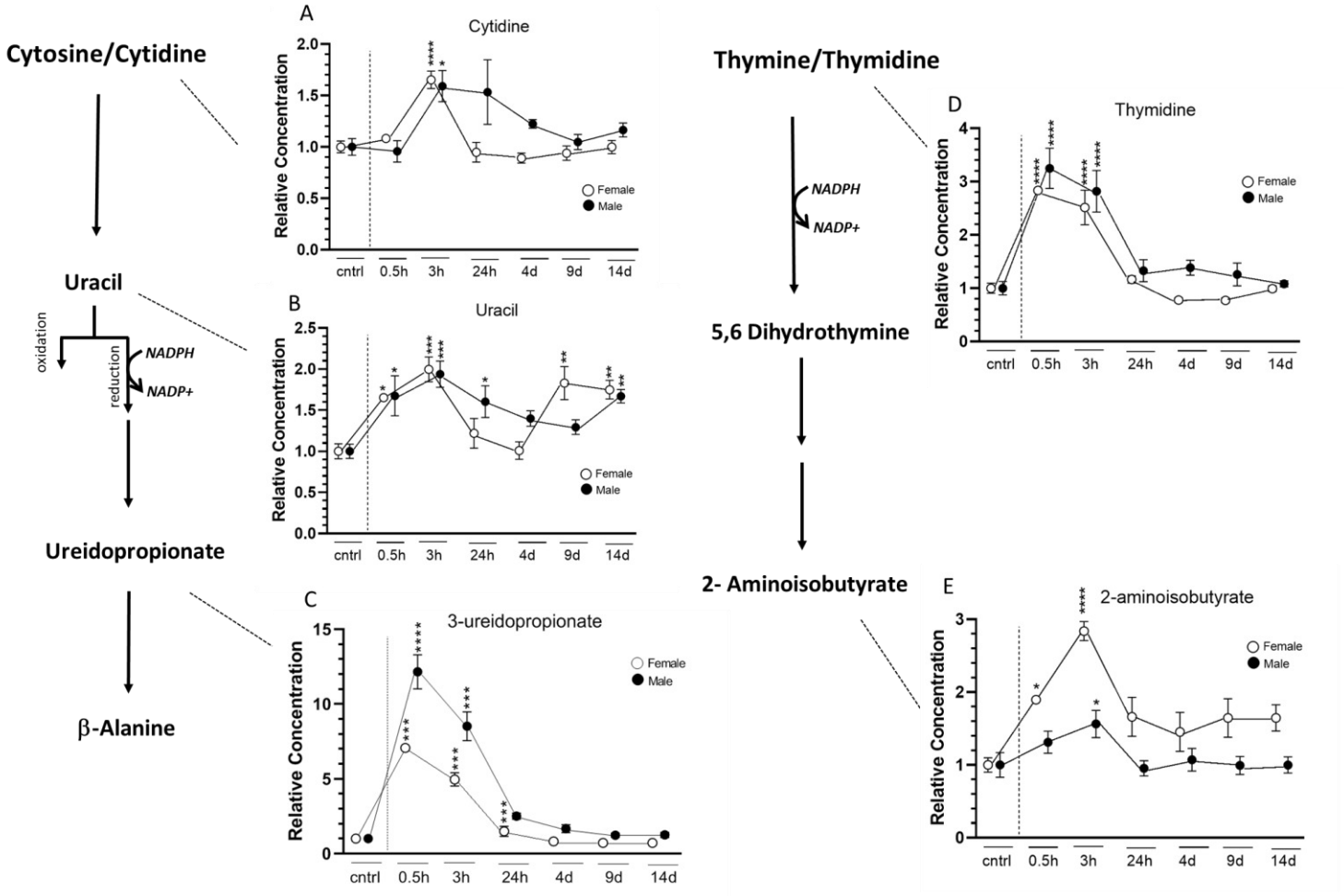


Free Fatty Acids, Ceramides and DAGs

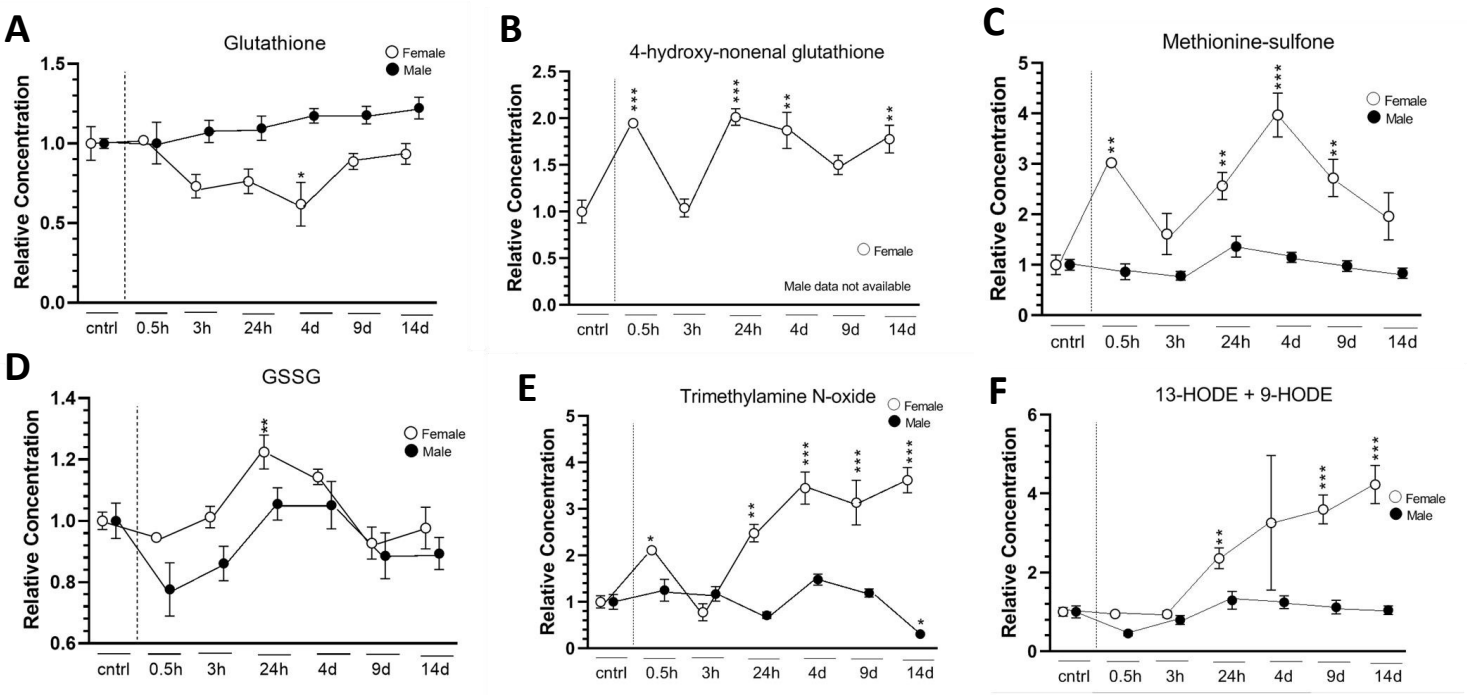


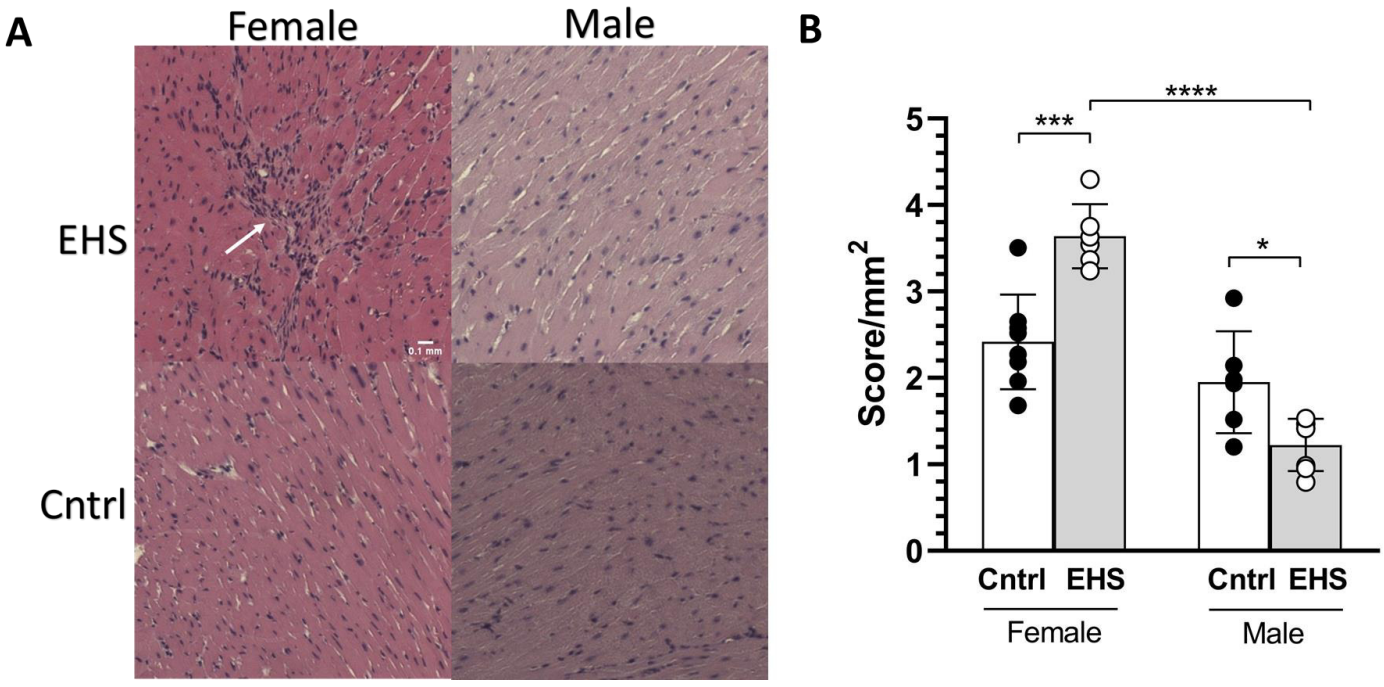

Purine metabolism
A
Adenosine monophosphate (AMP)
Relative Concentration
Female
Male
cntrl
0.5h
3h
24h
4d
9d
14d
AICAR
AMP
IMP
D
AICA Ribonucleotide (AICAR)
Adenosine
B
Adenosine
Hypoxanthine
E
Hypoxanthine
Xanthine
Guanine
C
Xanthine
Urate
Allantoin
F
Allantoin

Pyrimidine Metabolism



Indicators of Cell Stress




A
Female
Male
EHS
Cntrl
0.1 mm
B
Score/mm2
5
4
3
2
1
0
***
****
*
Cntrl
EHS
Cntrl
EHS
Female
Male

Medicine & Science in Sports & Exercise

Ibuprofen impacts heat tolerance but not gut injury in exertional heat stroke in mice

--Manuscript Draft--

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Abstract:	Intestinal injury is one of the most prominent features of organ damage in exertional heat stroke (EHS). However, whether damage to the intestine in this setting is exacerbated by ibuprofen (IBU), the most commonly used NSAID in exercising populations, is not well understood. PURPOSE: We hypothesized that IBU would exacerbate intestinal injury, reduce exercise performance and increase susceptibility to heat stroke. METHODS: To test this hypothesis, we administered IBU via diet to male and female C57/BL6J mice, over 48 h prior to EHS. Susceptibility to EHS was determined by assessing exercise response using a forced running wheel, housed inside an environmental chamber at 37°C. Core temperature (Tc) was monitored by telemetry. Mice were allocated into 4 groups: exercise only (EXC); EHS + IBU; EXC + IBU; and EHS only. Exercise performance and core temperature profiles were evaluated and stomachs, intestines and plasma were collected at 3 h, post EHS. RESULTS: EHS+IBU males ran ~87% longer when Tc was above 41°C ($P < 0.03$) and attained significantly higher peak Tc ($P < 0.01$) than EHS only mice. Histological analyses showed decreased villi surface area throughout the small intestine for both sexes in the EXC+IBU group vs. EXC only. Interestingly, though EHS in both sexes caused intestinal injury, in neither sex were there any additional effects of IBU. CONCLUSIONS: Our results suggest that at least in a preclinical mouse model of EHS, oral IBU at pharmacologically effective doses does not pose additional risks of heat stroke, does not reduce exercise performance in the heat, and does not contribute

	further to intestinal injury compared to EHS alone.
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Ibuprofen impacts heat tolerance but not gut injury in exertional heat stroke in mice

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Running Title: Ibuprofen impacts exertional heat stroke

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ABSTRACT

