

**Effects of Heat Stress on Hsp70, Sarcolipin, and Phospholamban Content and SERCA
Function in Female and Male Rat Skeletal Muscle**

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final reversions, as accepted by my examiners.

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Abstract

Regulation of cytosolic calcium (Ca^{2+}) levels in muscle is vital to the health and function of muscle due to its involvement in various signalling pathways and cell functions (Tu et al., 2016). Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps are found on the membrane of the sarcoplasmic reticulum (SR) and function to actively transport Ca^{2+} from the cytosol into the SR (Gamou et al., 2014; Rossi & Dirksen, 2006). Previous work has shown that in the face of heat stress (HS), a model for oxidative stress, heat shock protein 70 (Hsp70) expression is induced to protect the cytosolic domain and function of SERCA (Fu & Tupling, 2009; Tupling et al., 2004).. Having previously found that, sarcolipin (SLN) and phospholamban (PLN), which regulate SERCA in the transmembrane (TM) region of the pump, can also preserve SERCA function using both in-vitro and ex-vivo HS models (Fu et al., 2020) we wanted to assess if they can be stress-induced proteins like Hsp70. The purpose of this project was to assess the sex differences in basal protein expression of Hsp70, SLN, and PLN and the effect of *in-vivo* heat stress on SERCA function, total protein nitrosylation status, and gene and protein time-course expression pattern of the three proteins in male and female rat soleus and white gastrocnemius (WG) muscles. The heat stress protocol involved submerging the lower limbs of the animals either in a 37°C (control) or 42-42.5°C (HS) water bath to either maintain core temperature at 37°C (control) or between 41-41.5°C for 30 mins and were either allowed no recovery or were allowed to recover for 24 or 48 hours. Analysis of basal expression found that i) Hsp70 gene expression was higher in both muscles in the females, but its protein levels were higher in the male soleus, ii) SLN gene expression was also higher in both muscles in the females, but no sex differences were observed in the protein levels, and iii) PLN protein expression was higher in the male soleus. *In-vivo* heat stress i) did not affect SR Ca^{2+} uptake in the solei of both sexes whereas, in

the WG, SR Ca^{2+} uptake was increased after 24 hours in both sexes ii) did not have an effect on the protein nitrosylation status in the soleus of male and female rats and on WG of female rats, iii) induced both gene and protein expression of Hsp70 in the soleus and WG of both male and female rats, iv) induced protein expression of SLN in the male soleus but only gene expression of *Sln* in the male WG whereas the same effects were not observed in the females and v) did not induce *Pln* gene expression in either muscle, but did induce PLN protein expression only in the WG of males. The findings from this study show that even with a relatively mild acute stress model like the one used here, both SLN and PLN were upregulated at least in one muscle of males, indicating that these proteins may also function as stress induced proteins.

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Dedication

This thesis is dedicated to my grandfather Devendra. I know you were hoping I would choose the medical sciences, but I choose a different route, and I wouldn't have chosen it had it not been for all your "mini biology lectures" and all the stories of scientists you have told me since I was a kid! This thesis is the start of my journey into science and for that I owe you so very much. I am so thankful to you for inciting a passion for science in the young me!

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List of Abbreviations

Ca²⁺ – Calcium

CaMKII – Ca²⁺/calmodulin-dependent protein kinase II

CON – Control

DMD – Duchenne Muscular Dystrophy

Hsp70 – Heat shock protein 70

HS – Heat Stress

HS0 – Heat Stress 0 hour

HS24 – Heat Stress 24 hour

HS48 – Heat Stress 48 hour

SERCA – Sarco/Endoplasmic Reticulum Ca²⁺-ATPase

SLN – Sarcoplipin

SR – Sarcoplasmic Reticulum

PLN – Phospholamban

TM – Transmembrane

WG – White Gastrocnemius

Introduction

Importance of Calcium in Skeletal Muscle:

Calcium ions (Ca^{2+}) are highly important signalling molecules in muscle that are at the centre of several pathways responsible for the function, growth, and maintenance of muscle (Tu et al., 2016). Force generation is proportional to the amount of cytosolic Ca^{2+} released from the sarcoplasmic reticulum (SR) during excitation-contraction coupling (Lamb & Stephenson, 2018); with its release allowing for cross-bridge formation (Huxley, 1969). By activating Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), calcineurin, and mitogen-activated protein kinase, Ca^{2+} promotes growth and structural alterations in muscle (Fajardo et al., 2017; Tu et al., 2016). Ca^{2+} also activates calpains and caspases (Ca^{2+} activated proteases) which are vital to protein turnover and maintenance of muscle (Hyatt & Powers, 2020). In healthy states, when cytosolic Ca^{2+} levels are well regulated, these signalling pathways serve to benefit muscle (Hyatt & Powers, 2020; Tu et al., 2016; Valentim et al., 2022).

However, in disease states, where cytosolic Ca^{2+} levels are dysregulated, aberrant Ca^{2+} signalling can be damaging to muscle (Valentim et al., 2022). Particularly in muscular dystrophies, abnormally elevated cytosolic Ca^{2+} levels will lead to abnormal elevations in proteolytic activity (Gailly et al., 2007; Murphy et al., 2006) and reactive oxygen species (ROS) generation (Altamirano et al., 2012). In muscle, the SR bound sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump is a major regulator of cytosolic Ca^{2+} levels. In these diseased states, SERCA is often damaged, and its function is often impaired which then future contributes to calcium dysregulation (23-25). Using mouse models of limb-girdle muscular dystrophy and Duchenne's muscular dystrophy, Goonasekera and colleagues showed that increasing SERCA expression in these mice allowed for faster cytosolic Ca^{2+} removal leading reduction in

centralized nuclei and fibrosis. Similar findings have been shown using a pharmacological activator of SERCA (CDN1163) (Nogami et al., 2021), or a Hsp70 inducer (BGP-15) to protect SERCA (Gehrig et al., 2012) in other mouse models of muscular dystrophy; by allowing for better cytosolic Ca^{2+} regulation, pathology is being ameliorated. The preservation of SERCA function in the face of physiological stress is essential in allowing for proper regulation of cytosolic Ca^{2+} levels and thus preserving the health and function of muscle.

SERCA Structure and Function:

SERCA is a 110 kDa P-type ATPase which is embedded in the membrane of the longitudinal SR where it functions to transport Ca^{2+} against its concentration gradient in an ATP-dependent manner; ideally, at a ratio of 2 Ca^{2+} ions transported for every ATP hydrolysed (see figure 1) (Gamut et al., 2014; Rossi & Dirksen, 2006). Structurally, SERCA is composed of 3 regions: a cytosolic region which contains the phosphorylation (P-domain), actuator (A-domain) and nucleotide-binding (N-domain); a transmembrane (TM) region with 10 helices (M1- M10), and a luminal region (Toyoshima et al., 2000). Within the TM region lies the two Ca^{2+} binding sites – site I, formed by M5, M6, and M8 helices, and site 2, formed by M4 and M6 helices. Ca^{2+} binding is cooperative whereby binding of the first ion to one site increases affinity for the second ion (Lee & East, 2001). Furthermore, Ca^{2+} binding also occurs in a sequence – binding to site I first, followed by site II (Zhang et al., 2000). SERCA's catalytic cycle involves large conformational changes in which there is an E1 (facing the cytosol) and E2 state (facing the SR lumen) state (Gamut et al., 2014; Gorski et al., 2017). In the E1 state, the adenosine moiety of ATP binds the N-domain and upon Ca^{2+} binding, the A-domain assists in bringing the N-domain closer to the P-domain in which the Asp 351 residue is phosphorylated (MacLennan & Green,

2000; Mueller et al., 2004; Toyoshima et al., 2000). Then transitioning into the E2 state, the transmembrane region also undergoes conformational change so that the Ca^{2+} binding sites which have occluded the Ca^{2+} ions within, will face the SR lumen to allow for Ca^{2+} release (Gamut et al., 2014; Gorski et al., 2017). Within striated muscle, 2 primary SERCA isoforms exist – SERCA1a and SERCA2a, which are derived from two distinct genes: *ATP2A1*, and *ATP2A2* (Rossi & Dirksen, 2006). SERCA1a is found primarily in adult fast-twitch muscle in association with type II MHC isoform while SERCA2a is found primarily in adult slow-twitch in association with type I MHC isoform and cardiac muscle (Rossi & Dirksen, 2006; Tupling, 2009).

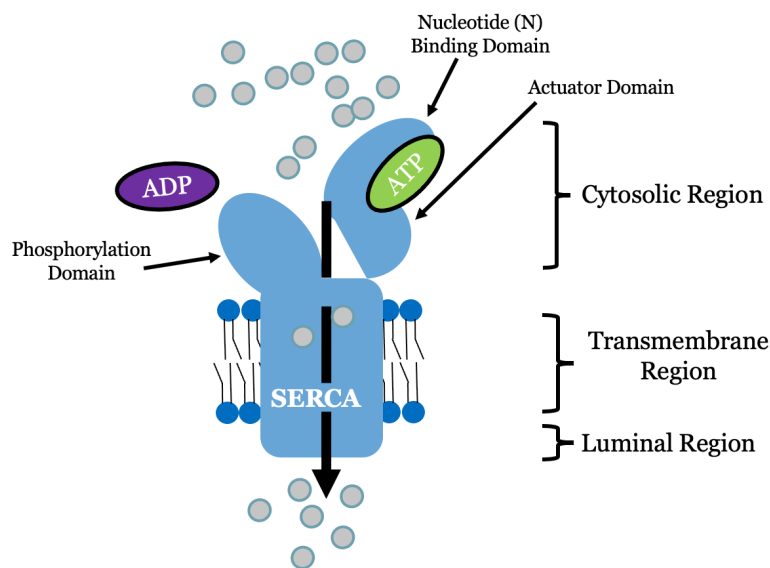


Figure 1: Sarco/Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) Structure

SERCA is a transmembranous protein with 3 regions – a cytosolic, transmembrane, and luminal region. The cytosolic region contains a nucleotide (N) binding domain, a phosphorylation (P) domain, and an actuator (A) domain.

Sarcolipin and Phospholamban – Regulators of SERCA:

The two most widely studied inhibitors of SERCA are sarcolipin (SLN) and phospholamban (PLN) (see figure 2). SLN and PLN are 31 and 52 amino acids in length,

respectively, and have three domains to their structure – SLN has a cytosolic, transmembrane, and luminal domain whereas PLN has two cytosolic domains (IA and IB) and a transmembrane domain II (Bhupathy et al., 2007; Gorski et al., 2017). The two proteins share significant homology within the transmembrane domain, with 7 identical and 7 hydrophobically conserved residues (Bhupathy et al., 2007). The expression patterns and associations of SLN and PLN with SERCA isoforms in skeletal muscle vary between different models. To date, Fajardo and colleagues are the only group to characterize the expression patterns of SERCA and its regulators. In human vastus lateralis, they found that SLN tends to associate more closely with SERCA1a in type II fibres while PLN tends to associate more closely with SERCA2a in type I fibres; however, it is likely that there is some crossover in expression between the two SERCA regulators (Fajardo et al., 2013). In rodent skeletal muscle, however, SLN and PLN are both expressed in mainly slow-twitch soleus, whereas neither are detected in the fast-twitch quadriceps (Babu et al., 2007). The mouse soleus contains approximately 50% type IIa fibres and 30% type I fibres (Bloemberg and Quadrilatero 2012) and therefore contain both isoforms of SERCA (Smith et al. 2013) which would explain the presence of both regulators in this muscle. The mouse vastus intermedius and white vastus lateralis, however, contain nearly 97 and 100% type II fibres (Bloemberg and Quadrilatero 2012), and thus carry the SERCA1a isoform (Babu et al. 2007), yet no expression of SLN has been found here. The rat soleus on the other hand, is made up of approximately 97% type I fibres (Bloemberg and Quadrilatero 2012) which would explain the presence of PLN, but not of SLN. In the atria, SLN is highly expressed and is the main regulator of SERCA2a in this tissue (Babu et al. 2007); so, it is not exactly clear what determines the expression pattern of SLN.

Both SLN and PLN inhibit SERCA by interacting at the M2, M6, and M9 helices (Gorski et al., 2017; Toyoshima et al., 2003; Toyoshima et al., 2013); however, their method of inhibition is different. At low cytosolic $[Ca^{2+}]$, the transmembrane domain of the unphosphorylated PLN will bind SERCA in the E2 state after Ca^{2+} ions have been released into the SR lumen to slow the transition back to E1 and lower SERCA's affinity for Ca^{2+} (Shaikh et al., 2016). This inhibition will be removed at higher cytosolic Ca^{2+} or by phosphorylation of PLN at either Ser16 by protein kinase A or at Thr17 by CaMKII (James et al., 1989; MacLennan & Kranias, 2003; Shaikh et al., 2016). In contrast, SLN, remains bound to SERCA at all concentrations of Ca^{2+} and inhibits its function by trapping SERCA in its E1 state with two magnesium ions bound at an incompletely formed site II and thus reducing SERCA's affinity for Ca^{2+} (Toyoshima et al., 2013; Tupling et al., 2011; Winther et al., 2013). Furthermore, SLN also promotes Ca^{2+} slippage from SERCA during the transition between the E1 and E2 states leading to uncoupled ATPase activity (Bombardier et al., 2013; Smith et al., 2002) which has been shown to promote muscle-based thermogenesis in mammals (Bal et al., 2012).

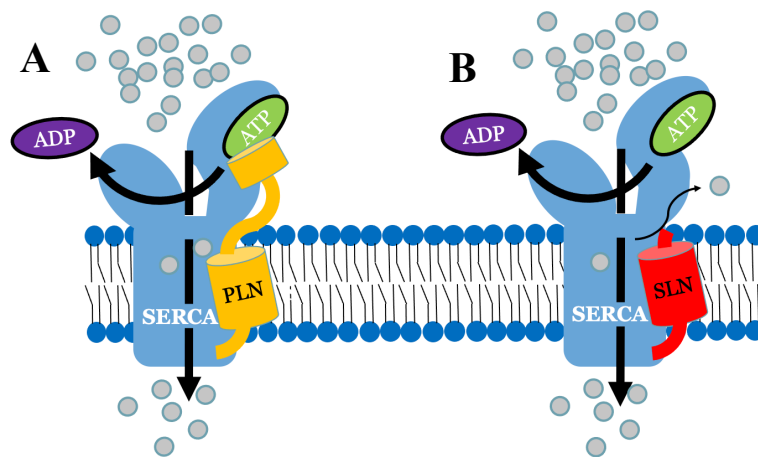


Figure 2: Sarcolipin (SLN) and Phospholamban (PLN) Regulate SERCA Function by Interacting with the Pump in the Transmembrane Region
 (A) PLN and (B) SLN bind to the same groove in SERCA's transmembrane region to inhibit Ca^{2+} uptake into the sarco/endoplasmic reticulum.

