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# Analyzing the Longevity of Sperm Within the Female Japanese Quail by Assessing Sperm Penetration of the Perivitelline Layer Under Optimal and Suboptimal Conditions.

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By

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Analyzing the Longevity of Sperm Within the Female Japanese Quail by Assessing Sperm Penetration of the Perivitelline Layer Under Optimal and Suboptimal Conditions.

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## Abstract

After mating, birds have the ability to store semen within the female reproductive tract. The sperm storage tubules will store and subsequently release semen to travel up the oviduct. Sperm cells that make the trek up the oviduct have a chance to fertilize the ovum. These sperm cells will bind to the perivitelline layer of the ovum and hydrolyze a hole in the perivitelline layer, where it has the possibility to fertilize the female sex cell. Analyzing the number of penetration points on the perivitelline layer is an effective way to analyze reproductive efficiency. Many environmental factor has its effect on reproductive efficiency, however, only a few research trials have been done that analyze how environmental variables affect sperm penetration in itself. A population of 120 twelve week old random bred Japanese coturnix quail was separated into breeding ratios of three hens per cock making 30 pens with four birds in each. Treatments of optimal nutrition, suboptimal nutrition, and mild heat stress 75-80°F were utilized. These treatments were compared to a control group, where the males were not removed from the pen after 14 days. Males were left in the breeding pens for 14 days and then taken out, except for the control group where the males resided for the entire duration of the study. Sperm penetration assays were taken and analyzed every other day until no fertile eggs were laid. On every other consecutive day, eggs were collected and set to incubate until hatch. After hatch, percent hatchability was calculated. It was observed according to

Davis' Correlation coefficients that day of the trial has a substantial negative correlation on sperm penetration and percent hatch by (-0.65871) and (-0.5058) respectfully. Sperm penetration and percent hatch show a very strong positive correlation of (0.76404). This population of quail was observed to store semen in sufficient quantities to maintain at ≈30 sperm penetration points (SPP) for 3 days before dropping significantly in SPP ($p < 0.0001$). Where this same population could lay hatchable eggs for 8 days before dropping in hatching percentage, ($P < 0.0024$). Additionally, it was observed that proper nutrition had a more significantly greater effect by increasing SPP and slightly increasing percent hatch than the effects of heat stress. Overall, it appears that similar trials that observe the longevity of fertility could be used to model the environmental effects on SPP, transversely affecting hatchability.

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# Chapter 1

## Introduction

*Coturnix coturnix japonica,* the "Japanese coturnix" quail has been domesticated since the twelfth century and it has been bred for meat and eggs. This has formed coturnix quail that are egg type birds, meat birds, as well as dual-purpose birds (Thear, 2007). Although, there is sufficient published research focused on quail to gain a baseline, all-around quail research focused on poultry production is minimal. The avian species has the capacity to store semen within the female reproductive tract. The sperm gland or spermatozoa tubules, which are responsible for said storage, are located between the uterus and the vagina (Figure 1). A tubule or tubules will contract at a physiologically determined time allowing the stored sperm to travel up the oviduct to the infundibulum, which is the site of fertilization (Etches, 1996). The spermatozoa will attach to the perivitelline layer above the germinal disc, and penetrate it (Figure 3). From there, one sperm will meet the ovum and fertilization will occur (Bramwell et al., 1995). A determination of sperm concentration and fertility can be measured by counting the number of sperm penetration holes within the perivitelline layer (Santos et al., 2013). There is a significant body of research within reproduction in chickens *Gallus gallus domesticus* and turkeys *Melegris gallopavo domesticus* with an emphasis on production in poultry science, yet not so much within quail. This study was be performed to gain an understanding of the efficiency of the sperm-egg interaction within the female quail.

This research provides insight onto how a breeder flock's reproductive status can alter under different environmental conditions. Additionally, the research gathered data to provide information on the longevity of stored quail semen in the spermatozoa tubules. This study has the potential to provide quail breeding operations with information on how to increase their flock's reproductive capacity. A better understanding of both the male and female reproductive system in quail and other species, aids in the improving of their reproductive parameters.