Intestinal injury is one of the most prominent features of organ damage in exertional heat stroke (EHS). However, whether damage to the intestine in this setting is exacerbated by ibuprofen (IBU), the most commonly used NSAID in exercising populations, is not well understood. PURPOSE: We hypothesized that IBU would exacerbate intestinal injury, reduce exercise performance and increase susceptibility to heat stroke. METHODS: To test this hypothesis, we administered IBU via diet to male and female C57/BL6J mice, over 48 h prior to EHS. Susceptibility to EHS was determined by assessing exercise response using a forced running wheel, housed inside an environmental chamber at 37°C. Core temperature (Tc) was monitored by telemetry. Mice were allocated into 4 groups: exercise only (EXC); EHS + IBU; EXC + IBU; and EHS only. Exercise performance and core temperature profiles were evaluated and stomachs, intestines and plasma were collected at 3 h, post EHS. RESULTS: EHS+IBU males ran ~87% longer when Tc was above 41°C ($P < 0.03$) and attained significantly higher peak Tc ($P < 0.01$) than EHS-only mice. Histological analyses showed decreased villi surface area throughout the small intestine for both sexes in the EXC+IBU group vs. EXC only. Interestingly, though EHS in both sexes caused intestinal injury, in neither sex were there any additional effects of IBU. CONCLUSIONS: Our results suggest that at least in a preclinical mouse model of EHS, oral IBU at pharmacologically effective doses does not pose additional risks of heat stroke, does not reduce exercise performance in the heat, and does not contribute further to intestinal injury compared to EHS alone.

KEY WORDS: Sexual dimorphism, fatty acid binding protein-2, NSAID, anti-pyrogenic, PGE2, hyperthermia

INTRODUCTION

There is widespread use of non-steroidal anti-inflammatory drugs (NSAIDs) throughout the US. For example, by 2004, ~8-12% of the entire US population >40 years old was regularly taking non-aspirin NSAIDs (1). In the US Army, by 2014, 82% of active duty Army received prescriptions for NSAIDs (2), with ibuprofen (IBU) comprising ~49% of all drug prescriptions (2). In one sample of collegiate football, ~70% of athletes were regularly taking NSAIDs during the season, and of these, ~80% were using IBU (3).

There are concerns with heavy use of non-steroidal anti-inflammatory drugs in individuals exercising intensely in the heat. IBU is considered a nonspecific NSAID, suppressing both cyclooxygenase 1 (COX-1) and COX-2 (4), but with ~15 times greater potency against COX-1 (5). This results in notable effects on the gastrointestinal (GI) barrier, reviewed in ref. (6). In addition, IBU also has other important side effects. For example, IBU ingestion in long endurance events is associated with higher incidences of kidney injury (7) and the US Food and Drug Administration recently issued new warnings regarding increased risks of thrombotic cardiovascular disease and stroke with frequent NSAID use, guidelines that include warnings regarding IBU (8).

