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HISTORY OF STRESS AFFECTS CELL-MEDIATED IMMUNITY

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ABSTRACT

Following exposure to stressors, energy resources are reallocated towards immediate responses, and diverted from functions such as the immune system. Which systems are suppressed may be altered by an animal's evolutionary history. The eastern fence lizard (*Sceloporus undulatus*) is a native species impacted by the predatory invasive fire ant (*Solenopsis invicta*). We examined how history of invasion by fire ants and exposure to acute stress affected the cell-mediated immune response of post-gravid lizards. Lizards were captured from sites with long (>70 years) histories of fire ant invasion and correspondingly higher levels of corticosterone, and sites not yet invaded by these ants. All lizards were treated while gravid with either a physiologically relevant dose of the stress-relevant hormone corticosterone, to simulate the corticosterone response to a fire ant attack, or a vehicle control. We measured the cell-mediated immune response of females post-laying with the phytohemagglutinin skin test. We found that history of exposure to stress (associated with fire ant invasion) and the contemporary corticosterone treatment affected cell-mediated immune response. Lizards from high-stress fire ant invaded sites had reduced immune response compared to those from low-stress uninvaded sites. CORT-treated lizards from uninvaded sites also had decreased immune response when compared to control lizards from uninvaded sites, but CORT-treated lizards from invaded sites showed no difference when compared to control lizards from invaded sites. This suggests that evolutionary history of stress alters the immune response to short-term stress. Future work on how different branches of the immune system respond to environmentally-induced stressors will be informative for predicting and managing these threats.

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Chapter 1

Introduction

An animal's response to environmental, social, and physical stressors often involves physiological alterations such as energy reallocation (Sapolsky et al., 2000), digestion (Bhatia and Tandon, 2005), changes to reproductive cycles (Wingfield and Sapolsky, 2003), and immunosuppression (Franchimont, 2004; Tuckermann et al., 2005). These changes can have both immediate and long-term consequences on the animal's survival (Cabezas et al., 2007; Romero and Wikelski, 2001). Important mediators of these functional modifications are the stress-relevant glucocorticoid hormones, which are released during activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (Bateman et al., 2018). Glucocorticoids are involved in the reallocation of energy resources to help with acute reactions to a stressor, and this can result in immunosuppression, especially of lymphocytic immunity (Tuckermann et al., 2005). While immunosuppression may be energetically favorable for immediate survival of a stressor, chronic immunosuppression could be disadvantageous in some environments, including those with a high risk of wounding from predation.

In an increasingly connected world, the introduction of invasive species is becoming a common pressure on native species and environmental systems. Eastern fence lizards (*Sceloporus undulatus*) are one of many native species adversely affected by the introduction of a new predator, the red imported fire ant (*Solenopsis invicta*), into their habitat (Langkilde, 2009). As the fire ant's range continues to expand, understanding the effects of this invader on native species is of utmost importance. The invasive fire ant was accidentally introduced to the southeastern United States between 1933 and 1945, in the southern portion of the fence lizard's range in Mobile, Alabama (Callcott and Collins, 2010). Fire ants pose a predatory threat to eastern fence lizards; as few as twelve stings is enough to cause paralysis and death in this species (Langkilde, 2009). The presence of invasive red fire ants in the eastern fence lizard's environment for over seventy years has caused behavioral and morphological changes in exposed populations of fence lizards not seen in populations whose habitats remain uninvaded (Langkilde, 2009). Previous work has shown that these species frequently encounter one another in areas where their ranges overlap (Langkilde, 2009). The invasion of fire ants has also caused changes in stress levels, as measured by plasma concentrations of corticosterone (CORT), the primary glucocorticoid stress hormone in this species. In the field, eastern fence lizards captured from sites invaded by fire ants display higher baseline CORT concentrations and higher restraint stress CORT concentrations compared to those from uninvaded sites (Graham et al., 2012), and even in captivity lizards from invaded sites have enhanced CORT responsiveness (McCormick et al., 2017).