**Statement of Problem**

This research is significant due to the lack of intensive research on quail reproduction focused on meat and egg production. Quail have been well examined within lab situations. Such laboratory trails provide a baseline for understanding the bird, yet, their application to production schemes can be questionable (Huss et al. 2008). To determine the Japanese quail's reproductive efficiency research like this can be beneficial to not only poultry production but also avian wildlife conservation. This study can provide data on the longevity of male to female interaction within the breeding process as well as environmental factors considering fertility. With the use of said data, quail production can become more advanced, widespread, and efficient within its breeding and hatching facilities.

## Objective

The objective of this study was to determine the longevity of fertility within the female reproductive system of Japanese quail, *Coturnix coturnix japonica*. Fertility and sperm concentration was determined with sperm penetration assays of the perivitelline layer within the germinal disc. Multiple breeder pens were utilized, one set of pens was set to be the control where a male was with the hens throughout data collection and had optimal feed and temperature conditions. The additional blocks had males taken off at the end of a fourteen day breeding period to track the longevity of fertility. The additional pens had blocks of optimal nutrition and others lacking in nutrition. Additionally, other pens were subject to slight heat stress and others were under ideal temperatures. The collection and analysis of data can help determine how effective Japanese quail reproduction is under multiple environmental scenarios.

# Chapter 2

# Literature Review

Fertility within the breeding stock of any species of poultry is crucial; without the ability to produce quality chicks, the poultry industry as a whole would come to a standstill. Any percent drop in fertility and/or hatchability will cost producers while failing to meet production needs. Fertility meaning the males ability to inseminate an ovum and a females ability to lay a fertile egg (Bourdon and Richard, 2000). Hatchability being the eggs ability to hatch (Bell et al., 2002). In order to meet economic returns, producers must ensure the breeding stock has the highest possible reproductive efficiency. Monitoring reproductive success through estimating ovulation, fertility, and hatchability can provide a hatchery or breeding facility precise information in order to make changes or keep the path to profitable poultry production (Etches, 1996; Hocking, 2009).

## Reproductive Physiology

Quail become sexually active at six weeks of age and based on sex remain active for different durations (Shanawany, 1994). After the onset of sexual maturity cocks,

the term for male quail remains sexually efficient for upwards of two years, while hens the term for female quail loose sexual efficacy around 12 months sooner (Etches, 1996).

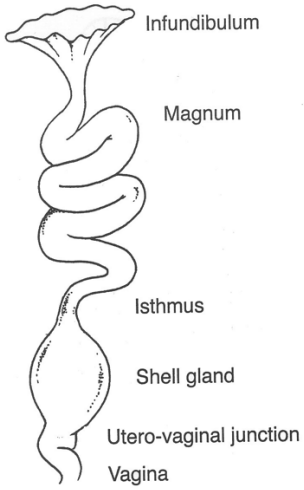



*Figure 1:* Diagram of the avian oviduct (Berg, 2000)

The avian female reproductive system is structured with one ovary connected to one oviduct. Within most of the avian species the left ovary and oviduct fully develop. Where the right will remain undeveloped. The ovary consists of many ova, typically at least 2,000, yet only a small percentage of these will actually be laid within its lifetime. The ova will mature within a follicular hierarchy, from largest to smallest. The largest follicle will ovulate first, then the next one in the hierarchy and so on until the clutch is laid (Whittow, 1999). The stigma or the least vascular tissue around the ova will split and release or ovulate the egg into the infundibulum. The infundibulum, a funnel-shaped organ will inherently engulf the ovum. Fertilization happens within the infundibulum due to the fact that this will be the only place where the ovum will remain pure, without

albumin that acts as a spermicide, membranes, or shell (Etches, 1996). The ovum will then pass into the magnum. Within the magnum, the four layers of albumin or egg white are secreted, attaching to the ovum. The chalaziferous layer of the albumin is comprised of thick strands of protein that anchor the ovum in the middle of the egg. The inner and outer thick albumin surround the egg. The thin albumin makes up the rest of the egg contents. The only significant difference between the four layers of the albumin is their water content (Bell et al., 2002). After upwards of three hours within the magnum, the egg with its yolk and albumin is transported into the isthmus. The isthmus is very distinguishable with a thick rounded muscle layer, layered with epithelial cells connected to tubular gland cells. As the egg passes through the isthmus, the inner and outer shell membranes of the egg are formed. Once the membranes are formed, the egg will pass into the uterus, better known in the avian species as the shell gland. The egg is plumped with a saline solution. Following the plumping, the calcification, or the application of the eggshell will occur (Leeson and Summers, 2000). As the egg rotates within the shell, a gland biochemical reaction occurs where, the egg exchanges electrolytes and water for calcium carbonate, “painting” the egg with a calcium layer (Etches, 1996). The finishing layers of calcium will also contain pigmentation. Every species of bird has its own variation of pigments in its shell. After the shell is formed, the first layers of a waxy organic matter known as the cuticle are applied (Morrison et al., 2018). The cuticle provides lubrication during the laying cycle, regulation of gasses and moisture during incubation, as well as protection against microbial contamination. The now shelled egg

