THE RELATIONSHIP OF REPRODUCTIVE HORMONES AND HOST SUSCEPTIBILITY TO THE OPPORTUNISTIC PATHOGEN, *VIBRIO VULNIFICUS*

by

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A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Biology

Charlotte

2015

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ABSTRACT

LESLIE M. MCKEE. The relationship of reproductive hormones and host susceptibility to the opportunistic pathogen, *Vibrio vulnificus.* (Under the direction of DR.YVETTE HUET and DR. JAMES OLIVER)

It is commonly found that males show a higher susceptibility to infectious diseases. While the underlying mechanism for this sex difference is not fully understood, one significant contributing attribute is the presence of specific sex hormones- e.g. Testosterone, Estrogen, and Progesterone. These hormones have been shown to moderate immune function in both protective and suppressive capacities. These sex steroids also have a direct effect on bacterial metabolism, although the range of responses (proliferation or inhibition) appears to be bacterial species-specific. *Vibrio vulnificus* is one example of a bacterium that produces an infection exhibiting a significant sex difference, with 85% of cases being male. Previous studies have suggested that the presence of estradiol protects against endotoxic shock caused by the lipopolysaccharide (LPS) of this bacterium, however, no studies have investigated the direct and indirect role of steroid hormones on the viability and proliferation of this bacteria in the bloodstream. The present study uses serum from rats as a model for septicemic infections. These sera were treated with different ratios of endogenous and exogenous sex hormones to determine the indirect and direct effect of viability of *V. vulnificus* in each condition, and survival assessed at various time points following bacterial inoculation.

Results indicate that presence of hormones in the sera do not appear to be a regulatory element for survival of this bacterium continuously. After 24 hours, there were no significant differences observed among any of the experimental groups. There do appear to be minor significant variations of proliferation for one ratio and time and in

male and female sera at early time points. The data presented gives us a further understanding the influence sex steroids may have on the viability of *V. vulnificus*, and suggests the investigation of alternative indirect effects of these hormones.

DEDICATION

This work is dedicated to my wonderful family. To my mother, Dr. Carolyn MacDonald, for always believing in me, and never losing hope… even when I did. To my father, Dr. Kelly T. McKee Jr., for always pushing me to be my best self and never letting me give up. To my sisters, Dr. Jocelyn Sweeney, Kelly E. McKee, and Kathleen Ramsey, for their positive attitudes and support throughout all of these years. Finally, to my niece and nephew, Anlyn and Jack Sweeney, for reminding me that it is ok if my data are not significant… because I already have the most important things in my life.

ACKNOWLEDGMENTS

I would like to thank Dr. Yvette Huet for all of the guidance, support, and knowledge that she has given me over the course of this project. She did not have to take me on as a student, but she took a chance on me, and because of her, I have become more than I ever thought possible. She helped me become my best self. I will forever be grateful for the opportunity that she has given me.

I would also like to thank my co-advisor, Dr. James Oliver. He has provided me with more knowledge than I could possibly ever realize. He pushed me to be the strongest version of myself, and that is what pushed me to succeed.

I wish to thank my other committee member, Dr. Jennifer Warner, for her encouragement throughout the last year, as well as for all of the time that she has put into helping me, not only as a member of my committee, but also as a teacher. I would also like to extend my gratitude to all of those who aided me and contributed to my project: Dr. Larry Leamy for all of his critical assistance with the statistical analysis; Rachel Gruner, for all of her hard work helping in the lab; and Alex Huet-Hudson, for all of her help with the gathering and examining of the rat vaginal cytology. I could not have completed this without you all.

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INTRODUCTION

It has been shown that men exhibit more susceptibility to infection (parasitic, bacterial, and viral) than women (3, 4, 12, 13, 24, 30). The reasons underlying this disparity are not well understood, but it has been demonstrated that responses to sex gonadal hormones, more specifically 17β- Estradiol- (E_2), Progesterone (P_4), and Testosterone (T_4), are contributing factors. All of these hormones differentially affect immune function. T_4 and P_4 can depress many steps in immune reactions, including the innate immune response of macrophages via expression of Toll-Like receptor 4 (TLR-4) (38, 45) and a reduction in production of pro-inflammatory cytokines, such as IL-6, NF-κB, IL-1β (45, 17, 15, 38). P_4 further expresses immunosuppression by inducing the development of anti-inflammatory cytokines, including IL-4 and IL-5 (35). While these two hormones have been shown to act as immunosuppressants (13, 24, 38), E2 has been found to have many protective effects by encouraging a higher level of immunological response (13, 24). E_2 has been shown to inhibit endotoxin-induced stimulation of cytokines (43), induce the expression of proinflammatory cytokines such as IL-1, IL-6, and TNF-α (9), and increase natural killer cell activity (42). It has been shown that E_2 is neuro-protective against viral and bacterial pathogens by moderating pro-inflammatory signaling in various regions of the brain (43). E_2 also safeguards against vascular disease by modulating nitric oxide production in humans and increasing vasodilation (11). It is important to note that while numerous studies have demonstrated the immunomodulation induced by these sex steroid hormones, the relative influence of each hormone is case dependent. The anti-inflammatory response of P_4 has been shown to be beneficial in response to injury (most notably, in neural trauma), by reducing cerebral edema (16), and

even aiding in re-mylenation of spinal nerves (46). However, the immunological suppression induced by this hormone can also alter host susceptibility to infectious diseases such Herpes Simplex Virus -2 (21) and *Chlamydia trachomatis* (22). Similar alterations in disease vulnerability are seen in response to T_4 as well. There are certain infections, such as shistosomiasis (caused by *Schistosoma mansoni*) in which T_4 appears to have a moderating effect on the proliferation or inhibition of disease progression (30). In contrast, it has also been shown that the same "protective" effects of E_2 may also impair certain aspects of immune function, such as inhibition of suppressor T-cells (34), production of excessive pro-inflammatory responses (13), and suppression of certain cytokine gene expression (43). Moreover, high doses of both T_4 and E_2 are toxic to lymphocytes (34).

Certain sex hormones can also have direct effects on bacterial metabolism, regulating growth, or expression of virulence factors (13). E_2 has been shown to encourage growth and proliferation of *Chlamydia trachomatis* by encouraging adherence of the elementary body form of the bacteria in the genital tract (6). There is also evidence to suggest that this hormone encourages the up-regulation of the *trpB* gene, allowing for the persistence of this bacterium in the viable but non-culturable state (2). P_4 has been shown to suppress the ability of *Helicobacter pylori* to absorb free cholesterol, thus inducing a bactericidal effect (19). *Bacteroides melaniogenicus* will use this hormone as a growth factor in place of certain vitamins (25). This hormone can also modulate the growth (both positively and negatively) of certain *Neisseria* species, depending on the type of medium used for incubation (29, 10).

The modulatory effects of hormones on the immune response as well as bacterial

metabolism suggests that the presence or absence of these steroid hormones may be used to explain the sex-based disparity of infection rates seen in certain illnesses. Previous studies have also supported this hypothesis by examining the response *in vivo* of sex steroids on bacterial lipopolysaccharide (LPS)-induced endotoxic shock. One study by Merkel et al. concluded that E_2 may impart a relative resistance to a particular sexually dimorphic disease initiated by *Vibrio vulnificus*. In this study, it was shown that exposure to E_2, endogenously or exogenously, significantly reduced the rate of death in rats due to the endotoxic shock produced in response to the LPS of this bacterium (28). This suggests an immunoprotective effect of E_2 as a contributor to the sex-differences seen in the infection rate of *V. vulnificus.*

Vibrio vulnificus is a gram-negative bacterium found in estuarine waters and the normal flora of many bivalve mollusks and other shellfish (32). This bacterium may be introduced into the human body via ingestion of undercooked shellfish or through an open wound, where it may present as an infection. Infection by *V. vulnificus* is responsible for approximately 95% of seafood related deaths (33). This bacterium can proliferate in the human body and cause a number of clinical manifestations, including gastroenteritis, primary septicemia, and purulent wound infection (32). These manifestations carry varying degrees of severity, the most critical being primary septicemia, which results in a 50% fatality rate (33).

Vibrio vulnificus can be sorted taxonomically into separate biotypes (1, 2, and 3) based on varying phenotypic factors (20, 32). Biotype 1 can further be classified into two genetically distinct groups, C-Genotype and E-Genotype, which typically correlate with the source of the isolate- Clinical (C stain) and Environmental (E strain) (20, 39).

Although the C-types are most often associated with human infections, all putative virulence factors are found in both genotypes (20). These virulence factors include, but are not limited to: extracellular proteases, which may cause tissue necrosis and lesions (20), LPS, a pyrogenic molecule that elicits endotoxic shock (20), and an iron acquisition system, which uses siderophores to extract iron from the host (41, 48). It has been determined that the amount of iron in a sample can dramatically increase host susceptibility to this organism (41). The exact mechanism responsible for this typical discrepancy in virulence between these two genotypes is still poorly understood.

As mentioned previously, one unique observation associated with human infection by this organism is the high level of sexual dimorphism. About 85% of those infected have been males, most over the age of 40, and most with underlying liver disease (28, 32, 33). While some may argue that there is a potential gender bias toward salt-water activities (e.g., fishing, recreation) such that there is a male predilection for these activities (and thus exposure to the pathogen), this would not explain the high rate of gender bias in disease associated with the ingestion of infected bivalves. While recent data do indicate that more men eat raw seafood (including oysters) than women, this may or may not correlate with the 85% of males that die from endotoxic shock (1, 40).

Women have higher levels of endogenous E_2 than men, and men have relatively higher levels of endogenous T_4 than women. This results in differences in sex hormone ratios in males and females. In both sexes, the levels of these primary steroid hormones decrease with age (12). Interestingly, the level of bioavailable E_2 in post-menopausal women is typically lower than that of elderly men (23). While E_2 and T_4 are considered the primary sex hormones, and thus hormones of interest in sexually dimorphic disease,

other hormones must also be considered: e.g. follicle stimulating hormone (FSH) and luteinizing hormone (LH) etc. In women, these hormone levels are variable and dependent on the timing of the stage of menstrual cycle (44).

Interestingly, to date, no study has been done to assess the relationship between varying levels and interactions of reproductive hormones and the propagation of the bacterium, *V. vulnificus*, in serum. While the definition of septicemia describes any microbial infection in a normally sterile site, there is no general quantification for this manifestation (26) – a person could have only a few hundred or millions of bacteria in the bloodstream, but both cases would still be considered “septicemic” under this umbrella term. The number of bacteria able to survive in the bloodstream could be critical in determining mortality rate. If the serum of males is able to support a higher level of bacterial propagation, this could be important in demonstrating a possible hormonal basis as a contributor to the observed gender disparity in this deadly disease, and would allow for the testing of the following hypothesis: *The production and conditioning effects of* E_2*,* P_4*, and* T_4 *allow for proliferation of the opportunistic pathogen, Vibrio vulnificus, in human blood.*

OBJECTIVES

The aims of this project were:

1. To determine if the sexual disparities seen in the development of endotoxic shock caused by the bacterium *Vibrio vulnificus* are due to the indirect ability of sera from males and females to support bacterial survival.
    a. The presence of endogenous hormones may alter certain aspects of the content of male and female sera (e.g. levels of protein, nutrient, antibodies, antigens, electrolytes, etc.), which may effect the survival of this bacterium in this environment.
2. To determine if said sexual disparities are due to an indirect effect of relative ratios of endogenous sex hormones in sera to allow bacterial survival based on varying hormone levels induced by the estrus cycle.
    a. Varying levels of endogenous steroid hormones may have an additive or synergistic effect, influencing the content of the sera to enhance or diminish bacterial survival.
3. To determine if there is a direct effect of particular exogenous steroid hormones and the encouragement/inhibition of bacterial survival in sera.
    a. Exogenous hormones themselves may have an effect on certain aspects of bacterial metabolism, allowing for survival and proliferation, or producing a bactericidal effect.
4. To determine if there is a direct relationship between relative ratios of exogenous sex steroid hormones and the encouragement/inhibition of bacterial survival in sera.

a. An ideal ratio of exogenous hormones may have an effect on of bacterial metabolism, as host environments have more than on steroid hormone present.