With the classical understanding of the effects of glucocorticoids on the immune system, this history of high stress levels caused by the presence of a predatory invasive species would be predicted to result in immunosuppression. However, immune response may affected by evolutionary history (Lochmiller and Deerenberg, 2000). Previous work has shown that eastern fence lizard hatchlings from sites invaded by fire ants have increased natural antibody function when treated topically with a physiologically relevant dose of CORT, whereas those from uninvaded sites exhibited the classic signs of CORT immunosuppression (Langkilde and Mccormick, unpublished). This would be consistent with an evolutionary perspective, as lizards from high stress fire ant invaded sites, due to frequent fire ant encounters, would consistently suffer from immunosuppression if all immune function is reduced in response to CORT. This could be detrimental as lizards in these populations are frequently bitten and stung by fire ants, breaking their skin and leaving them at risk of infection. Instead, it is possible that certain parts of the immune system may be selectively downregulated, or even upregulated, in response to CORT to reduce associated costs.

Further examination of the effects of CORT on different branches of the immune system, particularly in aspects of immunity that directly respond to fire ant attack, is necessary to fully understand the effects of glucocorticoids in organisms with different evolutionary histories of stress. Previous studies have examined natural antibodies (Langkilde and Mccormick, unpubl.; McCormick et al., 2015; Mccormick and Langkilde, 2014) which function to nonspecifically combat pathogens and toxins (Ochsenbein and Zinkernagel, 2000). However, there are many other aspects of the immune system which could also be affected by glucocorticoids and may be important for surviving fire ant envenomation. For example, cell-mediated immunity, particularly T-cell immunity, plays an important function in the adaptive immune responses to novel antigens such as fire ant venom, and is influenced by life history as well as genetics (Abbas et al., 1996; Newport et al., 2004). The phytohemagglutinin (PHA) skin test is a good measure of cell-mediated immunity, and PHA-L in particular preferentially stimulates T-lymphocyte response (Tylan and Langkilde, 2017). An advantage of using this test as a measure of cell-mediated immunity is that is a skin test, and skin immunity is likely to be particularly important to organisms that are exposed to frequent skin wounding, such as from frequent fire ant attacks.

Our study aimed to examine the effects of CORT treatment and multigenerational history of stress (i.e. fire ant invasion status) on cell-mediated immunity in eastern fence lizards. Our goals for the study were to: 1) determine the effects of multigenerational of stress on cell-mediated immune response; 2) evaluate the effects of CORT treatment on cell-mediated immune response; and 3) examine the potential interactions between stress history and CORT treatment in regard to immune response. We predicted that fence lizards from sites invaded by fire ants would have cell-mediated immune response compared to those from uninvaded sites, due to an evolutionary history of stress associated with exposure to invasive red fire ants. We also predicted that lizards treated with CORT would have a lower cell-mediated immune response compared to the control group. Finally, we predicted that the effects of CORT-treatment on cell-mediated immune response would differ based on evolutionary history, with lizards from high-stress, fire ant invaded sites showing an upregulated immune response when treated with CORT, compared to lizards from uninvaded sites following the same treatment. Understanding how cell-mediated immunity responds to CORT, and how this is affected by evolutionary history of stress, is critical for understanding the consequences of invasive species introduction and the health effects of stress.

Chapter 2

Methods

Field Capture

Gravid female eastern fence lizards (*Sceloporus undulatus*) (n = 58) were captured by noosing or by hand from six sites in the southeastern United States: four sites were in areas with long histories (55-70 years) of fire ant invasion ("invaded"): Site A (31˚10' N, 86˚20' W, which includes Florala and Geneva State Forest; n = 6;) Site B (31˚20' N, 86˚60' W, which includes Blue Lake State Park, Open Pond, Solon Dixon, and Conecuh National Forest; n = 20); Site C (30˚26' N, 87˚64' W, which includes Gulf State Park; n = 1); and Site D (30˚74', 87˚91', which includes Blakely State Park; n = 2), and two sites were in locations that have not yet been invaded by fire ants ("uninvaded"): Site E (36°28'N, 85°25'W, which includes Standing Stones State Park, TN; n = 7); and Site F (36°50'N, 88° 5'W, which includes Land Between the Lakes National Park, KY; n = 22). Females from invaded and uninvaded sites were both captured as part of two separate studies exploring the effects of maternal stress on offspring (MacLeod et. al., unpublished; Owen et. al., unpublished).