Both intense exercise and hyperthermia may exacerbate the effects of NSAIDs on the GI tract. Acute GI disturbances during competition are frequently experienced among endurance athletes which is often associated with GI barrier dysfunction (9). Barrier dysfunction is aggravated proportionately in humans with increasing core temperature (reviewed in (10)) and by pre-ingestion of NSAIDs such as IBU (11, 12). Hyperthermia alone can induce GI injury in animal models of passive heat stroke (13, 14) and exertional heat stroke (15). When indomethacin, a predominant COX-1 inhibitor, was given at a high dose immediately prior to

exposure to passive heat stroke in mice, 47% of the animals died compared to no deaths in controls or compared to mice given very low dose indomethacin (16). The mice treated with high dose indomethacin showed clear evidence of overt GI bleeding. Therefore, concern over NSAID use before exercising in the heat appears warranted, but the specific effects of different NSAIDs (particularly IBU) in conditions of heat stroke are not well defined.

Since IBU is by far the NSAID of choice in the US for both athletes and active military personnel, whom often exercise in hot environments, we tested whether IBU treatment affects overall exercise performance and whether it contributes to further development of GI barrier dysfunction in an animal model of EHS. Important differences from previous work include: 1) the use of an EHS model in mice with neurological endpoints resembling clinical definitions of heat stroke in humans (15). The endurance exercise component of the model may have unique influences on GI barrier function compared to models of passive heat stroke. 2) The incorporation of IBU into the chow of the animals for a 48-hour period prior to the EHS exposure. This method ensured that the IBU, in principle, could be distributed throughout the GI tract, that it was not associated with GI injury or stress from gavage procedures, as well as being mixed with a normal fiber content. The latter is a critically important variable in both preventing and promoting NSAID-induced GI injury (17). This administration method more closely resembles the prescribed administration in humans, i.e. taken with food. 3) The use of a dosage of IBU within chow that has previously been identified in male C57bl/6 mice to be tolerated well and to be highly effective in reducing pain and platelet thromboxane production in serum samples (18), and 4) comparisons of treatment between both males and females undergoing EHS, as male and female mice have profound differences in exercise performance leading up to EHS (19), and sex differences have been identified regarding sensitivity to IBU (20). Our

working hypothesis was that IBU treatment would reduce exercise performance in the heat in both sexes and exacerbate the extent of GI damage that occurs in this model.

METHODS

Animal Subjects

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) and upon arrival, housed on a 12:12-h light/dark cycle at 20–22°C and 30–60% relative humidity (RH). Standard chow (2918 Envigo; Teklad, Madison, WI) and water were provided ad libitum. All mice were ~12-13 weeks old upon arrival and were ~18-22 weeks at the end of the protocol. Mice were randomized into 4 groups for the EHS experiments: EHS+IBU, IBU+exercise (EXC), EXC Only and EHS Only. Groups initially were a minimum n = 6 but the EHS+IBU groups were an N = 12-16 because they were doubly paired to EHS only and IBU+EXC during the experiment. EXC mice were match-controlled to EHS mice in terms of the duration of the exercise protocol and time of tissue collection, except they were kept at 22.5°C and 50% RH during exercise.

Surgery and exercise training

Implantation surgery of telemetry devices was performed under sterile conditions. Under isoflurane anesthesia, a laparotomy was performed to place temperature telemetry emitters into the abdominal cavity (G2 E-Mitter; Starr Life Sciences, Oakmont, PA) for measurements of core temperature (Tc). The abdominal cavity was closed, and mice were then placed in single-housing during recovery and the rest of the experiment. They were monitored for pain and behavior for

48 h, and subcutaneous injections of buprenorphine were given every 12 h for analgesia. Mice recovered from surgery for 2 weeks and then were given in-cage running wheels (model 0297–0521, Columbus Instruments, Columbus, OH) to allow voluntary exercise training for 3 weeks. During the third week, animals were exercised on a forced running wheel on four occasions over four consecutive days (Lafayette, model 80840, Lafayette, IN; powered by a programmable power supply, as described previously (19)). Once training was completed, mice were given 2-3 full days of rest prior to either the EHS or EXC trial. In-cage running wheels were made available during that time.

Feeding Protocol

IBU was administered within the feed as described by Salama et al. (18). Food was weighed to confirm the feeding amount needed for each mouse. The feed was prepared in 2918 Envigo chow with IBU using a commercial enrichment company (Bio-Serv, Flemington, NJ). Mice given IBU began dosing with IBU 48 hours prior to the scheduled EHS protocol. The dose used was 375mg/kg, the highest dose previously tested to be effective by Salama et al. (18). We removed the original food from EHS-only mice and replaced it with identical feed but without IBU (Placebo). Initial feed intake data was measured to replicate feeding values in Salama et al. (18).

EHS Protocol

The EHS protocol was carried out as shown in the idealized schematic in Fig. 1. Mice were brought to the laboratory the afternoon before EHS. Tc was recorded in 30-s intervals throughout the night to ensure normal temperature profiles before EHS. The 12:12-h light/dark cycle was maintained. The EHS procedure was run in the early morning beginning at ~ 7:30 AM. Mice

remained in their cages with Tc being monitored while the environmental chamber (Thermo Forma, 3940, Thermo-Fisher, Waltham, MA) heated to a set point of 37.5°C, and 30–40% relative humidity (RH). Environmental chamber temperature and humidity were measured and recorded at the location of the running wheel. Once the temperature equilibrated (30–45 min), the mice were placed in the enclosed forced running wheel within the chamber. Mice were given >5 min to recover from the stress of being handled and then, once Tc stabilized again to 36–37.5°C, the running wheel was started on a pre-programmed and standardized incremental protocol (Fig. 1). Speed began at 3.1 m/min and increased 0.3 m/min every 10 min until the mouse reached a Tc of 41°C. Once 41°C Tc was reached, the "steady-state" exercise phase began with speed maintained until the symptom-limited end point (Fig 2). The EHS end point was previously defined by loss of consciousness, specifically, 3 consecutive revolutions of the wheel with no physical response by the mouse. After symptom limitation, mice were checked for responsiveness to tactile touch and then quickly removed from the exercise wheel and placed in their cage with ad libitum access to food and water. The environmental chamber door was opened to room air during the recovery period and the temperature regulator for the incubator changed to room temperature. Tc was recorded continuously pre EHS for ~10-12 h, and after EHS until tissue collection at 3 h post EHS, at which time the mice were euthanized.

Sample collection and analyses

At 3 hours post EHS, while under isoflurane anesthesia, whole blood samples were drawn via transthoracic cardiac stick, using a Luer lock syringe and a 27-gauge needle, preloaded with EDTA. Hematology analysis was done on whole blood using a HESKA Element HT-5 Veterinary Hematology Analyzer according to manufacturer's instructions (HESKA Corp., Colorado, USA). Whole blood was then centrifuged at 4˚C for 10 min at 2000 x g, the plasma

transferred to microtubes and immediately snap frozen in liquid nitrogen. Gastrointestinal tissues were immediately obtained for histological analyses. The 3-hour time point was used because of previous experience with development of GI injury in this model (15). The entire surface of the stomach was evaluated for micro-hemorrhage or other abnormalities. The small intestines were prepared using a bundling method as described by Williams et al. (21). Sections of the duodenum (DUO), jejunum (JEJ), and ileum (ILE) were independently analyzed. Histological slides were prepared by the University of Florida Molecular Pathology Core Laboratory (4 μm thick). Imaging was done with a 10X objective and when the entire intestine was too big for one image field, multiple images were merged together for analyses. The original Chiu method was used for scoring villi size and injury (22). Measurements of damage, intestinal villi and crypt depths were done by two blinded graders and averaged. Surface area was calculated from these measurements as $(2\pi)(VW/2)(VL)$, where VW = villi width and VL= villi height (23) and this was the primary outcome variable reported. Gastrointestinal damage was further verified using a biomarker for small intestinal damage, intestinal fatty acid binding protein-2 (IFABP2). An ELISA kit for PGE2 (R&D Systems Minneapolis, MN, USA) and FABP2 (Cloud-clone corp., Houston, Texas, USA) were used with plasma samples following manufacturer's instructions

Statistical and analytical approach

Statistical testing and graphics were performed using SAS-JMP ® and/or GraphPad Prism. Though many of the experiments were matched in terms of exercise performance between control and experimental animals, there was no statistical advantage to pairing the final statistical data. Therefore, the data were handled as independent samples in each group, with nonsymmetrical sample sizes. For single measurements of performance in IBU-EHS vs EHS only animals, data were analyzed using a two sample T-test for normal data with well-matched

variances, a Welch's correction for groups with uneven variances (Fisher's test) or a Wilcoxon signed ranked test for nonparametric data determined by Shapiro-Wilkes test. For multiple groups of data, groups were usually tested with one-way ANOVA with appropriate post hoc analyses such as Tukey. For villi surface area, effects of IBU treatment and region were tested with two-way ANOVA with interactions, followed by post hoc contrasts. FABP2 data were nonparametric and analyzed by Kruskal-Wallis followed by Steel Dwass or Steel nonparametric post hoc tests as appropriate.