In looking at these particular objectives, I was able to determine the primary objective of this thesis:

To determine if the presence of sex steroid hormones increases host susceptibility to septicemic infections by the organism *Vibrio vulnificus,* by allowing the proliferation of this bacterium in rat sera, a model for the human bloodstream.

MATERIALS AND METHODS

1. Animals: The animal model used in these experiments was the rat for the following reasons:

    a. Rats exhibit a gonadal hormone profile similar to that of humans (43).

    b. Sprague Dawley (SD) rats have been shown to have a sexually dimorphic response to endotoxin similar to that of humans (app. 80% of males vs. 20% of females die from *Vibrio vulnificus* endotoxic shock in both rats and humans (27).

    c. Rats provide a larger amount of serum per animal (compared to other laboratory rodents) because of their size and thus limited the total number of animals required the experiments.

    12- Female Sprague Dawley Rats (Charles River, Wilmington, MA) were used (110 to 150 grams). These rats were acclimated to the Vivarium for 9 days prior to use.

2. Estrous Cycle Stage Determination: The main stages of the estrous cycle of rats (diestrus, proestrus, and estrus) may be determined by the physical appearance of the vaginal epithelial cell types: diestrus manifests with small leukocytes, proestrus has nucleated epithelial cells, and estrus occurs with anucleated, cornified epithelial cells.

    In order to perform cytological examination, cells were obtained by lavage of the vaginal canal of the rat with approximately 100-200 microliters of saline (27). The samples were viewed under the dissecting microscope (70x magnification) for cell type identification.

3. Serum Sample Collection: Rat blood serum was acquired in several different ways:
    a. After two weeks of monitoring the estrous cycle in the SD rats, normally cycling rats were sacrificed in estrus, diestrus-1, or proestrus, and their blood acquired via exsanguination under deep isoflurane anesthesia (UNC Charlotte IACUC approved protocol). The blood samples were spun at 14,000 rpm for 12 minutes, and the serum collected and stored at -20.° C.
    b. Serum from ovariectomized females and orchidectomized males was purchased from Charles River (Wilmington, MA).
    c. Serum from normal female and male rats (separated and pooled) was purchased from Innovative Research (Novi, MI).
4. Hormone Quantification:

    Levels of T_4 and E_2 were measured in each serum samples from the cycling rats using ELISA kits purchased from Cayman Chemical (Ann Arbor, Michigan).
5. Iron Quantification:

    Iron levels in blood obtained from rats in different stages of the estrous cycle, as well as the gonadectomized male and female rat sera, were quantified using a colorimetric assay (Biovison, Milpitas, CA), and statistical analysis of growth curves utilized these data for normalization.
6. Bacterial Preparation: One strain of *Vibrio vulnificus* was used for this experiment:

a. C-Strain: C7184 (obtained from the laboratory of James D. Oliver). Bacteria was grown overnight from a frozen stock culture in 3 ml Heart Infusion (HI) broth at room temperature (21° C). The cells were then be spun down at 6600 rpm/10 minutes, and the supernatant removed. These cells were washed in 10 PPT Artificial Sea Water (ASW) to remove nutrients, and respun down at the same speed. The supernatant was removed, and the cells were then resuspended in 1 mL of ASW. Approximately 10^{5-7} cells in this culture were transferred into 3 ml of 10 PPT ASW, and untended for ~48 hours to induce a state of nutrient deprivation.

7. Spot Plate Analysis: Each culture was grown on a spot plate in order to determine bacterial survival rates. This was done at different time points, using HI agar (13).

   a. Hormone Stripping of Serum: The various types of rat sera were stripped of steroid hormones by a modified dextran-coated charcoal solution (DCS) methodology (18). A DSC solution was made using 5g activated charcoal, 0.5 g dextran, 0.44g NaCl, and 50mL of ddH_2O. This was added in a 1:20 ratio to thawed rat sera and incubated at 55 °C for 30 minutes. The solution was then centrifuged at 180xg for 5 minutes, and the supernatant was moved to clean conical tubes. The previous step was repeated on the supernatant to further separate the heavier charcoal pieces. The remaining supernatant was filtered using a 0.2µm filter.

8. Hormone Preparation/Addition: T_4, P_4, and E_2 hormones were purchased from Sigma Chemical Company (St. Louis, MO). Solutions of these hormones were

prepared at various concentrations. All steroid hormones were initially prepared in 100% ethanol. Final concentrations were:

a. Progesterone- 5µg P_4/2.5µl ethanol

b. Testosterone- 65ng T_4/5µl ethanol

c. Estradiol- 60pg E_2/5µl ethanol

9. Testing the indirect effects of sex hormones on bacterial proliferation in serum:

   a. 5 µl of the prepared bacterial culture was added to 500 µl (1:100 ratio) of each sera collected from rats at the different stages of the estrous cycle (proestrus, diestrus, and estrus). These were then incubated at 37° C and bacterial survival quantified using individual serial dilution spot plates at four time points (0 hours, 30 minutes, 2 hours, and 6 hours). This experiment was repeated four times for replication.

   b. 50 µl of the prepared bacterial culture was added to 500 µl (1:10 ratio) of the purchased normal male and female rat sera. These were then incubated at 21° C and bacterial survival quantified using a serial dilution spot plates at two time points (0 hours and 24 hours). This experiment was repeated three times for replication.

10. Testing the direct effects of sex hormones on bacterial proliferation in serum:

   a. 50 µl of the prepared (as described in part 6) bacterial culture was added to 500 µl (1:10 ratio) of the purchased, hormone-stripped (as described in part 8), sera of ovariectomized females and orchidectomized males. 5µl E_2 and T_4 solutions (made as described in part 9) were added to each aliquot in the following ratios: 1) 75%- T_4, 25%- E_2; 2) 25%- T_4, 75%-E_2; 3)

50%- T_4, 50%- E_2; 4) 0%-T_4, 0%-E_2 (control- ethanol only). These were then incubated at 21° C and bacterial survival quantified using serial dilution spot plates at four time points (0 hours, 30 minutes, 2 hours, and 6 hours). This experiment was repeated three times in the female sera, and twice in the male sera.

b. 50 µl of the prepared bacterial culture was added to 500 µl (1:10 ratio) of the purchased, pooled, hormone stripped (as described in part 8) rat sera. 5µl of P_4 solution (made as described in part 9) was added to these aliquots, or 5µl of ethanol (control). These were then incubated at 21° C and bacterial survival quantified using a serial dilution spot plates at two time points (0 hours and 24 hours). This experiment was repeated three times for replication

c. 50 µl of the prepared bacterial culture was added to 500 µl (1:10 ratio) of the purchased, hormone stripped (as described in part 8) sera of the ovariectomized female and orchidectomized male rats. 5µl of E_2 solution (made as described in part 9) were added to these aliquots, or 5µl of ethanol (control). These were then incubated at 21° C and bacterial survival quantified using serial dilution spot plates at two time points (0 hours and 24 hours). This experiment was repeated three times for replication

d. 50 µl of the prepared bacterial culture was added to 500 µl (1:10 ratio) of the purchased, hormone stripped (as described in part 8), purchased, sera of the ovariectomized female and orchidectomized male rats. 5µl of

T_4 solution (made as described in part 9) were added to these aliquots, or 5μl of ethanol (control). These were then incubated at 21° C and bacterial survival quantified using serial dilution spot plates at two time points (0 hours and 24 hours). This experiment was repeated three times for replication

e. 50 μl of the prepared bacterial culture was added to 500 μl (1:10 ratio) of the normal, purchased, pooled, rat sera and hormone stripped (as described in part 8), pooled, rat sera. These were then incubated at 21° C and bacterial survival quantified using serial dilution spot plates at two time points (0 hours and 24 hours). This experiment was repeated three times for replication.

11. Statistical Analysis:

Analysis of all data was done using a one, two, or three-way ANOVA. Tukey's multiple comparison test was also performed on all data.

RESULTS

Overall iron concentrations in the ovariectomized female, orchidectomized male, and cycling female rat sera were quantified using a colorimetric assay. The orchidectomized male sera had higher amounts of iron than that of the ovariectomized females. Iron concentrations in the sera of the cycling female rats were all similar (Tables 1 and 2).

To determine the differential, indirect ability of sex steroids from males and females to support bacterial survival in sera, *V. vulnificus* was added directly to aliquots of normal male and female rat sera. Bacterial cell counts were assessed at 0 hours (at inoculation dose) and at 24 hours by dilution spot plating method. There was no variation of survival based on source, males vs. females, on *V. vulnificus* (Figure 1).

The sera of ovariectomized female rats and orchidectomized male rats were stripped of residual sex hormones, and bacteria were added to each of these sera. Bacterial cell counts were assessed at 0 hours and at 24 hours by dilution spot plating method. Significance was seen with time ($P<0.05$), however no statistical significance was observed between treatment groups (Figure 2). This suggests that there are no indirect effects of steroid hormones (e.g. immune responses) in sera that alter *V. vulnificus* proliferation.

To determine if sex steroids directly alter viability of bacteria in sera, the stripped sera from the ovariectomized female and orchidectomized male rats were treated with various doses of hormone solutions. *V. vulnificus* was added to aliquots of the serum of the ovariectomized female rats. The serum was then inoculated with the 100% E_2 solution (60pg-E_2/5µlEthanol) and bacterial cell counts were assessed at 0 and at 24 hours using

dilution spot plating method. Survival rates were statistically significant over time ($P<0.05$), but there was no effect due to the addition of this hormone (Figure 3). This experiment was repeated using the serum of the orchidectomized male, and again, there was no statistical difference observed due to hormone exposure (Figure 4).

To determine if T_4 had a direct effect on bacterial proliferation, *V. vulnificus* was again added to the serum of the ovariectomized female rat to which the 100% T_4 solution (60ng-T_4/5µlEthanol) was added. Bacterial cell counts were assessed at 0 and at 24 hours using dilution spot plating method. Statistical significance was observed with time ($P<0.05$) only. (Figure 5). This experiment was repeated using the serum of the orchidectomized male, with similar results (Figure 6).

The direct effect of the steroid hormone P_4 was also investigated. The sera of pooled (male and female) rats were stripped of endogenous hormones. The P_4 solution (5µg-P_4/2.5µl-ethanol) was added to this sera along with *V. vulnificus.* Bacterial cell counts were assessed at 0 and at 24 hours using dilution spot plating method. P_4 did not alter survival rates (Figure 7).

To determine if there is a direct relationship of sex on bacterial viability at earlier time points, the 100% E_2 and 100% T_4 solutions were again (separately) added to the sera of ovariectomized female and orchidectomized male rats inoculated with *V. vulnificus*. Bacterial cell counts were assessed at 0, 0.5, 2, and 6 hours using spot plate dilutions. In the female sera, statistical significance in survival was observed over time ($P<0.05$) (Figure 8). In the male sera, no significance was observed (Figure 9). However, sex from which the sera was acquired did alter survival rates significantly ($P<0.05$) (Figure 10).