Animal Husbandry

Lizards were transported to the lab at the Pennsylvania State University and housed in plastic tubs (56 x 40 x 30 cm L x W x H) with damp sand for egg-laying. Each tub was equipped with a plastic water bowl and shelter that served as a refuge and basking perch. Females from uninvaded sites were housed in pairs, and females from invaded sites were housed individually. Lamps with 60W bulbs were hung above each tub over one end to allow a thermal gradient and provided heat for thermoregulation for 8 h each day. Overhead lights were set on a 12:12 hr light:dark schedule. Lizards were fed to satiation with mineral dusted crickets (*Acheta domestica*) three days a week, and water was available *ad libitum*.

Experimental Design

All female lizards were randomly assigned to either the CORT-treated or control group. CORT-treated animals were topically dosed with a 4 mg/ml solution of CORT (≥92%, Sigma C2505, Saint Louis, MO) in sesame seed oil, which is rapidly absorbed into the blood stream through the skin (Josabel et al., 2004). The volume of solution administered was adjusted based on lizard body weight, with 0.2 µl CORT solution per gram lizard body mass, for a final dosage of 0.8 µg CORT/g lizard body mass (Trompeter and Langkilde, 2011). Females in the control group received the sesame seed oil vehicle control only, the volume of the solution adjusted based on lizard body weight to match the CORT treatment.

CORT or control solutions were applied to the middle of the lizards' backs between 19:00 and 21:00 hrs, which is after the lizards had become inactive for the day, to avoid the need to handle the animals. Females from uninvaded sites were dosed with CORT or vehicle control every day, and females from invaded sites were dosed every other day due to logistic constraints associated with other studies; the effect of dosing regime was accounted for in the statistical models. Dosing continued until parturition for lizards from invaded sites, and until two days after parturition for uninvaded lizards.

Testing Immune Function

The phytohemagglutinin (PHA) skin test was used to measure cell-mediated immune response of these lizards. When performed using PHA-L, the PHA skin test preferentially stimulates a T-lymphocyte response, the magnitude of which is determined by measuring the swelling of the injected area (Tylan and Langkilde, 2017). As reproduction status is known to affect multiple measures of immune function (French and Moore, 2008), all PHA injections were performed 24 hours after parturition in order to synchronize reproductive cycles.

Post-laying females all had one of their hind feet measured in triplicate with a pressure sensitive micrometer (Mitutoyo dial thickness gauge, # 7301, to the nearest 0.01 mm ± 15 µm), then the foot pad was subcutaneously injected with 10µl of 2mg/ml PHA-L (>92%, Sigma, L2769, Saint Louis MO). The foot to be injected (right or left) was randomly selected. Twenty-four hours after injection, the thickness of the injected foot was measured again in triplicate with the same pressure sensitive micrometer.

Statistical Analysis

Triplicate measures of foot thickness were averaged, and pre-injection foot thickness was subtracted from post-injection values to determine the amount of swelling response. Body condition was calculated as the residuals of a linear regression of log-snout-to-vent length and log-post-partum mass.

To determine the effects of the invasion status of a lizard's site of capture and of CORT treatment on cell-mediated immunity, we used an Analysis of Variance (ANOVA) test with the magnitude of the PHA swelling response as the dependent variable, and fire ant invasion status (invaded vs. uninvaded), experimental treatment (CORT vs. vehicle control), and an interaction between invasion status and experimental treatment as factors. We included body condition, date of PHA skin test, clutch mass, number of doses a lizard received and time since capture, site of capture nested within invasion status, and number of days since last dose as covariates in initial models. All but the last two of these variables did not significantly explain the variation in our data and were removed to preserve degrees of freedom. Our final model thus included site identity nested within invasion status, and the number of days since last dose as covariates.

To avoid confounding our results with uninformative comparisons, we set LS Means Contrasts to compare specific factors of interest. In order to determine the effect of invasion status on PHA swelling response, we set LS Means Contrasts to compare the swelling of invaded and uninvaded lizards within each treatment (i.e. within controls and within CORT-dosed lizards, rather than comparing invaded controls with uninvaded CORT-dosed lizards, for example). To determine the effect of treatment on PHA swelling response, we set LS Means Contrasts to compare the swelling of CORT-treated and control lizards within each invasion status (rather than comparing control lizards from invaded sites and CORT-treated lizards from uninvaded sites or CORT-treated lizards from invaded sites and control lizards from uninvaded sites). Statistical analyses were performed using JMP Pro (version 12.0.0, SAS Institute Inc., Cary NC), with $\alpha=0.05$. Swelling responses were Johnson SI transformed via the JMP program to meet assumptions of normality.