RESULTS

Effects of IBU on Exercise Performance in hyperthermia

As reported previously (19), females consistently outperformed the males in terms of their running endurance, resistance to hypethermia and speed during the EHS trial. As shown in Fig. 2A, male mice treated with IBU reached symptom limitation at a higher Tc,max compared to untreated EHS controls (mean = 42.31 vs. 41.97, $P < 0.01$) and exercise time during the steady-state phase, (Tc >41°C) increased 87% compared to placebo-treated male EHS mice (Fig. 2B). In females, effects of IBU treatment appeared similar but did not quite reach statistical significance for either variable. In both males and females, IBU had no impact on the degree or timing of hypothermia during the 3-hour recovery from EHS (data not shown).

Effects of IBU on gastrointestinal damage

Fig. 3 illustrates the effect of EHS or EXC on a plasma biomarker for intestinal epithelial injury, FABP2. EHS elevated FABP2 in both males and females compared to exercise matched

controls, but no significant effects of IBU treatment in either group were observed. Females, in general, had higher levels of FABP2 during EHS compared to males ($P < 0.02$).

Histological measurements of gastrointestinal damage were evaluated using average villi surface area as an indicator of damage. In exercise controls, IBU treatment in both males and females consistently resulted in smaller villi throughout the three regions of the small intestine (Fig 4 A & B), which is an indicator of recent damage and rapid restitution. After EHS exposure, males demonstrated further reductions of villi size in the duodenum, as expected (15), and in females in the ileum compared to exercise controls (analysis not shown), but IBU treatment had no significant effect on villus size in any region, in either sex. Measurements of gastric injury (micro bleeding) and intestinal villi damage scores were not significantly different in response to IBU in any group (data not shown).

Effects of EHS and IBU on immune cell populations

Acute exercise (EXC) resulted in significant reductions in total white blood cell (WBC) counts compared to identically treated control animals allowed to recover for 4 days (Fig, 5A, males). Samples for female 3 h exercise controls were not available due to equipment failure. There were no significant effects of acute exercise on %neutrophils, %lymphocytes or %monocytes (Fig. B, C, D, males). However, EHS exposure reduced total WBC counts, greatly elevated the %neutrophils in both males and females (Fig 5B), reduced the %lymphocytes and elevated % monocytes.

IBU treatment in exercise control males significantly elevated the total blood WBC counts (Fig. 5A, males). However, there were no other significant effects of IBU on % cell phenotypes in EXC or in EHS.

Effects of IBU treatment on serum PGE2

We used plasma PGE2 at the time of sample collection (3 h post EHS) to test the efficacy of the IBU at this time point. In time-matched exercise controls (Fig. 6A), IBU-treated male mice showed marked inhibition of plasma PGE2. However, we did not observe such inhibition in females (Fig 6B). In males, EHS appeared to independently suppress plasma PGE2, with IBU treatment having no additional effects. In females, there was no effect of either EHS or IBU on plasma PGE2. In general, females had markedly higher PGE2 values in all conditions compared to males.

DISCUSSION

In contrast to our working hypothesis, we found that IBU treatment did not reduce exercise performance in the heat, in fact, male mice significantly increased their tolerance to acquiring heat stroke. This manifested as a higher attained Tc,max at symptom limitation and an increased duration of exercise during the steady-state stage >41°C. In contrast, in female mice, there was no statistical effect of IBU on performance, though they trended in the same direction as the males. IBU treatment in control mice of both sexes exhibited the expected injury throughout the small intestine. However, following EHS there was no additional GI injury induced by IBU treatment in either sex. This result suggests that the IBU administration protocol was effective in delivering the drug to the target but the IBU stimulus was either not strong enough to induce further damage or some aspect of exercising in the heat prevented IBU from further contributing to damaged villi.

IBU Administration

We faced several challenges in employing an effective drug delivery method in this experiment. We considered using intraperitoneal injection (IP), oral gavage, mixed in drinking water, mixed in chow and mixed in a single dose within a treat. Since our interest was in studying effects on the GI tract, the IP route was not an option. In our experience, oral gavage is extremely stressful to mice and can delay gastric emptying. Therefore, providing the IBU with food, over time (18), would more closely mimic conditions experienced by athletes and the military. This tactic would also help avoid peak serum levels, allow better distribution through the GI tract and include the presence of dietary fiber, which can both contribute to and suppress small intestinal damage induced by NSAIDs, depending on the type of fiber, and the effective bioavailability of the drug (24). Furthermore, the dose given was previously shown to be the high end of an effective delivery method to reduce pain over several hours post ingestion (18).

Timing and rate of metabolism of IBU was also a concern. Mice eat primarily during their waking hours, i.e. intermittently throughout the night. Our mice are on a 7:00/7:00 light cycle, and we began their EHS protocol or exercise protocol at ~7:30 AM with a 30 min equilibration phase. The entire EHS protocol then requires an average of 2 h in males and 3 h in females (19), with a 3 hour recovery period. Therefore, IBU had to be active for a minimum of 3-4 hours to impact the mice throughout the EHS exposure. The half-life of IBU in the blood of mice when given orally in water is ≈ 170 min (25), but the ability to inhibit responsiveness to pain continues for at least 6 hours (26). The fact that we observed clear reductions in PGE2 (Fig 6) in male exercise control mice when measured in the blood at ~7 hours after their last ingestion, and the profile of immune cell populations in the blood (particularly % lymphocytes) were still evident at this time point (Fig. 5) confirms that the drug was still likely to be

effectively working at this time, at least in males. However, we are less confident that it was still effective in females, where tissue sample collection was yet another 1.5 hours beyond the males because of the much greater exercise capacity and resistance to heat stroke, as described previously (19) .

Effects of IBU on Performance

The males treated with IBU were better able to resist the onset of heat stroke compared to their placebo-treated controls (Fig. 2). We have no direct evidence for a mechanism but wish to discuss several possibilities. First, there are mixed reports that IBU and other related analgesics have the capacity to improve endurance exercise performance in both rodents and humans. In exercise trained rats, IBU increased time to exhaustion using a swimming model (27). Another study showed that chronic IBU treatment results in longer distances of voluntary wheel running in mice (28). However, in human studies there is sparse evidence that IBU improves endurance exercise (29–31). In contrast, several human studies have shown that acetaminophen administration results in greater performance during endurance exercise (32, 33) and notably during cycling exercise to exhaustion in a hot environment (30°C/50% relative humidity), where endurance time was increased and skin temperature and Tc reduced by acetaminophen (34). Though not technically categorized as a NSAID, acetaminophen is a well-known PGE2 inhibitor and an anti-pyrogenic. One potential mechanism that has been proposed is that acetaminophen and IBU can cross the blood brain barrier and reduce brain PGE2 levels. PGE2 acts as a pyrogen in the brain that elevates the thermoregulatory set point, particularly in conditions of fever. Some investigators have proposed that pyrogenic effects of brain eicosanoids contribute to elevations in body temperature during endurance exercise (34).

