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Our preliminary studies show that an established model of systemic lupus erythematosus (SLE), the female *NZBWF1* mouse, had worsened indices of disease later in life when the mice were shipped to our institution at an early age during the summer. We hypothesized that interleukin (IL)-6-induced release of heat shock protein 90 (HSP90) is upregulated in response to this summer early-life stressor, thus accelerating autoimmunity and renal disease in female SLE mice. To begin to study this, we measured renal IL-6 and HSP90 in 6-week-old female *NZBWF1* mice that were shipped in winter or summer months and found that both were elevated immediately following summer compared to winter travel. Our findings indicate that the mediators associated with early-life travel/seasonal stressors may predict the progression of autoimmunity in SLE-prone mice. Other findings here within highlight the specificity of this effect in the kidney and describe sex differences in the observed phenomena.

THE IMPACT OF EARLY LIFE STRESSORS ON THE PROGRESSION OF SLE

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THE IMPACT OF EARLY LIFE STRESSORS ON THE PROGRESSION OF SLE

THESIS

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LIST OF ACRONYMS

Abbreviation	Meaning
SLE	Systemic lupus erythematosus
dsDNA	Double-stranded DNA
NFκB	Nuclear factor-kappaB
HPA	Hypothalamic pituitary adrenal
IL-6	Interleukin-6
NZW	New Zealand White
NZB	New Zealand Black
THI	Temperature-Humidity Index
BUN	Blood urea nitrogen
MAP	Mean arterial pressure
HSP	Heat shock protein
ER	Estrogen receptor

CHAPTER I

INTRODUCTION

I.I Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder that is an accelerating healthcare burden in the United States and around the world [1]. The urgency in understanding the pathogenesis of SLE is evident by the US government's recent increase in funds and programs targeted for SLE research [2]. Advancements in the understanding of the complicated pathogenesis of this multisystem autoimmune disease will likely benefit the entire field by exposing deleterious mechanisms relevant to all autoimmune diseases.

SLE predominantly affects young women in fertile years and women are nine times more likely to develop SLE than men [3, 4]. Alarmingly, there are other health disparities associated with SLE since Black and Hispanic Americans are three-to-five times more likely to develop SLE than White Americans [3, 4]. Notable symptoms of SLE include renal, musculoskeletal and neurological complications [5], and chronic inflammation is the usual underlying factor. The inflammation in SLE is the result of complex-forming autoantibodies, e.g., double-stranded DNA (dsDNA) autoantibodies, that form following a loss of tolerance and hyperactive T and B cell activity [6-8]. Elevated dsDNA autoantibodies are found in up to 90% of all SLE patients, so these prognostic autoantibodies are ideal for tracking the progression of SLE [9].

I.II Impact of Stress and SLE

The cause of SLE is largely unknown, but there are known risk factors associated with SLE such as a person's genetic background, along with environmental [10-12], immunoregulatory, hormonally and/or epigenetic factors [13, 14]. When contemplating the impact of stress on SLE, one generally considers recent stressful events, for example daily life stressors, sleep disturbances and/or unemployment that have the potential to cause a flare and can exacerbate disease activity [15]. However, there is ample evidence that stress events that occur before puberty, rather it be physical, sexual, or emotional stress [16], can contribute to healthcare complications later in life [17-21]. One study that monitored patients with major depression and maltreatment in childhood found these stressors were associated with increases in IL-6 and nuclear factor-kappaB (NFκB) DNA binding, an indicator of inflammatory pathway activation [22]. Another study linked early life stressors to a blunted hypothalamic-pituitary-adrenal (HPA) axis in the adult, which could delay the anti-inflammatory actions of endogenous cortisol [23]. A third study linked early life stress to heightened interleuken-6 (IL-6) responses later in life [24]. Together, these studies depict the influence of early life stress on an individual's immune health upon adulthood and highlight a need for studies to understand the impact of stress on the progression of SLE.

I.III Significance of Study

Determining the impact of early life stress on the pathogenesis of SLE specifically could help elucidate mechanisms that contribute to spontaneous overactivation of the immune system and autoantibody production in the disease. Studies within this thesis used a physiological approach to study the impact of seasonal environmental factors associated with early life stress that correlate to disease progression in SLE. The results of this study will reveal causative stress-

induced mechanisms that influence the accelerated progression of SLE disease severity. On a larger scale, this study may identify useful biomarkers that can predict the likelihood of females and minority groups to develop SLE and other autoimmune diseases. Studies have shown that women are twice as likely to develop depression like disorders from early life stress compared to males [25, 26]. Additionally, there is evidence that Black American and Hispanic racial groups have heightened stress exposure due to stressors related to their occupation, finances, relationships, racial bias and prejudice [27, 28]. These stressors can have negative impacts on the health and wellness of individuals, so it is imperative that more research is performed on early life stress and disease outcomes. Ultimately, understanding how early life stressors escalate autoimmunity in SLE-prone mice could provide insight into why Black and Hispanic women are more susceptible to SLE and other autoimmune diseases [29, 30].

I.IV Murine Model

Our laboratory uses the female *NZBWF1* mouse model of SLE to elucidate mechanisms involved in stress-induced autoimmunity. This model is produced from a first generation cross between New Zealand White (*NZW*) and New Zealand Black (*NZB*) mice and has been used to study SLE for over 50 years [31]. The *NZBWF1* model closely mimics characteristics of human SLE including spontaneous production of plasma dsDNA autoantibodies, renal injury and inflammation (i.e., lupus nephritis), and impaired renal function [32, 33]. Our laboratory has worked with this model for over ten years to study how chronic inflammation promotes to hypertension [34-39].

I.V Preliminary Data

The *NZBWF1* mice (referred to as SLE mice throughout) used in our studies typically arrive to our university in Fort Worth, TX early in life (5-6 weeks of age) from Jackson Laboratories in Bar Harbor, ME. We have noticed that past cohorts of female SLE mice develop varying disease severity later in life based on the season they are shipped and arrive to our campus early in life. Driven by these anecdotal findings, we placed mice from previous studies (unpublished and published studies [37-39]) into two groups based on their month of arrival, summer (April-September) and winter (October-March), and conducted a retrospective analysis. It is important to note that during the travel to our university, mice were packaged up to 20 per crate, however specific details involved in the packaging of the mice at Jackson Laboratories and the events leading to shipping are unknown. The mice traveled via an environmentally controlled truck (63 ± 3°F and 40-60% humidity) to our university in Fort Worth, TX and the transport took around 36 hours. The mice had free access to food and gelled water throughout the trip.

Upon arrival to our university, the crates were placed in an environmentally controlled holding room for 1-3 hours (68-79°F and a 30-70% humidity); however, the crates were not ventilated during this holding period, which allowed for varying levels in temperature and humidity based on the length of time in the holding room. The actual temperature and humidity the mice experience during this unspecified time is dependent upon the temperature and humidity outside. Female SLE mice arriving to Fort Worth, TX in summer (April-September 2015-2019; n = 38) were subjected to increased temperature and temperature-humidity index (THI), a measurement of comfortability, respectively (78.5 ± 1.8 vs. 52.5 ± 1.9 °F, $P < 0.001$ and 73.6 ± 1.2 vs. 53.0 ± 1.5 $P < 0.001$; **Figure 1A** and **1C**) compared to mice arriving in winter months (October-March 2015-2019; n = 51). Humidity did not differ between summer and winter groups (61.1 ± 2.5 vs. 62.3 ± 3.4%, $P = 0.7908$).

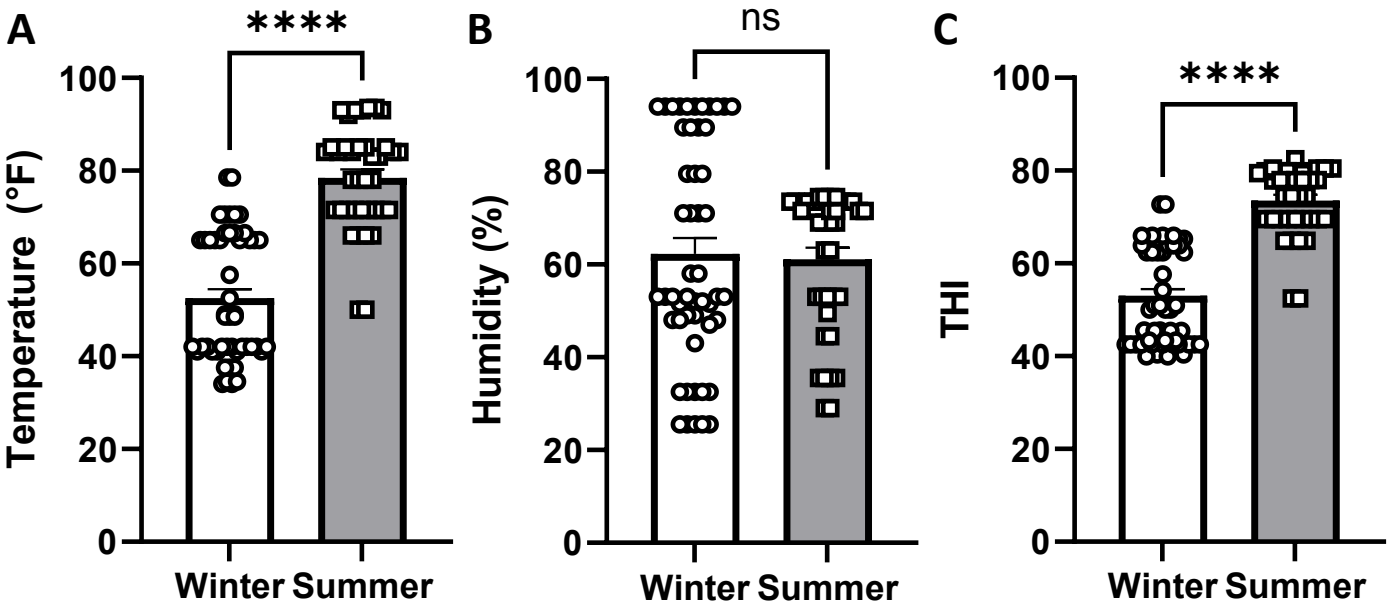

Figure 1*: Temperature and humidity in Fort Worth, Texas on specific animal arrival dates*. Temperature, humidity and temperature-humidity index (THI) on the day of arrival in winter (October-March; n = 51) and summer (April-September; n = 38). Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences. THI was calculated using this equation: THI = .8 (avg Temperature °C) + ((avg Humidity/100) (avg Temperature °C – 14.4) + 46.4)

Mice were placed in new clean cages with bedding at five per cage. The mice, again, had free access to food and water and were placed in a ventilated, environmentally-controlled room (68-79°F and a 30-70% humidity) and were mostly not manipulated until week 30, with the exception of normal husbandry and ear punches before 10 weeks of age. Body weight did not differ between summer and winter groups as the mice aged from 30 to 35 weeks (data not shown; P = 0.1370). Plasma dsDNA autoantibody concentration, a marker of SLE disease severity, was higher in aged female SLE mice that were shipped to our university while young in summer (n = 38) compared to winter (n = 51) months (6.0 ± 8.6e4 vs. 4.1e5 ± 5.1e4 U/mL; P = 0.04855; **Figure 2**). Urinary albumin concentration, an index of renal injury, was significantly higher in aged female SLE mice (34-35 weeks of age) that were shipped while young in the summer (n = 27) versus winter (n = 42) months (13263.7 ± 2555.4 vs. 5968.8 ± 1457.2 μg/mL; P = 0.0096; **Figure 3**). Other indices of renal injury, blood urea nitrogen (BUN) and urinary creatinine, were similar in female SLE mice that arrived in the winter and summer groups (n = 9-10 per group; 28.97 ± 5.98 vs. 29.64 ± 6.26 mg/dL; P = 0.9392, **Figure 4**; 78.79 ± 4.38 vs. 77.98 ± 10.72 mg/dL; P = 0.9473, **Figure 5**). Finally, there was no significant difference in mean arterial pressure (MAP) between mice that arrived in summer (n = 17) and winter groups (n = 39) (143.6 ± 3.5 vs. 140.2 ± 2.2 mmHg; P = 0.4096; **Figure 6**).

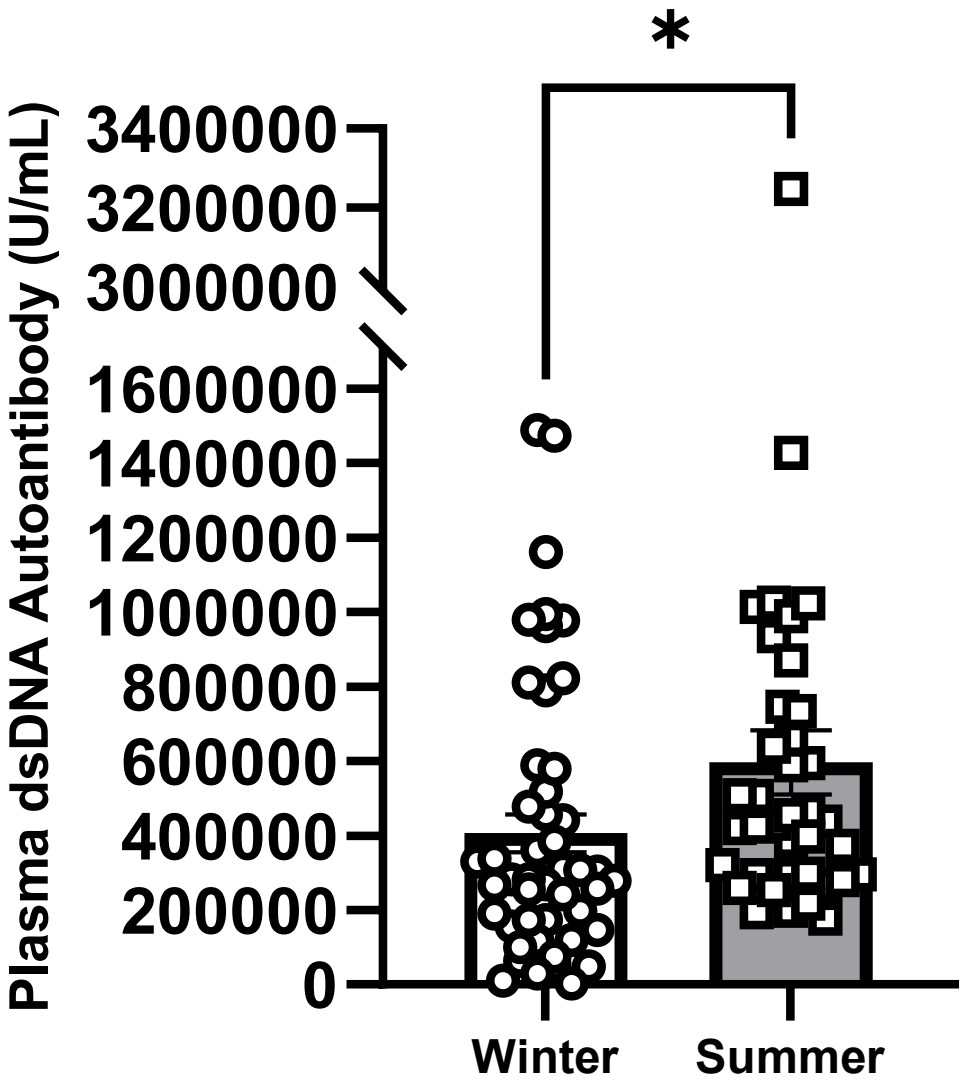

Figure 2*: Disease severity is worse in aged female SLE mice that were shipped while young in the summer.* Plasma dsDNA autoantibody concentration, an index of disease severity, measured via ELISA in blood samples collected in female SLE mice at 34-35 weeks of age and grouped based on their date of arrival to our institution: winter (n = 51) and summer (n = 38). Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences (P* = 0.04855).