Protection of SERCA by HSP70, SLN and PLN:

Several studies have reported the impact of stress on SERCA structure and function (Fu et al., 2020; Fu & Tupling, 2009; Gutierrez-Martin et al., 2004; Klebl et al., 1998; Senisterra et al., 1997; Tupling et al., 2004; Viner et al., 1999), and our lab has shown the protective effects conferred by heat shock protein 70 (Hsp70) in such conditions (Fu et al., 2020; Fu & Tupling, 2009; Tupling et al., 2004). Given SERCA's susceptibility to oxidative/Nitrostatic damage, we have found that that SERCA nitrosylation and carbonylation is increased alongside a reduction in its maximal activity in the face of heat shock (Fu et al., 2020; Fu & Tupling, 2009). In line with their role as stress-induced chaperone proteins, Hsp70 proteins are highly sensitive to changes in redox state, protein oxidation, and increased levels of unfolded proteins caused by cellular stress (Kalmar & Greensmith, 2009; Larkins et al., 2012; Liu & Steinacker, 2001). Under basal conditions, Hsp70 is bound to heat shock factor 1 (HSF-1), however, upon detection of a stressor, it dissociates from HSF-1 to perform its chaperone role by binding and repairing damaged and unfolded proteins (Kalmar & Greensmith, 2009; Liu & Steinacker, 2001; Noble et al., 2008). The now free HSF-1 can then trimerize and enter the nucleus to bind the heat shock element DNA sequence to upregulate Hsp70 expression in the cell to further combat the damage caused by the stressor (Kalmar & Greensmith, 2009; Liu & Steinacker, 2001; Noble et al., 2008).

We have shown in both SR membrane fractions and in HEK293 cells, that a lowered or lack of expression of Hsp70 leads to decreased fluorescein isothiocyanate (FITC) binding to SERCA's N-domain, which is indicative of denaturation of the domain (see figure 3) (Fu & Tupling, 2009; Tupling et al., 2004). In the SR membrane fractions incubated in a 37°C water bath, maximal SERCA1a activity was significantly greater after 30- and 60-minutes of incubation in the presence of Hsp70 (Tupling et al., 2004). Similarly, Hsp70 overexpressing

HEK293 cells showed significantly greater SERCA2a maximal activity after 60 minutes of HS (41°C) compared to cells only constitutively expressing Hsp70 (Fu & Tupling, 2009). By binding to and preserving the structural and functional integrity of SERCA, Hsp70 allows for better regulation of cytosolic Ca^{2+} levels so that muscle can adequately handle or at least minimize the damage caused by stress.

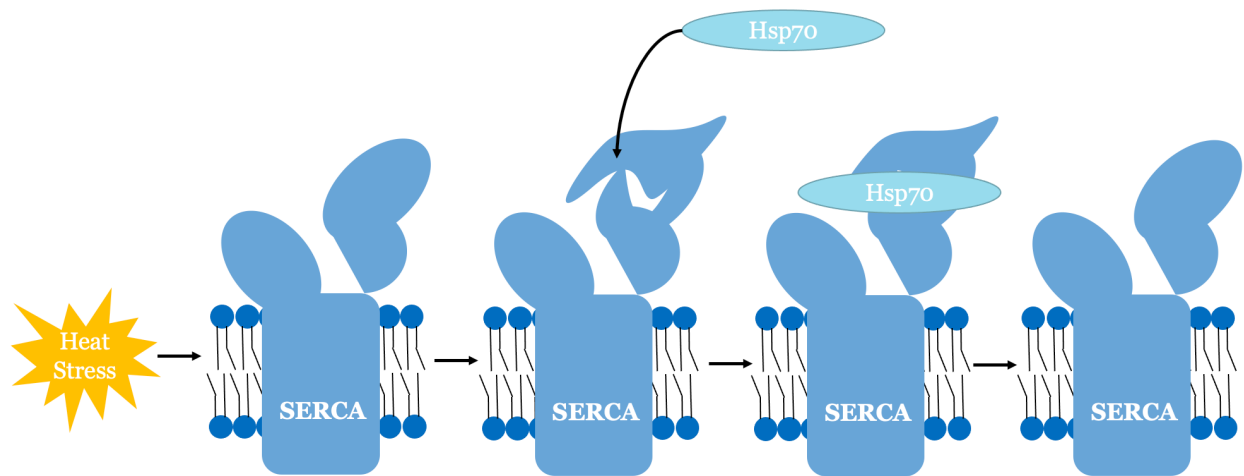


Figure 3: Hsp70 Mechanism for Protection of SERCA From Oxidative/Nitrostatic Stress Generated by Heat Stress

Heat stress leads to deanturation of the N-domain in the cytosolic region of SERCA and thus impairment of the pump. Hsp70 binds to the denatured N-domain to promote refolding and thus protects SERCA structure and function.

Despite the protection provided by Hsp70 on SERCA in the face of HS, with increased severity of stress, the protection subsides, and it was postulated that this may be because the transmembrane domain, which is inaccessible to Hsp70, was being denatured (Tupling et al., 2004). SLN and PLN are two proteins that interact with SERCA within the transmembrane domain and so it was hypothesized that they may provide protection to the pump in this region (Fu et al., 2020). *In-vitro* experiments in HEK293 cells showed that SERCA maximal activity was preserved in the face of a 60-minute exposure to HS if either SLN or PLN were present (Fu

et al., 2020). In addition, *ex-vivo* experiments using left ventricle and diaphragm also showed that endogenous expression of SLN and PLN preserves maximal SERCA activity in the face of both a 30- and 60-minute exposure to HS (Fu et al., 2020). The functional protection they provide here is much like that of Hsp70, but it remains to be seen whether SLN and PLN are stress-induced proteins like Hsp70.

Several studies have shown upregulation of SLN expression in various models of disease and a common feature amongst them is increased oxidative stress (Chambers et al., 2022). When myotube cultures which are isolated from mdx mice, a model of Duchenne muscular dystrophy (DMD), were subjected to pro-oxidant compounds, there was greater cell death, indicating an increased susceptibility to oxidative damage (Rando et al., 1998). In mdx and other mouse models of this disease, SLN expression is increased several fold compared with wild type mice in both the diaphragm and soleus (Schneider et al., 2013). In another study, Fajardo and colleagues reported significant elevations in makers of oxidative stress (protein nitrosylation and 2,7-dichlorofluorescein staining) in the soleus of PLN overexpressing mice which display a centronuclear myopathy like phenotype, alongside a 9-fold increase in SLN expression (Fajardo et al., 2015). Furthermore, in a model of muscle atrophy due to disuse, mitochondrial ROS emission was significantly elevated (Tomiya et al., 2019) and this finding coincides with significantly elevated SLN expression seen in this model (Fajardo et al., 2017; Tomiya et al., 2019). Evidence for increased PLN expression in disease states is lacking or very sparse. However, there have been several reports of decreases in SERCA:PLN ratio along with decreased phosphorylation of PLN in human, dog, and guinea pig models of heart failure, both of which lead to increased interaction with SERCA (Hasenfuss et al., 1994; Jiang et al., 2002; Kiss et al., 1995; MacLennan & Kranias, 2003; Schwinger et al., 1999). Furthermore, it has been

shown that ablation of PLN in DMD cardiomyopathy led to worsened membrane damage and diminished systolic and diastolic function (Law et al., 2018). Considering that both SLN and PLN bind to the same site within SERCA's TM domain (Gorski et al., 2017; Toyoshima et al., 2003; Toyoshima et al., 2013), it is possible that they both increase their interaction with SERCA to protect its structure and function within such highly oxidative environments.

Sex Differences in the Heat Shock Protein 70 Response:

Sex differences are found in muscle structure, metabolism, and function. In mouse solei, we have found that females have more type I fibres than in the males (data unpublished), whereas the males have more type II fibres than the females. Similar findings were observed in biopsies from human vastus lateralis muscle (Staron et al. 2000). Females have a greater intramyocellular lipid (IMCL) content than males and therefore are more reliant on lipid metabolism during exercise (Beaudry and Devries 2019). An exercise study done on mice demonstrated that females have a two-fold greater expression of genes involved in fatty acid metabolism compared to males which could explain the better performance on the exhaustive treadmill exercise test (Holcomb et al. 2021).

Sex differences in the basal expression of Hsp70 and its response to physiological stress have been done largely in cardiac tissue. Voss and colleagues found higher levels of basal Hsp70 in female Sprague-Dawley rats compared to males coinciding with greater cardioprotection in the females (Voss et al 2003). Using an ovariectomized female rat (OVX) model, they found that estrogen plays a key role in determining basal Hsp70 expression in the heart. At 9 weeks post-ovariectomy, Hsp70 levels were reduced to levels that were similar to males. However, when half of OVX rats were provided estrogen replacement therapy using a slow release 17 β estradiol

pellet, they maintained Hsp70 levels similar to that of intact females. Sex differences in the Hsp70 response in the heart to a physiological stressor and the role of estrogen is somewhat controversial. One study found that following a trauma-hemorrhagic shock, male rats treated with an estrogen- β receptor agonist showed higher levels of Hsp70 than vehicle treated and sham rats (Yu et al. 2006). HSF-1 DNA binding activity, which is an indicator of HSP induction, was also increased which would explain the increased Hsp70 levels (Yu et al. 2006). Furthermore, functional markers of cardiac function (cardiac output, stroke volume, and $\pm dP/dt_{\max}$) were also restored in estrogen- β agonist treated rats (Yu et al. 2006). Another study using isolated cardiomyocytes from female rats showed that following hypoxia-reoxygenation, cells treated with 17 β estradiol agonist or tamoxifen (estrogen-receptor modulator) induced Hsp70 expression via HSF-1 activation (Hamilton et al. 2004).

In contrast to the aforementioned studies, two very similar studies found that after just one session of 60-minute treadmill run (Paroo et al 2006) and whole-body hyperthermia (Shinohara et al 2004), male rats showed a more robust Hsp70 induction compared to the females who did not show a response at all. To assess the role of estrogen in this response, both studies also repeated the same experiment on OVX rats in which half the animals were treated with 17 β estradiol or placebo treatment, and found that, like their male counterparts, OVX rats also had a robust Hsp70 induction whereas the estrogen treated OVX rats did not. The physiological implications of these sex-differences in the heat shock response were best indicated by improved left ventricular (LV) function in the hearts isolated from exercised or heat treated male and OVX rats compared to those from female rats following ischemia-reperfusion protocol. In addition, Shinohara et al also exposed male rat cardiomyocytes to H₂O₂ either in the absence or presence of 17 β estradiol and while both groups of cells displayed increased Hsp70

expression compared to H₂O₂ untreated cells, estrogen treated cells showed a lower increase compared to cells not treated with estrogen. Furthermore, after a 48-hour washout period of the estrogen treated cells, they displayed showed a recovery in increased Hsp70 expression. When taking the findings of these studies together, it seems that estrogen is playing a modulatory role in females whereby under control or non-stressed conditions, it drives the higher basal Hsp70 expression in the heart compared to males, but as a result, they either do not display or have a less robust Hsp70 induction in the face of a physiological stressor with estrogen playing either an inhibitor or attenuator role.

Unpublished data from our lab indicates that SLN expression is greater in the solei of female mice compared to that in males. The same investigation has not been done on PLN but given that we see the difference in the homologous SLN, it is pertinent that it is looked at. Based off our current understanding of the sex difference in the heat shock response in the heart, it seems important to investigate whether there are sex differences in the response of both SLN and PLN to physiological stress (e.g., heat stress).

Rationale for a Heat Stress Model:

This study implemented an in-vivo heat stress model to generate oxidative stress within mouse hindlimb muscles. Heat has been shown to be an effective producer of oxidative stress (Bruskov et al., 2002; Flanagan et al., 1998) and as a result is a widely used stressor used to study cellular stress (Demirel et al., 2003; Gupte et al., 2011; King et al., 2002; Tolson & Roberts, 2005; Von Schulze et al., 2021). Heat produces H₂O₂ by activating oxygen atoms to a singlet state whereby all electrons are paired increasing likelihood of superoxide radical production leading to dismutation to H₂O₂ (Bruskov et al 2002). Using laser scan confocal

microscopy, heat stress was found to produce both intracellular and extracellular ROS (Zuo et al. 2000) thereby indicating that it produces global oxidative stress within the cell and is not just confined to one region. 24 and 26 cysteine residues found within the SERCA1a and SERCA2a structure, respectively, are susceptible to oxidative damage (Senisterra et al., 1997; Viner et al., 1999) in the form of nitrosylation and carbonylation (Gutierrez-Martin et al., 2004; Klebl et al., 1998). Aforementioned studies from our lab which implemented an *in-vitro* heat stress model showed that SERCA nitrosylation and carbonylation are increased with this model (Fu et al., 2020; Fu & Tupling, 2009). Furthermore, the induction of Hsp70 expression, which occurred in these studies, is itself a marker for increased oxidative stress (Noble et al., 2008; Tupling, 2008).

Research Purpose

In-vivo HS models involve application of heat via hot water, heating pad, or heated beads (Tolson & Roberts, 2005) and have been shown to effectively induce expression of Hsp70 and other proteins within its family in rodents (Demirel et al., 2003; Gupte et al., 2011; King et al., 2002; Tolson & Roberts, 2005; Von Schulze et al., 2021). However, to date, no study has looked at the effect of *in-vivo* HS on SERCA function or on the expression of the SERCA-regulatory proteins, SLN and PLN. Furthermore, with unpublished data from our lab showing higher SLN expression in female compared to male soleus and taking the pre-existing male bias in literature in the study of these regulatory proteins into account, we felt it was pertinent to compare the effects in both sexes. Therefore, this study investigated:

- i) The difference in basal gene and protein expression of Hsp70, SLN and PLN in the soleus and white gastrocnemius of male and female rats;
- ii) the effects of HS on protein nitrosylation status and SERCA function (Ca^{2+} uptake) in male and female rat soleus and white gastrocnemius (WG) muscles using an *in-vivo* model;
- iii) whether *Sln* and *Pln* are stress-inducible genes like Hsp70. Specifically, this study will investigate the time-course (0, 24, and 48 hours) of SLN and PLN gene and protein expression in response to HS using an *in-vivo* model.

Hypotheses

Objective 1:

- i) As shown previously (Voss et al. 2003), we expect that basal gene and protein expression of Hsp70 will be higher in the solei of male compared to female rats in both muscles.
- ii) Based on the higher basal SLN protein expression observed in female soleus in mice, the same difference in rats for both protein and gene expression in both muscles were expected.
- iii) Due to a lack of investigation into PLN expression differences between sexes, no differences in the basal PLN expression were expected.

Objective 2:

- i) Immediately following HS, protein nitrosylation will be increased and SERCA function will be reduced in both muscles but to a greater extent in the WG compared to soleus due to the higher levels of endogenous Hsp70, SLN, and PLN in the more oxidative soleus. Over the course of the next 24 and 48 hours, protein nitrosylation and SERCA function will decrease and increase, respectively.
- ii) It was expected that protein nitrosylation and SERCA function will be increased and decreased, respectively, but to a greater extent in the males compared to females due to predicted higher endogenous levels of SLN in the latter.

Objective 3:

- i) Immediately after treatment, and over the course of the next 24 and 48 hours after treatment, the mRNA and protein content of Hsp70, SLN, and PLN will be increased in both the soleus and WG.

- ii) The extent of increase in gene and protein expression of Hsp70 over the time course will be higher in the females compared to males due to higher basal Hsp70 expression in the latter.
- iii) The extent of increase in gene and protein expression of SLN over the time course will be higher in the males compared to females due to the predicted higher basal SLN expression in the females.
- iv) There will be no differences in the extent of increase in gene and protein expression of PLN over the time course between the two sexes.