To determine if there is a direct effect of ratios of sex steroid hormones on bacterial survival, various ratios of E_2 and T_4 were added into the sera of the ovariectomized female and orchidectomized male rats inoculated with *V. vulnificus*. Bacterial cell counts were assessed at 0, 0.5, 2, and 6 hours using spot plate dilutions. In the female sera, statistical significance was seen over time ($P<0.05$), and treatment ($P<0.05$). Significance was observed specifically at the $25\%E_2:75\%T_4$ and the control at the 2-hour time point (Figure 11). In the male sera, no significant differences were observed (Figure 12). Analysis of all the data demonstrated a significant variation in sex ($P<0.0001$) and time ($P<0.0001$). A significant interaction was also observed between sex vs. time ($P<0.0001$), more specifically at the 0, 0.5, and 2 hour time points (Figure 13). These data were then transformed into percent survival and re-analyzed. This analysis revealed significance overall variations of sex ($P<0.005$), treatment ($P<0.005$), and time ($P<0.0001$), however, no significant interactions of any of these traits (Figure 14).

To determine the indirect relationship between endogenous sex steroid hormones and bacterial survival, bacteria were added to sera from live, female rats in estrus, proestrus, and diestrus. The sera from rats in a particular stage of the cycle were pooled. An EIA kit was used to determine the levels of endogenous E_2 in each aliquot and were within previously reported ranges (data not shown). Bacteria were added to each of these samples, and the bacterial cell counts were assessed (via dilution spot plate technique) over several time points (0, .5, 2, and 6 hours). No significant differences were found (Figure 15).

To determine the overall effect (direct and indirect) of endogenous steroid hormones and bacterial viability, the pooled sera of male and female rats was split into

two experimental groups: stripped of all endogenous hormones (via DCC) or control. Survival was observed at 0 hours (at inoculation dose) and at 24 hours by dilution spot plating method. Differences in survival were statistically significant were only over time ($P<0.05$), but there was no significant difference by treatment (Figure 16).

TABLE 1: Serum total iron concentrations of gonadectomized male and female rats

Serum Type	Iron concentration ng/µL
Ovariectomized Female 1	1.31
Ovariectomized Female 2	0.95
Orchidectomized Male 1	2.09
Orchidectomized Male 2	3.27

TABLE 2: Serum total iron concentrations of cycling female rats

Serum Type	Iron concentration ng/µL
Proestrus	1.23
Diestrus	1.37
Estrus	1.23

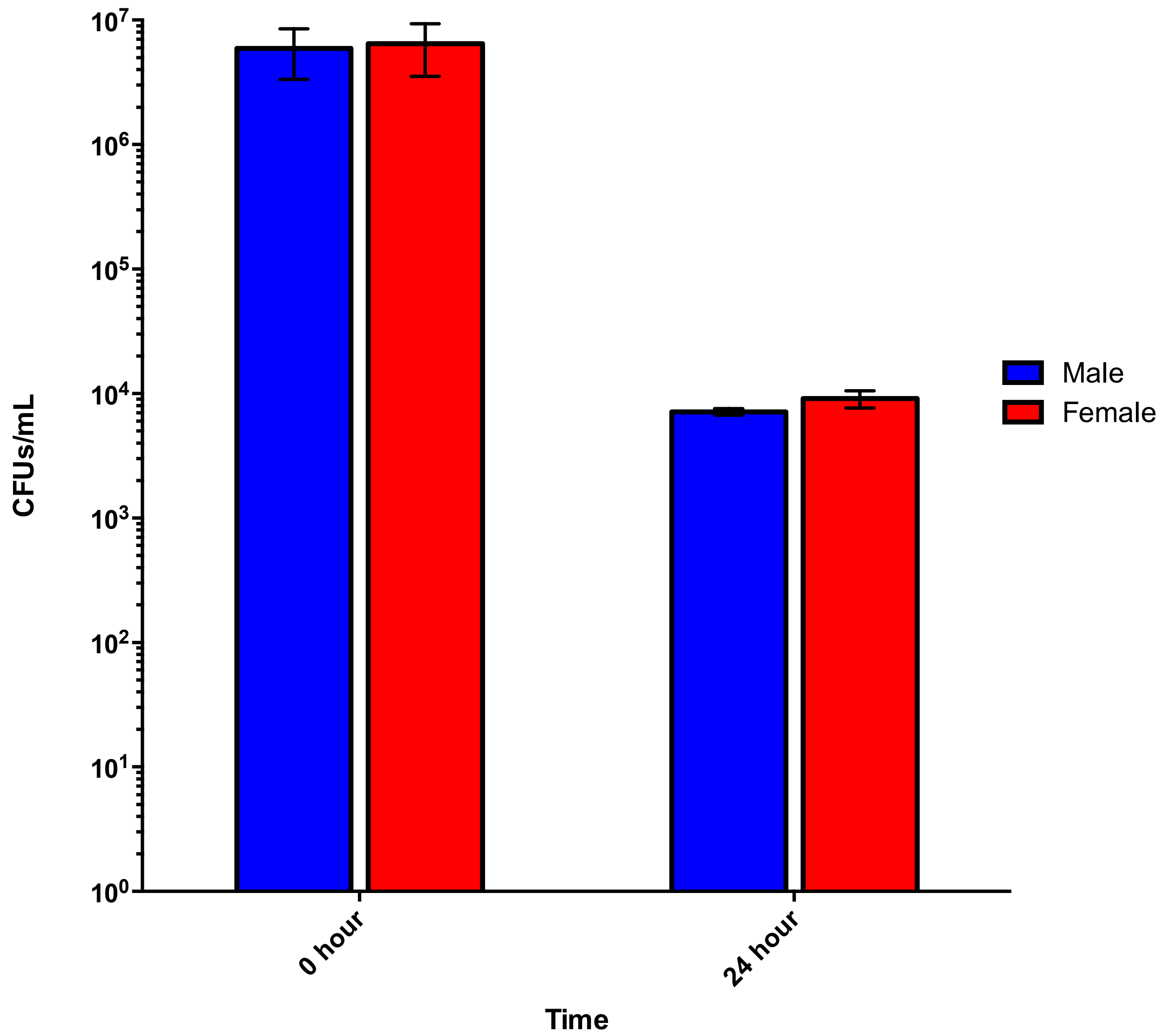

FIGURE 1: Survival of *V. vulnificus* in regular male and female rat serum at 0 hours (inoculation dose) and 24 hours. 2-way ANOVA was performed on the groups. No statistical significance was found ($p>0.05$).

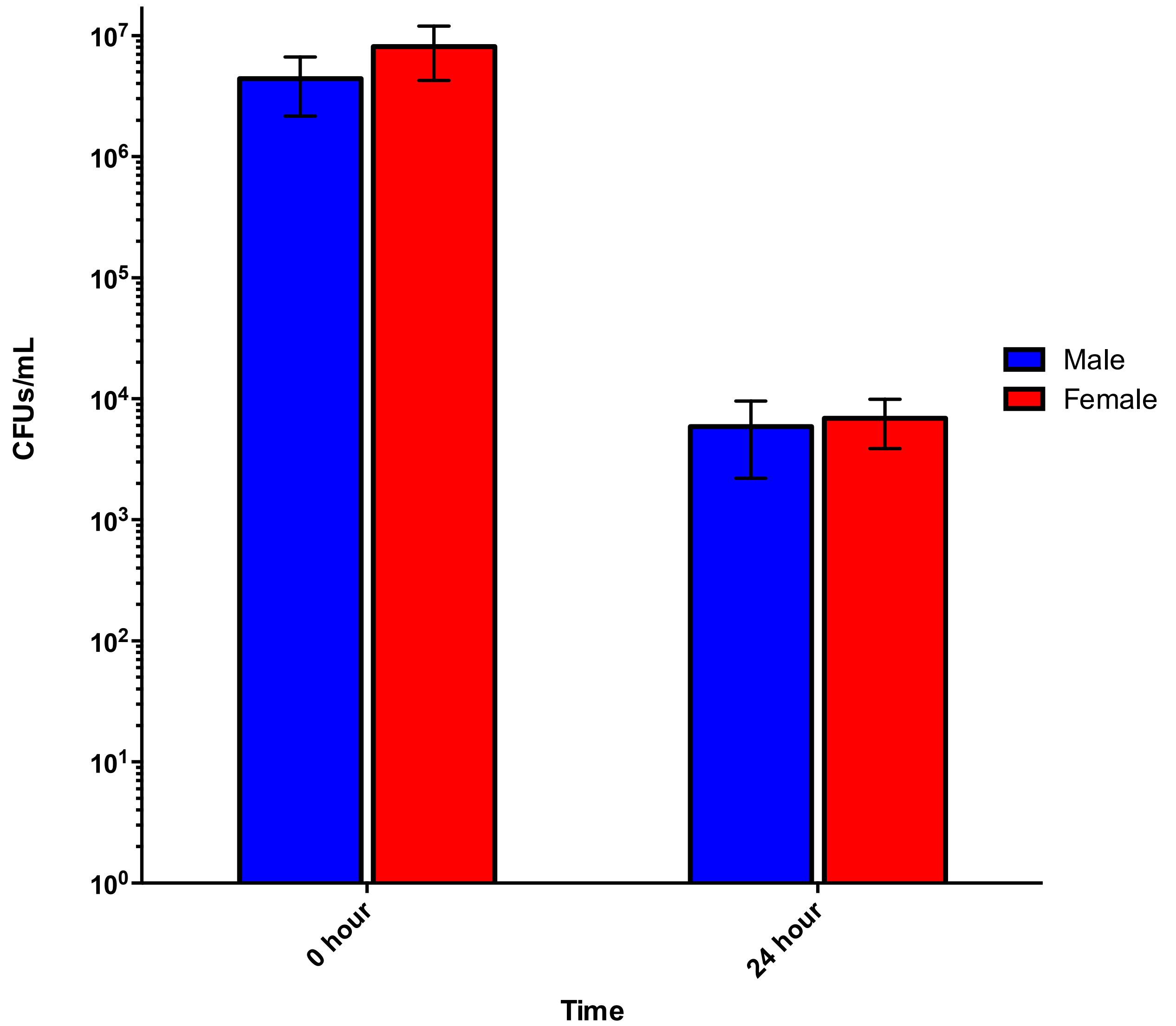

FIGURE 2: Survival of *V. vulnificus* in hormone-stripped, gonadectomized, male and female rat serum at 0 hours (inoculation does) and 24 hours. 2-way ANOVA was performed on the groups Statistical significance was only found in time variation * ($P<0.05$). No statistical significance between treatment, nor interaction was observed.