Chapter 3

Results

Females from invaded sites had a reduced swelling response to PHA injection compared to lizards from uninvaded sites (LS Means Contrast, invaded vs. uninvaded: $F_{2,49}$ = 4.95, p = 0.01; Fig. 1). The effect of CORT treatment on swelling differed between lizards from fire ant invaded and uninvaded sites. Within uninvaded sites, CORT-treated lizards had a lower swelling response than those treated with the vehicle control (LS Means Contrast, treatment within uninvaded: t = -2.50, p = 0.02), whereas there was no difference in swelling response between treatment groups in lizards from invaded sites (LS Means Contrast, treatment within invaded: t = -0.80, p = 0.43;-Fig. 1). However, this interaction between treatment type and site invasion status did not achieve statistical significance (ANOVA, treatment x invasion status: $F_{1,52}$ = 1.59, p = 0.21).

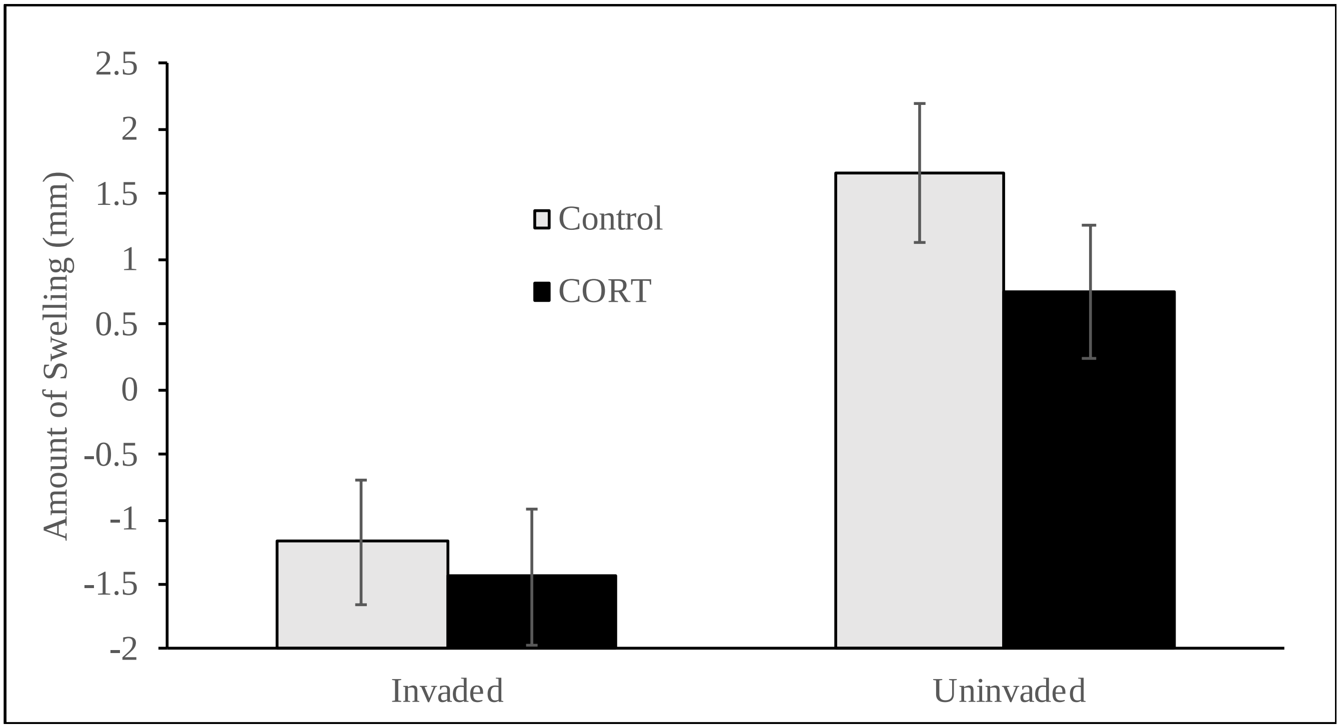

Figure 1: Figure showing LS Means of the statistical model accounting for site of capture and the number of days since last dose. Lizards from fire ant invaded sites (left) show reduced swelling response to PHA injection compared to lizards from uninvaded sites (right). CORT-treated lizards from uninvaded sites (right) show reduced swelling response compared to the control group; lizards from invaded sites (left) show no difference between CORT-treatment and control groups.