Methods

Animals:

Eight-week-old Sprague Dawley rats were purchased from Envigo and housed 2-3 per cage in an environmentally controlled room, under a reverse light/dark cycle (12/12 hr) in individual cages and were provided access to food and water *ad libitum*. There were 4 conditions with a male and female group in each condition, therefore totalling 8 groups overall (n=6 per group). The 4 conditions included a control (CON) condition, and 3 heat treated conditions, in which animals were either allowed no recovery and were euthanized immediately after treatment (HS0)- or were allowed to recover for 24 hrs (HS24), or 48 hrs (HS48) post-treatment. From these groups, soleus and WG were excised and processed for experimental analyses. All experiments within this study were reviewed and approved by the University of Waterloo Animal Care Committee in accordance with the Canadian Council on Animal Care.

In-Vivo Heat Stress:

In the control conditions, the lower limbs of the anesthetized (isoflurane) animals were placed in a control bath set at 37°C for 30 minutes with their core temperature (assessed with a rectal probe) being maintained at $37 \pm 0.5^\circ\text{C}$, and the tissues of interest were harvested immediately afterwards while the animal was still under anaesthesia. The 3 heat treated conditions involved submerging the lower limbs of the anesthetized rats in a hot water bath set at a temperature between 44-45°C and after either no recovery or a recovery period of either 24 or 48 hrs, the animals were once again anesthetized for tissue harvesting (see Figure 4 for image of protocol set up). All animals were euthanized by CO₂ exposure following tissue harvesting. The heat treatment consisted of two phases: 1) warm-up - the goal of this phase was to raise the core

within a range of 41-41.5°C, and 2) maintenance – during which core temperature was maintained between 41-41.5°C for a period of 30 minutes. Given that the rectum was submerged in the water bath as part of the lower body, the core temperature could not be assessed using the rectal probe for the entire duration of the procedure. Therefore, during the warm-up phase, the core temperature was monitored every 5 minutes and every 2.5 minutes during the maintenance phase. The difference in time intervals between the two phases is due to the difference in goals between the two phases; the warm-up phase involves larger jumps in core temperature while the maintenance phase involves keeping the core temperature within a very tight range.

To ensure the animals' safety, the following measures were put in place: 1) a pulse oximeter, which was attached at their front paw, was used to monitor the animals' blood oxygen (SpO₂) and heart rate (HR), 2) in the event that the core temperature during either phase rose above 41.5°C, the lower limbs were removed from the water bath while core temperature was continually monitored until it returned within the aforementioned range, and 3) they were provided with a 2-3 mL subcutaneous injection of saline for rehydration after heat treatment.

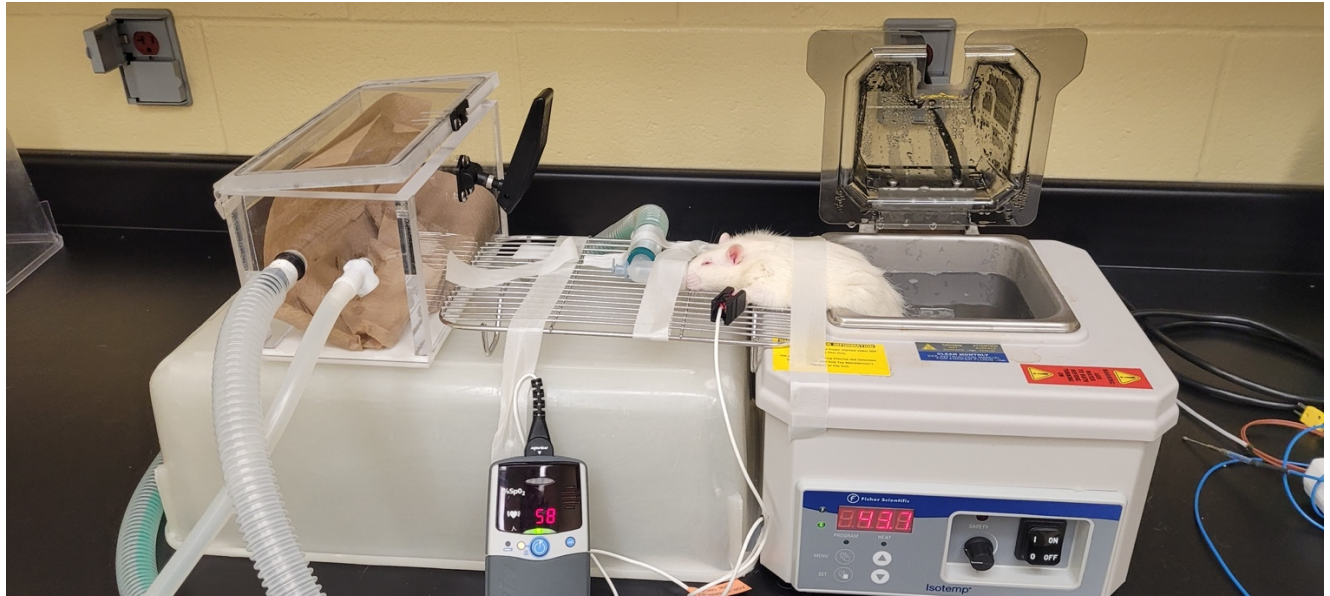


Figure 4: *In-Vivo* Heat Stress Set Up

The animal's hind limbs are submerged in the water bath while the animal is anesthetized using isoflurane which is supplied through a nose cone. The animal's heart rate and oxygen saturation are being measured by pulse oximeter attached to its front paw.

Western Blotting:

The protein expression levels of SLN, PLN, Hsp70, SERCA1a and, SERCA2a was assessed using standard western blotting techniques (Fajardo et al., 2013; Tupling et al., 2007). Upon excision, the muscle samples were detendonized and weighed to determine muscle mass. These samples were then be homogenized 1:10 (w/v) in ice-cold homogenizing buffer (250 mM sucrose, 5 mM HEPES, 0.2 mM PMSF and 0.2% [w/v] NaN₃) using a glass homogenizer and stored at -80°C. Subsequently, these muscle homogenates were solubilized into a 1x buffer, and the proteins were electrophoretically separated with the use of a tricine based SDS-PAGE (13% total acrylamide for PLN and SLN) and a standard SDS-PAGE (7.5% total acrylamide for Hsp70, and S-nitrosylated proteins, respectively). Upon electrophoresis, the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (PLN, SERCAs, Hsp70, and S-nitrosylated proteins) or nitrocellulose membrane (SLN). Semi-dry transfer was used to for

Hsp70 and S-nitrosylated proteins and wet transfer was used for SLN and PLN transfer buffer (25 mM glycine, 192 mM Tris base, 20% methanol, 0.1% [w/v] SDS). The membranes were then incubated in blocking solution (TBST buffer: 20mM Tris base, 137 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.5, with 5% [w/v] non-fat dry milk for SERCAs, Hsp70, PLN, and S-nitrosylated proteins or 5% [w/v] BSA for SLN) in order to prevent non-specific binding. It is at this point that the proteins were immunoprobed with the corresponding primary antibodies and subsequently immunoprobed with horseradish peroxidase-conjugated secondary antibodies (see table 1 for Western blot conditions). Luminata Forte™ was utilized to detect the resultant antigen-antibody complexes for SERCA2a, PLN, and Hsp70; whereas ECL Western Blot Substrate (BioVision) was used to visualize SLN, S-nitrosylated proteins. Chemidoc (Biorad) was utilized to quantify the resulting optical densities.

Protein Target	Transfer Method	Blocking Agent	Antibody Species of Origin	[1° Antibody]	[2° Antibody]
Hsp70	Semi-Dry	5% Skim Milk in TBST	Mouse	1:1000 Anti-mouse	1:2000
SLN	Wet	5% BSA in TBST	Rabbit	1:100	1:2000
PLN	Wet	5% BSA in TBST	Mouse	1:1000	1:2000
S-Nitrosylated Proteins	Semi-Dry	5% Skim Milk in TBST	Mouse	1:1500	1:2000

Table 1. Western blot conditions for each protein of interest.

A ponceau stain was used to normalize band densities to the amount of protein loaded for each respective band. For Hsp70, SLN, and PLN, ponceau stained proteins in the 37-50 kDa region were used for normalization, whereas total protein ponceau stain was used to normalize for S-Nitrosylated proteins. For each muscle, a total of 48 samples were collected across both sexes and as a result not all samples were able to fit on one gel or membrane. A total of 12

experimental samples were loaded on each gel (3 from each condition of the same sex) across 4 gels. Therefore, to account for differences in protein transfer or application of chemiluminescence, one sample from the first gel was used as a loading control and was loaded onto every subsequent gel used to blot for a particular protein within a particular muscle (see table 2 for specific loading controls used for each protein of interest). The ponceau normalized band density of each loading control sample in gels 2-4 were divided by the ponceau normalized band density of the sample in gel 1 to calculate a gel adjustment factor. Then, all final band densities of the experimental samples were normalized to the loading control by multiplying to the adjustment factor.

Protein Target	Soleus	White Gastrocnemius
Hsp70	CON male	HS48 male
SLN	CON male	CON male
PLN	CON female	CON female
S-Nitrosylated Proteins	CON male	HS48 male

Table 2. Loading control sample used to normalize band densities of experimental samples across multiple gels.

RNA Extraction, Reverse Transcription (RT) and Real-Time (q) PCR

Total RNA was isolated from rat skeletal muscle using TRIzol® Reagent (1 mL/≤100 mg tissue) using a glass homogenizer. Samples were then incubated at room temperature for 5 minutes, followed by the addition of chloroform (20% of TRIzol® volume) with vigorous shaking for 15-20 seconds, incubation at room temperature for 2 minutes, and centrifugation at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase of each sample was removed and mixed with isopropanol (50% of initial volume of TRIzol®) and incubated at room temperature for 10 minutes to precipitate the RNA. Samples were then centrifuged at 12,000 x g for 10

minutes at 4°C, pelleting the RNA. The supernatant was discarded, and the pellet was washed in 1 mL of 75% ethanol-25% DEPC-ddH₂O, centrifuged at 7,500 x g for 5 minutes at 4°C, then air-dried for 5-10 minutes prior to suspension in 20-50 µL DEPC- ddH₂O. The samples were then incubated at 58°C for 10 minutes to facilitate solubilisation, and stored long-term at -80°C. RNA samples were quantified using a Nanodrop 2000 Spectrophotometer and samples were adjusted to 2 µg/µL.

For the real-time PCR tests, complementary DNA (cDNA) was diluted 1:4, 2 µl was combined with 10 µl master mix of AccuStart PCR ToughMix® (from Quanta Bio, Beverly, MA), 1 µl of TaqMan® primers (Thermo Fisher Scientific) specific to the gene of interest (enumerated in Table 3), and 7 µl of ddH₂O. The cycling parameters were uniform across all genes: an initial cycle at 50 °C for 2 minutes, followed by another cycle at 90°C for 20 seconds, followed by 49 cycles at 95 °C for 3 seconds, and finally, 60 °C for 30 seconds. The relative expression level of the target gene was determined using the $\Delta\Delta C_t$ technique, with C_t values standardized against beta Actin.

Gene	Organism	Assay ID
<i>Hsp70</i>	Rat	Rn00596544_m1
<i>Pln</i>	Rat	Rn01434045_m1
<i>Sln</i>	Rat	Rn02769377_s1
<i>β-Actin</i>	Rat	Rn00667869_m1

Table 3. Primer sequences for qPCR analysis for *SERCA1*, *SERCA2b*, *Pln*, and *Sln*

SERCA Ca^{2+} Uptake:

Ca^{2+} uptake was determined in the presence of a precipitating anion, oxalate, with the use of the fluorescent dye Indo-1, as previously reported (Tupling & Green, 2002; Holloway et al. 2006; Tupling et al. 2001). Once thawed, muscle homogenate (100 μL and 60 μL for soleus and WG homogenate, respectively) was loaded into a clear plastic cuvette containing the Ca^{2+} uptake buffer (200mM KCl, 20mM HEPES, 10mM NaN_3 , 0.005mM TPEN, 15mM MgCl_2 , and 5mM Oxalate) which was maintained at a temperature of 37°C, followed by the addition of 1mM Ca^{2+} and 1mM Indo-1. The reaction then starts upon the addition of 250mM ATP in a spectrofluorometer that employs a monochromator to maintain the excitation wavelength at 355nm and two photomultiplier tubes to detect light emitted at 405 nm by the Ca^{2+} -bound Indo-1 (F) and at 485nm by the Ca^{2+} -free Indo-1 (G). As Ca^{2+} is being taken up by SERCA into the SR vesicles, the amount of Ca^{2+} -bound Indo-1 decreases while the amount of Ca^{2+} -free Indo-1 increases. Once peak SERCA Ca^{2+} uptake is reached, as indicated by a plateau in the levels of F and G, 40mM of SERCA inhibitor cyclopiazonic acid (CPA) was added to the reaction mixture to assess Ca^{2+} leak, followed by the addition of 5mM EGTA to assess maximum amount of free Ca^{2+} , and finally, 100mM CaCl_2 to assess maximum amount of Indo-1 bound Ca^{2+} . A background reading was taken in the absence of Indo-1 using just the buffer, sample, 1mM CaCl_2 , and 250mM ATP prior to the start of the reaction. Using equation 1 (see below and Table 4 for definitions of equation variables), a curve was generated to provide the final cytosolic free $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_f$) over time, and using the Savitsky-Golay algorithm, it was smoothed over 21 points (Holloway et al. 2006). For the soleus, Ca^{2+} uptake rates were best assessed at $[\text{Ca}^{2+}]_f$ of 1500 nM and at 2000 nM for the white gastrocnemius.

Equation 1: $[Ca^{2+}]_f = K_d \times (G_{max}/G_{min})(R - R_{min})/(R_{max} - R)$

Variable	Definition
K_d	Equilibrium constant for the reaction between Ca^{2+} and Indo-1=250
G_{max}	Maximum amount of Ca^{2+} -free Indo-1 after the addition of 5mM EGTA
G_{min}	Minimum amount of Ca^{2+} -free Indo-1 following the addition of 100mM $CaCl_2$
R	Ratio between F and G
R_{min}	Minimum value of R after the addition of 5mM EGTA
R_{max}	Maximum value of R after the addition of 100mM $CaCl_2$

Table 4: Definitions of $[Ca^{2+}]_f$ equation variables

Statistics:

The data were presented as means $\pm$ standard error. A t-test was used to determine differences in basal protein levels between males and females. A two-way ANOVA was used to assess differences in basal gene and protein expression with the two factors being sex and muscle. A two-way ANOVA was also used to assess responses of gene and protein expression and SERCA Ca^{2+} uptake to *in-vivo* heat stress with the two factors being sex and condition. Where significant differences were found, Bonferroni's post hoc test was used to compare specific means. Statistical significance was set at $P < 0.05$. R Statistical Software was used for statistical analysis and GraphPad Prism for graphical representation of data.

Results:

Basal Gene and Protein Expression in Soleus and White Gastrocnemius of Male and Female

Sprague-Dawley Rats

Hsp70

Fold-differences between muscles in *Hsp70* gene expression were calculated relative to male WG levels. Using a two-way ANOVA to compare relative gene expression between sexes and across muscles, we found a main effect of sex ($p<0.05$) and muscle ($p<0.05$) such that the solei in both sexes had significantly greater *Hsp70* gene expression compared to the WG ($p<0.05$), and the females had significantly greater expression compared to the males across both muscles ($p<0.05$) (Figure 5A). Basal *Hsp70* protein expression was significantly greater in the male soleus compared to female soleus ($p<0.05$) (Figure 5B). In the WG, we were not able to detect basal level of protein in both males and females.