will go into the vagina of the bird where the vagina will apply the last coating of the cuticle and, most importantly, it is the passageway for the excretion of the egg from the body or oviposition (Scanes, 2014). In between the shell gland and the vagina, there are tubular glands that store sperm (Figure 1). These glands are known as spermatozoa tubules. There are approximately 25,000 individual sperm tubules within a majority of females in the avian species (Etches, 1996). Each tubule has the potential to hold several hundred sperm, all facing the inner side of the tubule. The gland secretes lipids, glycogen, and acid phosphatases, yet their function for preservation of sperm cells and how significant these secretions are is unknown. Sperm release from a tubule or set of tubules is physiologically coordinated to have sperm within the infundibulum during ovulation (Billard, 1993).

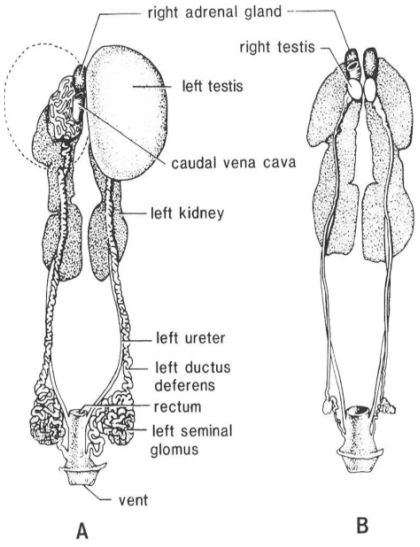



*Figure 2.* Diagram of the male avian reproductive system. (Orosz, 2019)
A- A sexually mature system
B- A sexually immature system

The avian male reproductive system starts with the testicles, located toward the ventral end of the body, dorsal to the body cavity. The testicles produce sperm via spermatogenesis within the spermatogonia of the seminiferous tubules. Exiting the testicles through the rete testis and epididymis, where sperm travel and acquire seminal fluid. Semen will then be transported and stored within the vas deferens until copulation when it is excreted through the cloaca and phallic folds of the phallus (Bell et al., 2002). Within Japanese quail, the proctodeal or cloacal gland produces a foamy solution that mixes with semen. The exact function of the foam is unknown. Speculation is that it aids in transport or acts as a aeration agent for spermatozoa. Other Galliformes like chickens and Turkeys also have this cloacal gland. Their cloacal gland is less developed and androgen independent. Where the Japanese quail's cloacal gland is highly developed and androgen dependent (Etches, 1996). Older literature states that extracting semen through abdominal massage is possible with Japanese quail. Where more recent studies state it is near impossible without the use of a teaser female. Still, even with a teaser female the results are not constant (Wentworth and Mellen, 1963; Chelmonska, et al., 2008). The highly developed and androgen dependent nature of the Japanese quail's cloacal gland is the most likely reason for their difficulty milking (Etches, 1996).