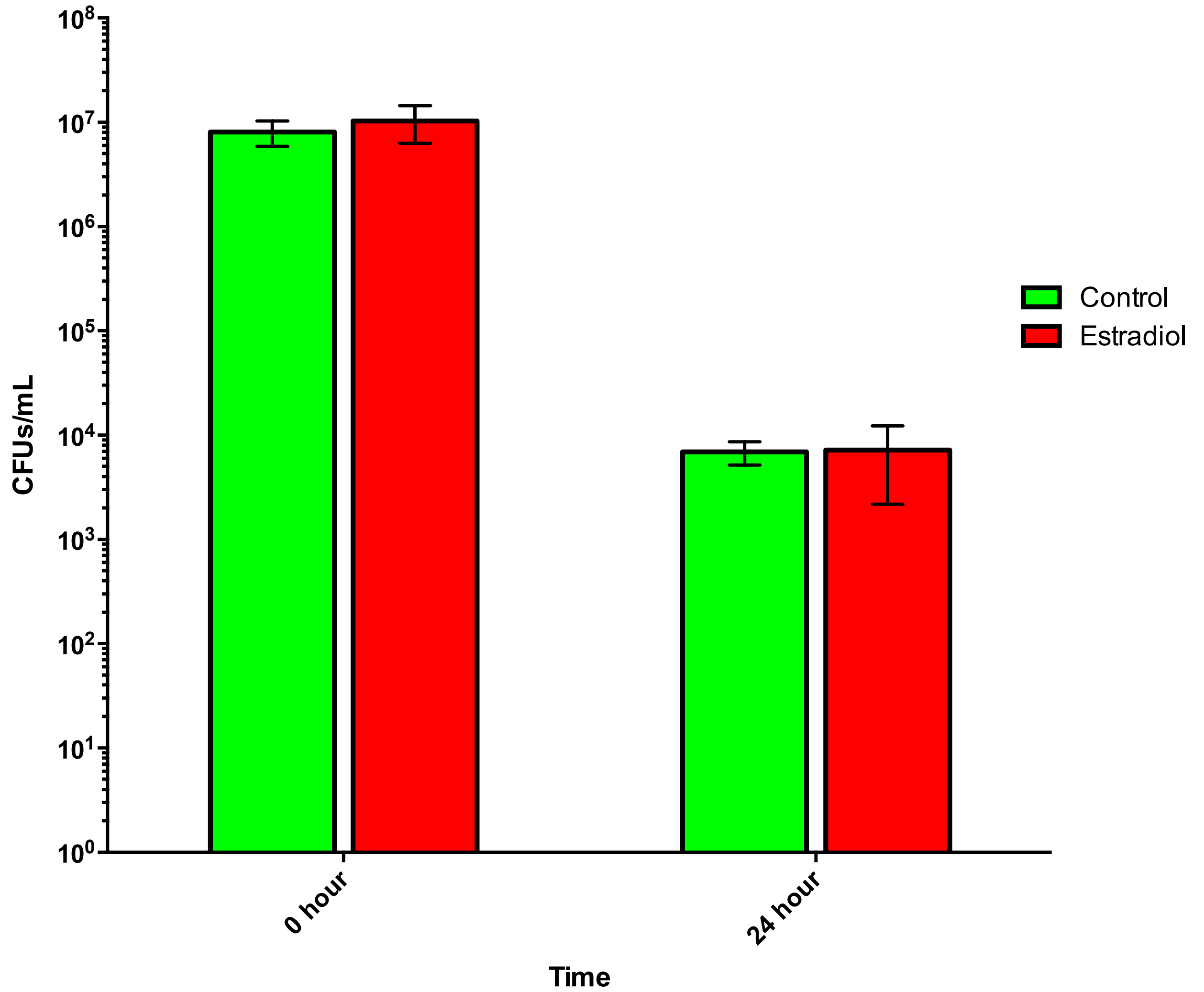

FIGURE 3: Survival of *V. vulnificus* in hormone-stripped, ovariectomized female rat serum -with and without treatment of exogenous E_2 at 0 hours (inoculation does) and 24 hours. 2-way ANOVA was performed on the groups. Statistical significance was only found in time variation * ($P<0.05$). No significance between treatment, nor interaction was observed.

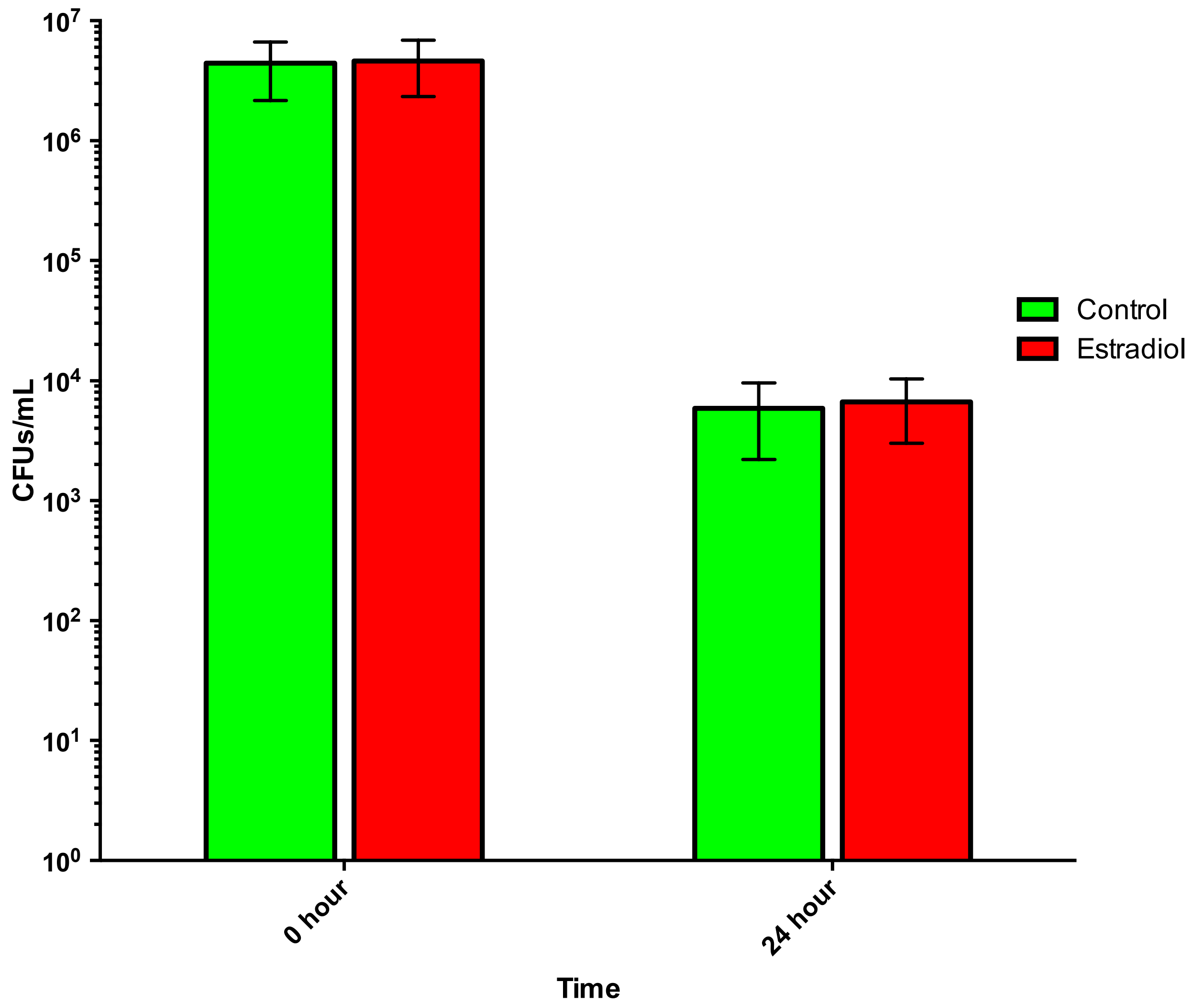

FIGURE 4: Survival of *V. vulnificus* in hormone-stripped, orchidectomized male rat serum -with and without treatment of exogenous E_2 at 0 hours (inoculation does) and 24 hours. 2-way ANOVA was performed on the groups. No statistical significance was found ($p>0.05$).

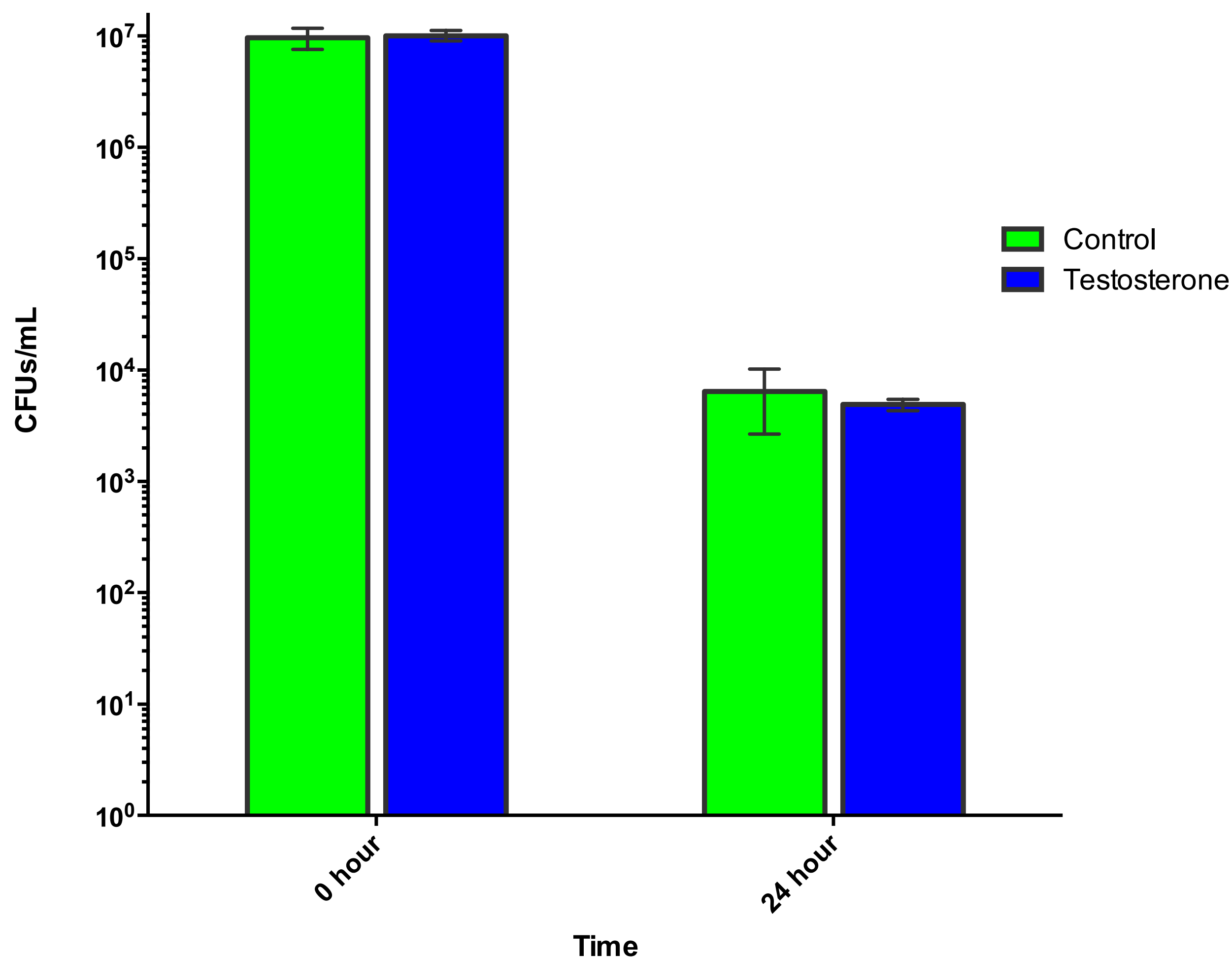

FIGURE 5: Survival of *V. vulnificus* in hormone-stripped, ovariectomized female rat serum -with and without treatment of exogenous T_4 at 0 hours (inoculation does) and 24 hours. 2-way ANOVA was performed on the groups. Statistical significance was only found in time variation * ($P<0.05$). No significance between treatment, nor interaction was observed.