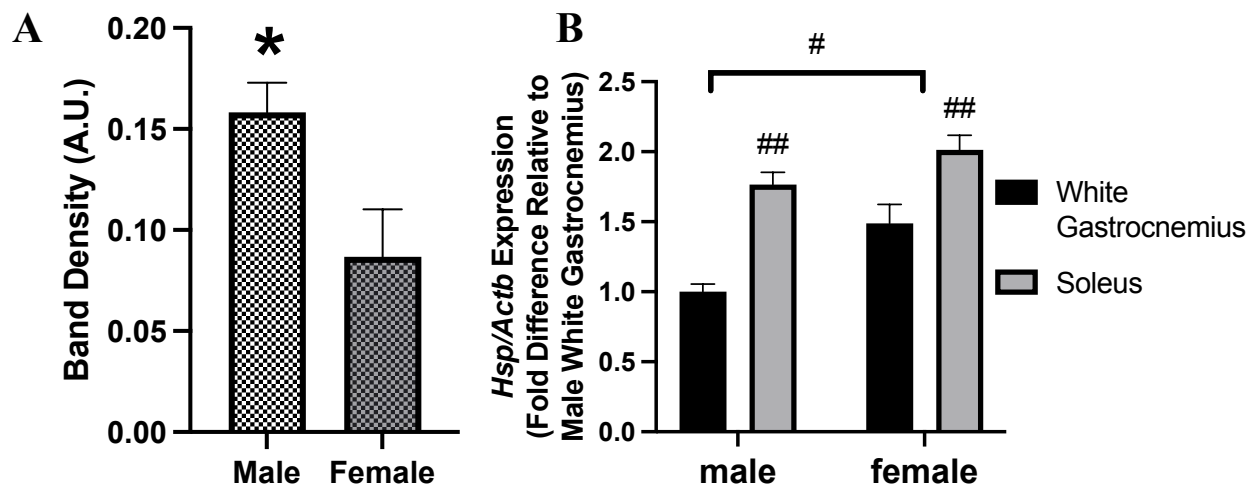


Figure 5: Basal Gene and Protein Expression of Hsp70 in Male and Female Soleus and White Gastrocnemius

(A) Comparison of *Basal Hsp70* gene expression between soleus and white gastrocnemius of male and female rats ($n=6$ in each group). Fold-differences were calculated relative to male WG levels. (B) Comparison of basal *Hsp70* protein expression between male and female soleus. (*) $p<0.05$ vs female WG, (#) main effect of sex, (##) main effect of muscle.

SLN

Two-way ANOVA of basal *Slm* gene expression between both sexes and muscles reported a significant interaction effect ($p < 0.05$) whereby *Slm* mRNA levels were highest in the female soleus, followed by the male soleus, female WG, and the male WG, with all groups being different than the other groups ($p < 0.05$) (Figure 6A). SLN protein expression, however, was not significantly different between the two sexes ($p > 0.05$) (Figure 6B). For the WG, despite loading 200 μ g of protein, we were not able to detect SLN expression in this tissue in either males or females.

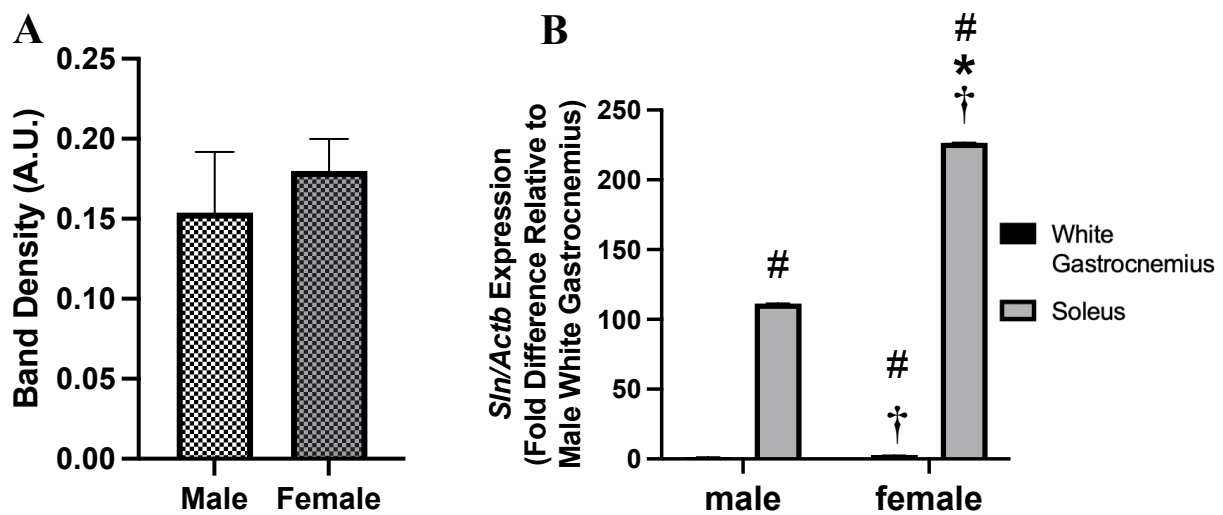


Figure 6: Basal Gene and Protein Expression of SLN in Male and Female Soleus and White Gastrocnemius

(A) Comparison of basal *Slm* gene expression between soleus and white gastrocnemius of male and female rats ($n=6$ in each group). Fold-differences were calculated relative to male WG levels. (B) Comparison of basal SLN protein expression between male and female soleus. (*) $p < 0.05$ vs female WG, (#) $p < 0.05$ vs male soleus, (†) $p < 0.05$ vs male WG.

PLN

When comparing gene expression across muscles using a two-way ANOVA, there was a significant interaction effect ($p < 0.05$) with subsequent pairwise comparisons showing that PLN mRNA expression was significantly higher in the male soleus compared to the WG ($p < 0.05$), however, no such difference was observed between muscles in females (Fig. 7A). Western blotting shows that the male soleus also had significantly more PLN protein than female soleus ($p < 0.05$) (Fig. 7B). In the WG, both the mRNA and protein expression patterns are in agreement whereby no significant differences are observed between the sexes, although the PLN protein levels are slightly higher in the females ($p > 0.05$) (Fig. 7C).

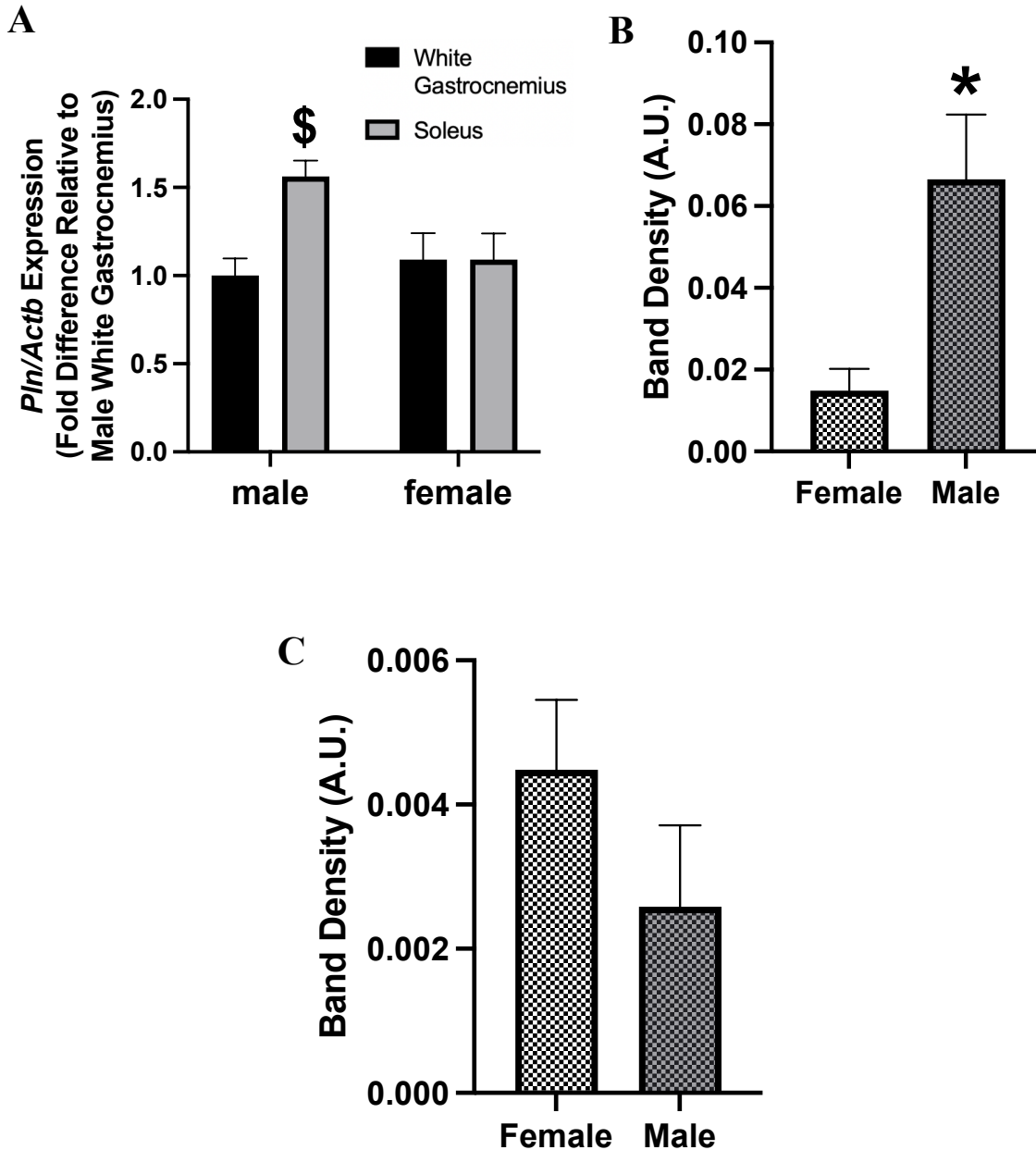


Figure 7: Basal Gene and Protein Expression of PLN in Male and Female Soleus and White Gastrocnemius

(A) Comparison of Basal *Pln* gene expression between soleus and white gastrocnemius of male and female rats. N=6 for gene expression and protein expression in female soleus, n=4 for male white gastrocnemius, and n=5 for male soleus. Fold-differences were calculated relative to male WG levels. Comparison of basal PLN protein expression between male and female soleus (B) and WG (C). (\$) $p < 0.05$ vs male WG. (*) $p < 0.05$ vs female soleus.

Gene and Protein Expression in Response to In-Vivo Heat Stress in Soleus and White Gastrocnemius of Male and Female Sprague-Dawley Rats:

HSP70

We used a two-way ANOVA to analyze differences in gene and protein expression in each muscle between HS conditions and sexes. In soleus, we found a significant interaction effect ($p < 0.05$) such that the females of the HS48 condition showed significantly elevated mRNA levels compared to all other HS conditions ($p < 0.05$) except the female HS24 group ($p > 0.05$), whereas there were no differences between HS conditions in the males (Fig. 8A). Hsp70 protein expression in soleus was assessed by Western blotting (Fig. 8C). A two-way ANOVA revealed a significant interaction effect ($p < 0.05$) and subsequent pairwise comparisons showed that the solei of both sexes had significantly greater protein expression in the HS24 condition ($p < 0.05$). Female solei of the HS48 condition expressed significantly higher levels compared to the male solei of the same HS condition ($p < 0.05$) whereas the male solei of the HS0 condition had significantly higher levels compared to the females of the same HS condition ($p < 0.05$) (Fig. 8B).

In the white gastrocnemius, a two-way ANOVA for gene expression revealed a significant main effect of HS condition such that mRNA expression was higher in the HS0 condition compared with all other HS conditions ($p < 0.05$) and was also higher in the HS24 condition compared to the HS48 condition ($p < 0.05$) (Fig. 9A). There were no differences between any of the other HS conditions and there no effects of sex ($p > 0.05$). Hsp70 protein expression in WG was assessed by Western blotting (Fig. 9C). A two-way ANOVA revealed a significant main effect of HS condition ($p < 0.05$) whereby the expression in the HS24 and HS48 groups was significantly greater than CON ($p < 0.05$) and HS0 ($p < 0.05$), where no protein could

be detected, but was not different from each other ($p>0.05$) (Fig. 9B). As with mRNA, there were no effects of sex for Hsp70 protein expression in response to HS.

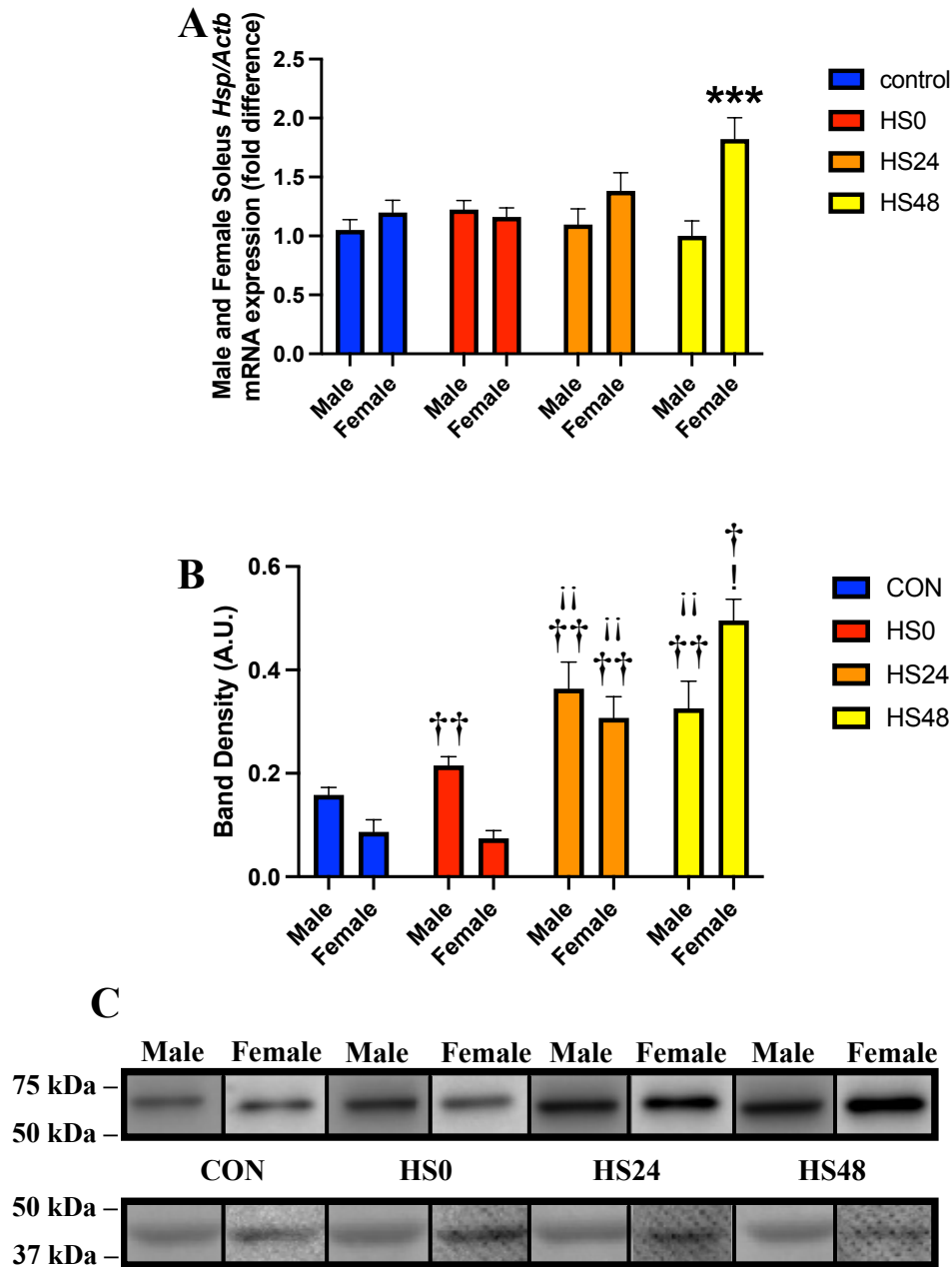


Figure 8: Male and Female Soleus Hsp70 Gene and Protein Response to *In-Vivo* Heat Stress
Soleus Hsp70 gene (A) and protein (B) response to *in-vivo* heat stress in males and females ($n=6$ in each group). (C) Western Blot representative images with ponceau staining and loading control (CON male). (j) $p<0.05$ vs male CON, (jj) vs female CON, (†) $p<0.05$ vs males HS0, (††) $p<0.05$ vs females HS0, (±) $p<0.05$ vs male HS24, (±±) $p<0.05$ vs female HS24, (!) $p<0.05$ vs male HS48, and (!!) $p<0.05$ vs female HS48.