Table 1. Fixed Effect Test from Statistical Model

Independent Variable	*Degrees of Freedom*	*F*	*P*
Invasion Status (Invaded or Uninvaded)	1,45	7.90	<0.01*
Treatment (CORT-treated or Control)	1,52	5.57	0.02*
Treatment x Invasion Status	1,52	1.59	0.21
Number of days since last dose	1,52	8.30	<0.01*

Asterisk (*) indicates statistical significance (α=0.05)

Chapter 4

Discussion

We found that CORT-treated lizards from sites uninvaded by fire ants had lower swelling response to the PHA skin test than lizards from the same sites treated with the vehicle control, which is consistent with the current understanding of the immunosuppressive effects of glucocorticoids (Tuckermann et al., 2005). Additionally, lizards from sites with long histories of fire ant invasion and associated stress had lower swelling responses to the PHA skin test compared to lizards from sites uninvaded by fire ants. This was also consistent with what we predicted, due to lizards from invaded sites having higher baseline levels of CORT in the field and higher CORT responsiveness in the lab (Graham et al., 2012; McCormick et al., 2017). However, among lizards from sites that were invaded by fire ants, CORT-treatment and vehicle control treatment groups did not differ in swelling response to the PHA skin test, which suggests that the effect of CORT on the immune system differs according to the history of exposure to fire ants and stress.

The reduction in the PHA swelling response seen in CORT treated lizards from uninvaded sites is a common response to stress (Dhabhar, 2006; Tuckermann et al., 2005). Immunosuppression can be beneficial for animals facing stressful situations as the immune system has a high energy requirement (Lochmiller and Deerenberg, 2000; Martin et al., 2008), consuming resources which could otherwise be used in fueling functions that would assist in immediate survival (Norris, 2000). In scenarios when the animal's stress response is activated, the energy cost of maintaining a fully-functioning immune system may be unnecessary, and perhaps counterproductive, for immediate survival in scenarios when the animal's stress response is activated (Lochmiller and Deerenberg, 2000; Martin et al., 2008; Norris, 2000).

Lizards from fire ant invaded sites were also immunosuppressed, having lower swelling responses to the PHA skin test compared to those from uninvaded sites. This downregulation of the immune response seems to be associated with a multigenerational history of high stress, caused by frequent encounters with fire ants throughout their lives. Environmentally motivated physiological trade-offs to conserve and reallocate energy resources are also seen in other species, such as seasonal (but not temperature-associated) variation in immune measures within the red knot bird (Buehler et al., 2008) and differing leukocyte counts between urban and rural tree lizards (French et al., 2008).

CORT-treated lizards from invaded sites did not show the classic signs of immunosuppression in the PHA skin test, as there was no difference in swelling response between the CORT-treatment and control groups. This suggests a change in the CORT responsiveness of the immune system between lizards of different stress histories.

Though lizards from invaded sites have lower swelling response compared to lizards from uninvaded sites, they were not further immunosuppressed by the CORT treatment. This may be explained by adequate immune function still being necessary for survival in the event of pathogen invasion and infection (Martin et al., 2008; Zimmerman et al., 2010). Pathogen invasion is particularly likely when an animal is wounded, such as from a fire ant bite and sting. In lizards from sites invaded by fire ants, their history of high stress being associated with fire ant attacks may have reprogrammed the immune response to stress, preventing further immunosuppression. This may be a response they've developed within their lifetime, after repeated encounters with fire ants, or it may be a product of multigenerational exposure to fire ants (Langkilde, 2009; McCormick et al., 2017; Schrey et al., 2016; Trompeter and Langkilde, 2011). Additional studies are required in order to determine if this is a heritable or plastic trait; it may be that lizards lacking a blunted immunosuppressive response to CORT treatments have historically survived and reproduced more successfully in invaded sites, thus selecting for this trait.

It is important to note that CORT-treatment schedule in this study was different between lizards of different invasion statuses, making effects of CORT-treatment regime and invasion status difficult to separate in the interpretation of our results. And dosage, frequency, and duration of treatment have been shown to have different effects of CORT on natural antibody levels (McCormick et al., 2015). Neither the number, frequency, nor duration of CORT doses administered before the PHA skin test were significant in our model, suggesting the results of CORT-treatment seen here are not due to differences in CORT dosing between lizards of different invasion statuses.