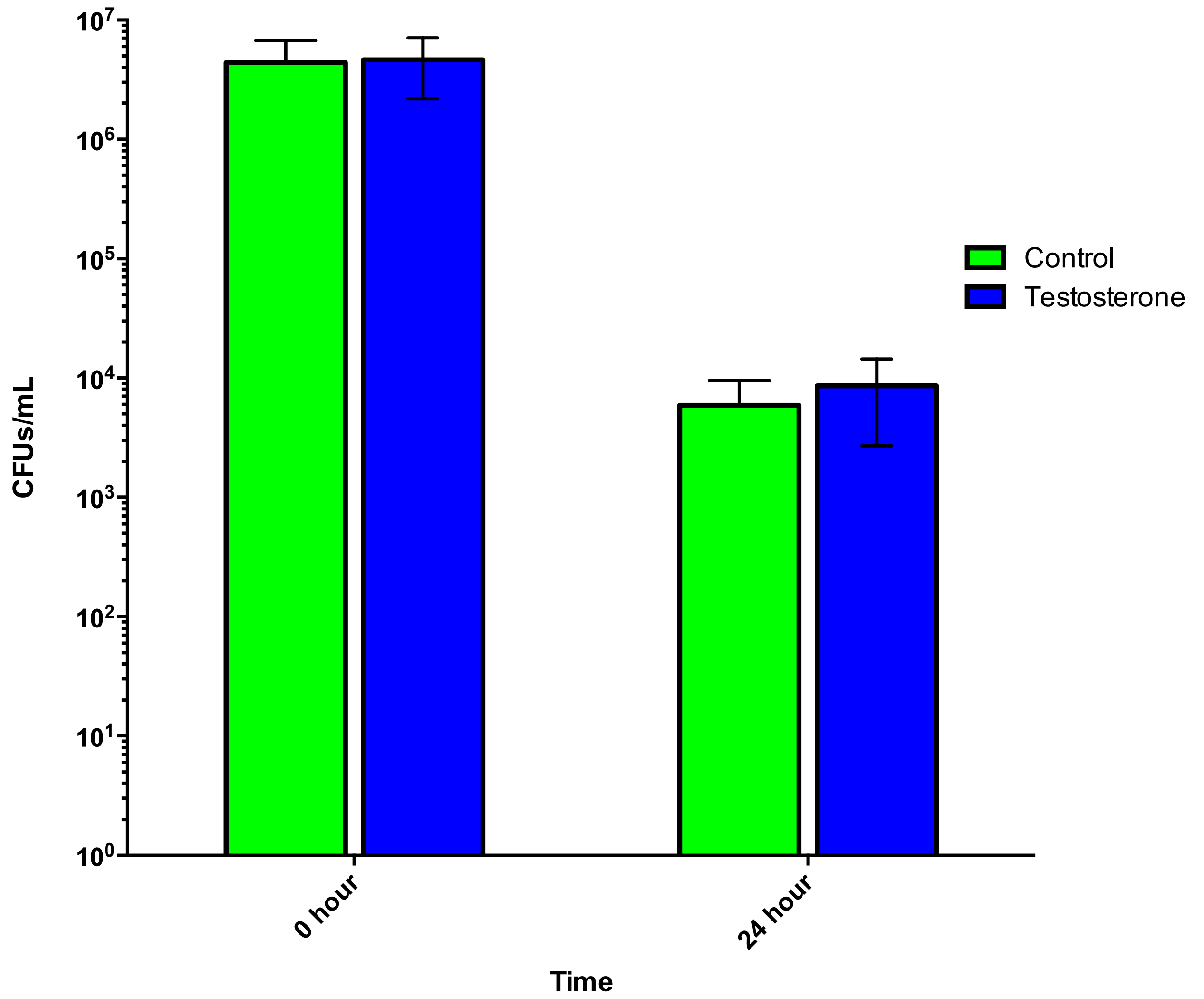

FIGURE 6: Survival of *V. vulnificus* in hormone-stripped, orchidectomized male rat serum -with and without treatment of exogenous T_4 at 0 hours (inoculation does) and 24 hours. 2-way ANOVA was performed on the groups. No statistical significance was found ($p>0.05$).

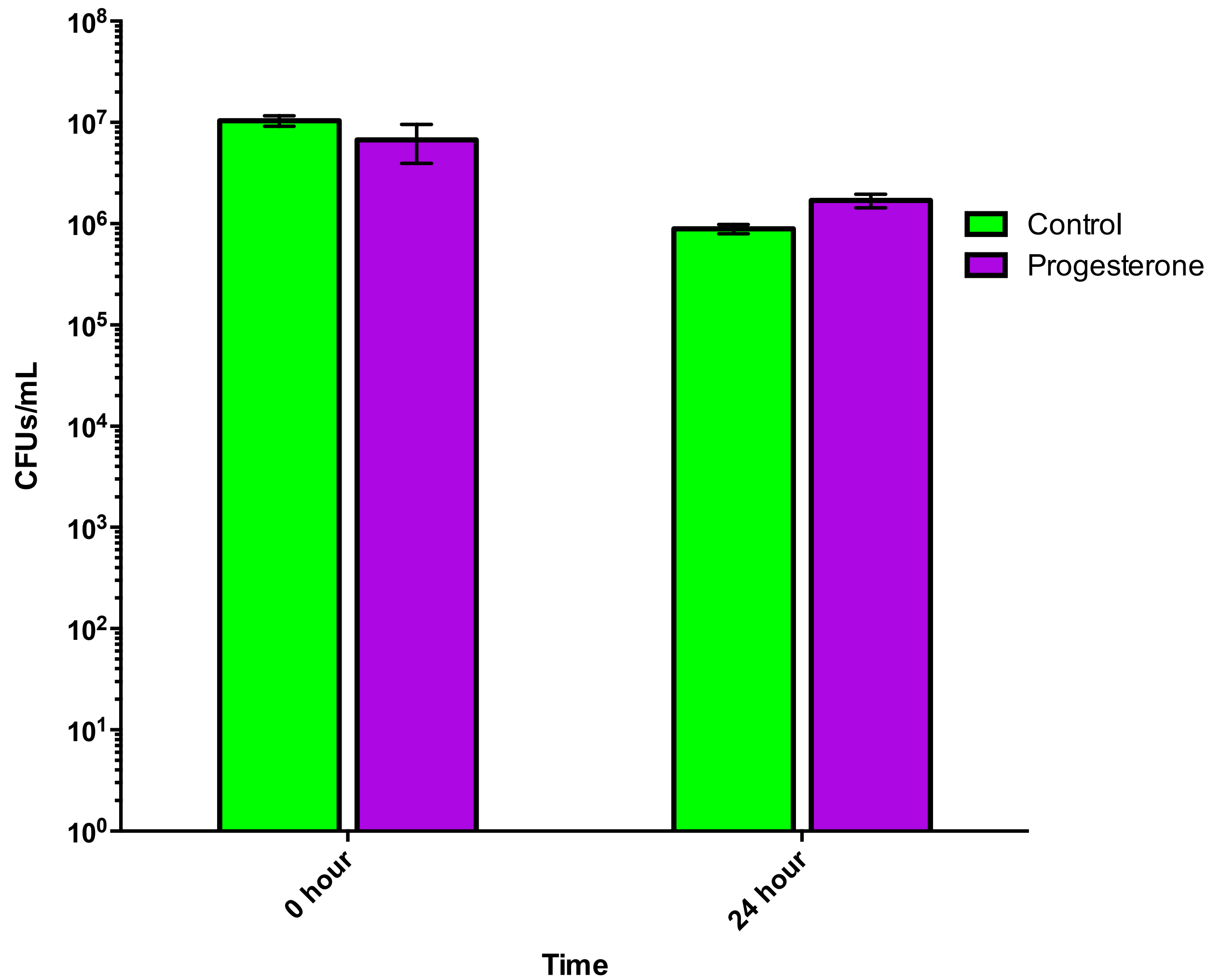

FIGURE 7: Survival of *V. vulnificus* in hormone-stripped, pooled (male and female) rat serum -with and without treatment of exogenous P_4 at 0 hours (inoculation does) and 24 hours. 2-way ANOVA was performed on the groups. No statistical significance was found ($p>0.05$).

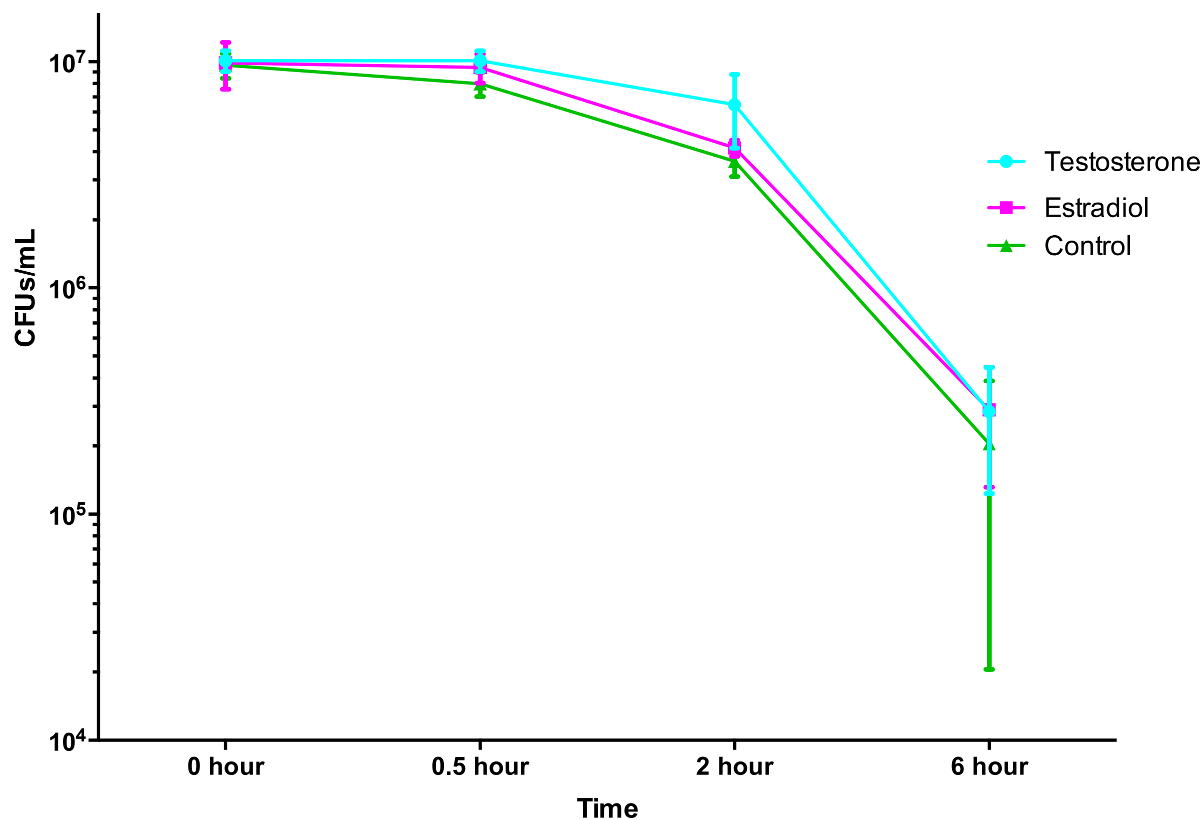

FIGURE 8: Survival of *V. vulnificus* in hormone-stripped, ovariectomized female rat serum -with and without treatment of exogenous T_4 and E_2 at various time points: 0 hours (inoculation does), 30 minutes, 2 hours, and 6 hours. 2-way ANOVA was performed on the groups. Statistical significance was only found with time *** ($P<0.05$). No significance between treatment, nor interaction was observed.