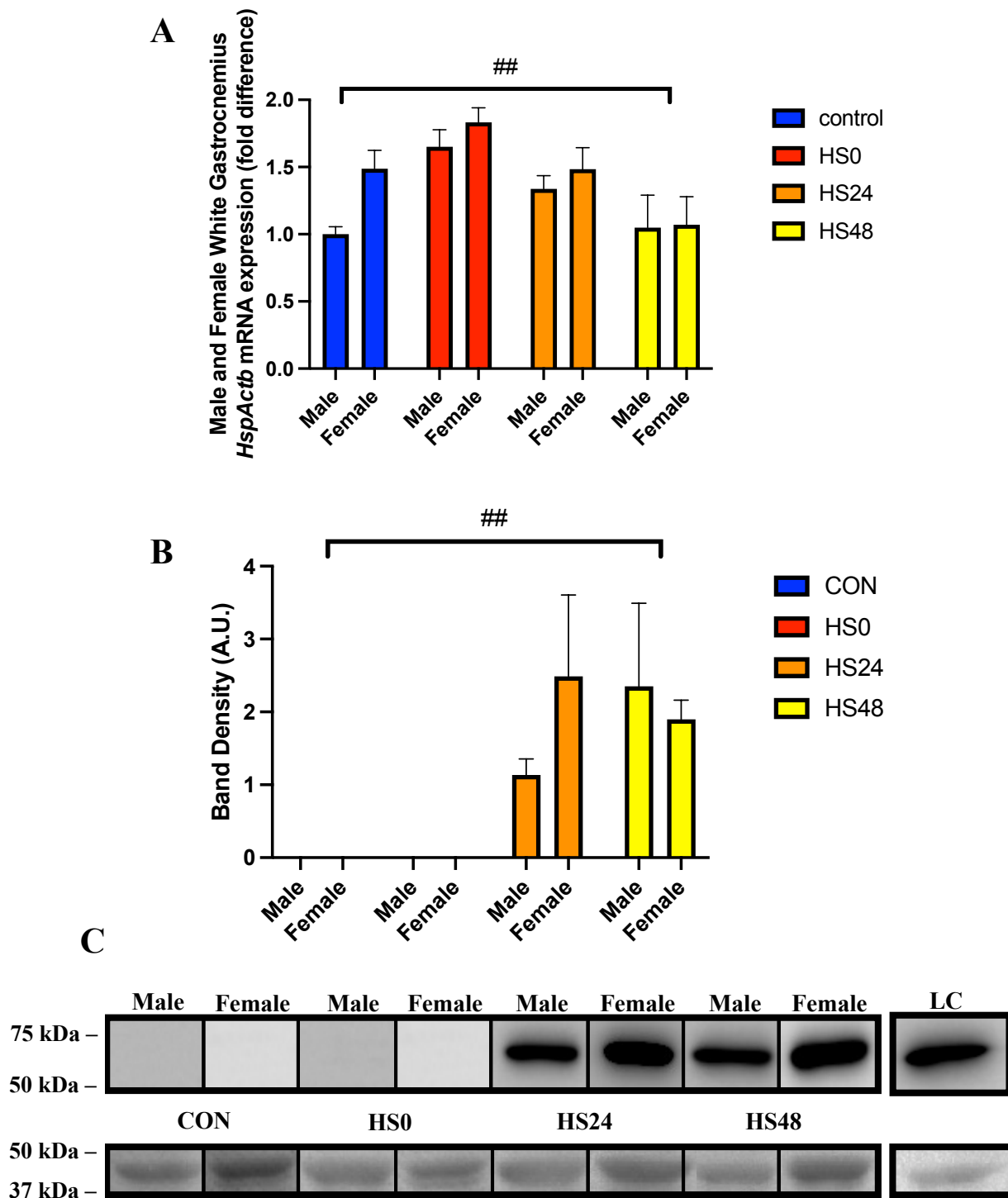


Figure 9: Male and Female White Gastrocnemius Hsp70 Gene and Protein Response to *In-Vivo* Heat Stress

WG Hsp70 gene (A) and protein (B) response to *in-vivo* heat stress in males and females (n=6 in each group). (C) Representative western blot with ponceau staining and loading control (HS48 male). (##) main effect of condition.

SLN

Two-way ANOVA for gene expression in the soleus revealed a significant main effect of sex and HS condition ($p < 0.05$). The female soleus had significantly greater *SLN* mRNA expression in comparison to males across all HS conditions ($p < 0.05$) (Fig. 10A). With respect to HS condition, *SLN* mRNA was lower in the HS24 and HS48 conditions compared with both the CON ($p < 0.05$) and HS0 ($p < 0.05$) conditions, but there were no differences ($p > 0.05$) between HS24 and HS48 or between CON and HS0 conditions. *SLN* protein expression in soleus was assessed by Western blotting (Fig. 10C). Two-way ANOVA found a significant main effect of sex, such that males expressed higher *SLN* protein than females in the heat stressed condition ($p < 0.05$), however, in the CON condition, *SLN* expression was higher in the females ($p > 0.05$) (Fig 10B). Despite this seemingly interactive effect, we did not see a significant interaction effect ($p = 0.071$).

Two-way ANOVA analysis for *SLN* gene expression in the WG found a significant interaction effect ($p < 0.05$) such that *SLN* mRNA levels in the male WG of the HS48 group were significantly greater than all other groups involved ($p < 0.05$) (Fig. 11). Despite loading 200 μ g of protein, we were not able to detect *SLN* protein in the WG of both males and females in any of the conditions.

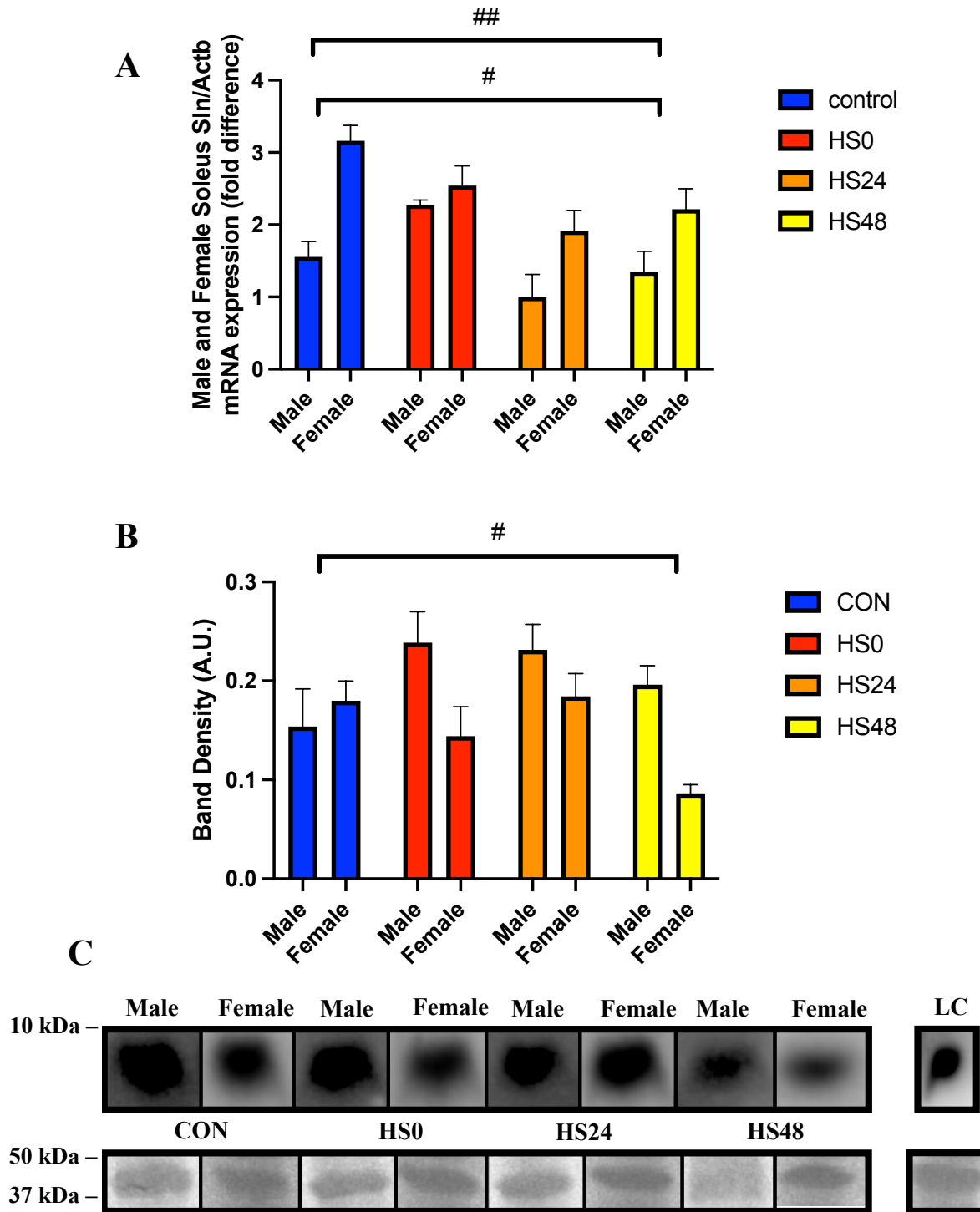


Figure 10: Male and Female Soleus SLN Gene and Protein Response to *In-Vivo* Heat Stress
Soleus SLN gene (A) and protein (B) response to *in-vivo* heat stress in males and females (n=6 in each group except HS48 male (n=5) protein expression). (C) Representative western blot with ponceau staining and loading control (CON male). (#) main effect of sex, (##) main effect of condition.

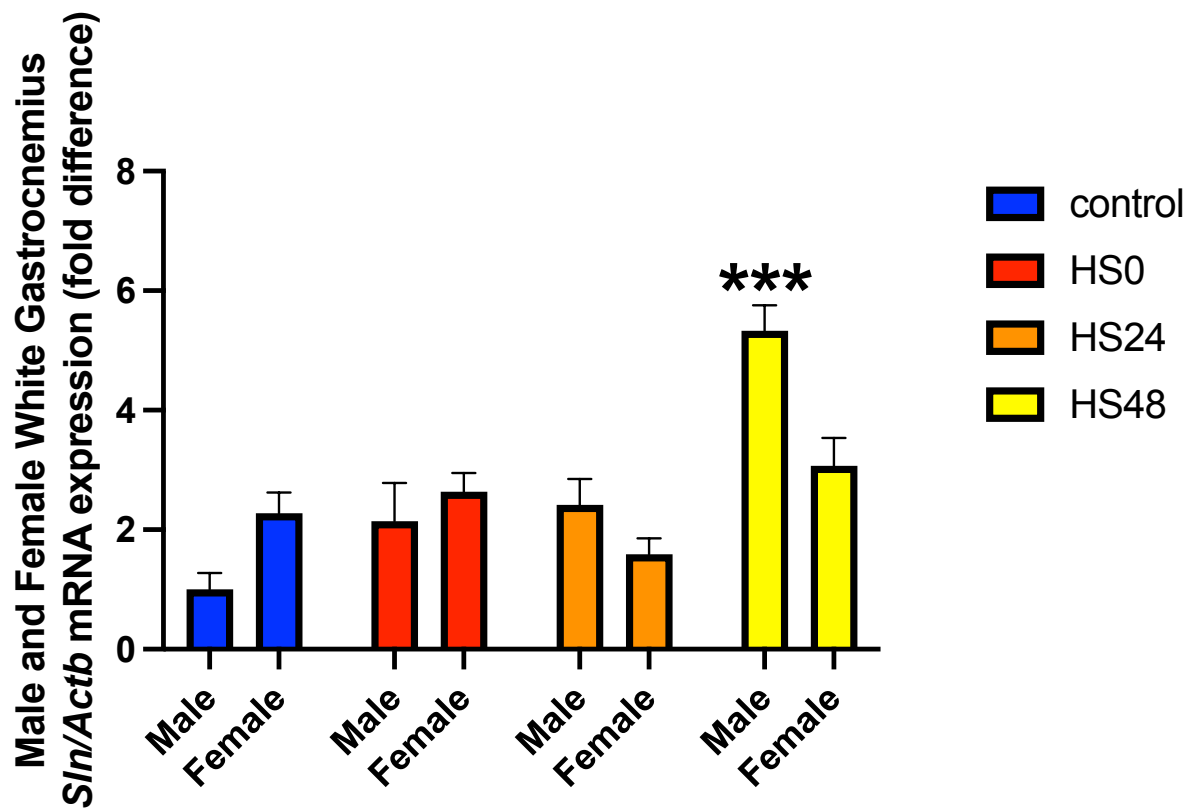


Figure 11: Male and Female WG *Sln* Gene Response to *In-Vivo* Heat Stress

WG *Sln* gene response to in-vivo heat stress in males and females (n=6 in each group). (***) $p < 0.05$ vs all other groups.

PLN

Two-way ANOVA for gene expression in the soleus revealed significant main effects of sex and HS condition, whereby *Pln* gene expression was higher in females compared to males ($p < 0.05$) and was higher in the CON condition compared to all other HS conditions ($p < 0.05$) (Fig. 12A). PLN protein expression in soleus was assessed by Western blotting (Fig. 12C). Here, only a main effect of sex was observed such that PLN expression was higher in males compared with females across all HS conditions ($p < 0.05$) (Fig. 12B). In the WG, there were no effects of sex or HS condition observed for *Pln* gene expression (Fig. 13A). PLN protein expression in WG was assessed by Western blotting (Fig. 13C). A two-way ANOVA for protein expression revealed a significant interaction, and subsequent pairwise comparisons found that protein expression in the male CON condition was significantly lower than the male WG of the HS0 ($p < 0.05$) and HS24 ($p < 0.05$) conditions as well as the female HS0 condition ($p < 0.05$) (Fig 13B). PLN levels were higher in the male WG of the male HS0, male HS24, and female HS0 condition compared to the WG of the HS48 condition.