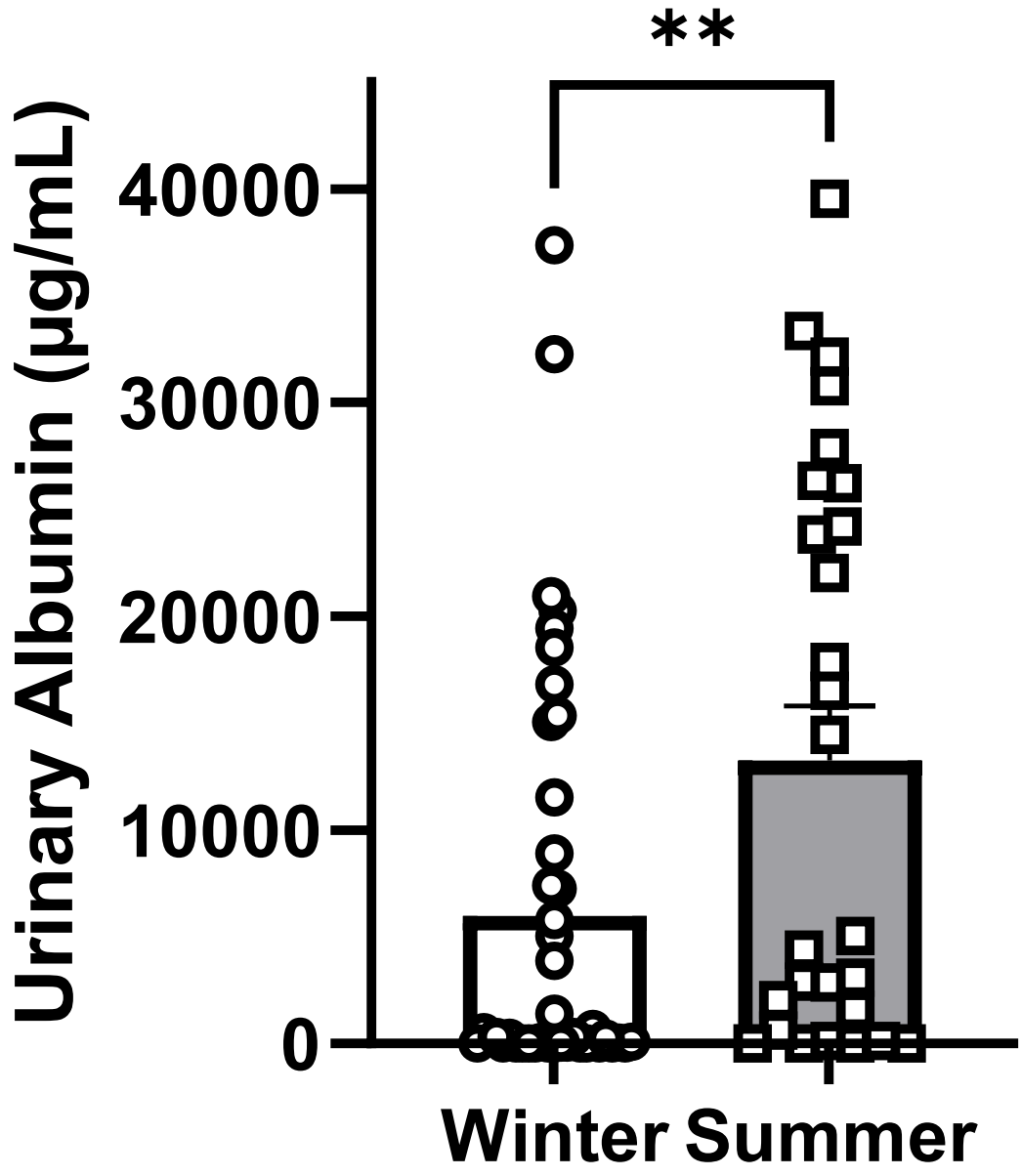

Figure 3*: Renal injury is elevated in aged female SLE mice that were shipped while young in the summer.* Albumin concentration measured via ELISA in urine collected at 34-35 weeks of age in female SLE mice that arrived in the winter (n = 42) and summer (n = 27) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences (P = 0.0096).

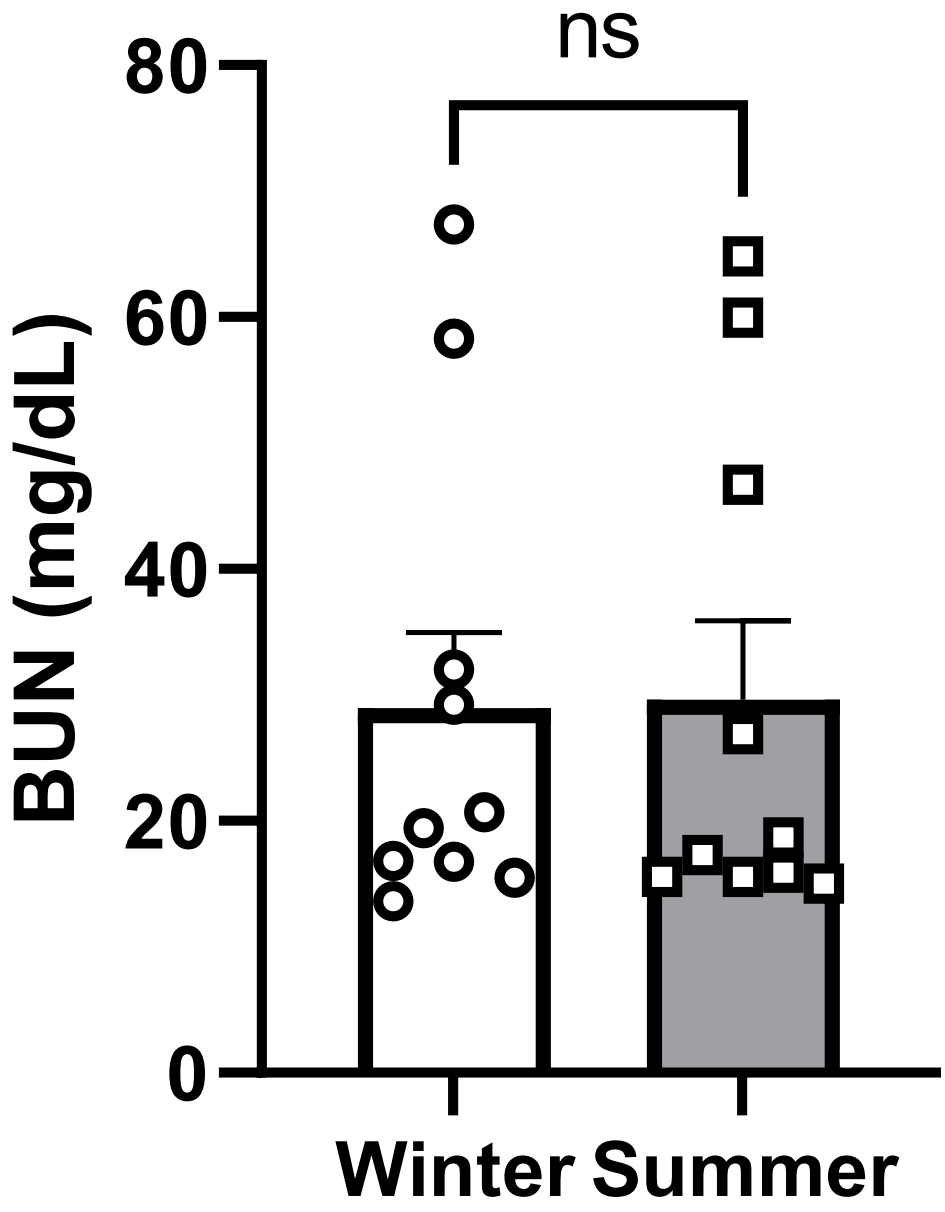

Figure 4*: Blood urea nitrogen is similar in aged female SLE mice that were shipped while young in summer and winter months.* Blood urea nitrogen (BUN) was measured via Quantichrom urea assay in blood collected at 34-35 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

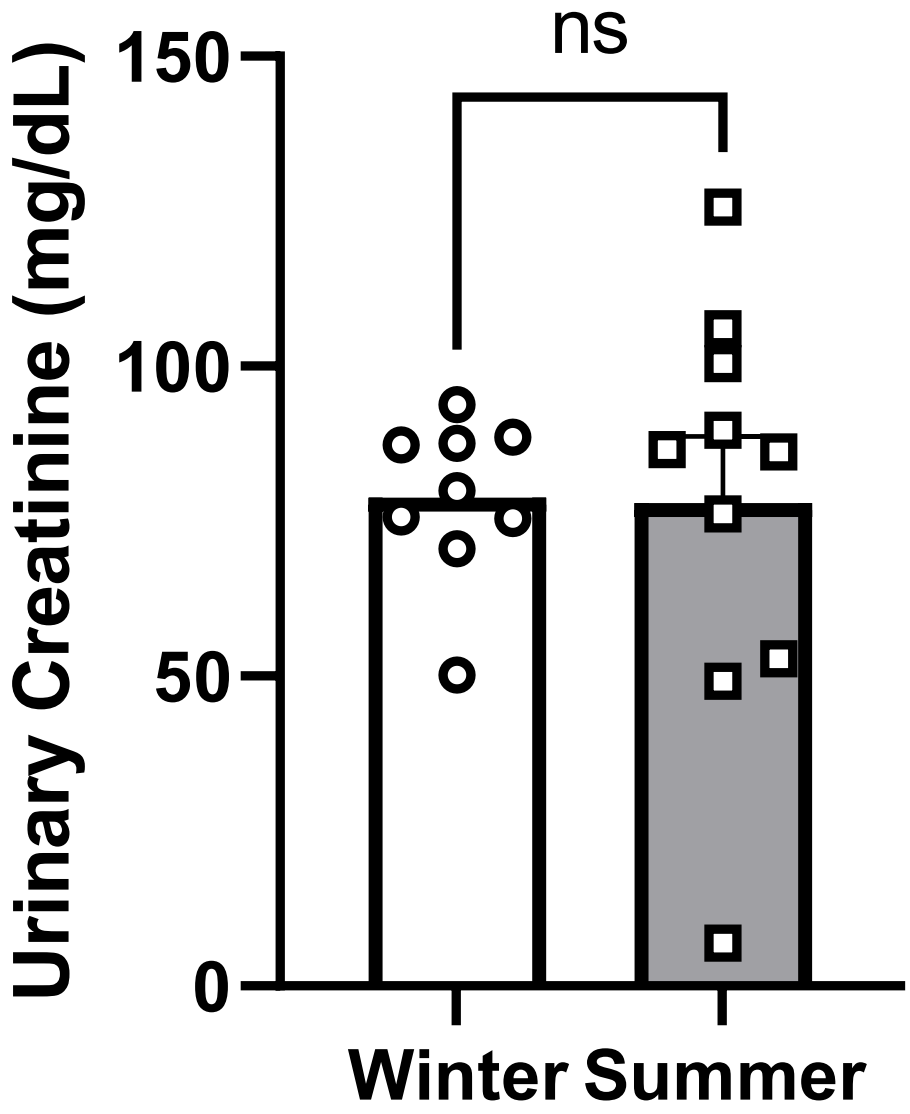

Figure 5*: Urinary creatinine is similar in aged female SLE mice that were shipped while young in summer and winter months.* Urinary creatinine was measured via creatinine assay in urine collected at 34-35 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

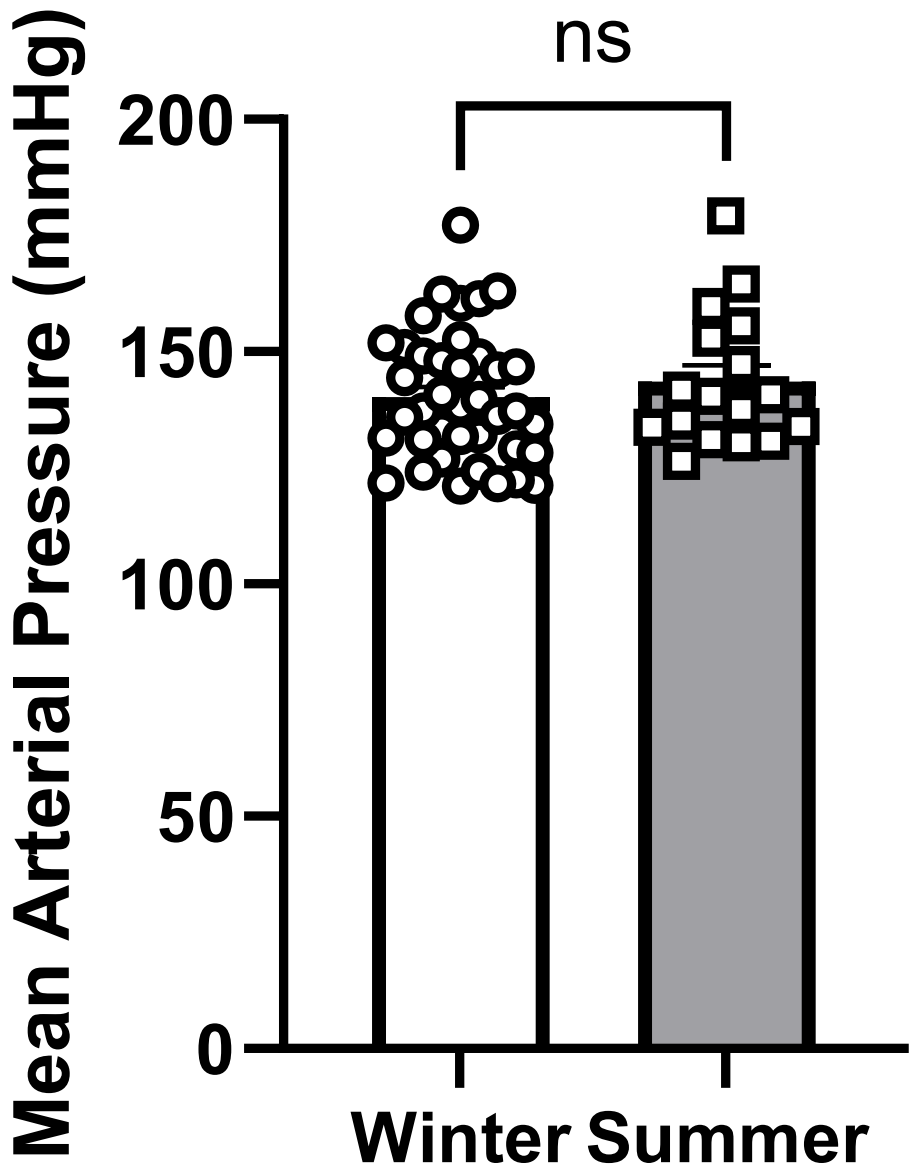

Figure 6: *Mean arterial pressure is similar in aged female SLE mice that were shipped while young in summer and winter months.* Mean arterial blood pressure measured in female SLE mice that arrived to our institution in the winter (n = 39) and summer (n = 17) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

