Investigating Antimicrobial Properties of Sceloporus virgatus Eggs Using Scanning Electron Microscopy

Grace Elliott
University of Puget Sound

Follow this and additional works at: https://soundideas.pugetsound.edu/writing_awards

Recommended Citation
https://soundideas.pugetsound.edu/writing_awards/83

This Natural Sciences and Mathematics is brought to you for free and open access by the Student Research and Creative Works at Sound Ideas. It has been accepted for inclusion in Writing Excellence Award Winners by an authorized administrator of Sound Ideas. For more information, please contact soundideas@pugetsound.edu.
Investigating Antimicrobial Properties of *Sceloporus virgatus* Eggs Using Scanning Electron Microscopy

by

Grace L. C. Elliott

This thesis is submitted to the faculty of the Biology Department of the University of Puget Sound in partial fulfillment of the requirements for the Bachelor of Science degree

January 6, 2020

Approved by:

____________________
Dr. Stacey Weiss
Thesis Advisor

____________________
Dr. Mark Martin
Reader
Abstract-

The eggshell is a vital structure that functions as a calcium reserve, site for gas exchange, and provides protection for the developing embryo. Looking specifically at the defensive properties of the eggshell, a combination of structural, chemical, and microbial properties helps protect eggs from pathogen-induced mortality. An initial aim of this review is to summarize the process of eggshell formation inside the reptilian oviduct and how this may contribute to egg protection. Another goal of this project is to explore the chemical and microbial defense mechanisms that are employed by the lizard *Sceloporus virgatus* to limit the risk of bacterial and fungal contamination of their eggs (D’Alba & Shawkey, 2015). By using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX), this project investigates the structural and chemical properties of the eggshell to gain a better understanding of how it helps protect the embryo. This study also explores the role of the cloacal transfer of beneficial microbes to the eggshell at limiting contamination by comparing the amount of bacterial and fungal growth on the surface of laid and dissected eggs using SEM imaging. The cloaca is the opening at the end of the digestive and reproductive tracts that functions in waste removal and reproduction and has been postulated to serve as an additional external barrier to infection (Martin, Gilman, & Weiss, 2010). Findings showed significantly more rod-shaped bacteria on the surface of laid eggshells (Day 0 and 25) in comparison to dissected eggs (p<0.05). Furthermore, dissected eggs were associated with a greater density of fungal hyphae than laid eggs (p<0.05), supporting the prediction that eggs that do not pass through the cloaca are more susceptible to fungal growth. These results support the prediction that dissected eggs are more vulnerable to fungal growth. Overall, these results indicate that passing through the cloaca may inoculate eggs with beneficial microorganisms that have the potential to protect eggs against fungal growth.

Chapter 1- General Overview of the Reptilian Oviduct and Eggshell Formation

The reptilian oviduct is recognized as a complex organ that is responsible for a number of critical reproductive functions such as eggshell formation, oviposition, and sperm storage (Girling, 2002). However, the intricacies of oviductal morphology and function are still far from being understood. In this chapter, I will focus on the structure of the oviduct of oviparous reptiles based on the body of information currently available. Following this synopsis, I will explore the function of these specific oviductal regions and the ways in which they are under hormonal control. Another aim of this review is to summarize the process of eggshell formation inside the reptilian oviduct and how this may contribute to the overall structure of the egg. By providing a
general overview of the oviduct of oviparous vertebrates and their role in eggshell formation I hope to highlight the complexities of the reproductive cycle of these reptiles.

**Oviductal Structure and Function**

The oviduct of oviparous reptiles’ functions as a passageway for eggs from the ovaries to outside of the body during oviposition (Palmer et al., 1993). It also serves as the primary site for fertilization to occur as well as sperm storage in some species of squamates (Girling, 2002). In terms of gross morphology, the oviduct can be separated into distinct regions based on differences in structure and function. These sections include (a) the infundibulum, (b) the uterine tube, (c) the isthmus, (d) the uterus, and (e) the vagina (Girling, 2002).

Starting with the infundibulum, this slender region of the oviduct helps receive the ovulated egg from the ovary through a funnel-like ostium opening in the coelomic cavity (Palmer et al., 1993). As a secretory region, the infundibulum has been associated with the deposition of unknown materials upon entry of the ovulated egg into the oviduct. Characterized by folds of connective tissue lined by mucosa, the uterine tube is associated with additional secretions onto the eggshell surface (Girling, 2002). While in alligators and turtles, the uterine tube is associated with albumen production, this is not the case for squamates (Palmer and Guillette, 1988). In squamates, the uterine tube is characterized by folded connective tissue lined with secretory epithelial cells and glandular crypts that are involved in sperm storage for some species (Palmer et al., 1993). Another under-studied region of the oviduct is the isthmus, an intermediate region that connects the uterine tube to the uterus. Its morphological similarities to these regions make it more of a transitional area rather than a distinct region in squamates. Generally, the uterus of oviparous squamates contains a thick layer of connective tissue with mucosal glands that facilitate the production of the eggshell (Palmer et al., 1993). These secretary cells play a role in determining the structural composition and thickness of the eggshell membrane among different species of lizards. Specifically, species containing loosely packed secretory granules in the uterus are associated with the production of hard, calcareous eggshells whereas loosely packed secretory granules are associated with soft, parchment-like eggshells (Girling, 2002). Underlying the mucosa are an inner layer of circular and outer layer of longitudinal muscles that expel the egg during oviposition. At the end of the oviduct is the vagina, characterized by a ciliated mucosal epithelium that leads to the cloacal opening of the lizard (Girling, 2002).
**Hormonal Manipulation of the Oviduct**

Over the course of the reproductive cycle, the oviduct experiences drastic changes due to seasonal fluctuations in hormones. During the preovulatory period the female begins to prepare for gravidity through increasing the secretory ability of cells in the epithelial layer lining the oviduct and increasing the weight of oviductal tissue (Girling, 2002). Regulation of this development requires activation via the endocrine system, specifically input from the hypothalamic-pituitary-gonadal and adrenal axes. Estradiol serves as one of the primary hormones regulating the developmental process of the oviduct prior to gravidity. Elevated levels of estradiol are associated with an increase in height and secretory activity in luminal and glandular epithelia, increased number and size of mucosal glands, and greater oviductal vascularity. An additional sex hormone is progesterone that is secreted via the corpora lutea and is believed to play a role in maintaining gestation during gravidity. Synthesis of progesterone has also been documented in the oviductal tissue of species of *Sceloporus* and *Urosaurus ornatus* (Weiss et al, 2003). Other hormones of interest in oviductal development during the breeding season include gonadotropins, androgens, and prolactin. However, the mechanism these hormones have on the oviduct requires further investigation (Girling, 2002). During the postovulatory stage, oviductal mass decreases significantly and is accompanied by reductions in epithelial cell height, glandular size, and secretory activity in glandular and epithelial tissues (Sarker, Sarker, & Maiti, 1995).