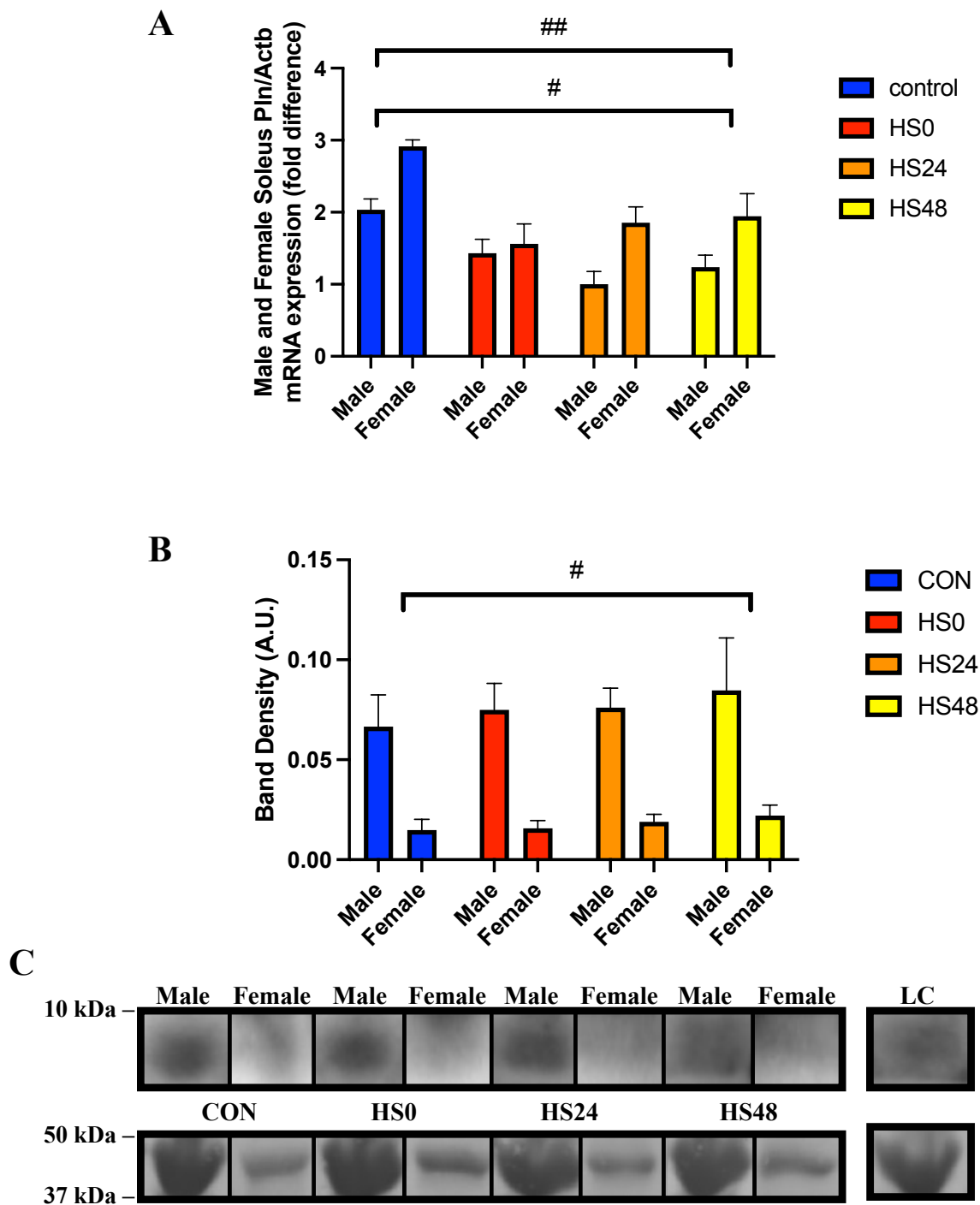


Figure 12: Male and Female Soleus PLN Gene and Protein Response to *In-Vivo* Heat Stress
 WG PLN gene (A) and protein (B) response to in-vivo heat stress in males and females (n=6 for all conditions across for both sexes except for CON male (n=4) protein expression). (C) Representative western blot with ponceau staining and loading control (female CON) (#) main effect of sex, (##) main effect of condition.

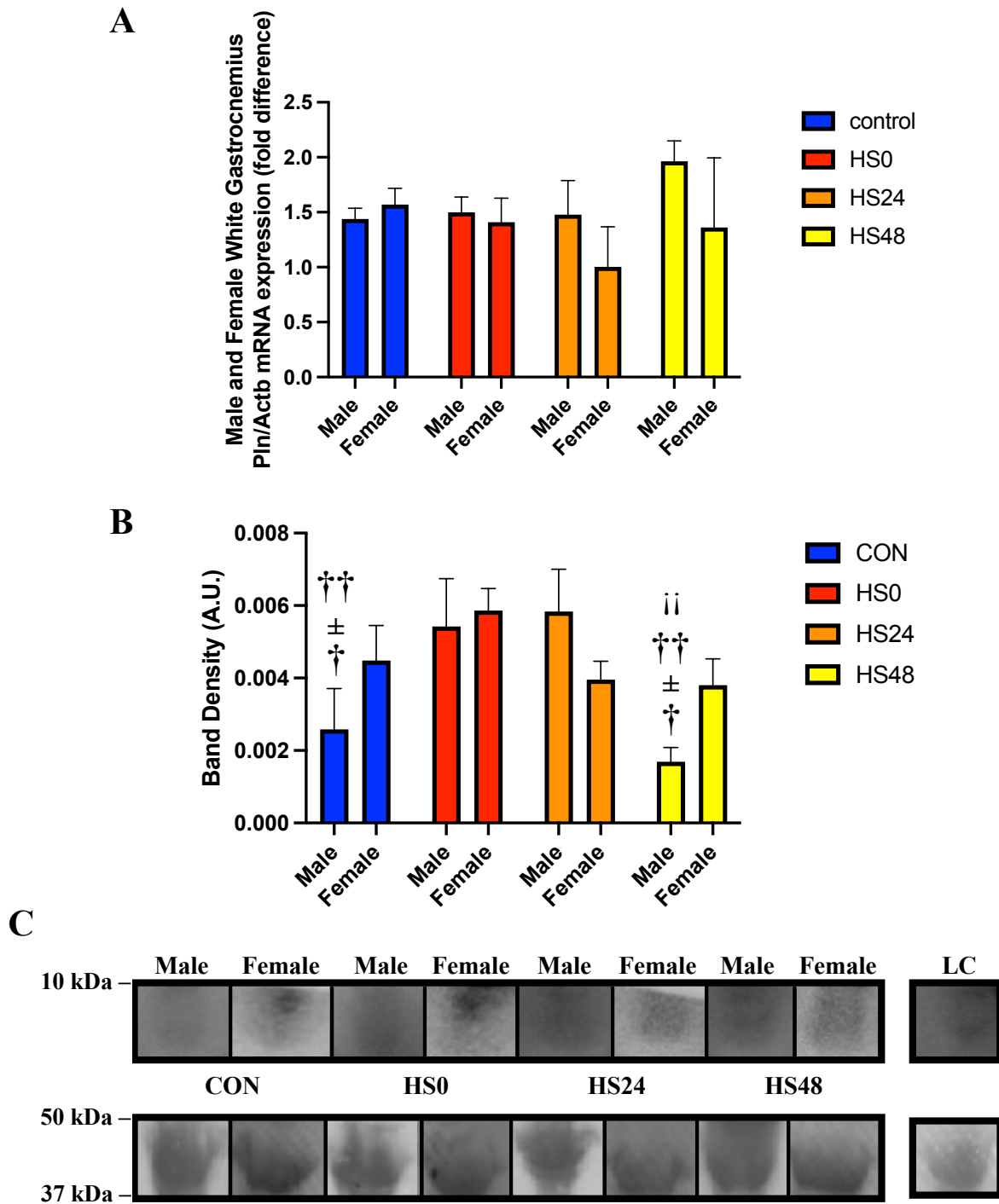


Figure 13: Male and Female WG PLN Gene and Protein Response to *In-Vivo* Heat Stress

WG PLN gene (A) and protein (B) response to in-vivo heat stress in males and females (n=6 for each group in all conditions, except CON male (n=4) and female (n=5) protein expression). (ii) vs female CON, (†) p<0.05 vs males HS0, (††) p<0.05 vs females HS0, and (±) p<0.05 vs male HS24.

Total Protein Nitrosylation Status in Response to In-Vivo Heat Stress:

Total protein nitrosylation was assessed by Western blotting (Fig. 15 and 16). Two-way ANOVA did not reveal any significant differences for the total protein nitrosylation status in the soleus (Fig. 14A). In the WG (Fig. 14B) however, there was a significant interaction effect ($p<0.05$) whereby protein nitrosylation was greater in the CON male WG compared to females in the same condition ($p<0.05$) and males WG of HS24 ($p<0.05$) and HS48 ($p<0.05$) condition.

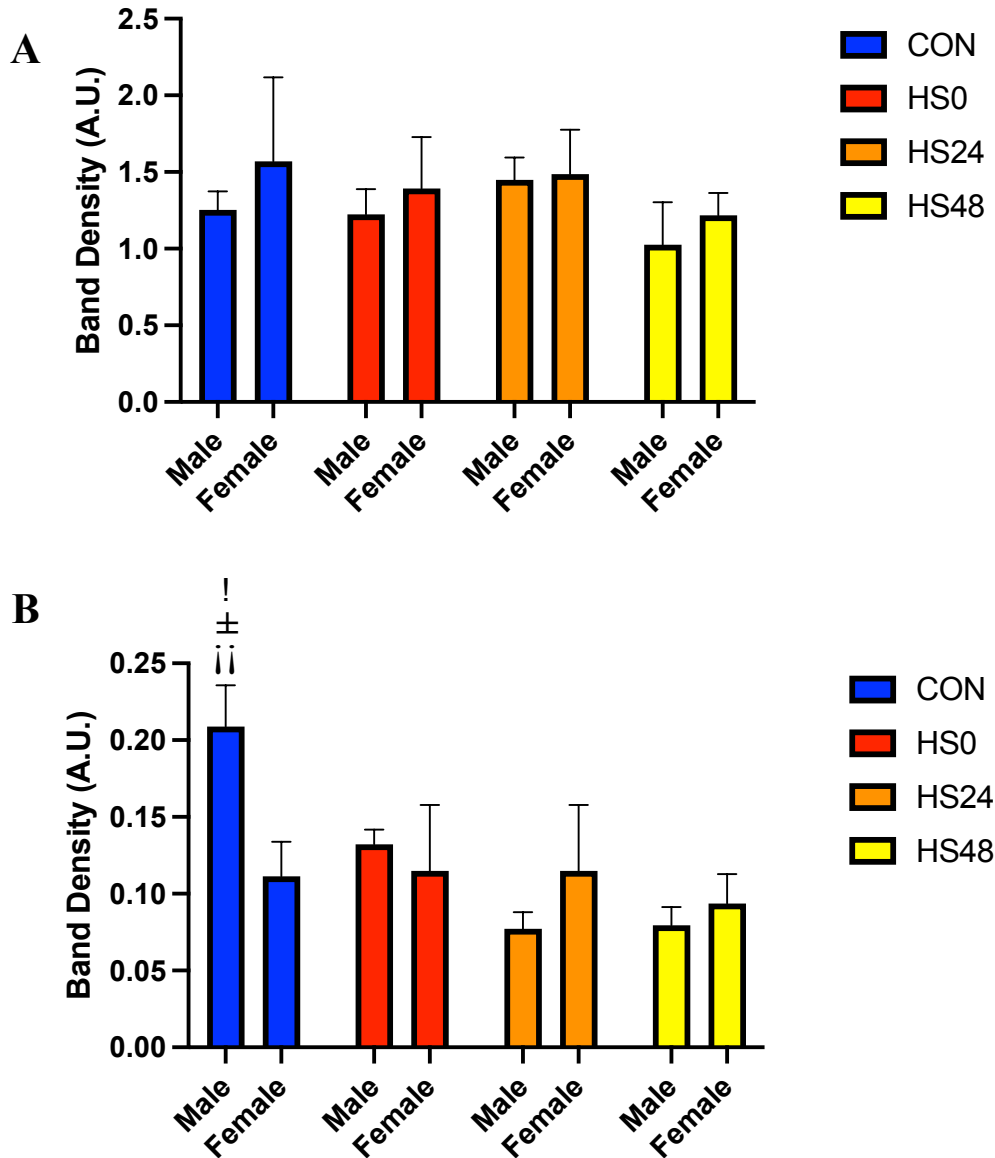


Figure 14: Male and Female Soleus and White Gastrocnemius Total Protein Nitrosylation Status Response to *In-Vivo* Heat Stress

Soleus (A) and WG (B) S-nitrosylated protein response to in-vivo heat stress in males and females (n=6 for each group across all conditions and muscles except HS48 male and female soleus protein expression (n=5)). (!) $p < 0.05$ vs male HS48, ($\pm$) $p < 0.05$ vs male HS24, ($¡¡$) vs female CON.

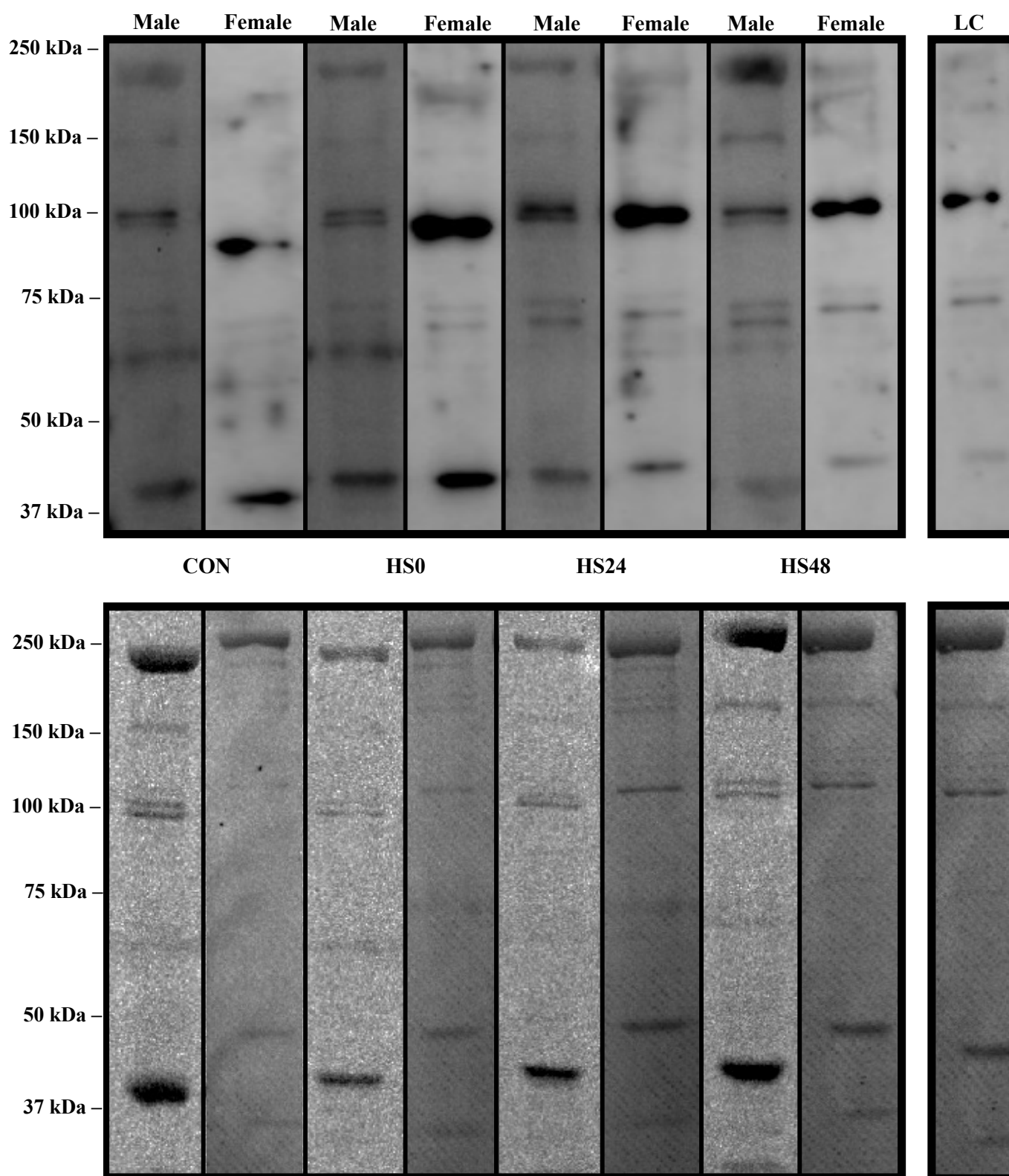


Figure 15: Western Blot of Male and Female Soleus Total Protein Nitrosylation Status Response to *In-Vivo* Heat Stress

Representative image of soleus total protein nitrosylation status response to in-vivo heat stress in males and females with loading control (male soleus) and ponceau staining.