I.VI Heat Shock Proteins

This retrospective analysis has uncovered an interesting phenomenon in the pathogenesis of SLE that is dependent on environmental/seasonal factors. Because temperature and THI seem to contribute to the accelerated disease severity following early life travel stress, we are curious about such environmental processes and how they may alter biochemical mechanisms that can promote to autoimmunity. Heat shock proteins (HSP) are a family of highly conserved proteins that act as chaperones in the folding and degradation of denatured proteins [40]. HSPs are activated in response to heat but also ultraviolet light, infection, and psychological and physical stressors [40-42]. Since both stress and temperature can activate heat shock proteins (HSP), the studies here within will look closely at the interaction between HSPs and SLE disease progression [40].

I.VII Heat Shock Protein 90

One HSP in particular, HSP90, is known to be elevated in SLE mouse models [43-45]. HSP90 enhances antigen presentation, activates lymphocytes and macrophages, matures dendritic cells, and specifically in SLE, elevates dsDNA autoantibody production [40, 46]. HSP90 has also been shown to bind to steroid hormone receptors, such as the estrogen receptor (ER) in the cytosol [47]. It is important to note that HSP90 is able to bind to all homodimer and heterodimers of ER [48]. This binding of HSP90 stabilizes the ER [49], allowing for estradiol to bind to its receptor, translocate into the nucleus and cause upregulation/downregulation of different transcription factors [49]. In doing so, the ER has the ability to increase the production of different cytokines [50]. Knowing this, HSP90 may have a larger role in females since estrogen is the predominant sex hormone in females.

HSP90 can also be expressed intracellularly and extracellularly [51]. Intracellular expression elicits a protective role on the cell, which allows for cell survival in lethal conditions [51]. However, when expressed extracellularly, HSP90 leads to modulation of immune responses that can involve the innate or adaptive immune system [51]. This extracellular expression can activate the immune system during stress and cause potential problems that can later lead to incidence of disease severity later in life. It has also been determined that there are two isoforms of HSP90: HSP90α and HSP90β [52]. HSP90α is 2-3 times more abundant than HSP90β in ERα containing complexes [52]. HSP90β is constitutively expressed, while HSP90α is only elevated in response to stress [52]. Evidence suggests that female mice have elevated ERα levels in kidneys upon aging and that ERβ is not expressed in kidneys [53]. In addition, male mice have shown decreased ERα levels with aging [53]. Because we have demonstrated an elevation of albuminuria, indicative of renal disease in female *NZBWF1* mice from our study, HSP90 may play a key role in SLE-induced renal disease, especially in females.

Studies suggest that elevated plasma HSP90 levels correlate to dsDNA autoantibodies in a SLE model of lupus known as MRL/lpr as well as other models of autoimmunity [43, 44, 46, 54, 55]. Additionally, elevated plasma interleukin-6 (IL-6), a cytokine involved with increased antigen presentation, leads to increased HSP90 concentrations [56]. A study by Stephanou et al. used peripheral blood mononuclear cells from SLE patients to look at this phenomenon, explaining that when IL-6 concentrations are elevated in these cells, HSP90 gene levels are consequently elevated [57]. Thus, elevation in IL-6 results in increased HSP90 concentration. Interestingly, general stress elevates circulating and renal IL-6 [58, 59] and another study demonstrated that by inducing psychological stress in mice, IL-6 was released from brown adipose tissue and increased

inflammation while eliciting a “fight or flight” response [60]. These IL-6 levels were detectible 18 hours after inducing this psychological stressor.

I.VIII: Hypothesis

Based on our understanding of the relationship between heat, stress, HSP90, the estrogen receptor and IL-6 described above, we hypothesize that early life stressors compounded with environmental triggers (i.e., increased temperature and humidity) cause upregulation of HSP90 levels and acceleration of autoimmunity and renal disease in female mice with SLE. Further, we hypothesize that early-life stress-induced increases in IL-6 guide this process.

CHAPTER II

METHODOLOGY

Murine Models: Female and male *NZBWF1* mice were used in this study. Female *C57* and female *NZW* mice, a parent strain of the *NZBWF1* model, were used as controls. All female mice arrived to our facility at 6 weeks of age. Male SLE mice arrived at 6-8 weeks of age. All mice came from Jackson Laboratories in Bar Harbor, ME by an environmentally controlled truck ride with free access to food and gelled water. Body weights were measured immediately upon arrival to the designated animal room and tissues were collected after euthanizing. All animal studies were approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Renal Cortex Separation and Homogenization: After euthanizing, kidneys were harvested and the cortex was isolated from the medulla of the right kidney. Cortices were then homogenized in 8 times their weight of RIPA buffer (1% Nonident P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). All steps were performed on ice.

Plasma dsDNA Autoantibody Detection: Plasma dsDNA autoantibody concentration was measured at 6-8 weeks of age via ELISA (Alpha Diagnostic, San Antonio, TX). Plasma samples were diluted (1:200), and 100 µL of sample diluent, standards, and samples were placed in a 96-well plate in duplicates. The samples were gently mixed and incubated for 60 minutes at room temperature. The wells were washed 4 times with a wash solution and patted dry. Next, 100 µL of diluted anti-mouse IgG HRP was added to each well and the samples were incubated for 30 minutes at room temperature. Once complete, the wells were washed 5 times and patted dry. 100 µL of substate solution was added to each well and were left to incubate for 15 minutes at room temperature in the dark. At this time, 100 µL of stop solution was added and gently mixed to stop the enzyme reaction. An absorbance reading of 450 nm was performed to determine the concentration of plasma dsDNA autoantibodies using an Epoch 2 microplate reader (BioTek Instruments, Winooski, VT).

Blood Urea Nitrogen Quantification: Blood urea nitrogen (BUN) was measured at 6-8 weeks of age via a QuantiChrom kit (BioAssay Systems, San Jose, CA). 50 µL of sample diluent, standards, and samples were placed in a 96 well plate in duplicates. 200 µL of working reagent was added to each well and samples were gently mixed. Samples were then incubated for 20 minutes at room temperature. Once complete, an absorbance reading of 520 nm was performed to determine the concentration of urea using an Epoch 2 microplate reader (BioTek Instruments, Winooski, VT). An analysis was performed to determine the amount of BUN in each sample.

IL-6 Quantification: Plasma and renal IL-6 was measured at 6-8 weeks of age via Mouse IL-6 Quantikine ELISA (R&D Systems, Minneapolis, MN). Plasma (1:2) and renal (1:4) samples were

diluted, and 50 µL of assay diluent was added to each well of the 96 well plate. 50 µL of diluent, standards, and diluted samples were placed in a 96 well plate in duplicates. The samples were gently mixed for 1 minute and incubated for 120 minutes at room temperature. The wells were aspirated and washed 5 times with a wash solution and patted dry. 100 µL of mouse IL-6 conjugate was then added to each well and samples were incubated for 120 minutes at room temperature. After completion, the wells were aspirated and washed 5 times, then patted dry. 100 µL of substate solution was added to each well and left to incubate for 30 minutes at room temperature in the dark. Lastly, 100 µL of stop solution was added and gently mixed to stop the enzyme reaction. An absorbance reading of 450 nm was performed to determine the concentration of plasma and renal IL-6 using an Epoch 2 microplate reader (BioTek Instruments, Winooski, VT).

HSP90 Quantification: Plasma and renal HSP90 was measured at 6-8 weeks of age via a mouse HSP90 ELISA kit (Novus Biologicals, Centennial, CO). Plasma (1:10) and renal (1:500) samples were diluted, and 100 µL of diluent, standards, and diluted samples were placed in a 96 well plate in duplicates. The samples were gently mixed, a plate sealer was added, and the samples incubated for 90 minutes at 37°C. Liquid was completely removed and 100 µL of biotinylated detection antibody working solution was added to each well. The samples were gently mixed, a plate sealer was added to the plate, and the samples incubated for 60 minutes at 37°C. After completion, liquid was removed and the wells were washed 3 times, then patted dry. Next, 100 µL of HRP conjugate working solution was added to each well. The plate was covered with a plate sealer and incubated for 30 minutes at 37°C. Liquid was removed and the wells were washed 5 times, then patted dry. 90 µL of substate solution was added to each well and left to incubate for 15 minutes at 37°C in the dark. Lastly, 50 µL of stop solution was added and gently mixed to stop the enzyme reaction.

An absorbance reading of 450 nm was performed to determine the concentration of plasma and renal HSP90 using an Epoch 2 microplate reader (BioTek Instruments, Winooski, VT).

Statistical Analysis: Statistical analysis was achieved using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Data was calculated as mean ± standard error of the mean. A $p < 0.05$ indicates significant difference. An unpaired Student's t test was used to determine statistical difference between two groups. Comparisons between multiple groups were analyzed via a two-way ANOVA along with post-hoc Tukey test for multiple comparisons.

CHAPTER III

RESULTS

Several comparisons were made in order to examine the impact of seasonal stressors on the progression of SLE. First, to determine if travel-associated stress accelerates SLE disease outcomes dependent on the season of travel, we compared indices of disease severity and renal injury in female SLE mice immediately after winter and summer travel to our university. Second, we compared female SLE mice to "normal" female mice to determine if the seasonal effects were specific to SLE mice. Finally, because our unpublished data suggest the progression of SLE differs between sexes, we compared the impact of seasonal stressors in male and female SLE mice.

III.I Impact of seasonal travel associated stressors on common SLE disease outcomes

To determine the stage of disease in the female SLE mice immediately after travel stress and if seasons impact this, we measured SLE disease outcomes at 6 weeks of age once the mice arrived to our institution. Plasma dsDNA autoantibody concentration was similar in female SLE mice that arrived in the summer and winter months (13027 ± 2029 vs. 12676 ± 2528 U/mL; P = 0.9150; **Figure 7**). BUN was also similar in female SLE mice arriving in the summer versus winter months (17.22 ± 1.26 vs. 15.00 ± 0.71 mg/dL; P = 0.1449; **Figure 8**). This suggests that stress associated with seasonal travel does not immediately alter prognostic autoantibodies or the integrity of the kidney in female SLE mice.

A comparison of dsDNA autoantibodies was made between female SLE mice and "normal" mice, and we found that when comparing these mice at early time point with control mice (*C57*) and the parental strain (*NZW*), the concentration of plasma dsDNA autoantibodies was significantly higher in female SLE mice ($P < 0.0001$; **Figure 9**); however, there was no seasonal effect ($P = 0.8987$; **Figure 9**). There was also no interaction in plasma dsDNA autoantibodies ($P = 0.1921$; **Figure 9**).

BUN was statistically different amongst female SLE, *C57*, and *NZW* mice at the early time point ($P < 0.0001$; **Figure 10**) and there was also a significant seasonal effect ($P = 0.0001$; **Figure 10**). However, there was no statistical interaction in BUN at the early time point in female mice ($P = 0.4018$; **Figure 10**).

To determine if there was a difference in SLE disease outcomes between sexes, we examined plasma dsDNA autoantibodies in male and female mice. Plasma dsDNA autoantibodies was lower in male SLE mice compared to female SLE mice ($P = 0.0119$; **Figure 11**). There was no significant seasonal effect on plasma dsDNA autoantibodies ($P = 7720$; **Figure 11**). There was also no interaction between groups ($P = 0.8992$; **Figure 11**). When comparing sex differences in renal injury at the early time point, we determined that male SLE mice had higher levels of BUN compared to females ($P = 0.0051$; **Figure 12**). There were no seasonal differences in BUN based on month of arrival ($P = 0.1392$; **Figure 12**). There was also no interaction seen between groups ($P = 0.3363$; **Figure 12**).