**Formation of Egg Components and Outer Shell**

An essential role of the oviduct in oviparous vertebrates is the formation of the eggshell (Girling, 2002). The structure and composition of the eggshell differs widely between species based on the properties of the oviductal regions that secrete the eggshell (Qualls, 1996). In general, there are two types of squamate eggshells, flexible and rigid. Specifically, flexible eggshells consist of an outer layer of calcium bicarbonate in the form of calcite that supply the embryo with a source of calcium during embryogenesis (Packard et al, 1982). In some cases, smooth spheres have been identified on the eggshell surface of oviparous squamates including *Lerista bougainvillii* and *Sceloporus virgatus* (Packard & DeMarco, 2004). Underlying the outer calcareous coating is the fibrous organic layer called the shell membrane. The inner boundary lies underneath this organic layer and is difficult to visualize even with Scanning Electron Microscopy (Qualls, 1996). Oviparous species with flexible eggshells often oviposit their eggs in
nests located in organic debris or the soil as a source of water during embryogenesis (Sexton et al., 2005). Conversely, species with rigid eggshells do not require an external source of water and therefore can be oviposited in a dry location (Sexton et al., 2005). In terms of structure, rigid eggs of the Gekkota have an extremely calcified shell with three distinct layers. These include an inner reticular boundary layer, a shell membrane, and a thick calcareous layer on the surface (Hallmann & Griebeler, 2015).

The exact mechanism associated with the production of the inner egg components, such as the albumen, inside the infundibulum and uterine tube is not fully understood. From this limited knowledge, it is recognized that following ovulation, the infundibulum is the first region of the oviduct to receive the egg, stimulating the secretion of proteins that cover the eggshell surface (Girling, 2002). However, the role of these secretions remains unknown. While the uterine tube produces the albumen layer in crocodilians and turtles, its role in squamates is inconclusive. This is mainly due to the lack of a distinct albumen layer separate from the yolk at oviposition in the eggshells of oviparous vertebrates. Despite no structural differences between the yolk and albumen layer, these proteins have been identified in the eggs of various squamates such as the lizards Sceloporus woodi, S. virgatus, and S. scalaris (Palmer & Guillette, 1991). Whether these proteins are synthesized in the oviduct requires further investigation. Another important question is whether the function of the albumen in squamates parallels that of species with a distinct albumen layer. In the case of crocodilians and turtles, the albumen is associated with various antimicrobial, nutritive, supporting, cushioning, and water-binding functions that facilitate embryonic development (Girling, 2002).

At the uterus, secretion of the fibrous eggshell membrane, followed by deposition of the outer calcareous layer occurs (Palmer, Demarco, & Guillette, 1993). It is believed that the uterine mucosal glands initially secrete thick layers of proteinaceous fibers that are wrapped around the eggs to produce the inner layer of the shell. As these fibers become thinner, they begin to form the outer organic layer of the eggshell. These predictions have been supported by investigating the process of eggshell formation in the lizard Sceloporus woodi (Girling, 2002). Within 24 hours of ovulation, observations confirmed that endometrial glands of the uterus secrete intact proteinaceous eggshell fibers that are oriented according to the rotation of the eggshells within the uterus (Palmer, Demarco, & Guillette, 1993). After several days post-ovulation, the shell membrane begins to take on a crest and trough morphology due to the secretion of additional
fibers and matrix on the eggshell surface (Packard, & DeMarco, 2004). Following membrane formation, calcification of the eggshell occurs through secretions by the luminal epithelium of the uterus (Palmer, Demarco, & Guillette, 1993). This prediction is founded on evidence that luminal epithelial cells have stained positive for calcium ions during eggshell formation in the lizard Crotaphytus collaris. Furthermore, the appearance of luminal epithelial cells becomes distended and hypertrophied during the period of maximum calcium deposition (Palmer, Demarco, & Guillette, 1993).

Time Period of Embryonic Development-

The time period of embryonic development in reptiles varies widely among species. While squamates often have extensive uterine retention times, chelonions and crocodilians oviposit eggs that are in the late gastrula stage (DeMarco, 1993). Therefore, squamate embryos are significantly more advanced in development at the time of oviposition than other reptilian orders. For instance, S. scalaris is characterized by a prolonged egg retention time that facilitates the development of an embryo that can hatch within a few weeks of oviposition (DeMarco, 1993). Within oviparous vertebrates, the shelling period is highly variable and likely explains the wide range of egg retention times exhibited by different species of squamates. Although some squamates oviposit their eggs briefly after shell deposition has occurred, other species such as S. virgatus are known to retain their eggs for prolonged periods (Mathies & Andrews, 2000). These species are recognized as intermediate to extreme egg retainers and often exhibit longer embryonic development inside the female (DeMarco, 1993). It is important to note that there are a limited number of intermediate, and very few extreme egg retaining species of squamates. This is likely attributable to the fact that the evolution of extreme egg retention is often followed by the adaptation of viviparity in which the embryo completes development inside the female. Specifically, it is hypothesized that the evolution of viviparity occurs concurrently with an increase in egg retention via the vascularization of the oviduct and chorioallantois (Mathies & Andrews, 2000). Thus, species such as S. virgatus that are distinguished by a prolonged egg retention time have a higher likelihood of evolving viviparity over evolutionary history.

Based on this general overview of oviduct morphology and eggshell formation, it is evident that there are still gaps in our existing knowledge of the reptilian reproductive system. While structural regions of the oviduct have been well described anatomically, the role of these structures in the process of egg shelling is not well understood. Notably, there is disagreement
regarding the presence of albumen in squamates and how these proteins are incorporated into the egg itself. In addition, the role of the luminal epithelium in the process of calcium secretion requires additional evidence to corroborate its function further (Girling, 2002). Another understudied topic relates to how specific properties of the eggshell help protect the developing embryo. It is possible that a combination of structural, chemical, and microbial properties defends eggs from environmental threats. In light of this missing body of information, it is important to continue to explore the complexities of eggshell formation and how eggshell structure contributes to embryo survival. In the following chapter of this thesis, I will explore this question in greater detail and introduce a number of possible structural and microbial defense mechanisms that have the potential to benefit eggshell survival. Specifically, I will investigate the physical and antimicrobial defenses of eggshells from the noteworthy species, *Sceloporus virgatus*. As previously mentioned, *S. virgatus* is recognized as a unique oviparous lizard due to its prolonged egg retention time. In addition, females provide no parental care following oviposition and eggs are exposed to a harsh environment that is conducive to bacterial and fungal growth (Abell, 1999). However, the eggs still have a high likelihood of hatching (Weiss et al., 2009) and appear to be protected from pathogens in the soil. Based on this information, it is evident that *S. virgatus* displays particularly interesting reproductive characteristics that are worth exploring further in the next chapter of this paper.
Chapter 2- Investigation of the Antimicrobial Properties of *Sceloporus virgatus* Eggshells