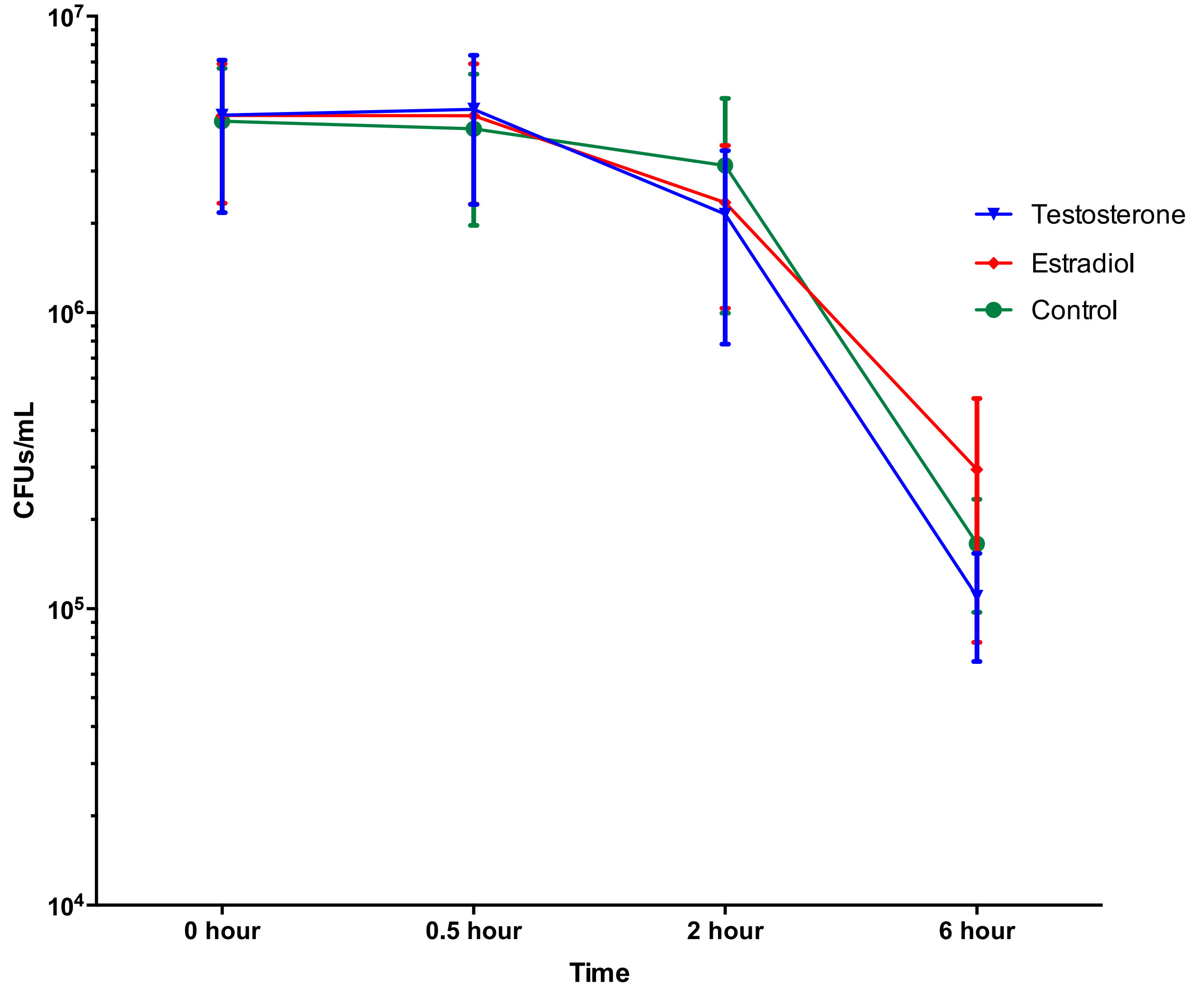

FIGURE 9: Survival of *V. vulnificus* in hormone-stripped, orchidectomized male rat serum -with and without treatment of exogenous T_4 and E_2 at various time points: 0 hours (inoculation does), 30 minutes, 2 hours, and 6 hours. 2-way ANOVA was performed on the groups. No statistical significance was found ($p>0.05$).

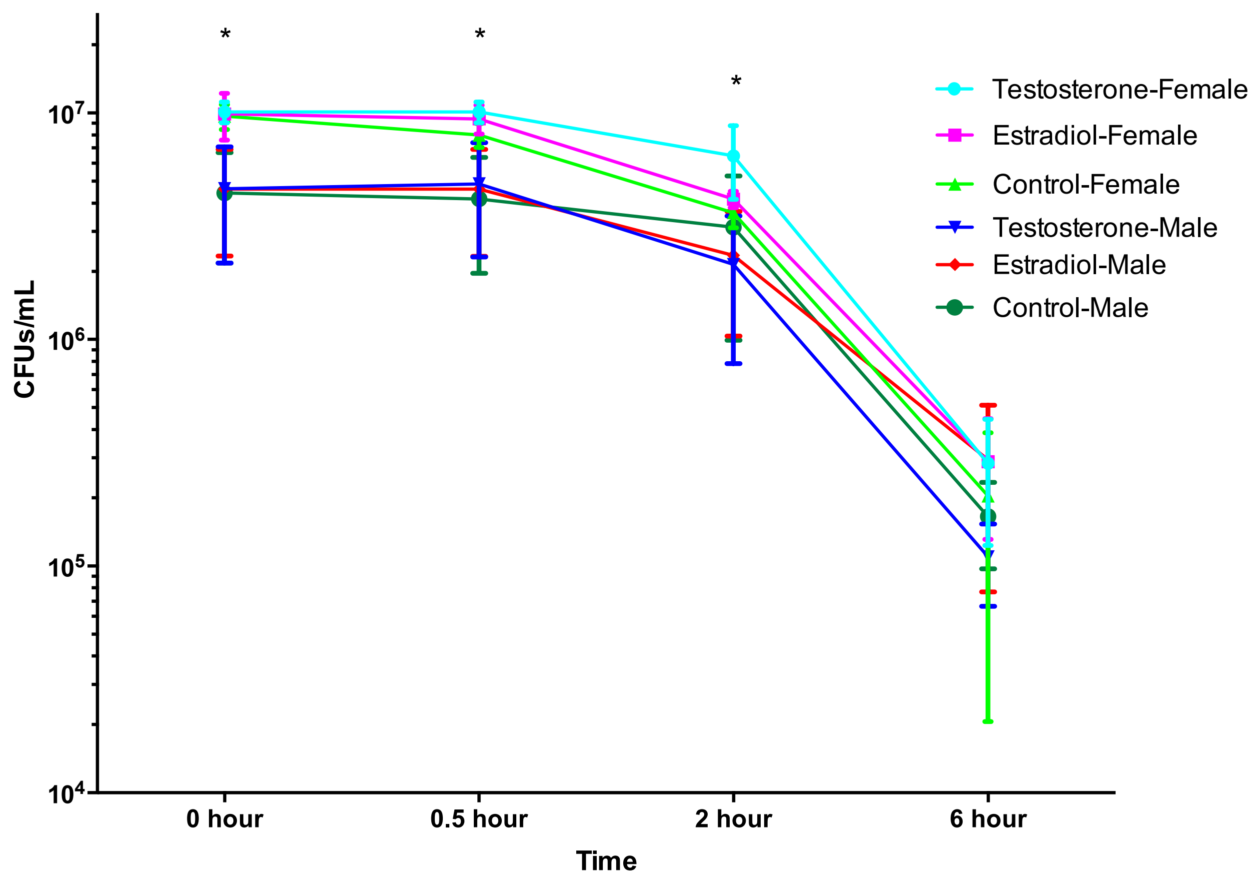

FIGURE 10: Survival of *V. vulnificus* in hormone-stripped, gonadectomized male and female rat serum -with and without treatment of exogenous T_4 and E_2 at various time points: 0 hours (inoculation does), 30 minutes, 2 hours, and 6 hours. Three-way ANOVA revealed significance in sex ($P<0.0001$) and time ($P<0.0001$), as well as a significant interaction between them ($P<0.05$). As the interaction is significant, the only point of relevance is the significance in that interaction. Tukey's post hoc test revealed that the time points of significant sex variation are 0, 0.5, and 2 hours.

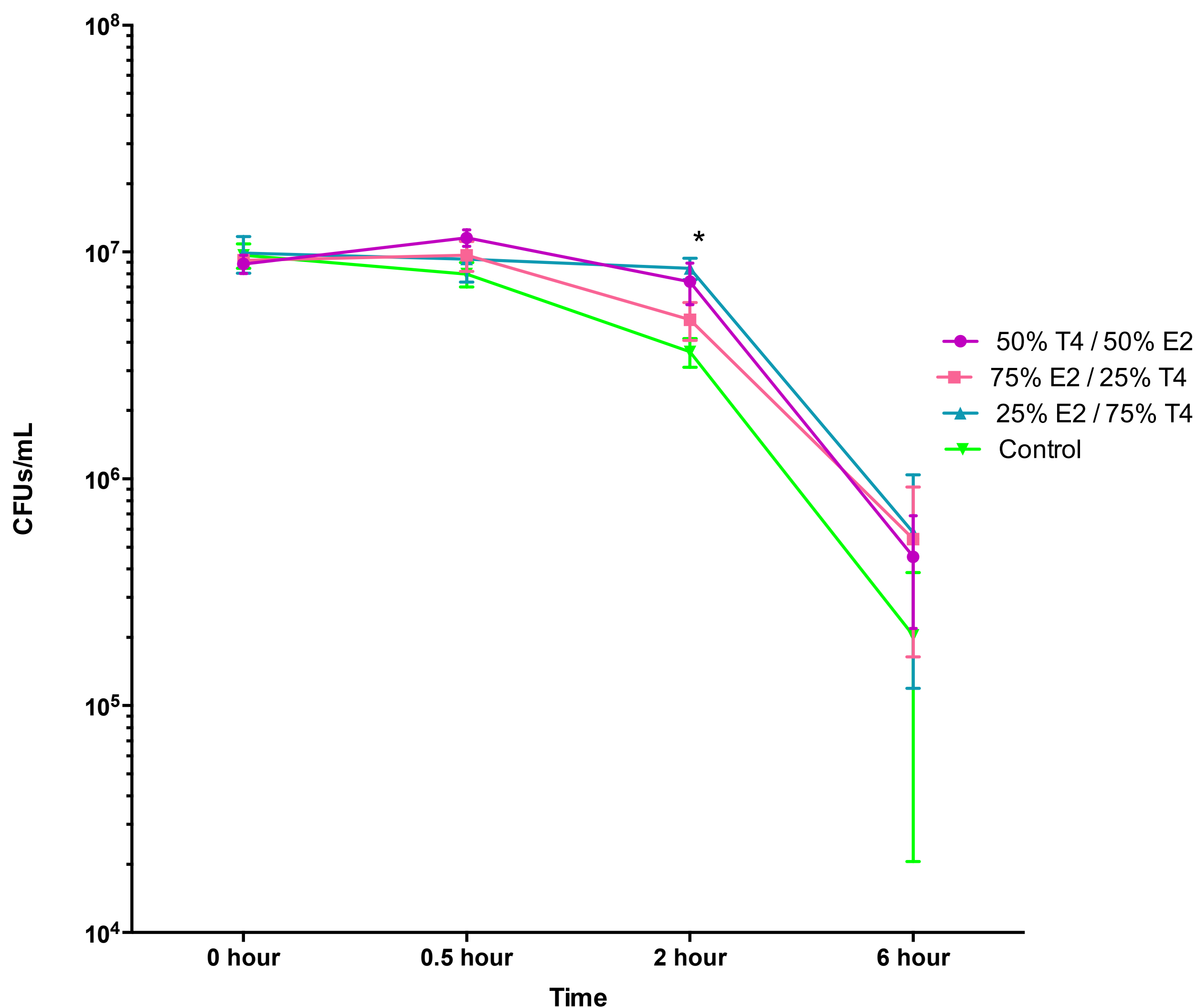

FIGURE 11: Survival of *V. vulnificus* in hormone-stripped, ovariectomized female rat serum -with and without treatment of varying ratios of exogenous T_4 and E_2 at various time points: 0 hours (inoculation does), 30 minutes, 2 hours, and 6 hours. 2-way ANOVA was performed on the groups. Statistical significance was seen in the variations of time ***($P<0.05$), and treatment *($P<0.05$). Tukey's Multiple Comparison revealed a significant difference between the $25\%E_2:75\%T_4$ and the Control at the 2-hour time point.