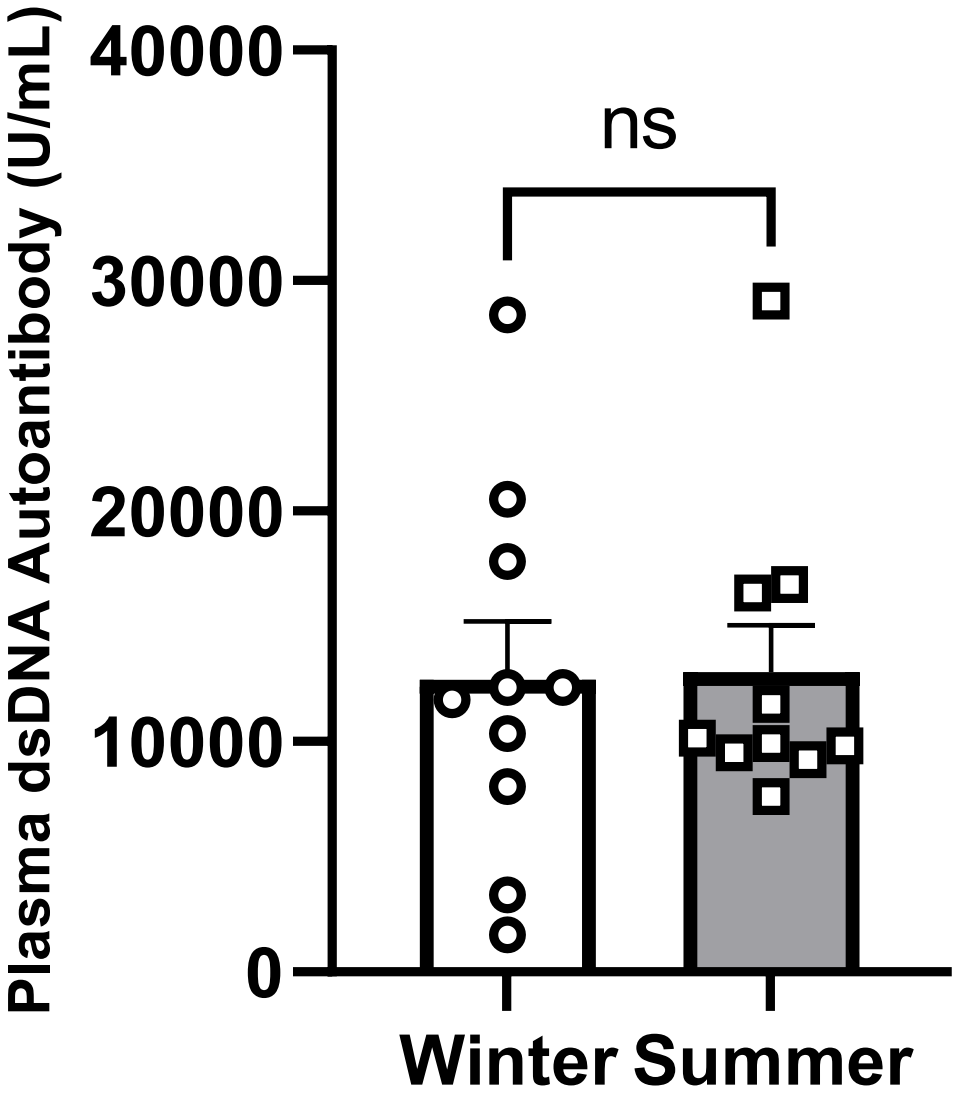

Figure 7: *Disease severity is similar in young female SLE mice that traveled to our university in the summer and winter months.* Plasma dsDNA autoantibody concentration was measured via ELISA in blood samples collected at the early time point of 6 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

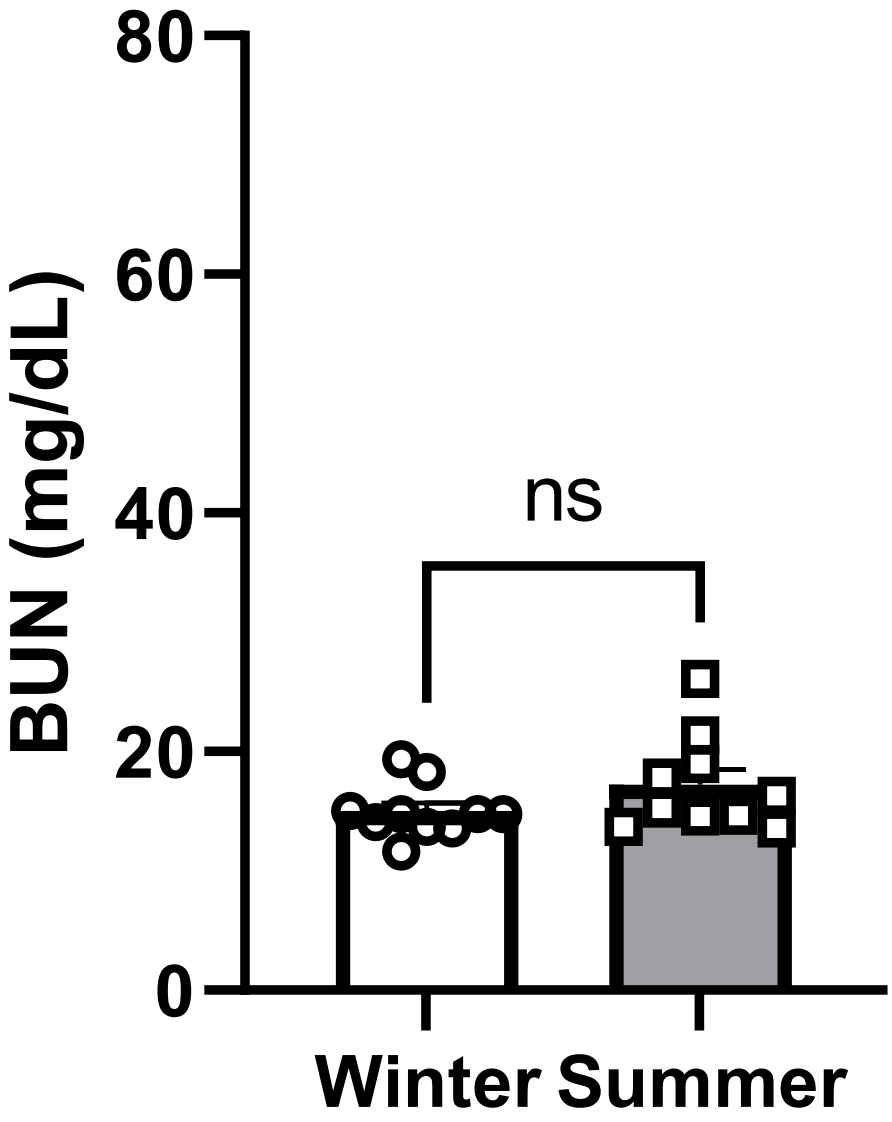

Figure 8: *Renal injury is similar in female SLE mice that travelled to our university in summer and winter months.* Blood urea nitrogen concentration was measured via Quantichrom Urea Assay in blood samples collected at the early time point of 6 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

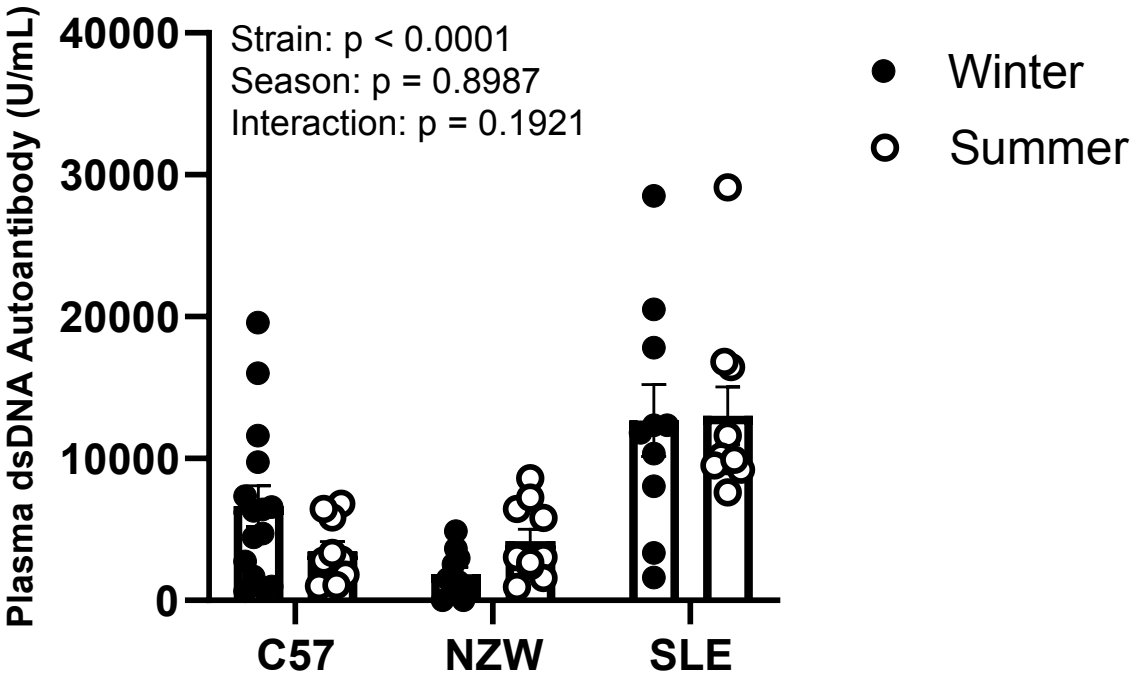

Figure 9: *Indices of SLE disease not present in control mice at early age immediately after travel.* Plasma dsDNA autoantibody concentration was measured via ELISA in blood samples collected at 6 weeks of age in *C57* (n =15, 10), *NZW* (n = 10, 10), and female SLE mice (n = 10, 10) in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of dsDNA autoantibodies in "normal" mice, we ran a separate unpaired Student's t test in *C57* and *NZW* mice separately. *C57* mice had no significant difference in plasma dsDNA autoantibodies in summer or winter months (3462 ± 683 vs. 6650 ± 1446 U/mL; P = 0.1013). *NZW* mice had significantly higher concentration of plasma dsDNA autoantibodies in the summer group compared to the winter group (4188 ± 831 vs. 1841 ± 514 U/mL; P = 0.0274).

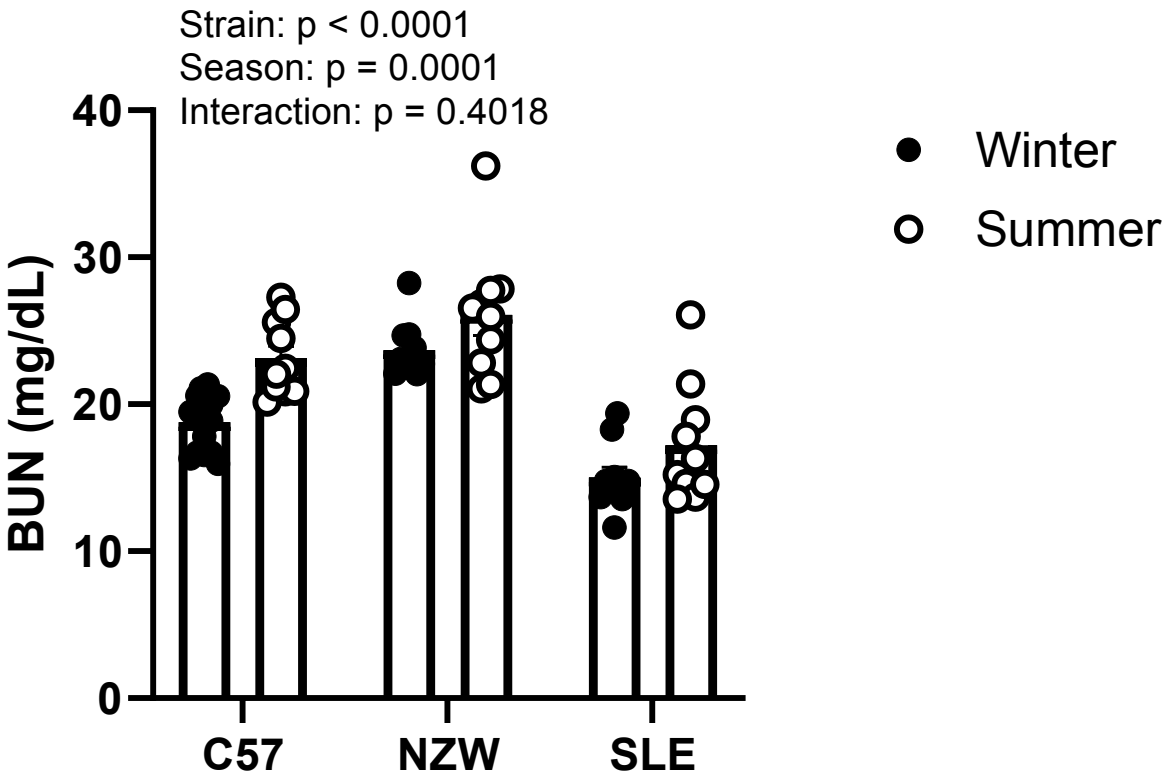

Figure 10: *Renal injury is less severe in female SLE mice immediately upon arrival.* Blood urea nitrogen concentration was measured via Quantichrom Urea Assay in blood samples collected at 6 weeks of age in *C57* (n =15, 10), *NZW* (n = 10, 10), and female SLE (n = 10, 10) mice in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of BUN in "normal" mice, we ran a separate unpaired Student's t test in *C57* and *NZW* mice separately. *C57* mice had significantly higher concentrations of BUN in summer months of arrival compared to winter (23.14 ± 1.38 vs. 18.79 ± 0.50 mg/dL; $P < 0.0001$). *NZW* mice did not have significantly different concentrations of BUN in either the summer or winter group (26.07 ± 1.38 vs. 23.68 ± 0.59 mg/dL; $P = 0.1276$).

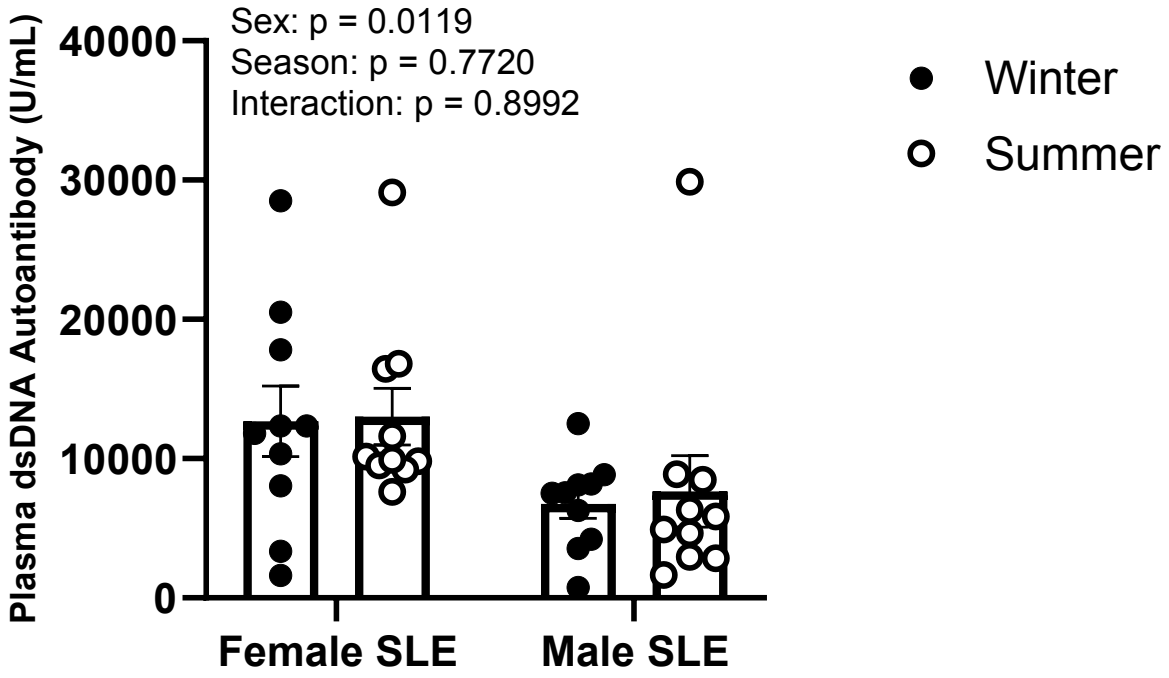

Figure 11: *Disease severity is lower in male SLE mice compared to females immediately upon arrival.* Plasma dsDNA autoantibody concentration was measured via ELISA in blood samples collected at 6-8 weeks of age in female SLE (n = 10, 10) and male SLE (n =10, 10) mice in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of dsDNA autoantibodies in male SLE mice, we ran a separate unpaired Student's t test. Male SLE mice had similar plasma dsDNA autoantibody concentrations in summer and winter months at 6-8 weeks of age (7648 ± 2579 vs. 6753 ± 1029 U/mL; P = 0.7508).