Introduction and Background

Eggs from oviparous vertebrates face a variety of environmental stressors that threaten their survival (D’Alba & Shawkey, 2015). During incubation, eggs are susceptible to contamination by bacteria and fungi that can lead to embryo mortality (D’Alba et al., 2014). In order to minimize possible infection, oviparous vertebrates have evolved a number of antimicrobial defenses. Notably, proteins within the egg serve as antibacterial chemicals to limit infection (Hincke et al., 2000). An initial barrier to pathogenic penetration is the eggshell itself. It functions as a physical barrier to the external environment and plays a critical role in preventing bacterial and fungal contamination (D’Alba & Shawkey, 2015). Furthermore, the hydrophobicity of the eggshell surface determines the ability of microbes to colonize the egg and form biofilms (D’Alba et al., 2014). Eggshells that are hydrophobic are associated with decreased bacterial attachment and therefore help prevent penetration and mortality of the embryo. However, microbes are still capable of entering the egg through pores designed for gas and water exchange with the external environment (Portugal, Maurer, & Cassey, 2010).

Microbial deposits on the surface of avian and reptilian eggshells, transferred via the cloaca, have also been hypothesized to serve as an additional external barrier to infection. The cloaca is an opening at the end of the digestive and reproductive tracts found in both reptilian and avian species that plays a role in excrement removal and reproduction (Martin, Gilman, & Weiss, 2010). For instance, beneficial microbes have been isolated from the cloaca of female sea turtles and the surface of sea turtle eggs (Sarmiento-Ramírez et al., 2014). In particular, members of the *Pseudomonas* species are recognized as a suppressor against fungal pathogens and may play a role in protecting sea turtle eggs (Sarmiento-Ramírez et al., 2014). The transfer of microbes to the contents of lizard eggs has also been documented in the common house gecko (*Hemidactylus frenatus*) but has not been linked to antifungal activity (Singh et al., 2014). Whether the cloacal microbiome plays a significant role as a defense mechanism remains unclear and further evidence is needed to support this hypothesis.

It is likely that a combination of structural, chemical, and microbial properties helps protect eggs from pathogen-induced mortality. In particular, eggs from the lizard *Sceloporus virgatus* are at a high risk of infection. This is attributable to the fact that eggs are laid during the
rainy season and are exposed to moist conditions that are favorable to bacterial and fungal
growth (Abell, 1999). Furthermore, females provide no parental care after oviposition of the eggs
into a soil burrow for incubation (Weiss et al., 2009). Despite these harsh embryonic conditions,
eggs still have a relatively high likelihood of hatching. In terms of the structural composition of
the eggshell, preliminary research by Arnett (2015) used scanning electron microscopy (SEM) to
determine morphological features of *S. virgatus* eggshells from 0 to 6 weeks of incubation. These
results documented the formation of large crystalline spheres, similar to the layer of calcite
spheres described on avian brush-turkey eggshells as the egg developed (D’Alba et al., 2014). In
addition, smooth spheres have been previously identified on the surface of *S. virgatus* eggshells
using SEM imaging (Packard & DeMarco, 2004; Arnett unpublished data 2015). However,
further research is needed to examine the chemical composition of the eggshell in order to gain a
more thorough analysis of the possible defensive properties of the egg.

Another antimicrobial property of *S. virgatus* eggshells is attributable to the female
transfer of beneficial microbes to the eggs as they pass through the cloaca. Previous experiments
using SEM have suggested that the microbial composition (microbiome) and fungal
susceptibility in *S. virgatus* lizard eggs differ between eggs laid naturally and those dissected out
of the female (Arnett unpublished data 2015). When eggs were dissected out, without cloacal
contact, they had greater fungal growth and less bacteria present. These differences may be
linked to the inoculation of eggs with microbiota from the cloaca when they are laid by the
female. However, these findings did not yield statistically significant results due to a limited
sample size and require further investigation to be properly elucidated. Preliminary research
conducted by Weiss (personal communication) suggests that eggs have a higher survival rate
when oviposited than when they are surgically removed from the lizard. These findings indicate
that the cloacae of female lizards may play a role in protecting eggs from succumbing to
infection and fungal growth. Further research has discovered that the cloacal microbiome of the
striped plateau lizard differs between male and females (Martin, Gilman, & Weiss, 2010).
Specifically, females had less diverse microbial communities than males and their cloacae were
dominated by the potentially antifungal bacterium *Serratia*. Its ability to degrade fungal cell
walls has been shown to effectively inhibit the growth and development of soil pathogens
(Gutiérrez-Román et al., 2015). During the reproductive season, it has been found that the cloaca
of female *S. virgatus* lizards tends to decrease in microbial diversity in favor of several types of
bacteria that may be beneficial to the eggs (Cox, 2015). From these preliminary results, it appears that the microbiome of the female cloaca plays a role in protecting the viability of eggs during incubation.

**Hypotheses and Rationale**

The existing literature on this topic has largely focused on the protective mechanisms of eggshells as it relates to structural and chemical defenses in a limited number of avian and reptilian species. However, little is still known about the antimicrobial properties of eggs and there is value to increasing the taxonomic representation of this understudied topic. Therefore, it is important to identify protective structural components of the eggshell cuticle in addition to the role of cloacal contact in inhibiting fungal growth. I hypothesize that structural analysis of eggshells using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX) will reveal crystalline spheres composed of calcium carbonate in the form of calcite as well as smooth spheres of an unknown elemental composition (Osborne & Thompson, 2005; Arnett unpublished data 2015). Due to preliminary evidence suggesting that cloacal contact transfers microbes that protect eggs from bacterial and fungal penetration, I predict that laid eggs will have a greater number of rod-shaped bacteria and fewer fungi on the surface of the eggshell than will dissected eggs. Diminished vulnerability to fungal infection in oviposited eggs suggests that cloacal contact may defend laid eggs from fungal penetration and embryo mortality (Weiss, personal communication). Therefore, I propose that laid eggs exposed to fungal assays will have fewer fungal spores attached to the surface of the eggshell than those that are dissected.