## Sperm/Egg Interaction

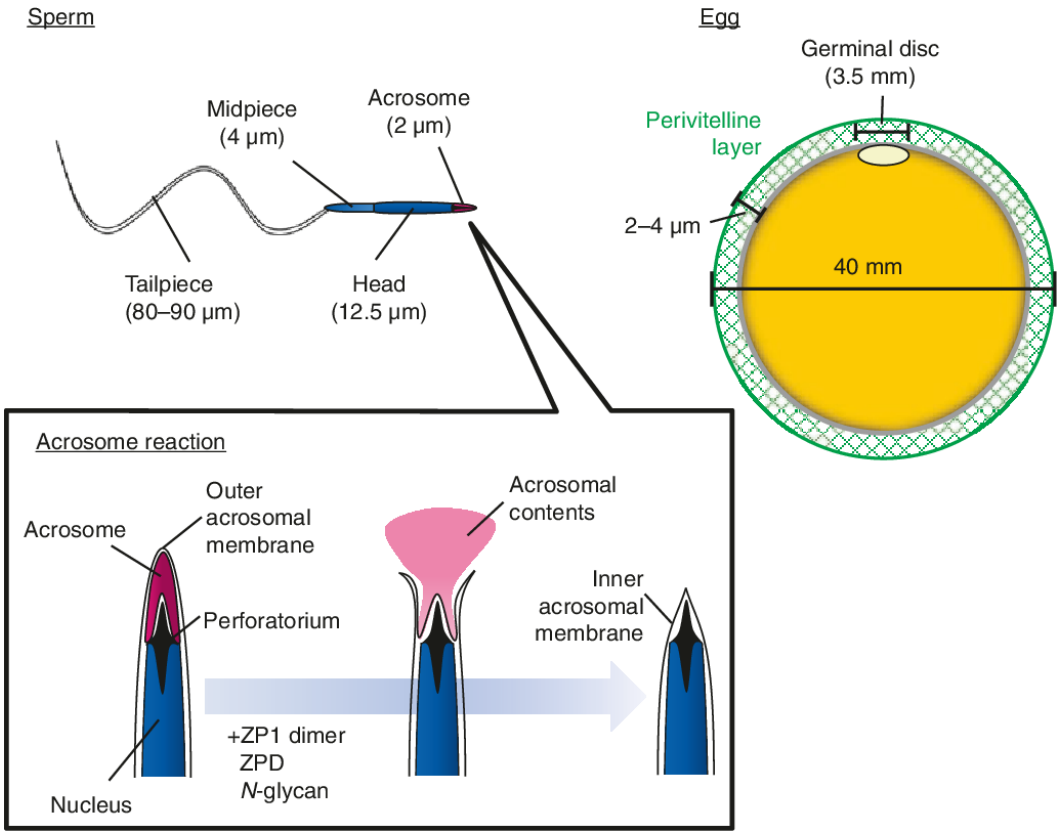



*Figure 3:* Diagram of the sperm egg interaction within poultry (Nishio and Matsuda, 2017).

After natural mating, the sperm transfers from the phallus of the male to the vagina of the female. Once in the vagina, it is unknown exactly how sperm cells are delivered to the sperm storage tubules. It is theorized that the sperm storage tubules draw up the sperm. To maintain fertility and ensure sperm is present during ovulation the storage tubules contract, excreting spermatozoa continuously throughout the day. What indicates the contractions, signaling when and how many, is unknown. Through contractions of the oviduct going caudal to cranial and sperms natural motility, the

spermatozoa will reach the infundibulum. Sperm is then stored within the infundibulum until ovulation (Sasanami et al., 2013). Once ovulation occurs, the spermatozoa will attach to the perivitelline layer of the ovum. The attached spermatozoa will hydrolyze a hole through the perivitelline layer by releasing the enzyme acrosin form the acrosome head. The sperm cell will them swim through the hole and puncture the vitelline layer with the head of the spermatozoa. The pronucleus of the sperm cell will then be released into the ovum connecting to the pronucleus of the ovum, commencing fertilization (Bakst and Howarth, 1977; Koyanagi et al., 1988). Sperm will penetrate the perivitelline layer in and around the germinal disc of the ovum. Only the sperm cells that penetrate the layer above the germinal disc have a chance of fertilization; out of the spermatozoa that do penetrate the perivitelline layer, only one actually fertilize the ovum. Fertility can be determined by assessing the indentation of the germinal disc on the perivitelline layer (Hocking, 2009). Eggs can be cracked and yolks separated from the albumen. With scissors separating a section of the perivitelline layer away from the yolk, while situating the germinal disc in the middle. Once separated, dying the membrane with a reagent will allow one to determine sperm penetration. Counting the number of sperm penetration holes around the indentation of the germinal disc can determine the longevity of quality sperm within any female of the avian species (Bramwell et al., 1996; Birkhead et al., 1994). Although not evaluating the semen itself, sperm/perivitelline interaction itself is a way of assessing the semen quality of individual males as well as flocks. The

spermatozoa's ability to interact itself is a way to analyze the sperms quality of end performance (Robertson, et al., 1998)