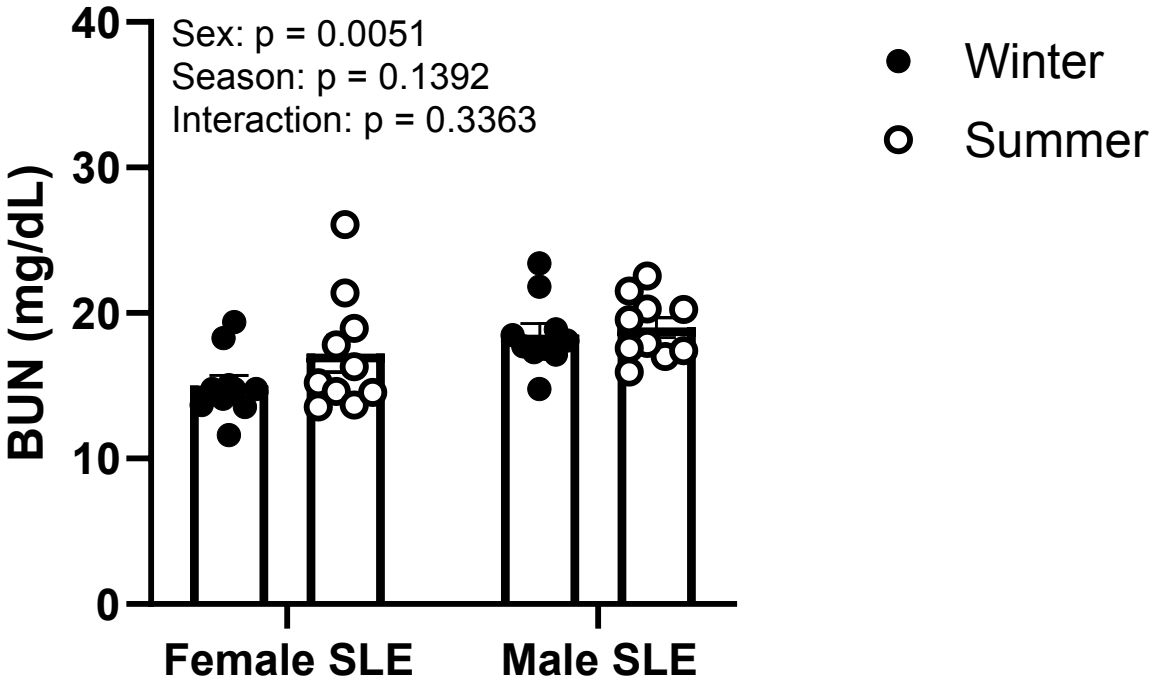

Figure 12: *Renal injury is similar in male and female SLE mice immediately upon arrival.* Blood urea nitrogen concentration was measured via Quantichrom Urea Assay in blood samples collected at 6-8 weeks of age in female and male SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of BUN in male SLE mice, we ran a separate unpaired Student's t test. Male SLE mice had similar BUN concentrations in summer and winter months of arrival (18.53 ± 0.77 vs. 19.00 ± 0.68 mg/dL; P = 0.6476).

III.II Impact of seasonal travel-associated stressors on inflammation in SLE

Circulating IL-6, a pro-inflammatory cytokine, was measured in female SLE mice as an index of full-body inflammation immediately upon arrival at 6 weeks of age in summer and winter months. Circulating IL-6 was paradoxically lower in female SLE and *C57* mice that arrived in summer months versus winter months (4.45 ± 1.50 vs. 19.02 ± 2.42 pg/mL; P = 0.0002; **Figure 13**) (2.91 ± 0.65 vs. 10.45 ± 1.45 pg/mL; P = 0.0021; **Figure 14**), respectively. Plasma IL-6 was lower in the female SLE mice that arrived in winter compared to *C57* mice that arrived in winter (P = 0.0190; **Figure 14**). Data for *NZW* mice is inconclusive since circulating IL-6 could not be detected in summer mice.

To determine if there was any significant difference in circulating IL-6 between sexes, we measured plasma IL-6 in female and male SLE mice at 6-8 weeks of age. There was no difference in plasma IL-6 in female and male SLE mice (P = 0.0813; **Figure 15**). There was a seasonal effect on plasma IL-6 in male and female mice (P < 0.0001; **Figure 15**), however, there was no interaction of IL-6 and no individual or across group comparison could be made (P = 0.7439; **Figure 15**).

To examine renal-specific inflammation immediately after travel stress we measured renal IL-6 in female SLE mice at 6 weeks of age. Renal IL-6 was higher in female SLE and *C57* mice arriving in summer versus winter months (41.41 ± 2.16 vs. 20.40 ± 2.84 pg/mL; P < 0.0001; **Figure 16**) (28.48 ± 2.61 vs. 16.91 ± 2.14 pg/mL; P = 0.0017; **Figure 17)**, but no difference was seen in *NZW*s (19.50 ± 1.94 vs. 20.71 ± 4.94 pg/mL; P = 0.8128; **Figure 17**). This indicates that renal inflammation is elevated in certain strains of female following travel stress in summer months at 6 weeks of age.

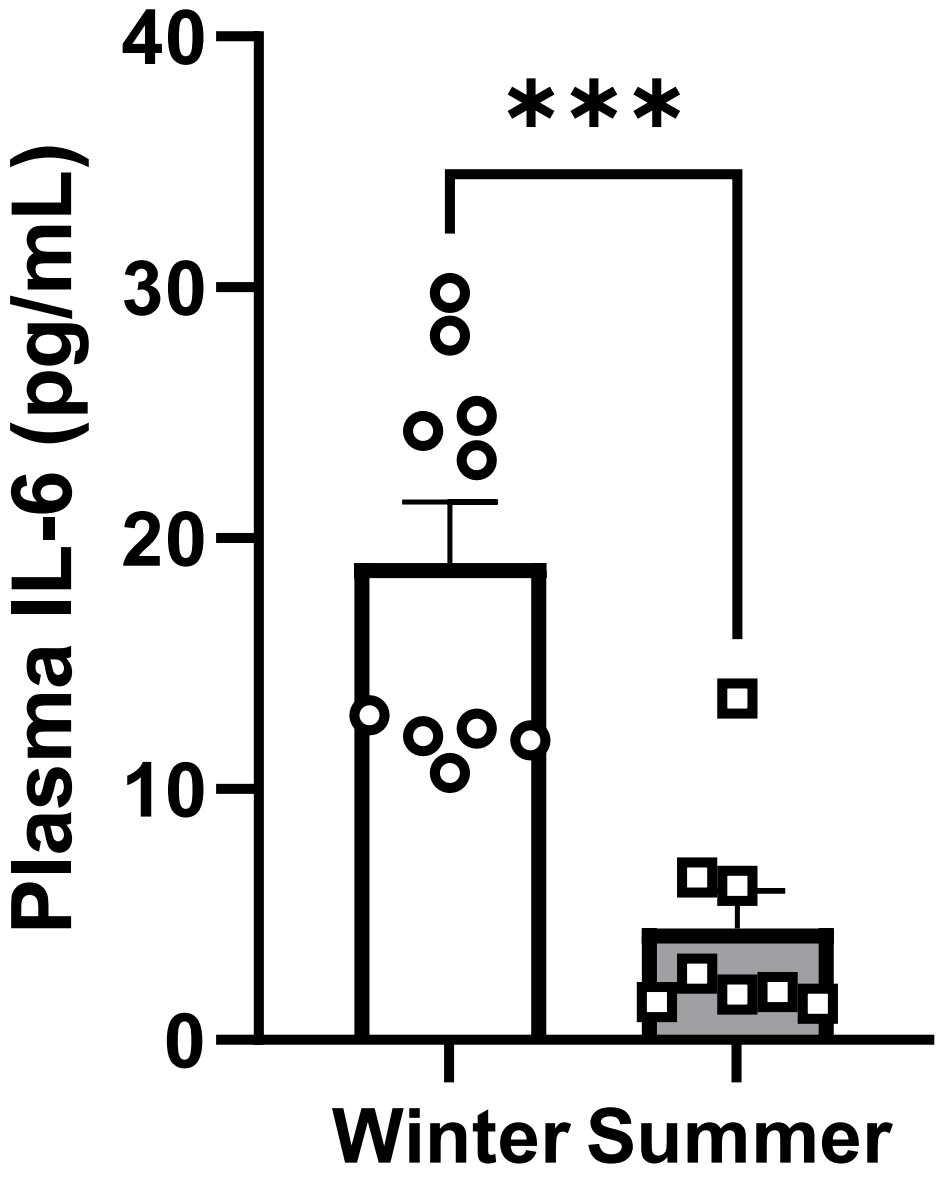

Figure 13: *Circulating IL-6 is lower in female SLE mice that traveled to our university in the summer.* Circulating interleukin-6 (IL-6) was measured via ELISA in blood samples collected at 6 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 8) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

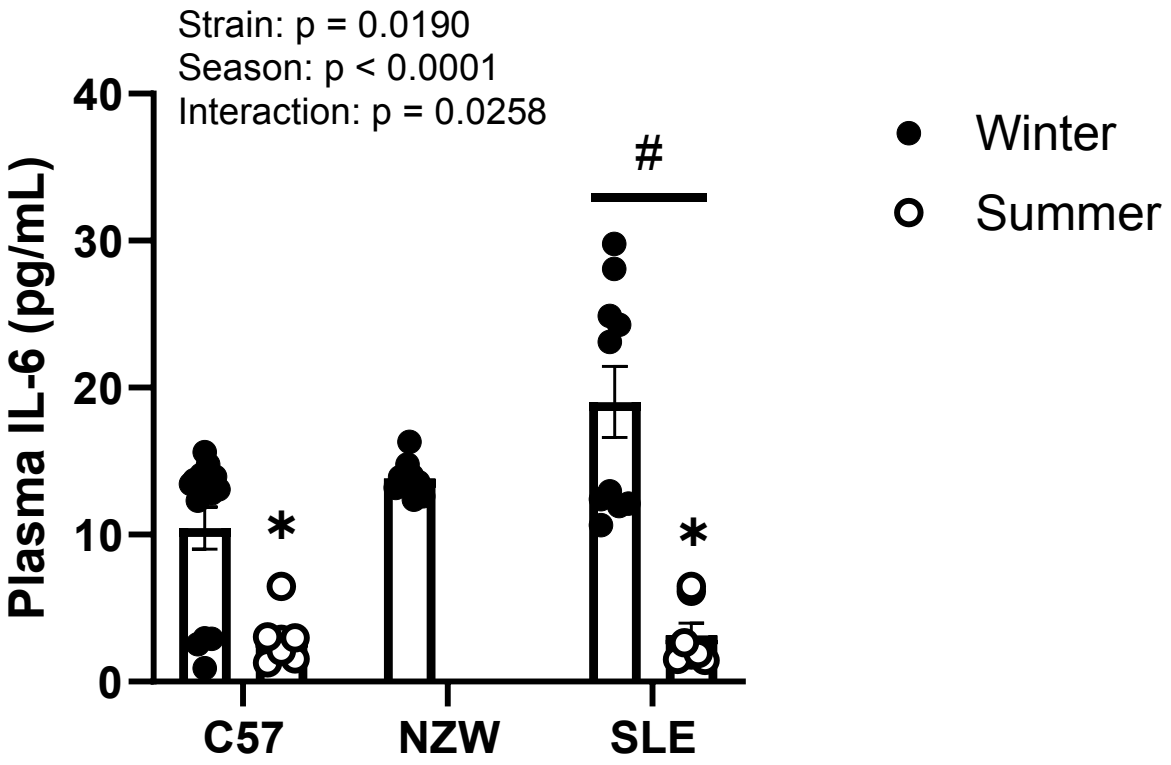

Figure 14: *Circulating IL-6 is higher in female SLE mice immediately upon arrival.* Circulating interleukin-6 (IL-6) concentration was measured via ELISA in blood samples collected at 6 weeks of age in *C57* (n =14, 7), *NZW* (n = 10, 0), and female SLE (n = 10, 8) mice in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. P values and accompanying symbols were determined using the results of Tukey post hoc analysis. (*P vs *C57*/winter ($p = 0.0205$) and SLE/winter ($p < 0.0001$); $^{\#}$P vs. *C57*).