**Specific Aims**

1. Use SEM and EDX to identify morphological features and chemical composition of *S. virgatus* eggs at Day 0 incubation.
2. Compare the amount of bacterial and fungal growth on the surface of laid and dissected eggs after 25 days incubation using SEM imaging.
3. Challenge laid and dissected eggshells in fungal assays to assess differences in attachment and penetration of fungal hyphae.
Methods

Egg Collection – Twenty-four female *Sceloporus virgatus* lizards carrying fertilized eggs were collected near the American Museum of Natural History’s Southwestern Research Station located in Cochise County, Arizona, USA. The lizards were collected in late June and housed in an outdoor enclosure to mimic their natural habitat. Gravid females were then shipped to the Weiss lab at the University of Puget Sound. Lizards were then randomly selected to be euthanized chemically using MS222 (n = 12; Conroy et al., 2009) in order to extract their eggs via dissection, or injected i.p. with 2 USP units of oxytocin in 0.1 ml aqueous solution (n=12) to induce egg-laying.

Egg Processing - Using sterile procedure, three eggs per female were randomly selected for SEM imaging (0 and 25 days) and fungal attachment assays. Eggs selected for later imaging were buried in individual 50 ml cups filled with sterile vermiculite with a slurry of AZ soil and water (1 g/ 0.8 ml) to mimic the natural environment. These cups were then covered in parafilm and placed in an incubator that was maintained at 28 °C (Andrews & Rose, 1994). For SEM imaging at 0 and 25 days, a small incision was made with sterile scissors at the tip of each egg, and the contents expelled. The empty eggshells were cut at the top and bottom to form a rectangular shape, a small portion was saved for Illumina sequencing for another study, and the remaining two halves of the eggshell separated. These eggshell halves were placed in ~2 mL of fixative (2% paraformaldehyde, 2% gluteraldehyde, 2% DMSO, and 1x phosphate buffer solution (PBS)) and left for 2 hours before being placed in individual vials of 1x PBS solution for a 48-hour period. After, eggshells were dried with a standard SEM specimen drying procedure, mounted on individual stubs, and placed in an airtight container with desiccant overnight. Mounted specimens were sputter-coated with gold palladium and observed with the scanning electron microscope.

For fungal attachment assays, eggs were initially incubated in sterile vermiculite for a total of nine days. Following incubation, a small incision was made with sterile scissors at the tip of each egg, and the contents expelled. The empty eggshells were cut at the top and bottom to form a rectangular shape, and the two halves of the eggshell separated. These shell pieces were then used to test for differences in fungal penetration, as described below.
**SEM Imaging** - The eggshells after 0 days of incubation (n = 24 eggs) were examined under SEM at 1.5K x magnification to measure the average density and diameter of smooth spheres found on the egg surface. The number of smooth spheres per sample were counted at thirty randomly chosen locations at 1.5K x magnification to quantify the mean number of spheres on the egg surface. The average number of rod-shaped bacteria found on the shell was quantified by scanning thirty randomly selected locations at 2.5K x magnification per egg. At this stage, no fungi were identified on the eggshell surface. In addition, the elemental composition of the eggshell was characterized using EDX at four locations per egg (n=9). This method utilizes X-rays released from the sample during bombardment by an electron beam to determine the elemental composition of eggshell (D’Alba et al., 2014).

The eggs selected after 25 days incubation (n = 20 eggs) were examined by scanning fifteen randomly selected locations at 2.5K x magnification to quantify the average number of rod-shaped bacteria and fungal spores found on the shell. Fewer locations (15 quadrats/shell) were selected for imaging at 25 days incubation because there was a greater abundance of bacteria on the surface than Day 0 eggs that required more intensive scanning to find. The number of rod-shaped bacteria and spores found on laid and dissected eggs were then compared to determine if there was a significant difference between these two groups (unpaired t-test).

**Fungal Assay** – To test whether the cloacal microbiome inhibits fungal attachment, fungal attachment assays were performed on eggshells of laid and dissected eggs. Halves of laid and dissected eggshells were put into 1 ml suspensions of a known concentration of *Neocosmospora rubicola* (2.48 x 106 hyphae/mL) or *Aspergillus protuberus* (1.04 x 106 hyphae/mL) fungus and incubated at room temperature (D’Alba et al., 2014). These species of fungi were cultured from soil samples taken in the natural environment where *S. virgatus* eggs would likely be found. As known pathogens of some plants such as the Pitaya *Hylocereus costaricensis* (Zheng et al., 2018), it was hypothesized that *N. rubicola* may also be capable of infecting *S. virgatus* eggs. As another known pathogenic fungus (Borsa et al., 2015), *A. protuberus* was also selected due to its potential to infect the eggshell. After 48 hours, shells were rinsed with deionized water and the number of fungi attached to the shell surface were quantified at 2.5K x magnification per egg. Fungal cell counts were performed on fifteen random locations across each shell piece and the
number of fungi on laid and dissected eggs were compared between these two groups (unpaired t-test).

Hydrophobicity Test- The hydrophobicity of both laid (n=1) and dissected (n=1) S. virgatus eggshells was quantified using contact angle goniometry. This technique involves measuring the contact angle ($\theta_c$) of droplets of deionized water placed on the eggshell surface (D’Alba et al., 2014). Contact angles were measured by Dr. Rachel Pepper by using a microscope with a commercial contact angle goniometer. A surface is considered hydrophilic if $\theta_c<90$ degrees, hydrophobic if $\theta_c>90$ degrees and superhydrophobic if $\theta_c>150$ degrees.

Results
Egg Morphology and Structure-
At 0 and 25 days of incubation, the eggshell surface was observed to have a crest and trough morphology characterized by the presence of raised polygons and ridges (Deeming and Ferguson 1991; Figure 1). Located on the eggshell surface, smooth spheres (Figure 2) and crystalline spheres (Figure 3) were also observed at Day 0 of incubation. Beneath the eggshell surface, a fibrous material was observed with irregular fibers that were closely opposed to one another (Figure 4). Cross sections of the eggshell confirmed the presence of proteinaceous fibers followed by an inner boundary membrane (Figure 5).
Figure 1. Surface of *S. virgatus* eggshell at Day 0 incubation. The eggshell is characterized by a crest and trough morphology that gives the surface a rough texture. A region covered by crystalline spheres can be seen in the center of the image. The image was taken on a scanning electron microscope at 120x magnification.
**Figure 2.** Smooth spheres found on *S. virgatus* eggshell surface at Day 0 incubation. The spheres vary in shape and size and are often located in the troughs that lie between the raised polygons of the shell surface. The image was taken on a scanning electron microscope at 1.5k x magnification.
Figure 3. Crystalline spheres covering *S. virgatus* eggshell at Day 0 incubation. The shape and size of the spheres is quite variable and they are present in only limited areas of the shell. Observations of the crystalline covering was irregular as some eggshells did not have surfaces covered by them. The image was taken on a scanning electron microscope magnified at 190x.
**Figure 4.** Fibrous material located underneath the smoother outside surface. Cracks in the outer layer revealed fibers arranged in an irregular manner that were closely opposed to one another. The image was taken on a scanning electron microscope magnified x 420.
Figure 5. Cross section of *S. virgatus* eggshell after Day 0 incubation. The outer surface is composed of thin fibers followed by proteinaceous fibers of the eggshell membrane and finally an inner boundary membrane. IM, inner boundary membrane; F, proteinaceous fibers; O, outer surface of eggshell membrane.