## Incubation

Once laid, the egg is the vessel for embryonic development. Thus, the quality characteristics of that vessel need to be monitored in order to ensure both long and short term success of a quail breeding operation. The mass of the egg correlates with the size of the yolk and days of incubation. Larger eggs take longer to incubate, where smaller eggs take less time to incubate (Morrison, et al., 2018). As embryonic development proceeds, the chick will absorb nutrients from mainly the yolk and absorb liquids mainly from the albumin (Scanes, 2014). The cuticle or the waxy layer of organic material will degrade over time. This degradation aids in air exchange in the incubating eggs. The embryo will absorb oxygen gas ($O_2$) and exhaust carbon dioxide ($CO_2$) through the shell. The process of the cuticle breakdown corresponds to the increased need of gas exchange as the chick grows (Morrison, et al., 2018). Eggs need to be turned during incubation, whether through mechanical force of an incubator or the biological force of a parent bird. The turning keeps the growing chick from becoming stuck to the shell. Additionally, it provides increased gas exchange to the whole surface area of the egg. The incubation time within Japanese quail is 18 days. From the first day of incubation to day 15-16, eggs are classified in the incubation stage. During the incubation stage eggs need to be turned

45 degrees at least once a day to insure the embryo does not stick to the shell (Bandy and Bakat, 2014). From days fifteen to the end of hatch the eggs are considered in the hatching stage and do not need to be turned. Days 16-20 are considered the hatching window, the allotted time when quail chicks should hatch. The growing chick needs an optimal environment. The optimal temperature and humidity for Japanese quail eggs during the incubation process is 37.5°C (99.5°F) and 50% humidity. While in the hatching period, temperature stays the same at 37.5°C (99.5°F), where, the humidity increases to 60%. This increase in humidity softens the shell membranes and shell. At the same time the growing chick has absorbed the majority of excess moisture in the egg. An increase in humidity insures the chick does not dry out during hatch (Shanawany, 1994). Hatchery management is essential for successful egg incubation. Through maintaining proper biosecurity, eggs and chicks have a decreased chance of coming into contact with pathogens. When the incubator and hatcher are running within the optimal settings, chicks have the best chance for embryonic development, hatching, and overall start on life (Bell et al., 2002).

**Nutrition**

Feeding quail breeders requires a balance between body weight and reproductive parameters. High body weight and/or fat content in both male and female quail decreases fertility and hatchability and increases the chance of embryo mortality during incubation

(Gebhardt-Henrich and Marks, 1995). Malnourished breeders loose reproductive effectiveness, or the ability to productively produce progeny. In order to find the perfect middle ground of reproductive soundness an *ad. libitum* to restricted 75-85% of *ad. libitum* feed intake is suggested; indicating quail can only require a slight restriction in feed intake (Hassan et al., 2003). From the time of hatch to onset of sexual maturity (six weeks), during the grower period Japanese quail require greater than 25% protein ration with at least 1% calcium. This provides the growing bird with enough protein and calcium to maintain growth and body structure. In contrast to other poultry such as chickens, ducks, and geese, quail have high protein requirements. After the six-week mark, their protein requirement shifts to 20-28% protein. This number varies due to what production system the quail are in as well as any environmental effects. A quail breeder diet should remain within 20-24% protein and greater than 2.5% calcium to ensure both males and females do not exceed a weight that would inhibit reproduction. Yet, there is enough protein and calcium to maintain all physiological functions, as well as to produce quality eggs for incubation (Scanes et al., 2004; Ensminger, 1992).

**Environmental Affects**

The environmental factors affecting quail production need to be taken into a consideration. A basic model equation for determining an animals performance is Phenotype = Genotype + Environment. Quail may have profound genetics, yet if their