The potential reprogramming of the immune system's response to CORT in lizards from fire-ant invaded sites was seen in response to injection with PHA, which induces cell-mediated immunity in the skin. It would be interesting to determine whether immune response in other parts of the body or other parts of the immune system are similarly or differently affected by stress history. Cell-mediated immunity in the skin is particularly relevant to skin wounding that results from fire ant attacks, though cell-mediated immunity in other body tissues may not be directly involved in responding to fire ant attacks. It is possible that immune tests of different tissues of lizards from invaded sites would show classic immunosuppression in response to CORT-treatment. Additionally, different branches of the immune system can be regulated differently in response to glucocorticoids such as CORT (Dhabhar, 2006). The immune test performed in this study measures cell-mediated immunity, particularly T-lymphocyte response, in the skin (Tylan and Langkilde, 2017). In a previous study, eastern fence lizard hatchlings from sites invaded by fire ants had increased natural antibody function in response to CORT treatment, while hatchlings from uninvaded sites had decreased natural antibody function in response to CORT treatment (McCormick and Langkilde, unpublished). In another study involving adult fence lizards, natural antibody function was upregulated in CORT-treated lizards from both invaded and uninvaded sites (Mccormick and Langkilde, 2014). These studies showed an upregulation of natural antibody immune function in response to a life history of stress and CORT-treatment, while the PHA skin test performed in this study showed a downregulation of cell-mediated immune function in response to life history of stress and CORT treatment. These differing results suggest that the alteration of immune response could vary between different aspects of the immune system.

Our results show that a life history of stress, caused by the presence of invasive fire ants, and treatment with the stress-relevant hormone CORT can both cause suppression of cell-mediated immunity in eastern fence lizards. While lizards collected from sites that have been invaded by fire ants showed a lower swelling response when compared to lizards from sites uninvaded by fire ants, lizards from these invaded sites did not show further immunosuppression when treated with CORT, unlike lizards from sites uninvaded by fire ants. This suggests that a life history of stress can alter immune function and energy reallocation by glucocorticoids. Energy reallocation in response to glucocorticoids and life history of stress can be complicated and may vary between different branches of the immune system, so future examination of different immune tests, such as wound healing or phagocytic response assays, would be important to further understand this complex system.

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WORK EXPERIENCE

Undergraduate Lab Personnel
Langkilde Lab — University Park, PA — *Sept. 2016-Present*

- Received Erickson Discovery Grant to head a project on immune and stress responses
- Designed and performed experimental protocol to measure cell-mediated immune response
- Read histology slides and collected data for a published study on PHA validation in lizards
- Extensively handled, provided care for, and performed behavioral studies on reptiles
- Gave oral presentations to biology peers in two national conferences: TREE 2017 and SICB 2018

Happy Tails Kennel Staff
Limerick Veterinary Hospital — Limerick, PA — *Jun. 2015-Nov.2016*

- Provided exercise, dietary, and general care for dogs, cats, and exotics in the facility
- Handled and controlled dogs in group play, on walks, and during medical procedures
- Worked behind the scenes of a veterinary clinic with customers and veterinarians

Chemistry Tutor & Communications Manager
Mehta Prep Academy — State College, PA — *Oct. 2016-Feb. 2017*

- Provided personalized tutoring services to peers struggling in Gen. Chemistry courses
- Worked with coworkers to expand and advertise a start-up company

Job Shadowing
Harleysville Veterinary Hospital — Harleysville, PA — *June 2009-Present*

- Observed companion animal care, diagnostics, and surgery in a small animal practice
- Completed senior project presentation focused on veterinary science careers

LEADERSHIP & ORGANIZATIONS

Istari Society
Founder and President — *University Park, PA* — *Sept. 2016-Present*

- Founded and led the first Tolkien and fantasy literature club on campus

Women in Science and Engineering
President — *University Park, PA* — *Aug.2014-Aug.2015*

Pre-Vet Club
Member — *University Park, PA* — *Aug. 2014-Present*

HONORS

Schreyer Honors College, Presidential Award (2014), Dean's List (every semester), Black Belt (Tae Kwon Do), Provost scholarship, Erickson Discovery Grant (2017), TREE 2017 American Society of Naturalists Best Oral Presentation, Eisenhower Memorial Scholarship, Theola F. Theovas Scholarship