At Day 0, laid and dissected eggs did not differ in smooth sphere density, sphere diameter, or sphere spatial distribution (p > 0.05 for all; Table 1). The average sphere density of dissected eggs (0.1 ± 0.02 spheres per 22,800 µm²) did not differ significantly from the average sphere density of laid eggs (0.5 ± 0.02 spheres per 22,800 µm²; Wilcoxon rank sum test, p-value = 0.452).
Table 1. Smooth Sphere Characteristics of Laid and Dissected Eggs at Day 0.

<table>
<thead>
<tr>
<th>Sphere Characteristic</th>
<th>Eggshell Type</th>
<th>Mean (± SE)</th>
<th>T-statistic (df)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere Density</td>
<td>Laid</td>
<td>6.0 (± 1.6)</td>
<td>0.77 (22)</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>Dissected</td>
<td>8.8 (± 3.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphere Diameter</td>
<td>Laid</td>
<td>7.6 (± 0.3)</td>
<td>-0.35 (22)</td>
<td>0.731</td>
</tr>
<tr>
<td></td>
<td>Dissected</td>
<td>7.4 µm (± 0.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Variance to Mean Ratio</td>
<td>Laid</td>
<td>54.2 µm (± 0.3)</td>
<td>0.11 (22)</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td>Dissected</td>
<td>57.8 µm (± 0.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The average sphere density was significantly greater on Day 0 eggs (9.0 ± 2.1 spheres per 22,800 µm²) than on day 25 eggs (0.2 ± 0.002 spheres per 22,800 µm²; 2 sample t-test, t = -16.82, df = 22, p = 0.0002; Figure 6).
Figure 6. Differences in average (±SE) smooth sphere density between eggs at Day 0 incubation and 25 days incubation. The average sphere density on Day 0 eggs (9.0 ± 2.1 spheres per 22,800 µm²) was significantly greater than the average sphere density of day 25 eggs (0.2 ± 0.002 spheres per 22,800 µm²; 2 sample t-test, t = -16.82, df = 22, p = 0.0002).

Smooth spheres contained Silicon, Nitrogen, Oxygen, Sodium, and Calcium (Table 2). Eggshells contained Silicon, Oxygen, Sodium, and Calcium. Smooth spheres had 377% more Silicon and 388% more Sodium but 68% less Calcium than the eggshell itself. The fibrous layer contained Sulfur, Sodium, and Calcium (Table 3). The inner membrane of the eggshell contained Sulfur, Potassium, Sodium and Calcium.
Table 2. Elemental Composition of Smooth Spheres and Eggshell Surface at Day 0 (n=9).

<table>
<thead>
<tr>
<th>Element</th>
<th>Location</th>
<th>Element Weight % (± SE)</th>
<th>T-statistic (df)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>Spheres</td>
<td>20.31 (± 2.76)</td>
<td>-4.83 (22)</td>
<td>p = 0.0001</td>
</tr>
<tr>
<td></td>
<td>Shell</td>
<td>4.25 ( ± 1.86)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Spheres</td>
<td>0.96 (± 0.96)</td>
<td>-1 (22)</td>
<td>p = 0.328</td>
</tr>
<tr>
<td></td>
<td>Shell</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>Spheres</td>
<td>37.12 ( ± 4.92)</td>
<td>-0.5766 (22)</td>
<td>p = 0.570</td>
</tr>
<tr>
<td></td>
<td>Shell</td>
<td>33.56 ( ± 3.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>Spheres</td>
<td>5.71 (± 1.32)</td>
<td>-2.97 (22)</td>
<td>p = 0.008</td>
</tr>
<tr>
<td></td>
<td>Shell</td>
<td>1.17 ( ± 0.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Spheres</td>
<td>15.18 ( ± 2.15)</td>
<td>5.54 (22)</td>
<td>P = 0.00006</td>
</tr>
<tr>
<td></td>
<td>Shell</td>
<td>48.33 ( ± 5.59)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Elemental Composition of Fibrous Layer and Inner Membrane of Day 0 eggshells.

<table>
<thead>
<tr>
<th>Element</th>
<th>Location</th>
<th>Element Weight % (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur</td>
<td>Fibers</td>
<td>22.42 (± 4.56)</td>
</tr>
<tr>
<td></td>
<td>Inner Membrane</td>
<td>19.75 (± 3.73)</td>
</tr>
<tr>
<td>Potassium</td>
<td>Fibers</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Inner Membrane</td>
<td>17.22 (± 2.23)</td>
</tr>
<tr>
<td>Sodium</td>
<td>Fibers</td>
<td>5.75 (± 3.36)</td>
</tr>
<tr>
<td></td>
<td>Inner Membrane</td>
<td>4.22 (± 1.62)</td>
</tr>
<tr>
<td>Calcium</td>
<td>Fibers</td>
<td>4.66 (± 4.51)</td>
</tr>
<tr>
<td></td>
<td>Inner Membrane</td>
<td>5.29 (± 4.41)</td>
</tr>
</tbody>
</table>

Preliminary measurements of the contact angle of *S. virgatus* eggshells showed hydrophobicity (106.27 ± 10.502 degrees; Table 4).

Table 4. Preliminary Contact Angle Measurements of Eggshell Surface.

<table>
<thead>
<tr>
<th>Egg Treatment</th>
<th>Location of Measurement</th>
<th>Left Hand Side</th>
<th>Right Hand Side</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissected</td>
<td>Top of Egg</td>
<td>124.00</td>
<td>124.510</td>
<td>124.255</td>
</tr>
<tr>
<td>Dissected</td>
<td>Side of Egg</td>
<td>72.851</td>
<td>124.921</td>
<td>98.886</td>
</tr>
<tr>
<td>Laid</td>
<td>Top of Egg</td>
<td>124.920</td>
<td>119.193</td>
<td>122.057</td>
</tr>
</tbody>
</table>
Bacterial and Fungal Loads on Eggshell Surface at Day 0 and Day 25-

At Day 0 incubation, bacteria were observed on the surface of laid and dissected eggs (Figure 7). The average number of bacteria on the surface of laid eggs (0.8 ± 0.1 per 1800 µm²) was 300% greater than the number of bacteria on dissected eggs (0.2 ± 0.04 per 1800 µm²; 2 sample t-test, t = -4.73, df = 22, p < 0.0001; Figure 8).