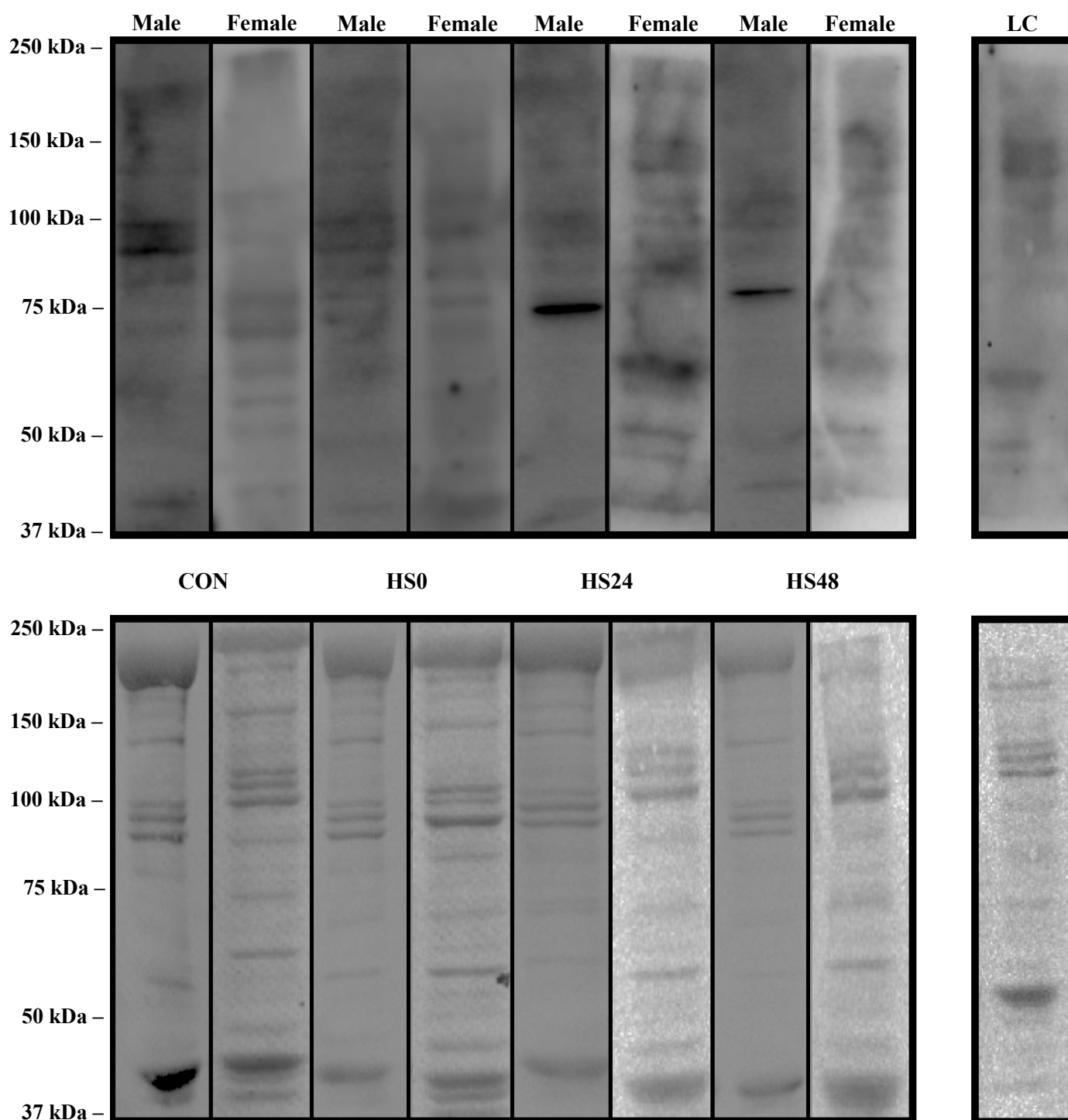


Figure 16: Western Blot of Male and Female White Gastrocnemius Total Protein Nitrosylation Status Response to *In-Vivo* Heat Stress

Representative image of soleus total protein nitrosylation status response to in-vivo heat stress in males and females with loading control (male WG) and ponceau staining.

SR Calcium Uptake

The mean starting $[Ca^{2+}]$ for the males was 2989.57 ± 70.32 nM and 2278.30 ± 101.66 nM for females and as a result Ca^{2+} uptake rates were best comparable at 1500 nM between the two sexes for the soleus (Fig. 17A). Two-way ANOVA analysis found a significant main effect of sex ($p < 0.05$) with females showing a significantly greater uptake rate compared to the males of their respective group ($p < 0.05$). For the WG, mean starting $[Ca^{2+}]$ in the males was 2880.10 ± 277 nM and 2901.59 ± 158.52 nM in the females, therefore we were able to make best comparisons at a $[Ca^{2+}]$ of 2000 nM (Fig. 17B). Two-way ANOVA comparing uptake rates at 2000 nM $[Ca^{2+}]$ found a significant main effect of condition ($p < 0.05$), such that uptake rates were highest in the HS24 condition, followed by CON, HS0, and HS48.

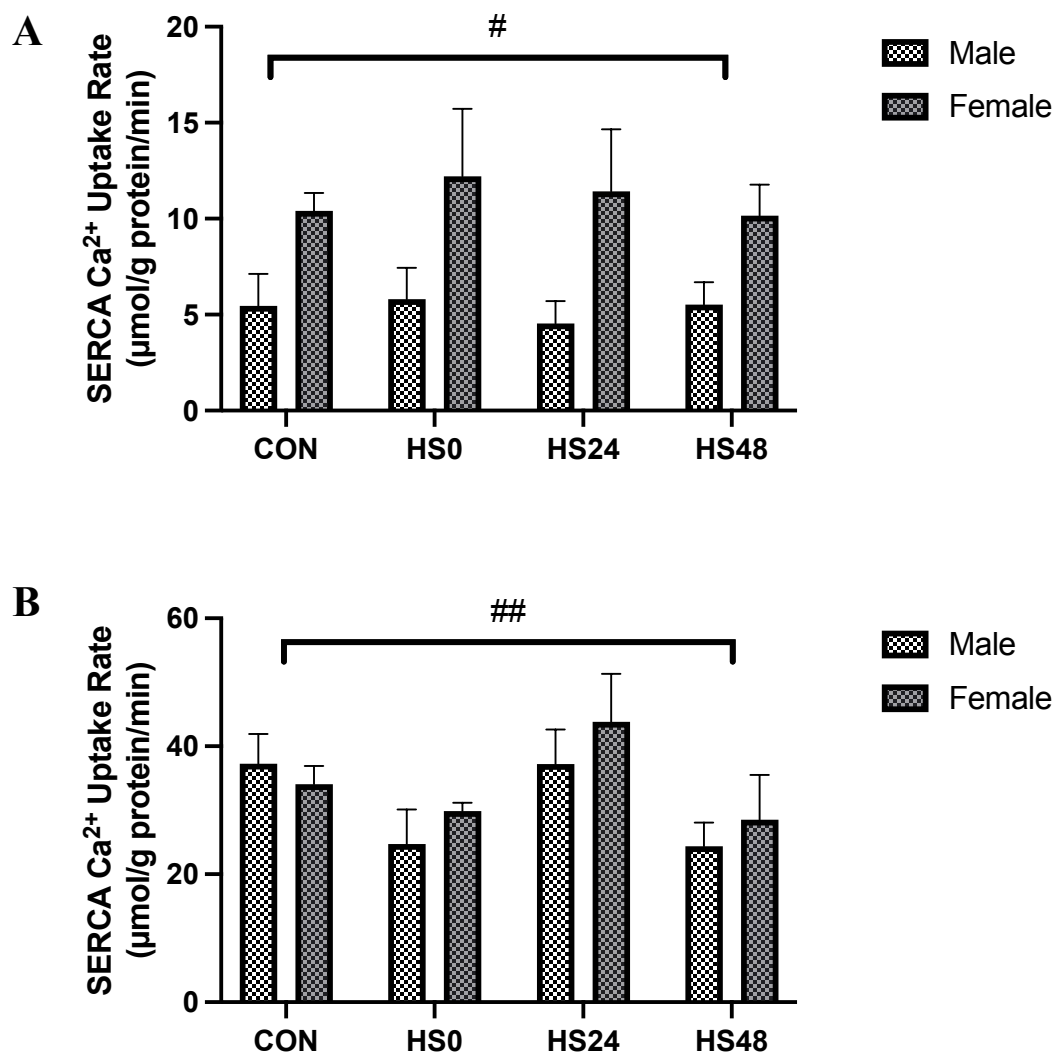


Figure 17: Male and Female Soleus and White Gastrocnemius SERCA Ca²⁺ Uptake Rate Response to *In-Vivo* Heat Stress

SERCA Ca²⁺ Uptake Rate at 1500 nM [Ca²⁺] (soleus) 2000 nM (WG) in male and female soleus (A) white gastrocnemius (B). N=5 for male CON, male and female HS0, and female HS24. N=3 for male and female HS48, female CON. N=4 for male HS0 and female CON. (#) main effect of sex. (##) main effect of condition

Discussion

The purpose of this project was to assess the sex differences in the basal gene and protein expression of Hsp70, SLN, and PLN expression in the rat soleus and white gastrocnemius and the effect of *in-vivo* heat stress on their subsequent time-course response, SERCA function, and total protein nitrosylation status in rat soleus and white gastrocnemius muscles. Basal expression pattern findings showed i) that despite greater *Hsp70* and *Sln* gene expression in both muscles in females, the male soleus had greater Hsp70 protein expression while no such difference was found for SLN in the soleus; and ii) PLN protein expression was greater in the male soleus. *In-vivo* heat stress: i) induced both gene and protein expression of Hsp70 in the soleus and WG of both male and female rats, ii) induced *Sln* gene expression but only in the male WG and increased SLN protein expression but only in the male soleus; iii) did not induce *Pln* gene expression, but did induce PLN protein expression in the WG of males; iv) did not affect SR Ca^{2+} uptake in the solei of both sexes whereas, in the WG, it increased uptake after 24 hours; and v) did not increase protein nitrosylation status in either muscle of either sex.

Variable Response of Stress Markers Suggest Inadequacies in Physiological Stress Model:

In this study, we used total protein nitrosylation status and Hsp70 gene and protein expression as markers of oxidative/nitrostatic stress. The Hsp70 protein expression pattern in response to heat stress in both sexes and muscles is quite similar with significance being reached at 24 hours after heat stress and remains elevated after 48 hours as well, which is in agreement with the pre-existing literature on the heat shock response (King et al. 2002). Sex differences in the response appear in both the gene and protein expression pattern in the soleus. Only in the females, do we see an increase in the *Hsp70* gene expression after 48 hours. As such these

differences are reflected by the Hsp70 protein levels, which in the females, although lower compared to males immediately after the stressor due to their lower basal levels, were now significantly greater compared to males after 48 hours. Although, both sexes display an Hsp70 response, it was greater in the females compared to the males. In the heart, females have a higher basal Hsp70 level compared to males and as such do not show a robust increase in expression when faced with a physiological stressor and this effect was shown to be likely mediated by estrogen (Paroo et al., 2002; Shinohara et al., 2004; Yu et al., 2006). Here, in the soleus, the sex difference in the heat shock response follows the same pattern – as the females have lower basal protein levels, there is less readily available protein to protect the proteins within the muscle and thus are more susceptible to oxidative/nitrostatic damage. Therefore, based on our current understanding of the heat shock response, it is possible that there is greater HSF binding activity with HSE in the females to promote greater upregulation of the *Hsp70* gene. The elevation in gene expression and continued elevation in Hsp70 protein levels after 48 hours in the females may be indicating this. Estrogen may also be mediating this effect in an opposite manner here in the soleus compared to what is observed in the heart, however, this mechanism was not studied here and would need to be further explored in future studies.

In the WG, there were no sex differences in the Hsp70 response to heat stress. The gene expression pattern displays an upregulation immediately after heat stress and then subsequently decreases after 24 and 48 hours. As a secondary finding, the WG of both sexes has very low basal levels of Hsp70 as we were not able to detect its presence in either the CON or HS0 conditions and was only detected after 24 hours. The *Hsp70* gene response displays a muscle difference. Basal *Hsp70* gene and protein expression is higher in the soleus compared to the WG and so in the face of heat stress, it would be more susceptible to damage and so there is a greater

need for Hsp70 induction in the WG and thus you see an immediate response to the stressor.

Whereas the soleus may not need an immediate response due to the more readily accessible basal stores that provide protection against stress. In addition, similar to the sex difference observed in the soleus, there is also a possibility of increased HSF binding activity in this muscle as well. However, given that we did not directly compare the response between muscles, these interpretations are merely a hypothesis that would need to be tested in a future study.

While the heat shock protein response shows that physiological stress was induced within the solei and WG, total protein nitrosylation results would suggest otherwise. In the solei of both males and females, nitrosylation status did not change across the different conditions. There were also no changes in the nitrosylation status in the female WG, however, in the male WG, there was actually a significant decrease in the nitrosylation status in all the heat stressed conditions in comparison to the CON condition. Interestingly however, basal protein nitrosylation was higher in the male WG compared to the female WG, which could potentially be related to differences in redox signalling between the two sexes within this muscle. These results, together with the induction of Hsp70 in both muscles suggest that the HS protocol employed in this study only induced a mild physiological stressor that was enough to upregulate Hsp70 expression, as seen in past studies (King et al. 2002) but was not severe enough to increase protein nitrosylation in the whole muscle. However, it does not explain the higher basal nitrosylation status in the male WG and the subsequent lowering in the heat stressed conditions.