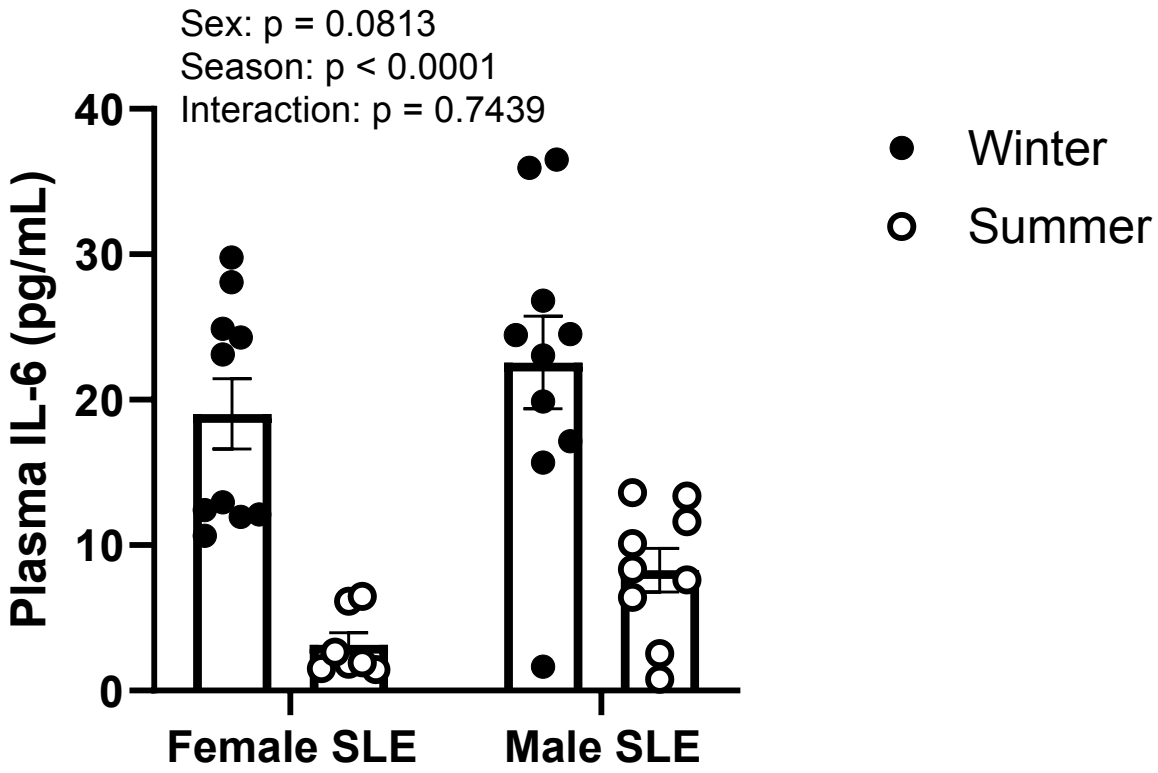

Figure 15: *Circulating IL-6 is lower in mice that arrive in the summer.* Plasma IL-6 concentration was measured via ELISA in blood samples collected at 6-8 weeks of age in female (n = 10, 8) and male (n = 10, 9) SLE mice that arrived in the winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of plasma IL-6 in male SLE mice, we ran a separate unpaired Student's t test. Male SLE mice had significantly lower concentrations of plasma IL-6 in summer versus winter months of arrival (8.27 ± 1.50 vs. 22.57 ± 0.3.20 pg/mL; P = 0.0012).

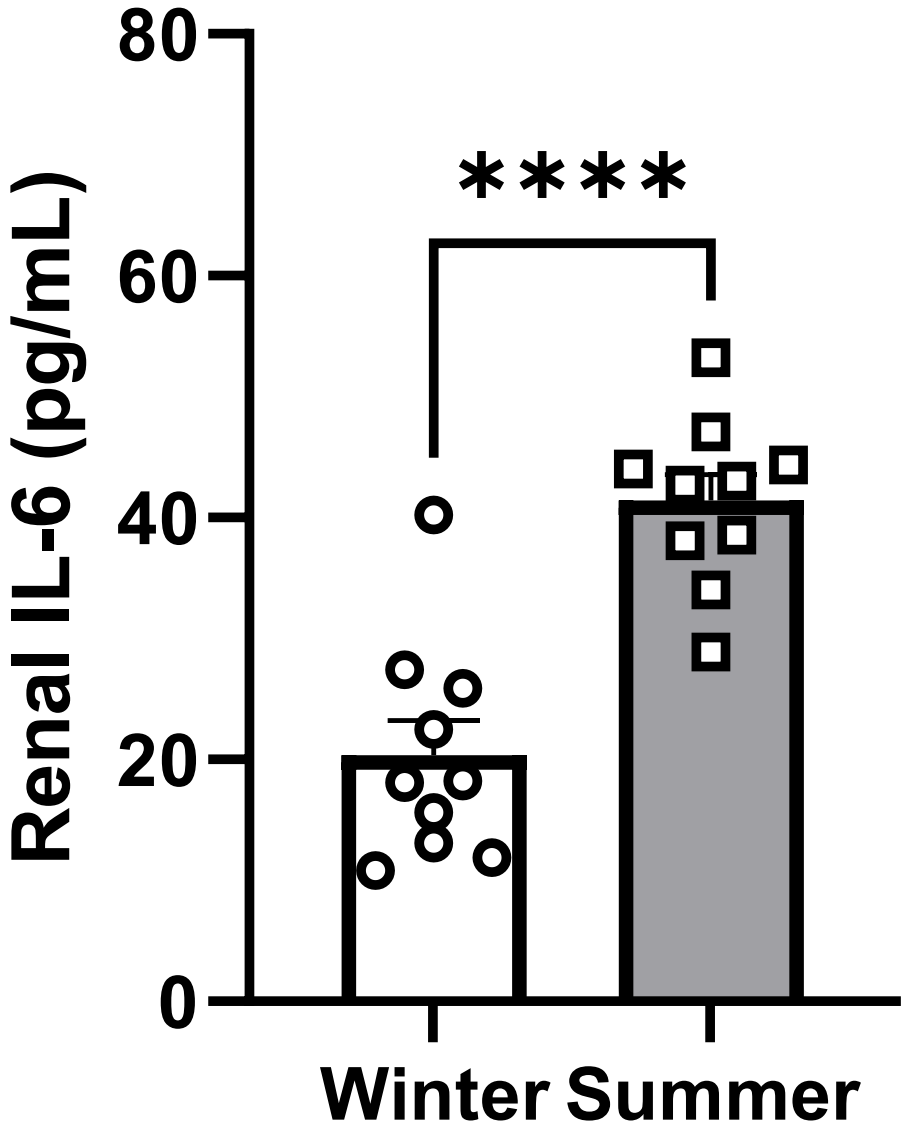

Figure 16: *Renal IL-6 is higher in female SLE mice that arrive in the summer versus winter.* Renal interleukin-6 (IL-6) was measured via ELISA in renal cortex homogenate samples collected at 6 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

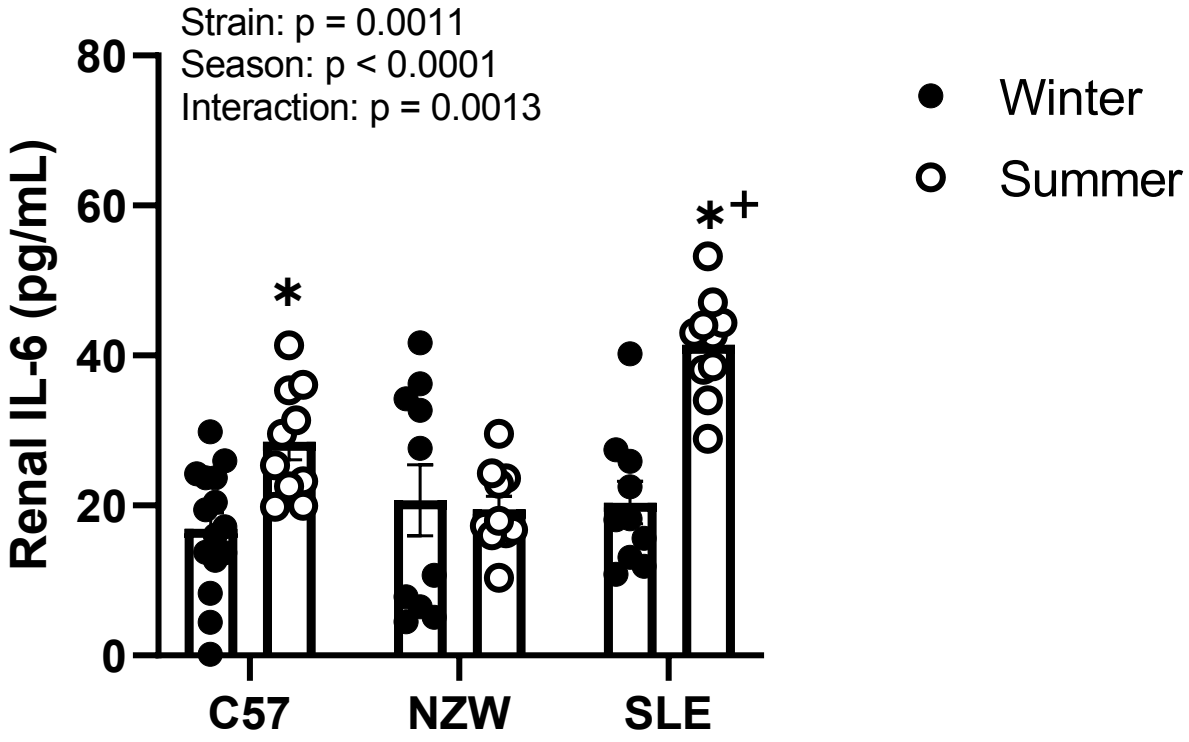

Figure 17: *Renal IL-6 is higher in female SLE mice immediately upon arrival.* Renal interleukin-6 (IL-6) concentration was measured via ELISA in renal cortex homogenate samples collected at 6 weeks of age in *C57* (n =15, 10), *NZW* (n = 10, 10), and female SLE (n = 10, 10) mice in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. P values and accompanying symbols were determined using the results of Tukey post hoc analysis. (*P vs corresponding winter P+ vs C57/winter).

To compare differences in renal IL-6 between sexes, renal IL-6 was measured in female and male SLE mice at 6-8 weeks of arrival in summer and winter months. There were seasonal ($P = 0.0006$; **Figure 18**) and strain specific differences ($P = 0.0330$; **Figure 18**), however, there was no interaction in renal IL-6 in these male and female mice ($P = 0.0618$; **Figure 18**).

III.III Mechanisms involved in worse outcomes to summer travel-associated stress in SLE mice:

Since both increased temperature [40, 42] and IL-6 [56, 57] can induce changes in heat shock proteins, we specifically investigated whether HSP90 is altered following seasonal travel. Although there was no significant difference in plasma HSP90 in female SLE mice arriving in summer and winter months (31.95 ± 2.68 vs. 30.35 ± 1.05 ng/mL; $P = 0.6179$; **Figure 19**), there was significantly higher renal HSP90 in female SLE mice immediately after travel to our institution in summer months (3014 ± 262.6 vs. 1710 ± 124.6 ng/mL; $P = 0.0003$; **Figure 20**). Circulating HSP90 was also measured in *C57* and *NZW* mice to determine differences in plasma HSP90 between female SLE and "normal" mice. There was a seasonal effect ($P = 0.0108$; **Figure 21**) and strain effect ($P < 0.0001$; **Figure 21**); however, there was no interaction of plasma HSP90 data ($P = 1.446$; **Figure 21**).

We also looked for differences in plasma HSP90 between female and male SLE mice at the early time point based on the season of arrival. There were significant differences in the season of arrival ($P = 0.0030$; **Figure 22**). There was no significant difference in plasma HSP90 in female versus male SLE mice ($P = 0.0743$; **Figure 22**). There was an interaction in plasma HSP90 overall though ($P = 0.0236$; **Figure 22**). Although female SLE mice had no differences in renal HSP90 in

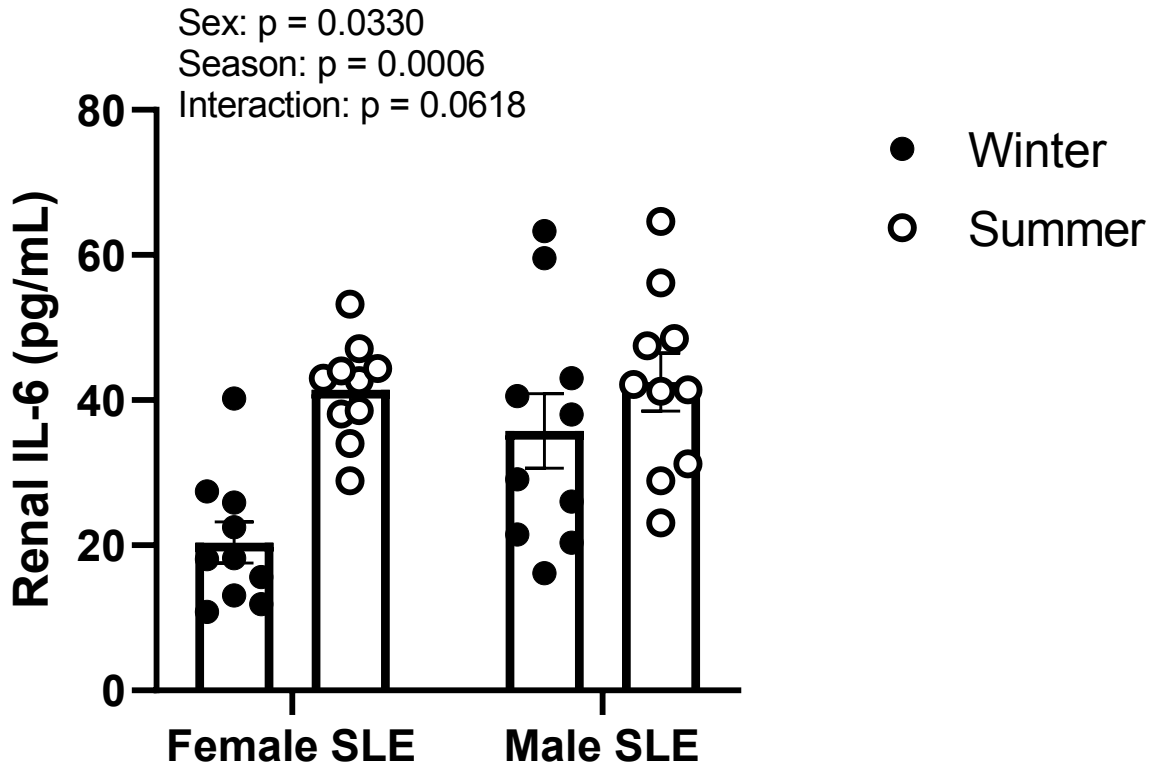

Figure 18: *Renal IL-6 is lower in female SLE mice than males immediately upon arrival.* Renal IL-6 concentration was measured via ELISA in renal cortex homogenate samples collected at 6-8 weeks of age in female and male SLE mice that arrived in winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of renal IL-6 in male SLE mice, we ran a separate unpaired Student's t test. Male SLE mice had similar renal IL-6 concentrations in summer and winter months of arrival (42.48 ± 3.99 vs. 35.77 ± 5.14 pg/mL; P = 0.3156).