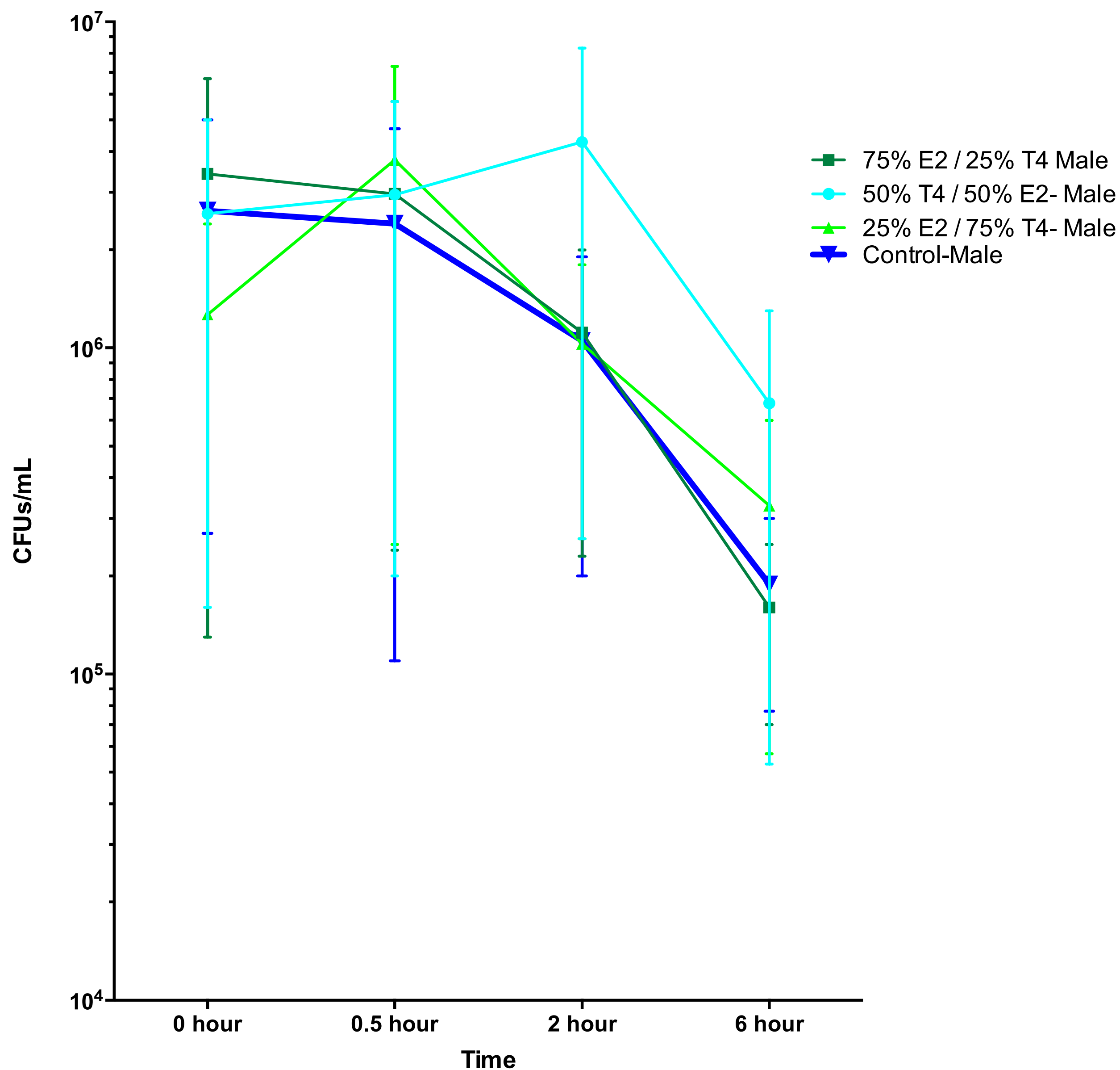

FIGURE 12: Survival of *V. vulnificus* in hormone-stripped, ovariectomized female rat serum -with and without treatment of varying ratios of exogenous T_4 and E_2 at various time points: 0 hours (inoculation does), 30 minutes, 2 hours, and 6 hours. 2-way ANOVA was performed on the groups. No statistical significance was found ($p>0.05$). Large variance in error bars is due to only having two replicates for this experiment.

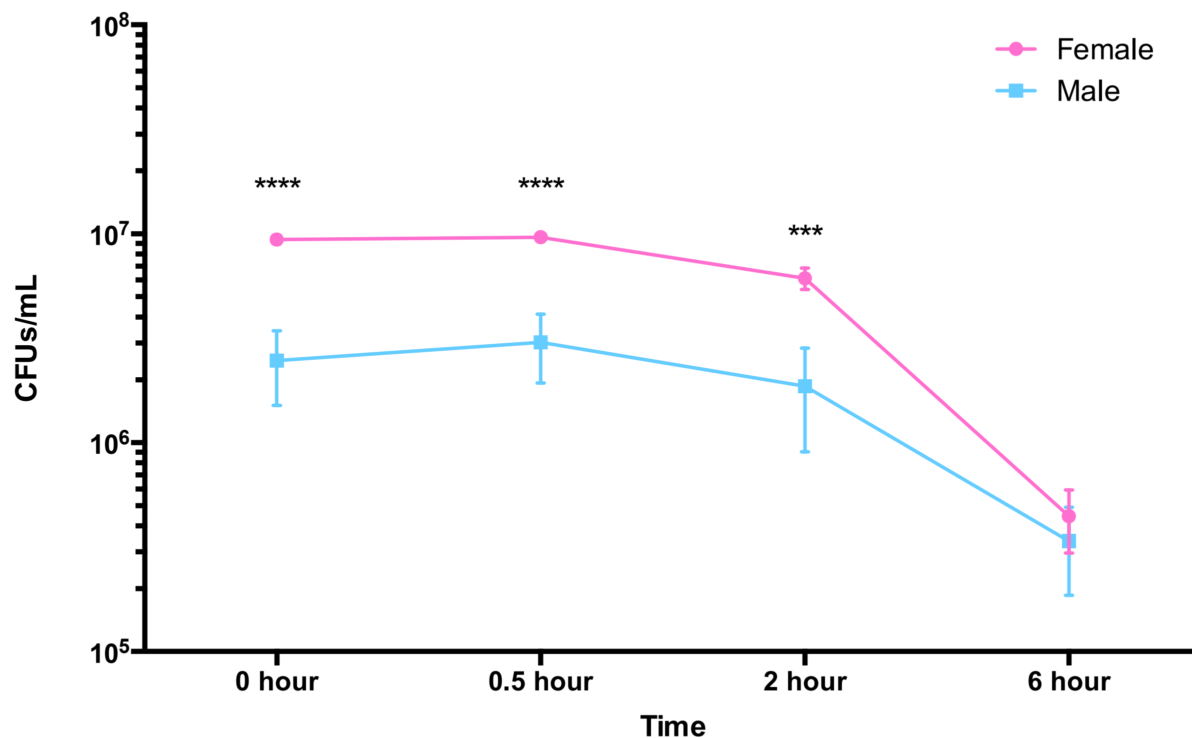

FIGURE 13: Survival of *V. vulnificus* in hormone-stripped, gonadectomized male and female rat serum -with and without treatment of varying ratios of exogenous T_4 and E_2 at various time points: 0 hours (inoculation), 30 minutes, 2 hours, and 6 hours. All treatment groups were pooled by sex. 2-way ANOVA was performed on the groups. Statistical significance was seen in sex ($P<0.0001$), and time ($P<0.0001$) along with an interaction of sex vs. time ($P<0.0001$). As the interaction between sex and time is significant, the individual variations are irrelevant. Tukey's Multiple Comparison revealed a significant difference between the sexes at the 0 hour, .5 hour, and 2 hour time points.

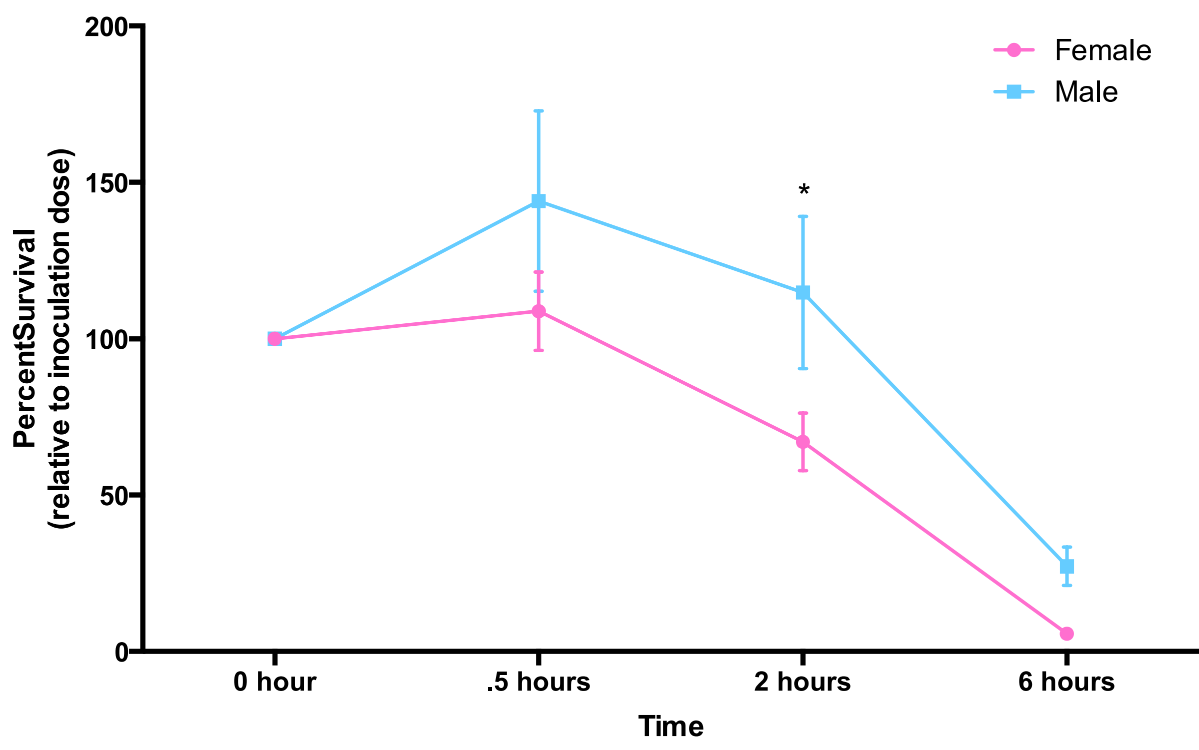

FIGURE 14: Percent survival of *V.vulnificus* in hormone-stripped, gonadectomized male and female rat serum -with and without treatment of varying ratios of exogenous T_4 and E_2 at various time points: 0 hours (inoculation), 30 minutes, 2 hours, and 6 hours. Two-way ANOVA was performed. Significance was seen in sex ($P<0.005$) and time ($P<0.0001$). No significant interactions were seen ($P>0.05$). Tukey's multiple comparisons test revealed significance at the two hour time point.