A further consideration that relates specifically to this model is that the males exercised to a higher Tc compare to their placebo-treated counterparts (Fig. 2), which was unexpected. Based on the pyrogen hypothesis discussed above, this would appear to be an opposite effect. However, it is important to note that the endpoint in this model is CNS dysfunction, not exercise exhaustion. We do not understand the origins of CNS dysfunction in heat stroke but it could reflect loss of cardiovascular function (35), attaining a pain or stress threshold, loss of blood brain barrier integrity or low nutrient delivery, all of which could be influenced in various ways by NSAIDs and analgesics.

Low responsiveness in females

In females, we saw no statistically significant effect of IBU treatment on exercise performance, no effect on PGE2 levels at the time of sample collection, and no apparent effects on immune cell profiles at any time. However, IBU-induced injury to the small intestine in the time matched exercise controls was nearly identical in both sexes (Fig. 4A & B), suggesting that the drug delivery system was effective in both. These results suggest that either the longer exercise time experienced by the females (1.5-2 h) reduced the bioavailability of IBU during the late stages of EHS or that female mice had a lower sensitivity to IBU. There is no available evidence for lower sensitivity to IBU in female rodents, but differences in effectiveness of IBU in pain management have been observed in females (20). Nevertheless, we believe the most likely explanation for our different results between the males and females is that the exercise time of the females extended beyond a period of IBU effectiveness.

Another interesting sex difference was that in male mice, post EHS, there was a marked suppression of plasma PGE2 in both IBU and placebo-treated mice (Fig. 6A), but the effect was not seen at all in females (Fig. 6B). Similar reductions in PGE2 have been observed following

human EHS in military populations, which were mostly male (36). The effects of heat and stress on eicosanoid production are complex, but there are several hormonal influences that may be relevant. For example, we have previously shown that both male and female mice respond to EHS with 400-500% increases in plasma corticosterone prior to 3 hours of recovery (19). Glucocorticoids have been shown to inhibit PGE2 production by several known mechanisms. Nevertheless, the high PGE2 levels in females, that appeared unaffected by either EHS exposure or IBU treatment (Fig. 6), remain a mystery. Though plasma PGE2 is known to be similar in control male and female mice, COX2 is under regulation by estradiol (37) which can result in sex differences. For example, PGE2 elevations following the stress of burn injury in female mice are much higher compared to males (38), an effect attributed to a reduced rate of PGE2 degradation and clearance in stress due to reductions in estradiol-regulated prostaglandin dehydrogenase.

Lack of effects IBU on small intestine injury following EHS

Another unexpected finding was that though IBU caused injury to the small intestine in all exercise control mice, there was no evidence of further injury in IBU treated EHS mice. It could be that we met some threshold for injury from EHS that was not further affected by superimposed IBU. However, another possibility is that the normal hormonal responses to EHS as compared to control exercise conditions may have prevented further loss of gut integrity. In a previous study we reported that EHS results in a rapid spike in IL-6 production immediately after EHS (19). We have also previously shown that the preconditioning injections of IL-6 protect the intestinal lining during the recovery period of EHS (14). Also, elevations in endogenous glucocorticoids that we have also reported at this stage (19), are protective of gastric injury induced from NSAIDs (39), though their effect on the small intestine is not as well defined.

Finally, in the female mouse, suppression of PGE2 degradation during stress may have further protected the intestine and/or promoted its rapid recovery (40). Therefore, we hypothesize that the confluence of factors related to exercising in the heat induced an overriding protection of the intestine from further NSAID-induced injury in EHS.

Conclusions

The results of these experiments are not consistent with the idea that IBU ingestion at analgesic doses prior to exercising in the heat necessarily comprises an added risk factor to developing heat stroke or suffering from further GI injury. However, these conclusions should be viewed with caution because humans may respond differently, human subjects often ingest higher than recommended doses of NSAIDs and may have extenuating conditions that could make them vulnerable to negative side effects. Sex differences, dosing and administration issues, contrasting actions of different COX inhibitors, effects of varying levels of endurance exercise and hyperthermia, uncertainties of the pharmacokinetics of the drug under different environmental conditions and the varying influence of the drugs on different tissue types provide just some of the challenges to our full understanding of what NSAID ingestion means to the health of the competing athlete or the active Warfighter.

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474

FIGURE LEGENDS

Figure 1. Experimental design of exertional heat stroke exposure and time of tissue collection. The first component of the treatement was an incremental exercise stage that lasted until the mouse attained a core temperature of 41°C. The mouse then performed steady-state exercise until it reached symptom limitation. The duration from the beginning of 41°C until reaching Tc,max and symptom limitation was considered the ability of the mouse to resist heat stroke. Samples were collected after three hours of recovey.

Figure 2. Effects of IBU treatment on A: peak core temperature attained during the EHS and B: The time of steady state exercise during hyperthermia >41°C, and indicator of resistance to heat stroke. (n= 14-16 EHS + IBU, n = 6 EHS only; Means ± SD, analysis two sample t test for either equal or unequal variances, depending on the sample distributions).

Figure 3. Effects of Ibuprofen and exertional heat stroke (EHS) on plasma fatty acid binding protein, (FABP2), a biomarker for intestinal injury. P = placebo, IBU = ibuprofen. Exer = time-matched exercise-matched control mice. Means ± SD, Welches ANOVA corrected for unequal variances, post hoc test comparisons of all groups using Prism. (n= 14-16 EHS + IBU, n = 6 EHS only; Means ± SD).

Figure 4. Effects of IBU treatment in A &B: exercise controls and C&D: exertional heat stroke animals. Measurements of average intestinal villi surface area in each region of the small intestine were computed as indicators of restitution response to villus damage. (N = 6 mice per groups, randomly selected). Results of two-way ANOVA are inserted into each panel. Individual P values are post hoc contrasts using (SAS JMP).

Figure 5. Whole blood cell counts with effects of IBU, EHS and acute exercise on the immune response. An additional set of sham control animals were added to the experiment which were allowed to recover for 4 days (4D) to test for the effects of acute exercise. The other is a time-matched exercise control (3h), only available for the male data sets because of technical difficulty. Results are post hoc following ANOVA for each sex.

Figure 6. Plasma [PGE2] in A: Females and B: Males in placebo (P) controls vs. ibuprofen (IBU) treated mice. (n= 14-16 EHS + IBU, n = 6 EHS only; Means ± SD). ANOVA followed by post hoc comparisons.