environment is improperly managed, the quail will never meet their genetic potential (Vali, 2008; Bourdon and Richard, 2000). After hatch, the brooder or nursery for the quail chicks needs to have a temperature of 35°C (95°F). Every consecutive week the temperature needs to drop by 5° till week five. After the fifth week, quail can be kept at their "preferred" temperature of 21-22°C (70-73°F) until the end of their production cycle. Whether meat, egg, or breeder type quail 21-22°C (70-73°F) is the known optimal temperature for Japanese quail (Vercese, et al., 2012). Where some unpublished literature suggest Japanese quail achieve their thermoneutral zone at 30.5°C (86.9°F) (Czarnoleski et al., 2018). Within the thermoneutral zone, the bird does not have to use energy to keep warm or cool off. It is crucial to stay at least close to the birds thermoneutral zone for welfare and production (Lovette and Fitzpatrick, 2016). Quail are extremely photosensitive. Naturally, quail would come into the mating season in spring, hence quail are long day breeders. Quail breed during days where day length exceeds night time. In the scientific literature, the photoperiods effect on quail reproduction has been analyzed extensively. Fourteen hours of light per day with 10 hours of night has shown to be the most effective at maintaining egg production and fertility. Suboptimal light hours will reflect in a decrease to a sudden stop in egg production within females. In males, their sperm count is significantly reduced due to the shrinkage in testicular size going out of their breeding season. Excessive light hours will decrease light sensitivity and lead to the same effects as too reduced light (Randall and Bolla, 2008; Follett and Pearce-Kelly, 1990). From the scientific literature, light intensity does show an effect on rate of

production. Definitive data to apply precise intensities with certain daylengths has not been determined (Etches, 1996). Ensuring the nutritional and environmental effects of a bird's reproductive efficiency will enhance the productive capacity of a quail breeder flock.

**Housing**

Quail breeders are in general kept in cages. This ensures the cleanliness of both bird and egg as well as breeding effectively in a cage system. Quail breeders require 16 to 6.45$cm^2$ (25 $in^2$) of floor space per bird. Mating ratios can vary due to the size of cage and floor space (Ensminger, 1992). Male to female ratios are decided through analyzing floor space, number of birds, fertility needs, and the social hierarchy. Male quail can be quite aggressive and bully both hens and other cocks. Paying careful attention to these factors is key to the welfare of breeders in addition to the performance parameters of the flock (Thear, 2007). Hen to cock ratio of 4:1 is common practice for commercial colony breeder systems with up to 50 birds in each pen. A 3:1 ratio demonstrated higher fertility within smaller breeding groups (Woodard and Abplanalp, 1967). Both a commercial and physiological understanding of production will help identify areas in quail reproduction that would benefit quail producers and efficiency within the proposed research.

# Chapter 3

# Materials and Methods

## Research Design

Random bred quail chicks were brooded under optimal conditions on pine shavings within a nursery style brooder box. At three weeks of age males and females were separated by the sexual dimorphism of the breast color pattern. At ten weeks, after the onset of sexual maturity, 120 Japanese quail were divided into 24 breeding pens. Each pen housed three virgin hens and one cock, having an optimal 3:1 female to male ratio as described by Woodard and Abplanalp (1967). Quail were then reared in a GQF breeder colony (Quail battery breeder pens, GQF; Savannah, GA) system at 6.45cm$^2$ (25 in$^2$) per bird. All breeders were fed *ad libitum* by their representative treatment. This study was conducted through a factorial design with four treatments and a control. Six pens were fed a formulated breeder diet with 20% protein and 3.50% calcium, under the known optimal temperature 21.1-23.3°C (70-74°F). Another six pens were fed the formulated breeder diet and were subjected to heat stress of 23.9-26.7°C (75-80°F) throughout the day. Additionally six pens were fed a suboptimal diet with 18% protein and 1.25% calcium, under optimal temperature 21.1-23.3°C (70-74°F). Where a subset of six pens were fed the suboptimal feed and under heat stress averaging 23.9-26.7°C (75-80°F) during the day. Finally six pens were left as a control with optimal feed and temperature, without the removal of males. Treatment 1- optimal feed /lower temperature.

Treatment 2- suboptimal feed/lower temperature. Treatment 3- optimal feed/higher temperature. Treatment 4- suboptimal feed/higher temperature. The control had optimal feed/lower temperature and cocks remained with hens throughout the duration of the trial.