<table>
<thead>
<tr>
<th>Laid Side of Egg</th>
<th>92.526</th>
<th>67.240</th>
<th>79.883</th>
</tr>
</thead>
</table>

**Figure 7.** Rod-shaped bacteria (white) on *S. virgatus* laid eggshell at Day 0 incubation. The bacteria are sometimes hidden in the rough-textured covering on the surface and are about 2 µm in length. The image was taken with a scanning electron microscope magnified x2.5k.
Figure 8. The average number of bacteria on the surface of laid eggs (0.813 ± 0.138 per 1800 µm²) was significantly greater than the number of bacteria on dissected eggs at Day 0 incubation (0.2 ± 0.04 per 1800 µm²; 2 sample t-test, t = -4.74, df = 22, p < 0.0001). Areas were measured with scanning electron microscopy by randomly selecting 30 areas on the eggshell surface and measuring the density of bacteria at x2.5k magnification.

After 25 days of incubation, bacteria were observed on the surface of laid and dissected eggs (Figure 9). The average number of bacteria on the surface of laid eggs (19.9 ± 2.1 per 1800 µm²) was significantly 315% greater than the number of bacteria on dissected eggs (4.8 ± 1.1 per 1800 µm²; 2 sample t-test, t = -6.48, df =18, p < 0.05; Figure 10).
Figure 9. Rod-shaped bacteria observed on *S. virgatus* dissected eggshell at 25 days incubation. Image was taken at 2.5k x magnification.
Figure 10. The average number of bacteria on the surface of laid eggs (19.9 ± 2.1 per 1800 µm²) was significantly greater than the number of bacteria on dissected eggs at 25 days incubation (4.8 ± 1.1 per 1800 µm²; 2 sample t-test, t = -6.45, df = 18, p < 0.05).

After 25 days of incubation, fungal hyphae were observed on the surface of laid and dissected eggs (Figure 11). The average number of fungal hyphae on the surface of dissected eggs (0.9 ± 0.2 per 1800 µm²) was 150% greater than the number of hyphae on laid eggs (0.4 ± 0.1 per 1800 µm²; 2 sample t-test, t = 4.07, df = 36, p < 0.0001; Figure 12).
Figure 11. Fungal hyphae found on *S. virgatus* dissected eggshell after 25 days of incubation. Hyphae varied in width (0.4 µm - 2 µm) and were characterized by their branched structure across the eggshell surface. The image was taken with SEM at 2.5k x magnification.
Figure 12. The average number of fungal hyphae on the surface of dissected eggs (0.9 ± 0.2 per 1800 µm²) was significantly greater than the number of hyphae on laid eggs (0.4 ± 0.1 per 1800 µm²; 2 sample t-test, t = 4.07, df = 36, p < 0.0001).

**Fungal Attachment Assay**

Fungal hyphae and bacteria were identified on both laid and dissected eggshells following incubation with *Neocosmospora rubicola* and with *Aspergillus protuberus*, two fungi previously isolated from soil in *S. virgatus* nesting habitat (Figure 13; Figure 14). The way that fungal species influenced the number of fungal hyphae did not depend on whether the egg was laid or dissected (2-way ANOVA, *F* = 0.21, df = 1, 30, *p* = 0.651, *n* = 19; Figure 15). The type of fungal species significantly influenced the density of fungal hyphae found on *S. virgatus* eggs (2-way ANOVA, *F* = 15.57, df = 1, 30, *p* = 0.0004). The effect of egg oviposition, laid or dissected, also significantly affected hyphae density (*F* = 23.46, df = 1, 30, *p* < 0.0001). The average (±SE) density of fungal hyphae on all eggs exposed to *Neocosmospora rubicola* (3.1 ± 0.3) was 121% greater than when eggs were exposed to *Aspergillus protuberus* (1.4 ± 0.2). The average density of fungal hyphae on dissected eggs exposed to *Neocosmospora rubicola* (3.9 ± 0.4) was 70% greater than laid eggs exposed to *Neocosmospora rubicola* (2.3 ± 0.4). The average density of fungal hyphae on dissected eggs exposed to *Aspergillus protuberus* (1.9 ± 0.4) was 111% greater than laid eggs exposed to *Aspergillus protuberus* (0.9 ± 0.2).
Figure 13. Fungal hyphae of *Aspergillus protuberus* on *S. virgatus* dissected eggshell after 9 days of incubation. Fungal hyphae cover the eggshell in a highly branched structure that spreads across the surface. The image was taken with SEM magnified 270 times.
Figure 14. Fungal hyphae of *Aspergillus protuberus* found on *S. virgatus* dissected eggshell after 9 days of incubation. Hyphae are covered in rod-shaped bacteria that are roughly 2 µm in length. The image was taken with SEM magnified 270 times.
Figure 15. Average (± SE) number of fungal hyphae on laid and dissected eggs exposed to the fungal species *Neocosmospora rubicola* and *Aspergillus protuberus*. The way that fungal species influenced the number of fungal hyphae did not depend on whether the egg was laid or dissected (p = 0.651, n = 19). The type of fungal species significantly influenced the number of fungal hyphae (p = 0.0004). Dissected eggs had significantly more fungal hyphae than did laid eggs (p < 0.0001).

**Discussion**

*Egg Morphology and Structure*—

Observations of eggshell morphology at 0 and 25 weeks incubation indicate that the outer surface of the membrane is organized into a series of crests and troughs. This type of organization increases the surface area of the eggshell that comes into contact with the external environment such as soil and water. Therefore, a crest and trough morphology is associated with an increased ability to absorb water and stretch as the eggshell swells over time (Packard et al, 1982). Oviparous squamates typically possess an outer inorganic layer that consists of calcium
carbonate in the form of calcite (Qualls, 1996). Elemental analysis of the outer inorganic layer of *S. virgatus* eggshells found high concentrations of calcium and low levels of silicon. These findings suggest that the outer layer of egg may consist of calcite that serves as a source of calcium during embryogenesis (Packard & Packard, 1984). Furthermore, the presence of silicon has also been described on the calcareous layer of Physignathus lesueurii eggshells (Osborne & Thompson, 2005). Small cracks in the shell were also observed that revealed a fibrous layer located below the outer membrane of the eggshell surface. Cross sections of the *S. virgatus* eggshell confirmed that the outer surface is composed of thin fibers followed by proteinaceous fibers of the eggshell membrane and finally an inner boundary membrane. These findings are consistent with other squamate eggshells that are composed of an outer inorganic layer underlain by an organic membrane composed of irregular fibers that are closely opposed to one another (Packard, & DeMarco, 2004).

Examination of the eggshell using SEM revealed the presence of crystalline spheres and smooth spheres covering the surface of laid and dissected eggs. Smooth spheres were consistently found on all eggshells and have been previously characterized on *S. virgatus* eggshells using SEM techniques (Packard & DeMarco, 2004). The presence of crystalline spheres was highly variable and even absent on some eggshells. A variable crust of crystalline material has been characterized in other oviparous squamates including the lizard, *Lerista bougainvillii* (Qualls, 1996). Crystalline spheres have also been described on the surface of avian brush-turkey eggshells (D’Alba et al, 2014).