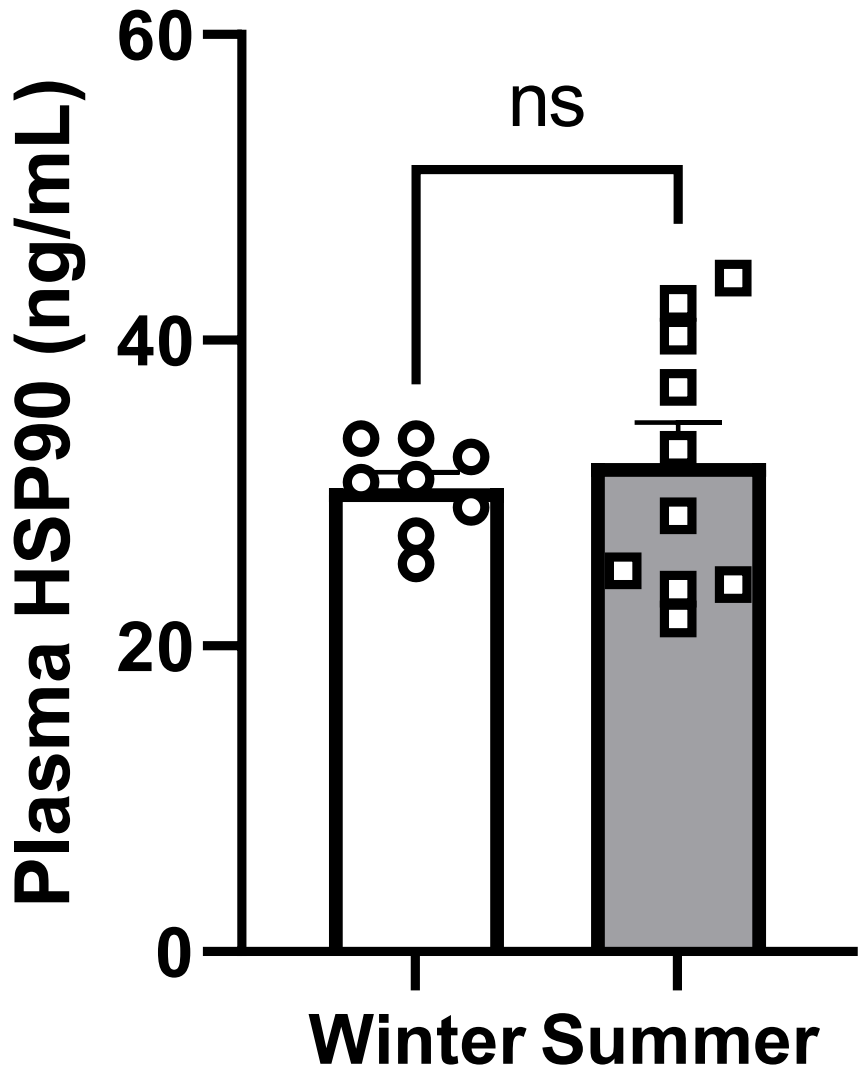

Figure 19: *Circulating HSP90 is similar in female SLE mice that arrive in summer and winter months.* Circulating heat shock protein 90 (HSP90) was measured via ELISA blood samples collected at 6 weeks of age in female SLE mice that arrived in the winter (n = 8) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

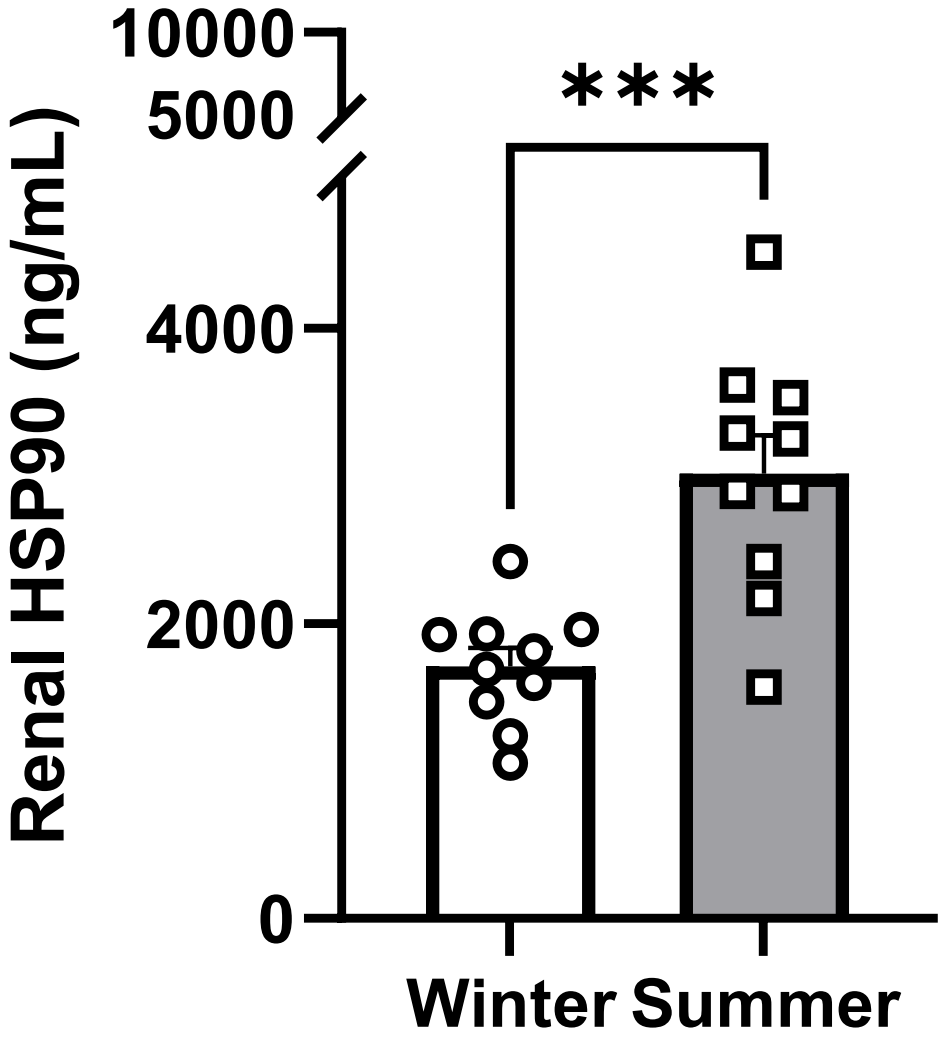

Figure 20: *Renal HSP90 is higher in female SLE mice that arrive in the summer versus winter*. Renal heat shock protein 90 (HSP90) was measured via ELISA renal cortex homogenate samples collected at 6 weeks of age in female SLE mice that arrived in the winter (n = 10) and summer (n = 10) months. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences.

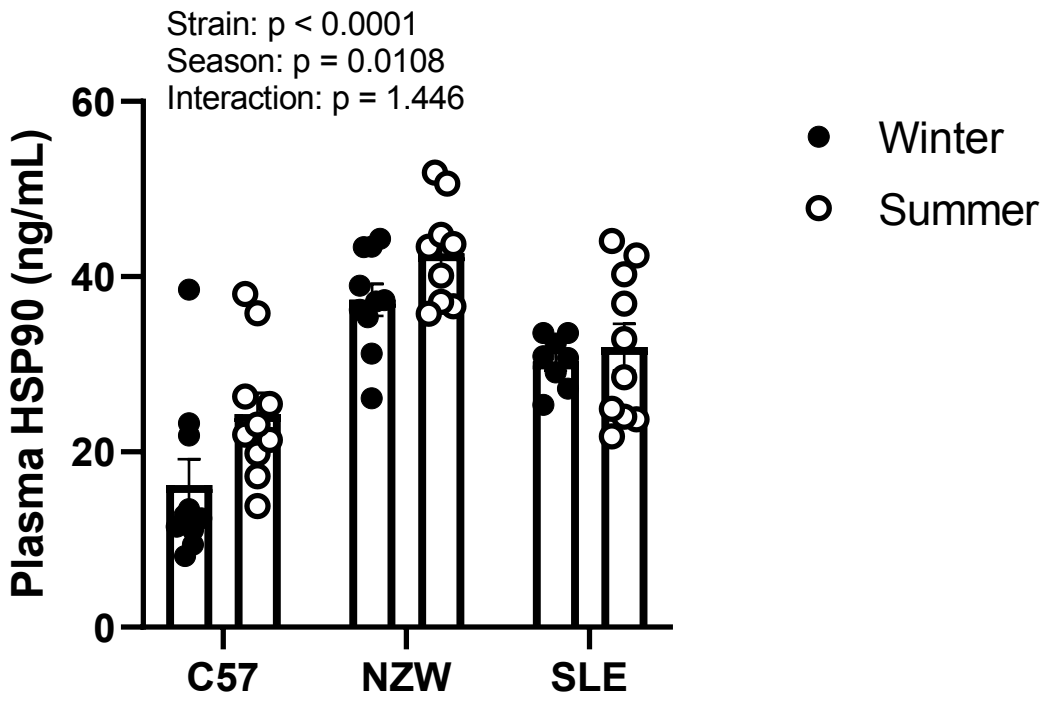

Figure 21: *Circulating HSP90 is similar in female SLE mice that arrive in summer and winter months.* Plasma heat shock protein 90 (HSP90) concentration was measured via ELISA in blood samples collected at 6 weeks of age in *C57* (n =10, 10), *NZW* (n = 10, 9), and female SLE (n = 8, 10) mice in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of plasma HSP90 in "normal" mice, we ran a separate unpaired Student's t test in *C57* and *NZW* mice separately. *C57* mice had significantly higher concentrations of plasma HSP90 in summer months of arrival compared to winter (24.30 ± 2.41 vs. 16.24 ± 2.93 ng/mL; P = 0.0476). *NZW* mice did not have significantly different concentrations of plasma HSP90 in either the summer or winter group (42.67 ± 1.95 vs. 37.35 ± 1.80 ng/mL; P = 0.0613).

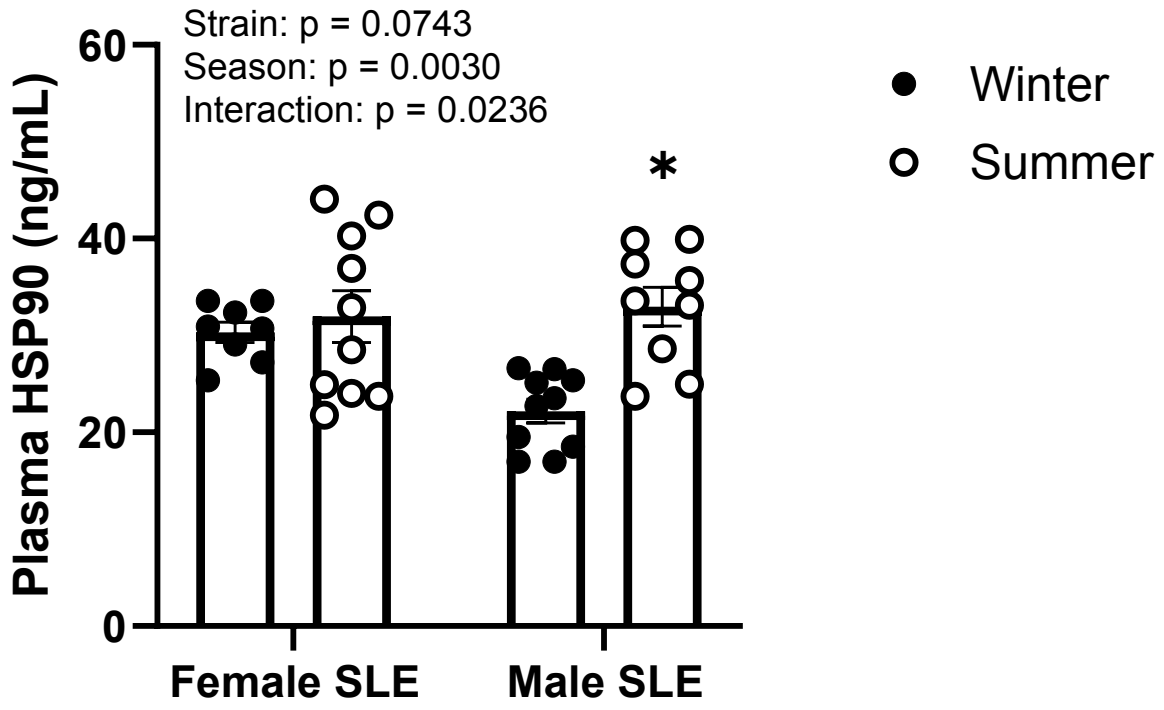

Figure 22: *Circulating HSP90 is higher in male SLE mice that arrive in the summer.* Plasma HSP90 concentration was measured via ELISA in blood samples collected at 6-8 weeks of age in female (n = 8, 10) and male (n = 10, 9) SLE mice that arrived in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. P values and accompanying symbols were determined using the results of Tukey post hoc analysis. (*P vs male SLE/winter; p = 0.0018).

mice that arrived in winter and summer months, male SLE mice had higher levels of plasma HSP90 in summer months (32.98 ± 2.01 vs. 22.19 ± 1.22 ng/mL; P = 0.0002; **Figure 22**).

To look at renal-specific HSP90 responses, renal HSP90 was measured in *C57* and *NZW* mice to determine if there were differences in renal HSP90 based on the season of travel. Although there was a statistical effect of seasons (P = 0.0471; **Figure 23**) and strain (P < 0.0001; **Figure 23**), there was no interaction of renal HSP90 (P = 0.8008; **Figure 23**).

Lastly, renal HSP90 was measured in female and male SLE mice at 6-8 weeks of age to investigate any difference in sex based on the season of arrival. There was significantly different effect of season (P = 0.0021; **Figure 24**) and strain (P = 0.0104; **Figure 24**), but there was no interaction overall in renal HSP90 (P = 0.9744; **Figure 24**).