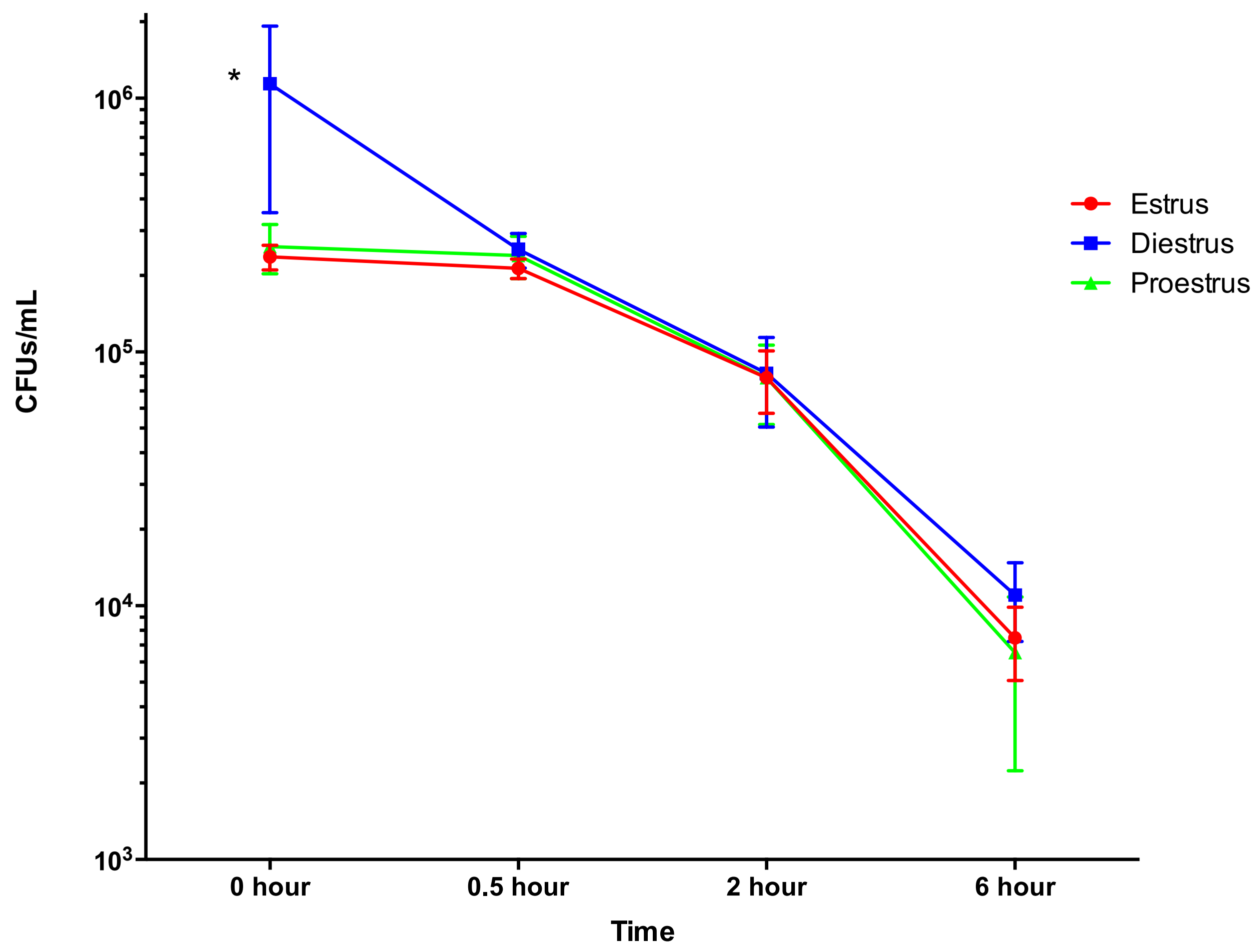

FIGURE 15: Survival of *V. vulnificus* in the serum of female rats at various stages of the estrous cycle at various time points- 0 hour (inoculation), 0.5 hour, 2 hour, and 6 hour. Two-way ANOVA was performed. No statistical significance was found (P>0.05). However, after performing Tukey's multiple comparison test, significance (*) was seen in the diestrus sera at the 0 hour time point (inoculation dose), against both proestrus and estrus.

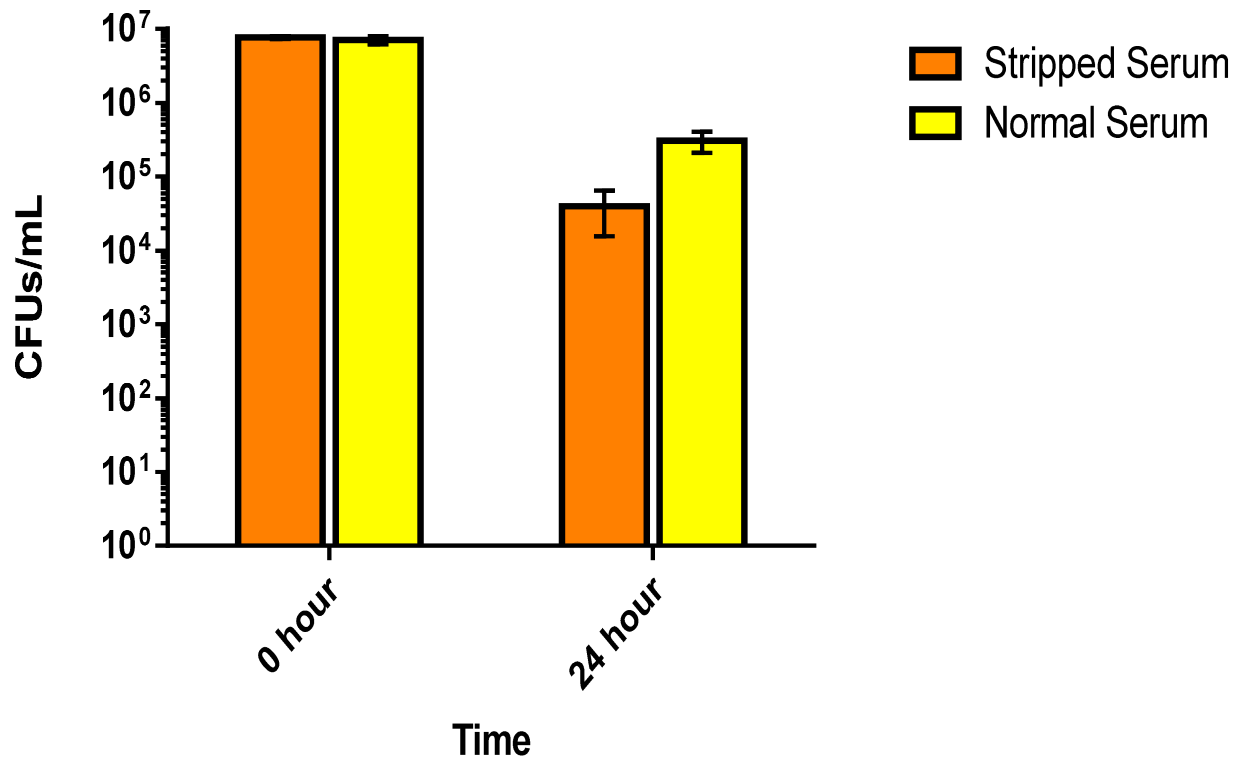

FIGURE 16: Survival of *V. vulnificus* in hormone-stripped and normal pooled (male and female) rat serum at 0 hours (inoculation) and 24 hours. 2-way ANOVA was performed on the groups Statistical significance was only found in time variation ** ($P<0.05$). No significance between treatment, or interaction was observed.

DISCUSSION

Sexual dimorphism in disease progression is a recognized phenomenon, however, it is poorly understood. There is speculation as to the cause, based on the effects of sex steroids on disease-influencing factors such as immune system regulation, or modulation of bacterial metabolism. In the case of *V. vulnificus,* it has been shown that there is a significant protective immunological effect of sex steroids against pathogenesis caused by this bacterium (28). The intent of this study was to further investigate a potential mechanism for the sexually dimorphic response associated with the induction of *V. vulnificus* pathogenesis. Specifically, the effect of sex steroid hormones on the viability of *V. vulnificus* in sera was investigated, in order to assess an "ideal" environment for bacterial proliferation, and potential differences in disease progression.

As noted in several other bacterial species, the presence of hormones in certain environments can have a direct effect on bacteria with respect to their metabolic processes, gene regulation, and survival (6, 2, 25, 10, 29). In this study, I investigated the direct effect of steroid hormones on bacterial survival in serum by adding known concentrations of hormones to sera from which sex hormones had been removed. In the conditions used, there is some evidence of hormone treatment having a significant effect on bacterial viability. The survival of bacteria in hormone-stripped serum of an ovariectomized female rat containing 25%E_2:75%T_4, exhibits a higher level of viability at the two-hour time point than other experimental groups. This differential effect of hormone tends to favor more of a male-like rather than a female-like hormone ration. This indicates that there may be a minor sex difference in bacteria proliferation at this particular time point under the conditions used. However, this is the only point of

significance, which indicates that further investigation is necessary to determine if this is relevant to sexual dimorphic disease outcomes.

Another mechanism by which hormones may alter survival of bacteria is through endogenous hormones indirectly acting on the host to alter the profile of the serum. In clinical cases, the mean time frame in which symptoms of infection typically manifest is ~24 hours (20). Results demonstrated that in the conditions used, after a 24-hour inoculation of the *V. vulnificus* into sera, there did not appear to be any significant variation in survival in any type of hormone-manipulated condition. However, when bacterial survival is observed at earlier time points, there is a significant interaction of sex over time. This observed variation is only apparent prior to six hours, after which the viability of bacteria in both sexes reach similar levels and remain constant.

Previous studies have examined the survival of *V. vulnificus* in serum (5, 47). One study by Williams, *et al.* highlights the high level of survival, even growth, seen in C-strains of this bacterium in sera, the opposite of the high rate of die-off seen in this study. Another study, demonstrated that the use of sera with active complement decreased survival of the bacteria while sera with inactivated complement induced growth (5). The differences in my data in comparison to these data may be explained by variations in methodology. My study used a higher inoculation dose (10^{5-7} CFU/ml vs. 10^{4-6}), rat sera (instead of human), and complement active sera (compared to heat-inactivated complement). It is likely that a variation in the initial condition(s) used, e.g. a lower inoculation dose and/or heat-inactivation, may result in a significant difference in results from those seen in the present study.

The research presented here demonstrates that the early percent survival of *V. vulnificus* is higher in the male sera than in the female sera. As stated previously these early differences in viability of *V. vulnificus* may be explained, at least in part, on the variation of iron present in the short-term gonadectomized male and female sera. Iron has been shown in previous research to be a contributing factor to the proliferation of *V. vulnificus* (5). One of the most critical factors of serum survival of *V. vulnificus* is the presence of iron, as it is required for the growth of this bacterium (41, 48). The availability of iron may be a relevant element to the sexually dimorphic response seen in this bacterium. Males innately have more iron in their serum than females (7). This has been shown to also be regulated in part by steroid hormones. The presence of E_2 has been demonstrated to moderate iron levels in females- the induced reduction of this hormone (by ovariectomy) significantly decreases serum iron concentrations, while the exogenous addition of the hormone increases it (37). T_4 has also been shown to increase blood hemoglobin levels, especially in older men. As hemoglobin is the primary carrier of iron, this would exponentially increase iron concentration in the blood, however not necessarily in serum (8). This indicates that while individual sex steroids may not have a direct effect on bacterial metabolism, they may still have an indirect effect on proliferation and survival of this organism *in vivo*. The difference in whole-blood iron levels, combined with the well-documented immunological response of E_2, may explain, at least in part, the sexual dimorphism seen in *Vibrio vulnificus*.

While differences in iron present (Table 1) in our short-term gonadectomized samples may explain the minor, early variances in observed survival, it is important to note that longer-term loss of sex hormones would alter these iron differences. Thus, it is

likely that in other models, e.g. sera obtained from long-term gonadectomized or reproductively senescent animals, there may be different survival responses of sera on *V. vulnificus*. Indirect effects of sex steroids on factors in sera may be one of several differences that have synergistic or additive effects in the process of initiation and progression of infection by *V. vulnificus*. Data presented here provides some evidence that ratios of sex steroids may be able to alter survival of the bacterium, however further research must be done to determine the interaction that different sex steroids at different ratios may have on *V. vulnificus* in the human host.

This thesis investigated the role of steroid hormones on the viability of *V. vulnificus* in serum. While minimal significance was observed in the conditions used, this study provides further information to expand our understanding of the underlying mechanisms that result in the sex disparity of this infection. The knowledge gained from this research provides new information to guide future research. This includes using lower inoculation doses of bacteria in studies that expand the experimental design to include further study of early time points; iron concentrations; ratios; and both heat inactivated and non-inactivated sera.

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