Findings indicate that there is no significant difference in the density or diameter of smooth spheres between laid and dissected eggs at Day 0 and at 25 days incubation. In fact, the distribution of these smooth spheres was clumped across all eggshells. However, there was a significantly higher number of smooth spheres on the surface of eggs at Day 0 incubation than those at 25 days incubation. This suggests that the spheres may have degraded or become absorbed by the eggshell during incubation. Elemental analysis of the spheres showed high concentrations of silicon, calcium, sodium, and oxygen. These results give useful insight into the elemental composition of the spheres and possible functions they serve on the eggshell surface. In particular, silicon is associated with the maintenance of albumen quality and reduction of weight loss in order to preserve egg integrity (Knight, Bowery, & Cooke, 1972). For instance, the use of synthetic silicone fluids is utilized to preserve albumen quality for at least 2 months of
egg storage. In addition, applying sodium silicate to salmon eggs reduces toxicity of copper and aluminum in the environment (Pessot et al., 2014). Thus, it is possible that the spheres serves a similar function by preserving the albumen and weight of the egg over the course of the incubation period.

Another potential role of the smooth spheres is the prevention of bacterial and fungal colonization of the eggshell surface. For instance, brush-turkey (*Alectura lathami*) eggshells are covered in calcite nano-spheres that are superhydrophobic in nature and therefore serve as an antimicrobial defense for eggs (D’Alba et al., 2014). Superhydrophobic spheres have also been characterized on the surface of brown-widow spider (*Latrodectus geometricus*) eggs that act as an effective defense strategy against bacterial colonization (Makover et al., 2019). Originating from the oviposition fluid, these spheres create electrostatic interactions with bacteria and therefore limit bacterial adhesion to the egg surface. Based on these studies, it is hypothesized that the smooth spheres on *S. virgatus* eggshells are also superhydrophobic in nature and thus limit bacterial and fungal adhesion to the egg surface. However, future studies are needed to elucidate the nature of the spheres and where exactly they originate from. The fact that there was no observed difference in sphere density on laid or dissected eggs suggests that they are deposited at some point in the oviduct prior to passing through the cloaca. Therefore, it is likely that these spheres are deposited on the surface of the eggs following production of the eggshell membrane in the uterus (Girling, 2002). In the future, it would be interesting to determine whether the activity of secretory granules in the uterus plays a role in the deposition of spheres on the eggshell surface.

**Hydrophobicity of Eggshells**

Hydrophobicity or wettability of a surface is recognized as a determining factor in bacterial adhesion (Makover et al., 2019). Based on preliminary results, *S. virgatus* eggshells appear to contain hydrophobic properties. Comparatively, the average hydrophobicity of *S. virgatus* eggshells (106.27 ± 10.502 degrees) is greater than that of chicken (*Gallus gallus domesticus*) eggs (66.5 ± 3.20 degrees) and smaller than that of brush-turkey eggs (135.3 ± 2.65 degrees; D’Alba et al., 2014). In addition, *S. virgatus* eggshells were observed to have relatively high hysteresis, meaning that when water droplets were placed on the eggshell they remain pinned to the surface and did not roll off. Therefore, the eggshell appears to trap water droplets on the surface, prohibiting water from dispersing across the eggshell. Known as the ‘petal
effect,’ this process is associated with limited biofilm formation and protects the eggshell from bacterial penetration (D’Alba et al., 2014). It is important to note that the technique used to measure hydrophobicity is still being refined in order to provide a more accurate representation of the eggshell surface. For instance, the contact angle likely varies between eggs and depending on the position of the water droplet on a single egg. In the future it would be useful to increase the sample size of eggs measured and to standardize the specific location the water droplet is placed on the shell. In addition, obtaining a head-on photo of the water drop without it being oriented towards or away from the camera would also be important for future experiments.

**Bacterial and Fungal Community of Day 0 and Day 25 Eggshells**

The average number of rod-shaped bacteria present on the surface of laid eggs was significantly greater than the number of bacteria on dissected eggs. Therefore, laid eggs may contain higher concentrations of rod-shaped bacteria than dissected eggs. These findings are consistent with preliminary evidence suggesting that cloacal contact transfers microbes to the eggshell surface during oviposition. It is important to note that the number of bacteria present on the eggshell surface at oviposition/dissection was likely not disturbed by the SEM fixing and drying process. By using an egg covered in a solution of *Serratia* bacteria as a positive control, it was possible to determine if SEM imaging had the potential to disturb bacterial colonies present on the egg. After going through the fixing and drying procedure, rod-shaped *Serratia* were clearly identified on the eggshell surface demonstrating the reliability of SEM imaging for the purpose of this project. Therefore, it is reasonable to conclude that the number of bacteria identified on laid and dissected eggs is an accurate representation of the number of microorganisms present on the eggshells. However, SEM imaging can be problematic due to possible shrinkage and distortion of bacterial specimens during the chemical fixation process (Golding et al., 2016). In order to address this issue, it would be helpful to utilize ionic liquids, (1-butyl-3-methylimidazolium tetrafluoroborate) diluted in water, instead of sputter coating to get better results with microbial imaging (Golding et al., 2016). These highly conductive salts have been shown to enable SEM image contrast at a higher resolution than sputter coating without damaging microbial specimens in the process. In the future, it would be useful to use this technique to facilitate more accurate identification of bacterial communities on the eggshell.

While the species of rod-shaped bacteria has not yet been confirmed, the observations made in this study suggest that the microorganism may be *Serratia*. Specifically, the eggshells
covered in *Serratia* showed similar characteristics to the morphology and size of the unidentified rod-shaped bacteria covering laid and dissected eggs. Both the individual colonies of *Serratia* bacteria and the unidentified microorganisms were rod-shaped and approximately 1-5 um in length. Furthermore, *Serratia* has been identified as the primary component of the female *S. virgatus* microbiota making up approximately 43.0% of the identified phylotypes isolated from the cloaca (Martin, Gilman, & Weiss, 2010). Due to the abundance of this microorganism in the cloaca, it is likely that colonies of *Serratia* were transferred to eggs during oviposition.