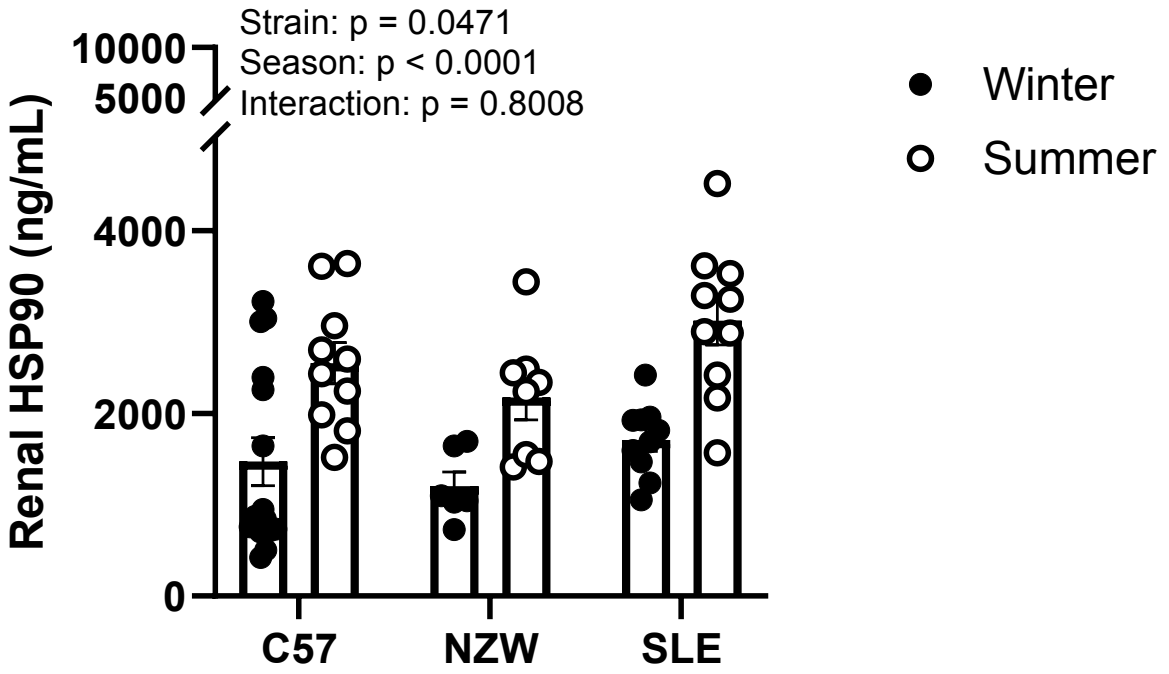

Figure 23: *Renal HSP90 is higher in female mice immediately upon arrival in the summer versus winter.* Renal heat shock protein 90 (HSP90) concentration was measured via ELISA in renal cortex homogenate samples collected at 6 weeks of age in *C57* (n =15, 10), *NZW* (n = 6, 8), and female SLE (n = 10, 10) mice in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of renal HSP90 in "normal" mice, we ran a separate unpaired Student's t test in *C57* and *NZW* mice separately. *C57* mice had significantly higher concentrations of renal HSP90 in summer months of arrival compared to winter months (2549 ± 225.1 vs. 1472 ± 264.5 ng/mL; P = 0.0084). *NZW* mice also had significantly higher renal HSP90 concentrations in summer months of arrival (2172 ± 241.5 vs. 1205 ± 155.9 ng/mL; P = 0.0091).

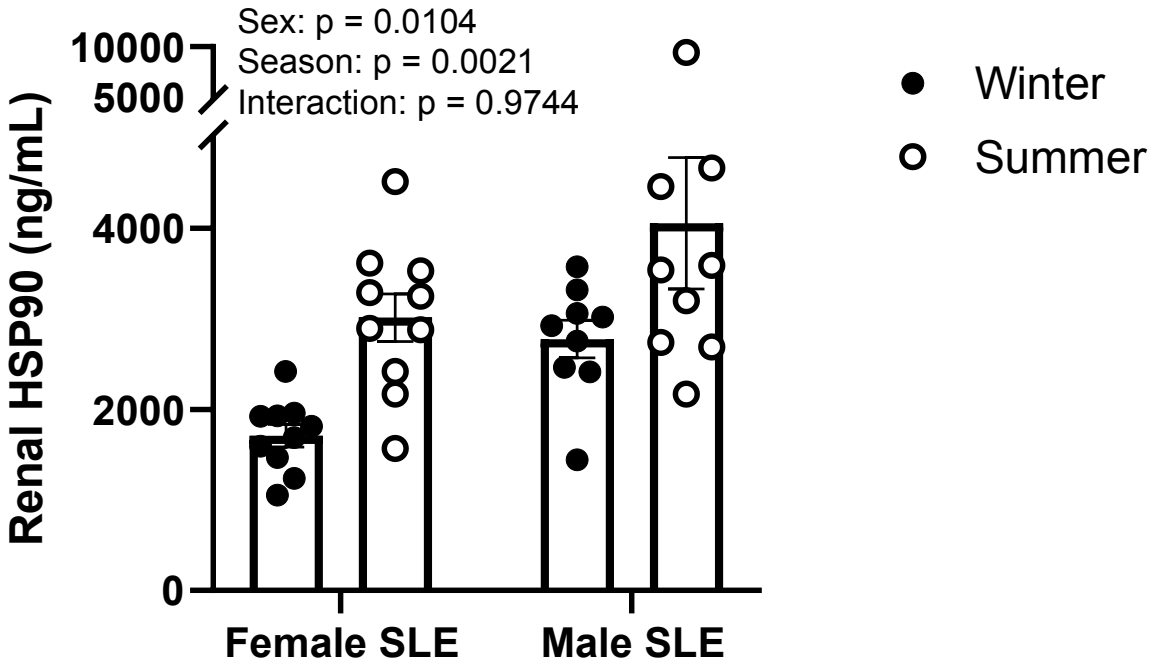

Figure 24: *Renal HSP90 is higher in SLE mice immediately upon arrival in the summer.* Renal HSP90 concentration was measured via ELISA in renal cortex homogenate samples collected at 6-8 weeks of age in female (n = 10, 10) and male (n = 8, 9) SLE mice that arrived in winter and summer months, respectively. Values are presented as mean ± SEM. A two-way ANOVA was used to detect statistical differences between groups and the results of the ANOVA are listed on the graph. To individually analyze impact of seasons of renal HSP90 in male SLE mice, we ran a separate unpaired Student's t test. Male SLE mice had similar renal HSP90 concentrations in summer and winter months of arrival (4055 ± 725.1 vs. 2821 ± 207.5 ng/mL; P = 0.1444).

CHAPTER IV

DISCUSSION

Preliminary data from our lab suggest that stressors associated with travel accelerate SLE depending on the season of travel. In the current study we determined that young female SLE mice subjected to travel stress in summer months developed elevated renal IL-6 and HSP90 concentrations that correlate with worsened disease outcomes later in life.

As expected, disease severity (**Figure 7**) and renal injury (**Figure 8**) was similar in female SLE mice that immediately arrived at our institution in summer and winter months at 6 weeks of age. Also, plasma dsDNA autoantibodies were elevated in female SLE mice compared to control mice (**Figure 9**) and male SLE mice (**Figure 11**). This is expected since female SLE mice develop greater disease severity than male SLE mice [61], *C57* mice develop low levels of plasma dsDNA autoantibodies [62], and *NZW* mice develop intermittent nonpathogenic autoantibodies [63]. Regarding our data from BUN in female SLE and control mice, BUN was significantly lower in female SLE mice (**Figure 10**). We also observed lower levels of BUN in female SLE mice compared to males (**Figure 12**).

It was interesting to see differences in circulating and renal inflammation, since plasma dsDNA autoantibodies and BUN were similar. However, this is evidence that seasonal stressors are playing a role in causing inflammation. IL-6 is an inflammatory cytokine that is produced and secreted by macrophages in response to acute stress and promotes antibody production by

promoting B and T cell activity [64]. This elevation in IL-6 overtime can lead to activated B cell differentiation into antibody producing plasma cells, thus leading to incidence of autoantibody production [65].

Looking specifically at IL-6, a cytokine that has both inflammatory and tissue remodeling mechanisms, there were differences in plasma and renal IL-6 in mice arriving in winter and summer months. Circulating IL-6 was significantly decreased in all strains that arrived in summer compared to winter months (**Figure 13-15**). This was not the case for renal IL-6 though. Renal IL-6 was elevated in female SLE mice that arrived in summer compared to winter months (**Figure 16**). This overexpression of IL-6 found in female SLE mice in summer months was significantly higher than those found in the control mice (**Figure 17**). It was also important to see that although renal IL-6 was higher in male mice, a seasonal effect was not seen in these mice (**Figure 18**). IL-6 is produced and secreted by kidney resident cells such as podocytes, endothelial cells, mesangial cells, and tubular epithelial cells (TECs) in response to different stressors [59]. It seems reasonable that IL-6 would be elevated in SLE mice compared to control mice due to the increased incidence of autoimmunity [32, 33]. The fact that circulating IL-6 is overexpressed in winter months of arrival while renal IL-6 is overexpressed in summer months of arrival is intriguing.

As stated previously, it is already known that IL-6 is elevated with elevated HSP90 gene levels [57]. From our results, we were able to determine that renal HSP90 and renal IL-6 had similar trends in summer compared to winter months of arrival in female SLE mice (**Figure 16, 20**). This trend coincides with our hypothesis that environmental factors in summer months of arrival early in life are elevating these molecules thus potentially causing increased incidence of disease severity and renal disease in these female SLE mice (**Figure 2-3**). This difference was not shown in circulating HSP90 (**Figure 19**), however, having us speculate that these environmental

stressors are only affecting mechanisms specifically found in kidneys of female mice. When looking specifically at plasma HSP90 in control mice, plasma HSP90 was only elevated in *C57* mice in summer months of arrival (**Figure 21**). Plasma HSP90 was also elevated in male SLE mice that arrived in summer compared to winter months of arrival (**Figure 22**). This elevation in circulating HSP90 may only affect male SLE mice. This, however, does not seem likely since plasma IL-6 was lower in male SLE mice in summer months of arrival (**Figure 15**). When looking specifically at renal HSP90, renal HSP90 was elevated in female mice immediately upon arrival (**Figure 23**). This was not the case for male SLE mice though (**Figure 24**), indicating that this elevation in renal HSP90 is specific to female mice. It was interesting to also see this similar trend in renal IL-6 (**Figure 17-18**).

This relationship between renal IL-6 and renal HSP90 has led us to ponder if this trend is shown at a greater extent at 34-35 weeks of age. To determine this, we performed experiments on renal HSP90 and renal IL-6 at 34-35 weeks of age in female SLE mice by using a randomized study from past samples. We determined that renal HSP90 at 6 weeks of age was significantly higher than that at 34-35 weeks of age (**Figure 25**).

Renal IL-6 and renal HSP90, seem to have a relationship implying that renal HSP90 may resemble an early biomarker for determining the extent of how severe renal injury and disease severity will be in female SLE mice. Multiple studies have already looked at HSPs as serological biomarkers for kidney disease [68, 69] and one study has even determined that using a vaccine against HSP90 can reduce SLE disease manifestations in SLE prone mice [45]. Knowing this, renal HSP90 may be used as a biomarker in detecting early onset kidney disease. Future studies will be conducted to determine if urine can be viable in detecting HSP90 in female SLE mice.

Our findings lead us to contemplate whether genetic etching due to seasonal stress amplifying HSP90 is playing a role in the enhancement of disease severity in female *NZBWF1* mice that arrive to our institution before puberty. Future experiments will focus on the role of renal HSP90 and IL-6 in the progression of SLE later in life. We plan to breed mice in house as well as acquire mice at 18-19 weeks of age, removing this variable of early life stress travel and early life, respectively, to determine if disease severity and renal injury differs between these groups. We predict that HSP90 and IL-6 will be less dramatic in these groups that are house bred as well as those that we obtain during adulthood. This will give more insight as to whether seasonal stress before puberty can cause adverse effects that cause later problems in life.

This and future studies will be influential in the field of clinical SLE because of the potential to identify biomarkers that accelerate autoimmunity that are enhanced by stress and the environment. Women are twice as likely to develop depression and other disorders from early life stress [25, 26] and 90% of SLE patients are females. In addition, Black and Hispanic Americans have documented heightened stressors compared to White Americans relating to occupation, finances, relationships, racial bias and violence [27]. These minority groups are predominantly affected by SLE in the US at a 3:1 compared to majority groups [3]. Understanding mechanisms involved may elucidate the cause of health disparities in SLE and other autoimmune and chronic inflammatory diseases.

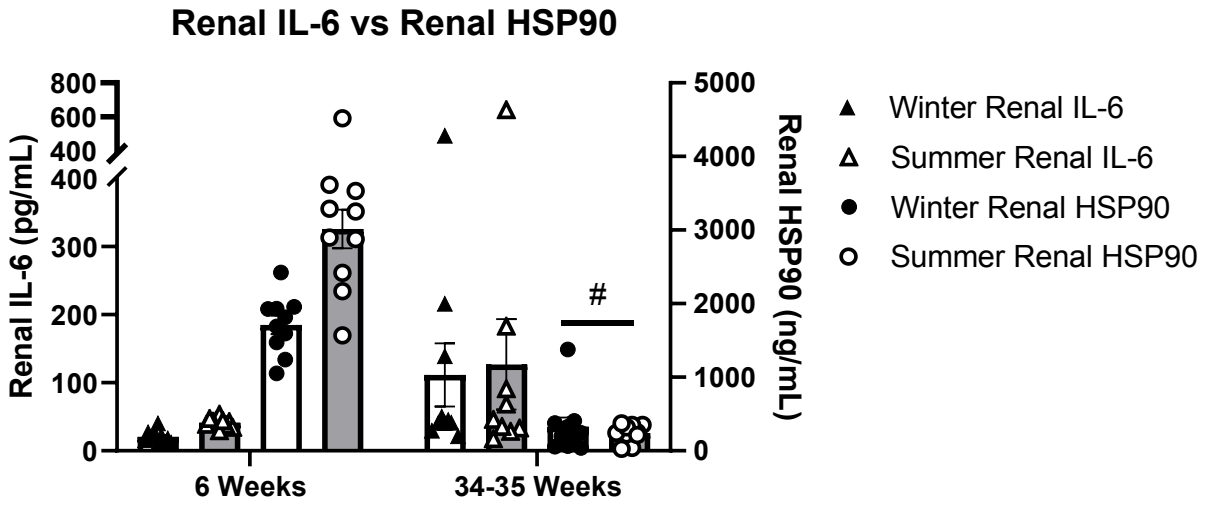

Figure 25*: Renal HSP90 is a potential biomarker for disease severity in female SLE mice.* Renal IL-6 was measured via ELISA in renal cortex homogenate samples collected at 6 weeks of age (n = 10, 10) and 34-35 weeks of age (n = 10, 9) in female SLE mice that arrived in winter and summer months, respectively. Renal HSP90 was measured via ELISA in renal cortex homogenate samples collected at 6 weeks of age (n = 10, 10) and 34-35 weeks of age (n = 10, 10) in female SLE mice that arrived in winter and summer months, respectively. Values are presented as mean ± SEM. An unpaired Student's t test was used to detect statistical differences. P values and accompanying symbols were determined using the results of Tukey post hoc analysis. ($^{\#}$P vs Female SLE/HSP90).

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