Figure 1

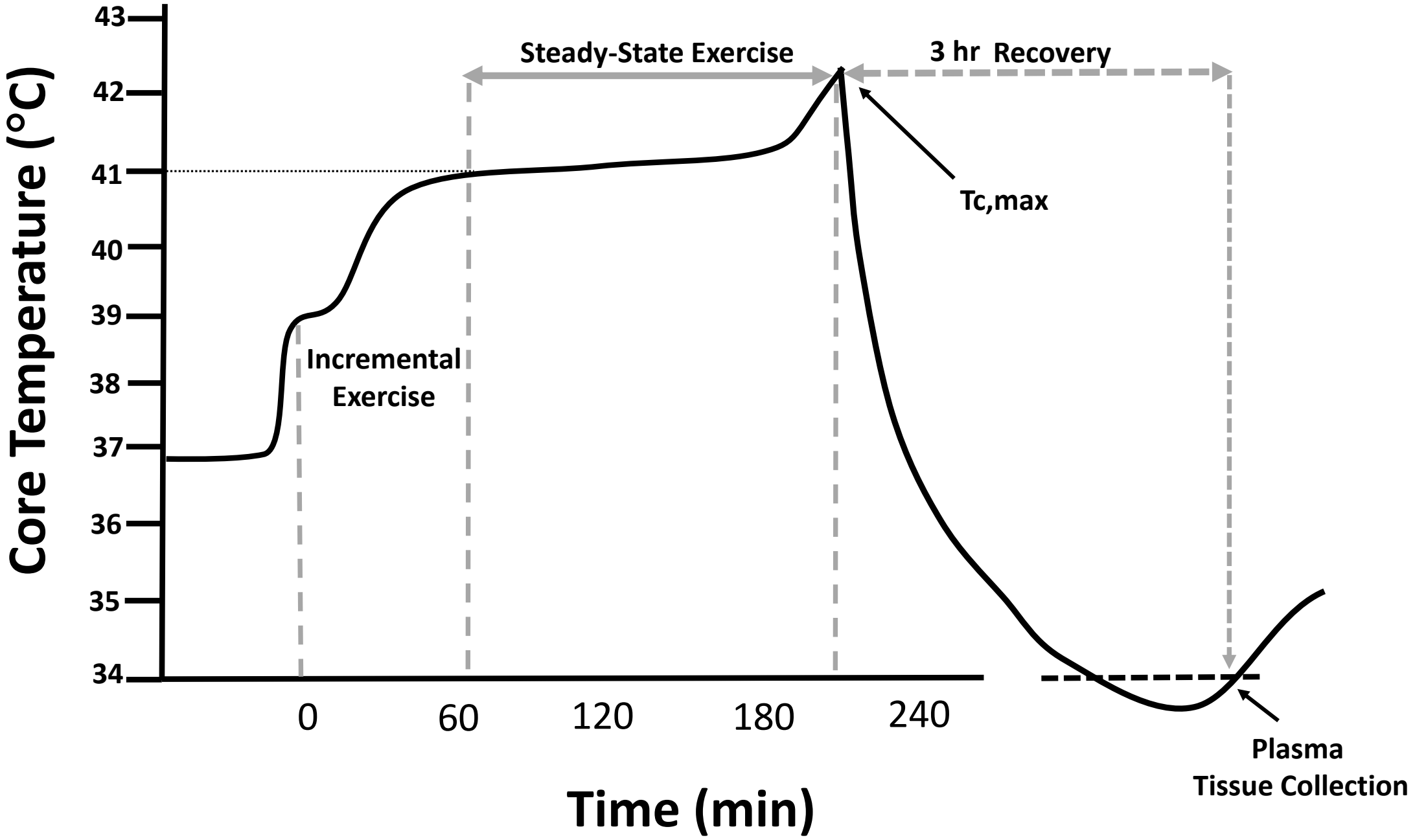

Core Temperature (°C)
43
42
41
40
39
38
37
36
35
34
Steady-State Exercise
3 hr Recovery
Tc,max
Incremental
Exercise
0
60
120
180
240
Time (min)
Plasma
Tissue Collection

Figure 2

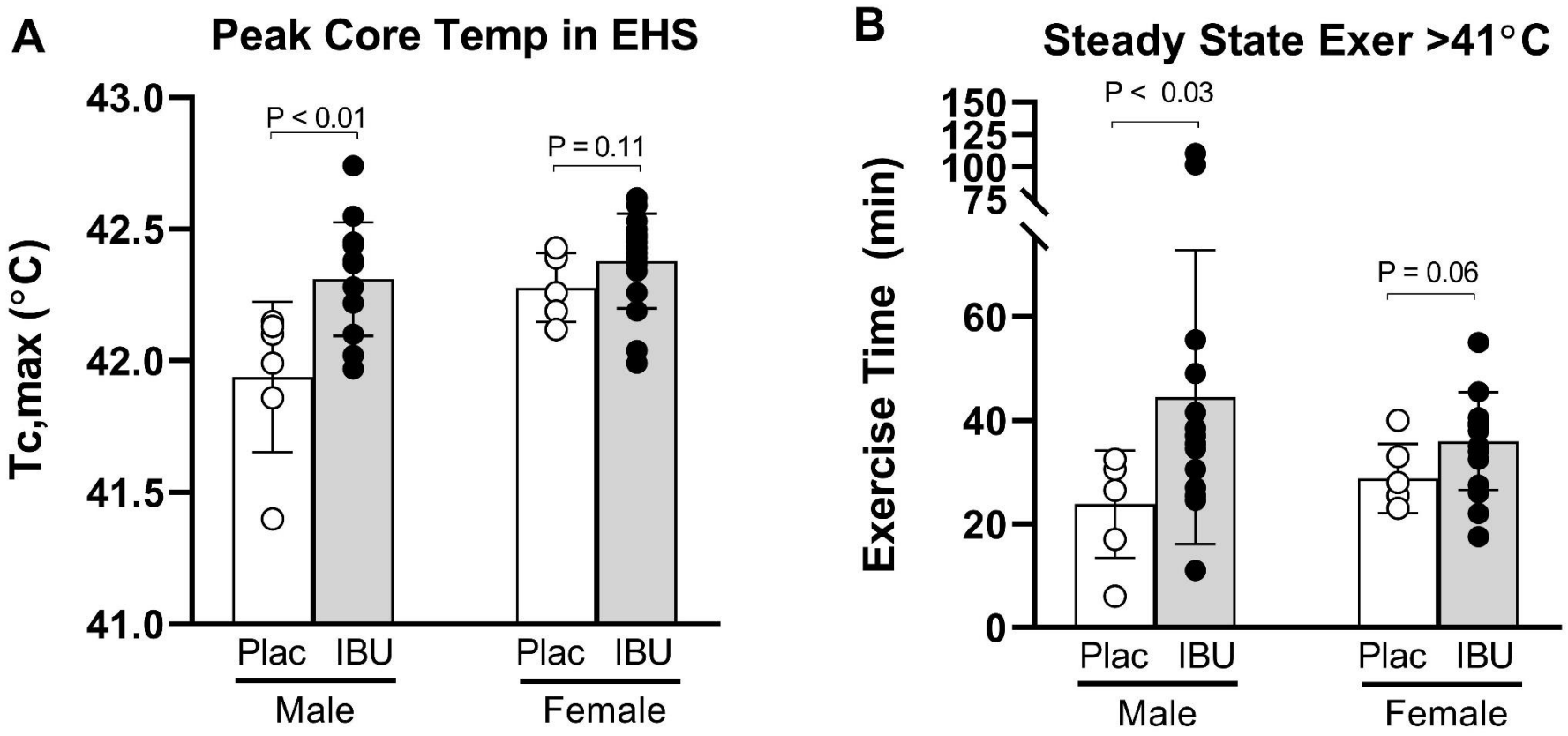

A
Peak Core Temp in EHS
Tc,max (°C)
43.0
42.5
42.0
41.5
41.0
P < 0.01
P = 0.11
Plac
IBU
Plac
IBU
Male
Female
B
Steady State Exer >41°C
Exercise Time (min)
150
125
100
75
60
40
20
0
P < 0.03
P = 0.06
Plac
IBU
Plac
IBU
Male
Female