Signs of Sarcolipin Induction in Response to In-Vivo Heat Stress:

Having previously shown that SLN protects SERCA structure and function in the face of *in-vitro* heat stress (Fu et al. 2020), we hypothesized that SLN would increase in response to *in-*

vivo heat stress. In the solei of both sexes, *Sln* gene expression decreased after 24 hours and remained lower after 48 hours, which is opposite to what was hypothesized. The *Sln* gene is being downregulated within this muscle in response to heat stress, which in comparison to the more established stress induced gene, *Hsp70*, seems to indicate that it is not a stress-induced gene. However, the protein expression data suggests the opposite. Despite not finding any significant changes in protein expression across the different conditions, differing trends were observed in the male and female soleus SLN response to heat stress. In the CON condition, the males had lower expression compared to females, however, in all heat stressed conditions, males show significantly higher expression. When a cell undergoes acute proteotoxic stress, as seen here in this study, chaperone genes such as those from the family of heat shock proteins are upregulated (Sawarkar, 2022; Tupling et al., 2004, 2007; Fu and Tupling 2009), while non-chaperone genes are downregulated in a phenomenon termed stress-induced transcriptional attenuation (SITA) (Sawarkar, 2022). In our aforementioned studies involving SERCA damage and its protection in the face of heat stress, we have shown that SERCA undergoes oxidative/nitrostatic damage and is denatured at the N-domain (Fu and Tupling 2009; Tupling et al., 2004). In those studies, we assessed both oxidative/nitrostatic damage and protein denaturation and showed that Hsp70 protected against both as is expected of a chaperone protein (Fu and Tupling 2009; Tupling et al., 2004). In the studies involving SLN's protection of SERCA, only oxidative/nitrostatic damage was assessed and no assessment was made on the structural integrity of the TM region of SERCA (Fu et al., 2020). Oxidative stress can either lead to specific redox modifications in the protein without necessarily changing the overall structure or non-specific damage that can denature the protein and impair its function (Bensuade et al. 1990). Therefore, it is difficult to say definitively if the TM region of SERCA was denatured as a

result of redox modifications caused by heat stress. As a result, it is not exactly clear if SLN is a chaperone protein like Hsp70 and new assays would need to be developed similar to FITC binding for the TM region to provide more definitive evidence. Under the basis of SITA (Sawarkar, 2022), if SLN is not acting as a chaperone protein, it would make sense that the *Sln* gene is being downregulated. However, given that we see a protein response in the males argues to the contrary. By interacting with SERCA, SLN can regulate cytosolic Ca^{2+} levels and thus modulate Ca^{2+} signalling in the muscle through calcineurin and CAMKII mediated gene regulation to promote mitochondrial biogenesis, myogenesis, changes in fibre type, and potentially SLN expression (Fajardo et al., 2017; 2018; Chambers et al. 2022; Maurya et al., 2018). Therefore, it is possible that in this study, we may potentially be seeing Ca^{2+} signalling by post-transcriptional means to upregulate SLN expression in the male soleus.

There also appears to be a sex difference in the response to heat stress as the SLN protein expression seems to be increased in the males but not in the females. Therefore, unlike the sex difference observed in the classically stress induced Hsp70 (Paroo et al. 2006; Shinohara et al. 2004), differences in basal SLN expression cannot explain the sex differences in the stress response of SLN within the soleus. Basal SLN gene expression in the soleus was higher in the females, therefore, according to the Hsp70 response, you would expect an increase in gene expression in the males and either no change or a smaller increase in the females, however, both sexes displayed a downregulation. Furthermore, basal protein expression levels were not different between the sexes, yet the male soleus shows an increase in expression while the female soleus did not. Estrogen, binding to the estrogen receptor-alpha, modulates the mRNA levels, and thus protein levels of Hsp70 in the heart (Shinohara et al. 2004; Yu et al. 2006). However, the effects of estrogen can also be mediated via both post-translational and post-transcriptional

mechanisms (Cignarella et al. 2023). On that basis, it is possible that estrogen could be mediating its effects on SLN protein expression by post-transcriptional mechanisms in response to *in-vivo* heat stress, but this needs to be further explored.

Despite a lack of protein response in the WG of both males and females, *SLN* gene expression seems to be responding to the heat stress in this tissue. As observed in the male WG, it was significantly elevated after 48 hours of recovery compared to all other groups, whereas the female WG did not show a clear response. Furthermore, *SLN* gene expression, which was higher in the female WG under basal conditions, was now significantly lower compared to the male WG after 48 hours. Unlike the soleus, the sex difference in gene response observed here for the WG may be related to differences in basal expression levels of the gene. The females have higher expression and therefore do not need an upregulation meanwhile the males have lower expression and therefore need to upregulate. This finding also displays differences in response across muscles.

Taking these findings together, we see signs of an SLN response in both the soleus and WG in the face of *in-vivo* heat stress and that its response varies between sex and across muscles. SLN gene and protein expression have been found to be significantly elevated in several models of disease in which the muscles are exposed to high levels of oxidative stress for a prolonged period of time, far longer than the 30 minutes observed here in this study (Chambers et al. 2020). Yet, even after just 30 minutes in this acute stress model, we do see a response in both gene and protein expression thus, providing support to the role of SLN as a stress-induced protein. If the severity or duration of this stress model was increased, we may see even larger increases in SLN gene and protein expression and may lead to ectopic protein expression in the WG.

Phospholamban Induction in Response to Heat Stress in the White Gastrocnemius:

The PLN response to *in-vivo* heat stress does not show quite as clear a trend as SLN or Hsp70. Here, it was found that the females expressed more *Pln* mRNA than the males in soleus under basal conditions and in both sexes, there was a significant downregulation in the mRNA levels in the heat stressed conditions compared with the control condition. In contradiction to the sex difference observed in the basal mRNA levels of the *Pln* gene, the protein expression of PLN was found to be significantly greater in the males in the soleus. Furthermore, a protein response was not observed in either sex, however, it does remain consistently elevated in the males compared with females across all conditions. The results here show that while the *Pln* gene is sensitive to stress it is perhaps not a stress-induced protein as it failed to demonstrate any kind of response to heat stress within this muscle.

This downregulation of the *PLN* gene is not observed in the WG, in fact there were no significant changes in gene expression in either sex, however the trend depicts a slight upregulation after 48 hours in the males and slight downregulation in the females after 24 hours with a return back to control levels after 48 hours. Unlike in the soleus, here in the WG we do see a protein response of PLN to heat stress. The protein trend in males showed an immediate increase in levels of PLN and remains elevated up until 24 hours after heat stress followed by a significant decline back to near to control levels after 48 hours, whereas the females did not show any significant changes but did show a trend with a slight elevation immediately after heat stress and return to near control levels after 24 and 48 hours.

PLN and SLN are homologous (Bhupathy et al., 2007), and while both can regulate either SERCA isoform (Fajardo et al. 2013), their expression patterns are usually opposing each other (Tupling 2009). In this study, we were able to detect PLN in both soleus and WG but were not

able to detect SLN in the WG. So, while we see protein response of SLN to heat stress in the soleus but not WG, we see a protein response of PLN to heat stress in the WG but not the soleus. This finding alludes to differences in regulator of choice being upregulated within each muscle to provide protection to SERCA; SLN protein may be preferentially increased in the soleus whereas PLN protein may be preferentially increased in the WG. The absence of change in *Pln* mRNA in the WG of either sex despite an increase in protein also alludes to potential post-transcriptional regulation. Alternatively, it could also allude to mobilization of PLN pentamer stores leading to increased monomer levels (Glaves et al. 2019) and not actual increase in total PLN protein itself. We have shown previously that PLN can also protect SERCA structure and function in the face of heat stress like SLN, and PLN too has been found to be upregulated in diseased states such as heart failure (Hasenfuss et al., 1994; Jiang et al., 2002; Kiss et al., 1995; MacLennan & Kranias, 2003; Schwinger et al., 1999). Therefore, it is not surprising to see a response of this protein under physiological stress.

SERCA Calcium Uptake Activity is Not Affected in the Soleus but was Elevated in the White Gastrocnemius 24 Hours in Response to In-Vivo Heat Stress

In the soleus of both males and females, *in-vivo* heat stress did not affect Ca^{2+} uptake indicating that SERCA function was not impaired in this muscle. Interestingly, we that found across all conditions, females had higher uptake rates than males. Since, the levels of SLN and PLN were not different between the sexes, differences in level of inhibition cannot explain this result. Although, not assessed in this study, an explanation behind this could be greater SERCA content in the female soleus. However, we could not find any direct evidence in literature which showed differences in SERCA content in rodent soleus. Another determinant could be SERCA

isoform content which would be determined by differences in fibre type composition; if the female soleus has greater type II fibre composition compared to males, then there would likely be greater SERCA1 (faster isoform) and thus greater uptake rate (Periasamy and Kalyanasundaram, 2007). However, a study in Lewis rats found no differences in fibre type composition between male and female solei (Novak et al., 2010). Furthermore, in Wistar rat soleus, males showed significantly faster twitch relaxation (Dryzmala-Celichowska & Krutki, 2015) which would suggest greater SERCA uptake rates which is contrary to what found in this study.

In the WG, we found that SERCA Ca^{2+} uptake in both sexes was affected by *in-vivo* heat stress as it was decreased immediately after heat stress followed by an increase in uptake to a peak after 24 hours and a return back to near control levels after 48 hours. In cells overexpressing Hsp70, we have previously found maximal SERCA ATPase activity to be significantly higher following heat stress (Fu and Tupling, 2009). It is possible that the same may be occurring in the WG because we see a large increase in the expression of Hsp70 in the muscle after 24 hours. Redox signalling is known to modulate SERCA activity (Valentim et al. 2022). In arterial smooth muscle, reversible S-glutathiolation of cysteine residues by peroxynitrite has been shown to activate SERCA activity, whereas irreversible oxidation of the same residues prevents this activation (Adachi et al. 2004). Hsp70 (Fu and Tupling 2009, Tupling et al. 2004) and PLN (Fu et al. 2020) have both been shown to prevent SERCA oxidative and nitrostatic damage in the face of heat stress. With Hsp70 protecting SERCA in the cytosolic region and PLN in the transmembrane region, the two proteins could be working in conjunction modulate the redox signalling leading to an increase in SERCA Ca^{2+} uptake activity after 24 hours in the WG.

The lack of a similar response in the soleus can possibly be explained by the differences in expression pattern of Hsp70, SLN, and PLN between the two muscles. In the WG, only PLN was detected under basal conditions, whereas in the soleus, all three proteins were detected under basal conditions which indicates higher basal SLN and Hsp70 protein levels within this muscle. So, up to 24 hours, in the WG, PLN would have to bear the responsibility of protecting SERCA from any redox changes until Hsp70 levels can increase to a sufficient level where they can assist PLN. In the soleus however, higher pre-existing levels of SLN and Hsp70, alongside PLN, can begin to provide protection immediately in the face of heat stress and therefore, SERCA may be protected to a greater extent from redox changes in the soleus which could prevent even the potential reversible redox changes that lead to increased SERCA Ca^{2+} uptake activity.

Limitations

This study has at least five limitations that could be impacting the outcomes assessed here. 1) The sample size for each group was six animals and with four conditions and two sexes, there were a total of 8 groups being analyzed. 2-way ANOVAs usually require a larger sample size to best find statistically significant differences. Furthermore, our power calculations (power = 0.8) using Cohen's f , indicated that we would need approximately 11 animals per group. 2) In this study, we used a manual method of homogenization with a glass-on-glass mortar and pestle whereas previous studies examining rat skeletal muscle have used mechanical method of homogenization using a Polytron device (Holloway et al. 2006; Tupling et al. 2001). Rat skeletal muscle is very dense and thus would better be broken down by a Polytron homogenizer. The SR Ca^{2+} uptake assay is a highly sensitive assay and therefore homogenate prep could affect the results we see here. Especially, considering that our uptake rates were much lower than

previously published in literature. 3) This issue of sample prep led to very low levels of or no SERCA Ca^{2+} uptake activity being detected in many of our samples which made it challenging to analyze uptake rates at higher $[\text{Ca}^{2+}]$ and therefore some of the groups in the analysis have smaller sample sizes, with some as low as 3. These smaller sample sizes could also be affecting our results and masking potential significant findings. 4) The white gastrocnemius muscle was excised by cutting the superficial portion of the muscle only, however, it is possible that some samples may have had more of the deeper, more oxidative red or mixed gastrocnemius and that may be impacting some of our analysis due to differences in fibre type between the white and red gastrocnemius (Bloemberg and Quadrilatero 2012). 5) In this study, we only assessed total protein nitrosylation as a global marker of physiological stressor and not any other markers such as DCF (Val's 2015 PLNOE paper), antioxidant enzyme activities (Superoxide dismutase, glutathione peroxidase, etc.) (Bouزيد et al., 2014), malondialdehyde levels (Bouزيد et al., 2014), and protein carbonylation (Fu and Tupling, 2007; Fu et al., 2020). Given that we do see an Hsp70 response, we can be certain some level of stress was generated, and so, it is also possible that our method of nitrosylation detection may not have been optimal for us to observe these changes. Including other assays in this study that are perhaps more sensitive may have allowed us to better see changes in the redox status.

Conclusion and Future Directions

In this study, we assessed the impact of an *in-vivo* heat stress protocol on male and female rat soleus and white gastrocnemius SERCA function and expression of its two regulators – sarcolipin and phospholamban. We found that our heat stress model was mild and was only able to induce an Hsp70 response after 24 hours in both muscles and did not increase total

protein nitrosylation. Here, we found that despite variable responses in gene expression of SLN and PLN in response to *in-vivo* heat stress, the protein expression of the two regulators are induced in response to physiological stress but differ when it comes to muscle. We found that SLN expression seems to be preferentially induced in the soleus, whereas PLN seems to be preferentially induced in the white gastrocnemius muscles. This response was also sex specific because SLN response was induced in the soleus whereby only the males displayed an increase in protein expression following heat stress. Furthermore, in the WG, we could not detect SLN protein expression, however, the males displayed an upregulation in *Sln* gene expression after 48 hours, whereas the females did not. In the WG, we found a sex difference in the PLN response whereby males increased expression, but the females did not.

Future studies should investigate the mechanistic aspects of the stress-induced role that SLN and PLN displayed in this study. Particularly, by looking at direct nitrosylation and carbonylation of SERCA to provide more direct evidence for the stress-induced role of these proteins. Furthermore, it would also be interesting to assess the effect of *in-vivo* heat stress on SR Ca^{2+} uptake in a knockout model to see if a lack of SLN or PLN or both makes SERCA more vulnerable to damage. Future studies should also consider using a more severe model of cellular stress because our model only generated very mild levels and perhaps may not have been enough to induce expression of SLN and PLN in the females. Having observed the sex differences in the SLN and PLN induction response, future studies should attempt to understand the mechanisms responsible. With the role of estrogen being shown in the Hsp70 stress response differences between males and females, it would be pertinent to also look at the role of estrogen in the induction of SLN and PLN as well. Particularly, looking at potential post-transcriptional mechanisms because here we found that despite no changes in the *Sln* or *Pln* gene, protein

expression was increased. Furthermore, we detected PLN monomer protein expression using western blot, however, because we do not see changes in the mRNA of PLN in either muscle, an alternate mechanism that should be explored is the ratio of PLN pentamer to monomer to investigate if PLN monomer levels are increasing due to post-transcriptional mechanisms or if it is just mobilization of the PLN pentamer stores.

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