After 25 days of incubation, the average number of bacteria on the surface of laid eggs was significantly greater than the number of bacteria on dissected eggs. These results are similar to those at Day 0 incubation, also finding a higher density of rod-shaped bacteria on laid eggs. An important difference between these two time points is the number of bacteria found across laid and dissected eggshells. Specifically, there was an increase in the number of bacteria on the eggshell surface over the 25 day incubation period. It is possible that the initial bacteria present on the Day 0 eggs proliferated on the eggshell surface so that there was a higher density of rod-shaped bacteria found at 25 days. The fact that the percent difference in bacteria between egg type was roughly similar at the two time points (300% vs. 315%) suggest that there are long-term differences in the density of bacteria on the surface of laid and dissected eggshells. Laid eggs were found to have significantly greater amounts of rod-shaped bacteria when they were first oviposited and after being incubated for weeks. If these rod-shaped bacteria are associated with fungal inhibition, then laid eggs are more capable of resisting fungal penetration than dissected eggs across the incubation period. In the future, it would be useful to look at the eggshell surface at 6 weeks incubation to determine if laid eggs maintain this higher density of rod-shaped bacteria.

Eggs incubated for a total of 25 days in vermiculite mixed with Arizona soil were exposed to fungal species native to the study sites where female *S. virgatus* were collected. Observations on the SEM revealed fungal growth on regions of both laid and dissected eggshells. However, the average number of fungal hyphae on the surface of dissected eggs was significantly greater than the number of hyphae on laid eggs. The fact that dissected eggs had a greater density of fungal hyphae on the eggshell surface supports the hypothesis that eggs that do not pass through the cloaca are more susceptible to fungal growth. These findings suggest that laid eggs are not as vulnerable to fungal growth as dissected eggs and may be protected by the
rod-shaped bacteria on their surface. It would be useful in the future to characterize the fungal species native to Arizona at the specific sites where female *S. virgatus* are collected. This would elucidate which fungal species are the most prevalent in the soil and whether or not they are pathogenic to lizard eggs.

**Fungal Assay of Day 9 Eggshells**

Whether eggs were laid or dissected had a significant effect on the number of fungal hyphae present on the eggshell following incubation with *Neocosmospora rubicola* and *Aspergillus protuberus*. Across both fungal species, dissected eggs had a greater number of fungal hyphae than laid eggs. A greater density of fungal hyphae on dissected eggs supports the prediction that eggs that do not pass through the cloaca are more susceptible to fungal growth. These results indicate that laid eggs are less vulnerable to fungal growth and potentially protected by the rod-shaped bacteria on their surface. Fungal species also had a significant influence on the density of fungal hyphae found on *S. virgatus* eggs at 9 days incubation. Specifically, the average density of fungal hyphae on all eggs exposed to *Neocosmospora rubicola* was greater than when eggs were exposed to *Aspergillus protuberus*. These findings may be attributable to the fact that the concentration of the *Neocosmospora rubicola* fungal suspension (2.48 x 10^6 hyphae/mL) was higher than the concentration of *Aspergillus protuberus* (1.04 x 10^6 hyphae/mL). Therefore, eggs that were exposed to a greater density of hyphae had a higher number of hyphae covering the surface of the eggshell.

While we cannot make any conclusions regarding the exact role of the rod-shaped bacteria on the eggshell surface, there are a number of protective functions that it may serve. Specifically, the rod-shaped bacteria may have antifungal properties that are capable of inhibiting the growth of *Neocosmospora* and *Aspergillus*. In fact, a strain of the bacteria *Serratia marcescens* isolated from rhizospheres of tea plants has been associated with the inhibition of nine different tea root fungal pathogens (Dhar Purkayastha et al., 2018). The bacterium is capable of breaking down the fungal mycelia by producing hydrolytic enzymes such as chitinase, protease, lipase, and cellulase (Palacios, 2015). An additional study investigating the interaction of soil bacteria with *Botrytis cinerea* found reduced pathogenicity of the fungus on the leaves of plants (Schoonbeek et al., 2007). Soil bacteria were capable of degrading oxalic acid produced by the *B. cinerea* which inhibited the growth and pathogenicity of the fungus. These findings showcase the capacity of different species of bacteria to impede fungal growth and reduce
pathogenicity. Therefore, it is possible that the rod-shaped bacteria on laid eggs is capable of producing hydrolytic enzymes or breaking down toxic byproducts that inhibit the growth of fungal hyphae on the eggshell surface. In order to explore these potential mechanisms, it would be useful to conduct additional studies using SEM to examine the interaction zone between pathogenic fungal hyphae and rod-shaped bacteria isolated from laid eggs to see if it can inhibit fungal mycelia (Dhar Purkayastha et al., 2018).

**Implications and Further Directions**

It is well known that microbes play a beneficial role in the survival and development of eukaryotic organisms (Bang et al., 2018). In spite of drastic differences in lifestyles and forms, bacteria and animals have evolved specialized relationships in the form of symbiosis or through shared environments (McFall-Ngai et al., 2013). Notably, the maternal transmission of beneficial microbes is now recognized as a widespread phenomenon in the animal kingdom, ranging from phyla such as Porifera, Mollusca, Arthropoda, and Chordata (Funkhouser & Bordenstein, 2013). The ubiquity of maternal symbiont transmission highlights the evolutionary significance of this mechanism as the first inoculation of advantageous microbes.

Despite recent advances in microbial research, there are still many aspects of the host-microbe interaction that remain undiscovered. The results of this future research will provide useful insight into the structural and microbial defense mechanisms of S. virgatus lizard eggs. In particular, findings will indicate whether dissected eggs have increased vulnerability to fungal/bacterial contamination in comparison to laid eggs, as suggested by my current research. These results may reveal mechanisms for how reptilian eggs can prevent pathogenic infection and have implications for understanding the importance of the microbiome to the reproductive success of organisms. Future studies will examine the genetic composition of the eggshell’s bacterial community to compare the diversity between laid and dissected eggs. These techniques are currently being refined to effectively isolate microbial DNA in order to examine the different microbial species that are associated with the surface of S. virgatus eggs. Additionally, these results will be compared to the microbial composition of the female cloaca to determine if there are overlapping species. By conducting further research using sequencing of microbial communities, it will be possible to investigate whether the cloacal microbiome plays a role in the transfer of beneficial microbes to the surface of the eggshell. Ultimately, the knowledge gained from both this project and future research will add to the current understanding of microbial
symbionts and elucidate the role of maternally acquired microbial communities to the survival of reptilian eggs.

Acknowledgements

This research was supported by the National Science Foundation (1755408) and the University of Puget Sound Enrichment Committee under IACUC PS18002. Field research was made possible by the Southwestern Research Station (SWRS) under AZGF permit SP649069 and SWRS Student Research Award. Special thanks to professors Stacey Weiss and Mark Martin who provided insight and expertise that greatly assisted with my research. I would also like to thank Amy Replogle for all of her assistance with learning SEM and EDX techniques and Michal Morrison-Kerr for supplying critical laboratory materials. Thanks to Helena Heyer-Gray for assistance in the field and other students in the Weiss lab for being so helpful this summer.
Works Cited