Figure 3

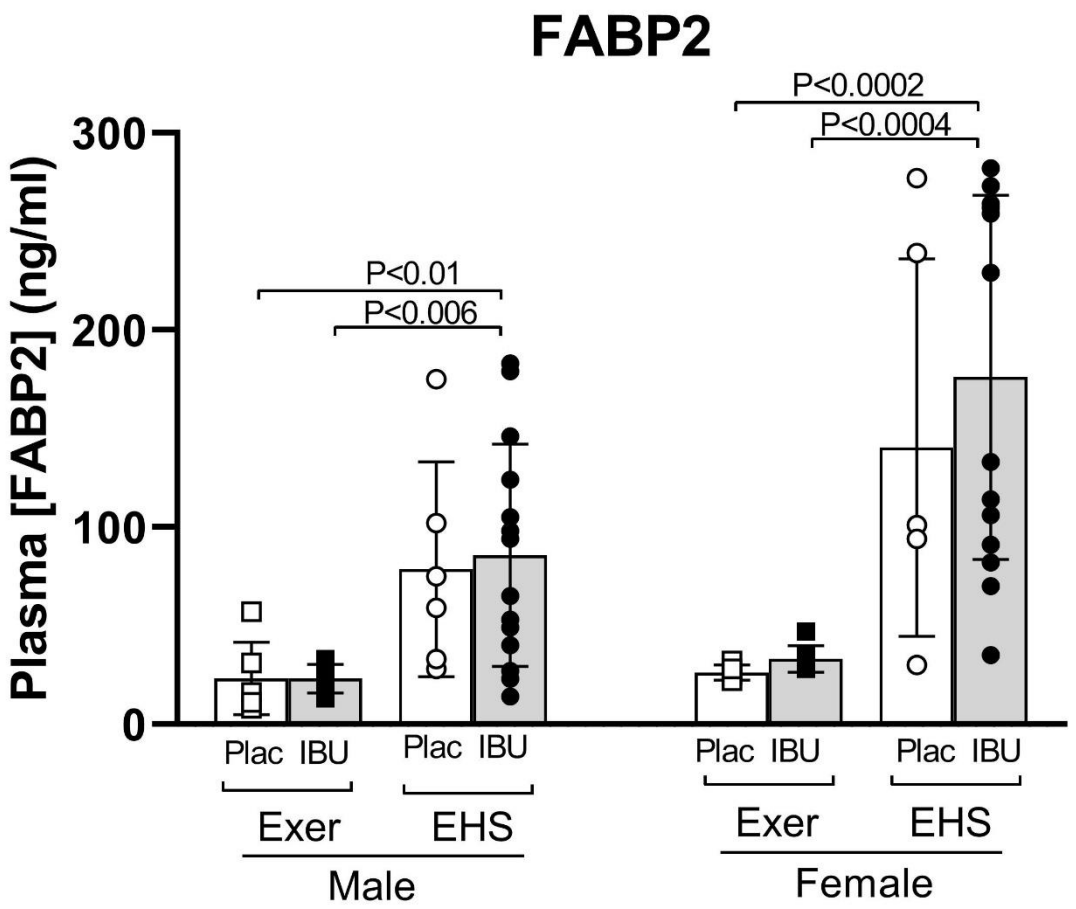

FABP2
Plasma [FABP2] (ng/ml)
300
200
100
0
P<0.01
P<0.006
P<0.0002
P<0.0004
Plac IBU
Plac IBU
Plac IBU
Plac IBU
Exer
EHS
Exer
EHS
Male
Female

Figure 4

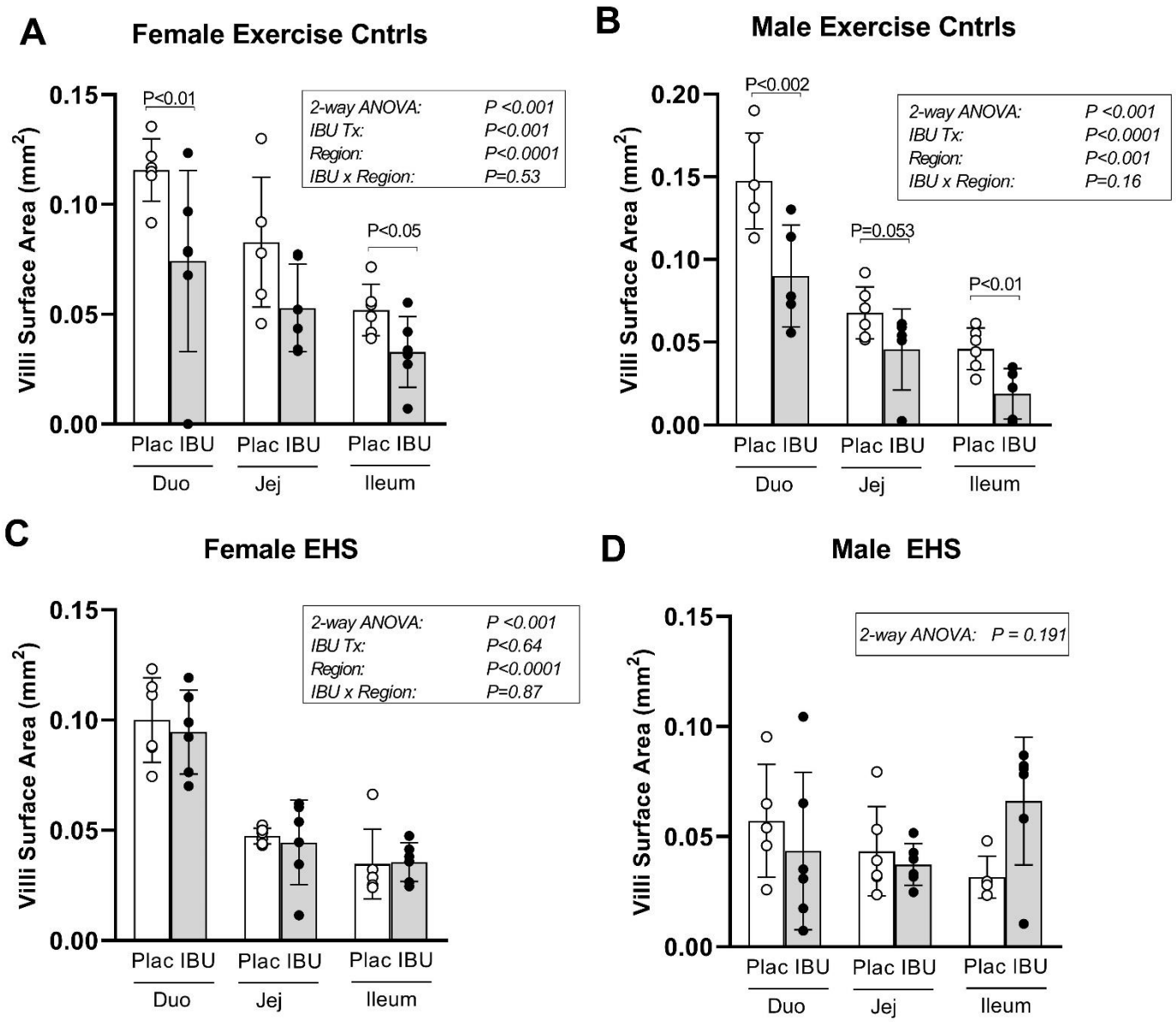

A
Female Exercise Cntrls
Villi Surface Area (mm²)
0.15
0.10
0.05
0.00
P<0.01
P<0.05
2-way ANOVA: P <0.001
IBU Tx: P<0.001
Region: P<0.0001
IBU x Region: P=0.53
Plac IBU
Plac IBU
Plac IBU
Duo
Jej
Ileum
B
Male Exercise Cntrls
Villi Surface Area (mm²)
0.20
0.15
0.10
0.05
0.00
P<0.002
P=0.053
P<0.01
2-way ANOVA: P <0.001
IBU Tx: P<0.0001
Region: P<0.001
IBU x Region: P=0.16
Plac IBU
Plac IBU
Plac IBU
Duo
Jej
Ileum
C
Female EHS
Villi Surface Area (mm²)
0.15
0.10
0.05
0.00
2-way ANOVA: P <0.001
IBU Tx: P<0.64
Region: P<0.0001
IBU x Region: P=0.87
Plac IBU
Plac IBU
Plac IBU
Duo
Jej
Ileum
D
Male EHS
Villi Surface Area (mm²)
0.15
0.10
0.05
0.00
2-way ANOVA: P = 0.191
Plac IBU
Plac IBU
Plac IBU
Duo
Jej
Ileum

Figure 5

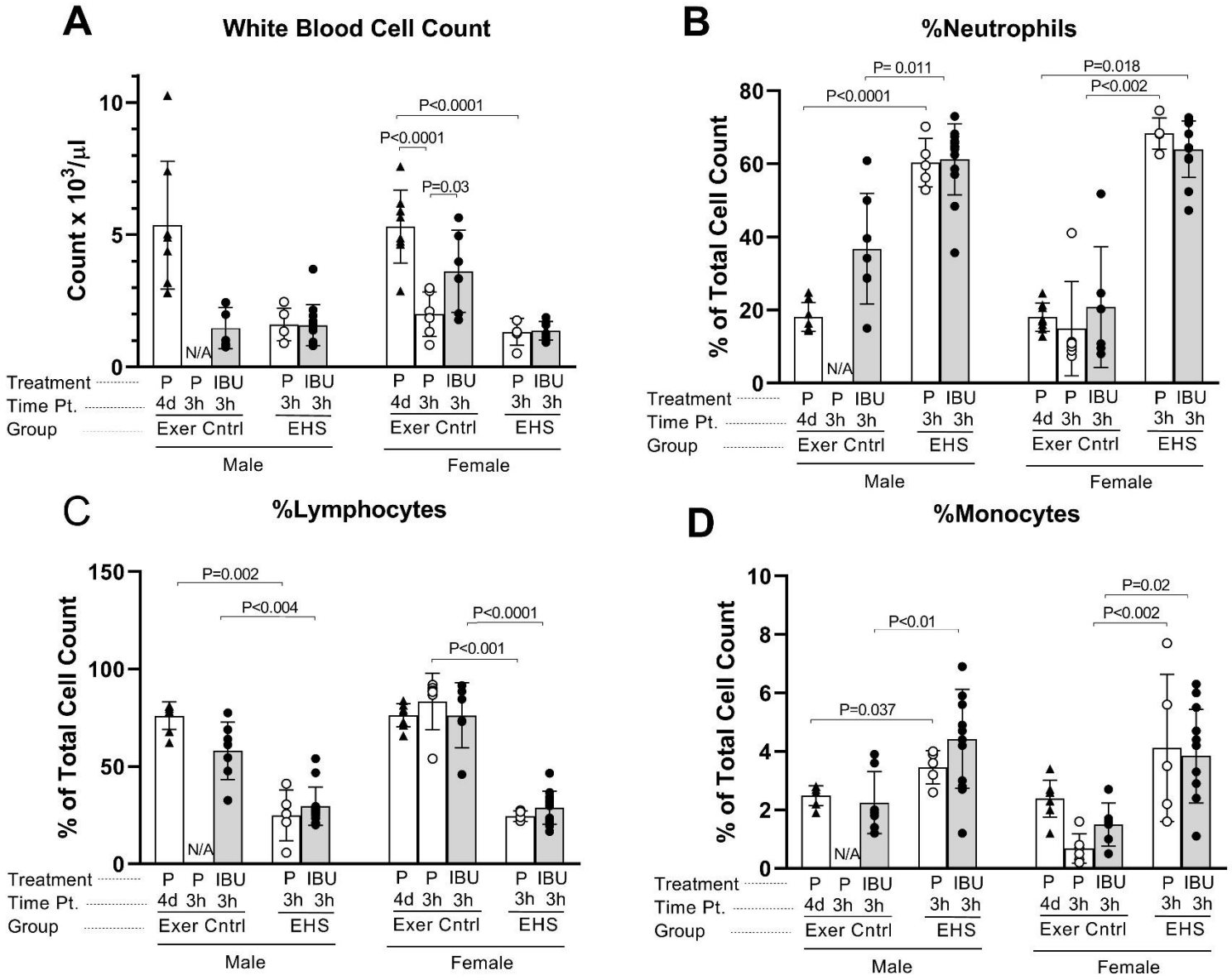
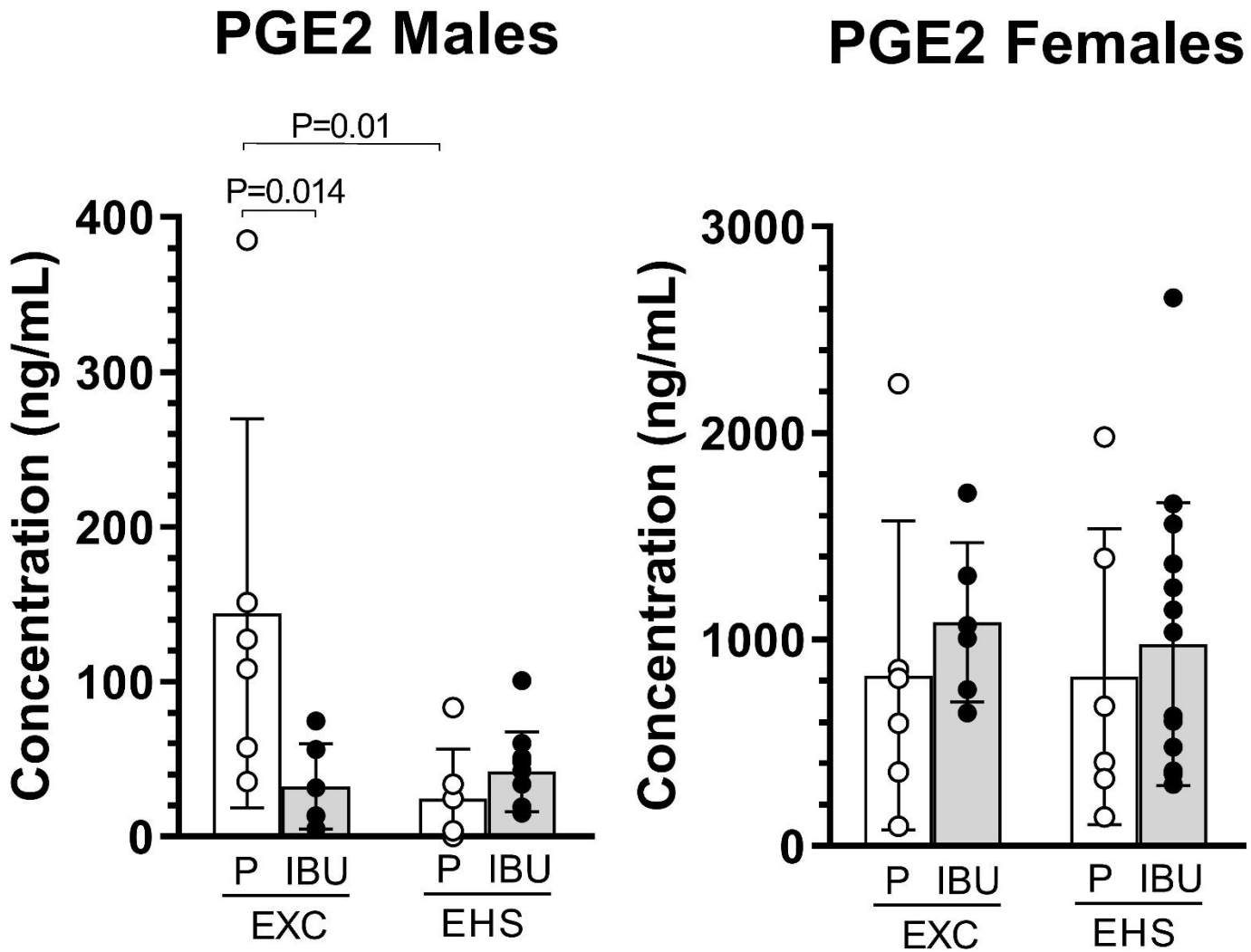

A
White Blood Cell Count
Count x 10³/µl
P<0.0001
P<0.0001
P=0.03
N/A
B
%Neutrophils
% of Total Cell Count
P= 0.011
P<0.0001
P=0.018
P<0.002
N/A
C
%Lymphocytes
% of Total Cell Count
P=0.002
P<0.004
P<0.0001
P<0.001
N/A
D
%Monocytes
% of Total Cell Count
P<0.01
P=0.037
P=0.02
P<0.002
N/A
Treatment P P IBU P IBU P P IBU P IBU
Time Pt. 4d 3h 3h 3h 3h 4d 3h 3h 3h 3h
Group Exer Cntrl EHS Exer Cntrl EHS
Male Female

Figure 6


PGE2 Males
P=0.01
P=0.014
Concentration (ng/mL)
400
300
200
100
0
P IBU
EXC
P IBU
EHS
PGE2 Females
Concentration (ng/mL)
3000
2000
1000
0
P IBU
EXC
P IBU
EHS