Table 1:
*The cage setup for the trial. Each colored bar represents three pens within a treatment.*

| | | |
|---|---|---|
| TRT 1 OF/LT | | TRT 3 OF/HT |
| TRT 2 SF/LT | | TRT 4 SF/HT |
| TRT 1 OF/LT | | TRT 3 OF/HT |
| TRT 2 SF/LT | | TRT 4 SF/HT |
| CON | | CON |

**Data Collection**

At twelve weeks of age, egg collection began after two weeks of breeding had commenced and males were removed from the breeding pens. After male removal, the laying period began. Sperm penetration assays were conducted for all eggs laid every other day (odd numbered days) as follows. As described by Bramwell et. al, (1998), the eggs were opened and albumen separated from the yolk by transferring the egg content back and forth from each half of the shell. After separation, the yolks were soaked in a batch of 1% sodium chloride NaCl aqueous solution for approximately 10 minutes. A centimeter square of the perivitelline layer (PL) around the germinal disc was then removed from the yolk with curved dissecting scissors and forceps. The section was

agitated within a clean 1% salt water solution to ensure a uncontaminated membrane. Once clean, the PL was placed on a slide and straitened with a pair of needles. Roughly three drops of buffered 10% formalin (Buffered Formalin, Health Link Family Medical Products; Jacksonville, FL) was placed on the PL and let to set for approximately 10 seconds. The formalin was drained and a drop or two of Periodic Acid Schiff's Reagent (Schiff's Reagent, Electron Microscopy Sciences; Hatfield, PA) was placed on the PL, and let to sit for approximately 60 seconds before drainage and very gentle rinsing with clean 1% salt water solution. The formalin, a penetrative preservative leaches into the PL. The Schiff's reagent reacts with the formalin, turning it purple; including the formalin which has penetrated the PL. Once the liquid is drained and the slide is rinsed, light can shine through the sperm penetration points. Sperm penetration was determined by counting the penetration holes around the outline of the germinal disc of the PL over the area of ($1.35mm^2$) under a 100× microscope (Bramwell 1998). In some lines of Japanese quail very little preferential attraction of the germinal disc occurs, making it hard to find under the microscope. In these cases the use of sperm penetration holes as a guide to the germinal disc area is helpful (R. K. Bramwell, 2019). A gridded slide sticker was used in the cases of indistinct germinal disc areas. Every other consecutive day (even numbered days), eggs were collected and incubated until hatch. Eggs were then placed in an incubator (1502 Sportsman incubator, GQF; Savannah, GA) set to 37.5°C (99.5°F) and 50% RH, separated utilizing a factorial design by treatment and day during the 15 day incubation process. On day 15, eggs were transferred to a hatcher (1550 Digital hatcher,

GQF; Savannah, GA) set to 37.5°C (99.5°F) and 60% RH for the pipping/hatching stage. All chicks hatched within the 16th day and 20th day period were recorded to calculate percent hatch by eggs laid through the following equation:

$$\%\ Hatch = \frac{\#\ of\ chicks\ hatched}{\#\ of\ eggs\ set} \times 100$$

To make a more accurate assessment chicks hatched were not divided by fertile eggs; as it was initially known that many eggs would not be fertile due to the nature of the trial. All unhatched eggs were recorded according to their grouping and disposed of. Every replication ended when all eggs of the treatments of that day were determined infertile, with no sperm penetration points.

**Statistical Analysis**

Data was analyzed in a nested factorial design using SAS 9.4 (Copyright © 2013 SAS Institute. Cary, NC). All data was statistically analyzed with a one-way ANOVA, Proc GLM to determine significance between replication, treatment, and day on sperm penetration and percent hatchability with a 95% assurance ($P < 0.05$). In addition a correlation analysis, Proc CORR was conducted to determine the level of correlation between variables. The Davis Correlation coefficient technique was used to interoperate the correlation tables (Davis, 1971). All means were separated utilizing LS means separator.

## Chapter 4

## Results and Discussion

At the completion of this trial, 3 replications were held where all eggs laid every other day were analyzed for sperm penetration. Eggs from every other consecutive day were set in the incubator and eventually hatcher. Each replication was conducted for 13 days to the point where no fertile egg was laid except for the control. Treatment 1- optimal feed /lower temperature. Treatment 2- suboptimal feed/lower temperate. Treatment 3- optimal feed/higher temperature. Treatment 4- suboptimal feed/higher temperature. The control had optimal feed/lower temperatures and males were not removed anytime throughout the trial.

### Sperm Penetration Assay

The following information was analyzed from the sperm penetration assays conducted during the trial. Days 1, 3, 5, 7, 9, and 11 were set for sperm penetration analyses. By the 13$^{th}$ day no fertile eggs were laid, completing the replication.

Table 2:
*ANOVA on the variables of replication, treatment and day on the reproductive parameters of Japanese quail sperm penetration points.*

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| **Replication** | 2 | 14133.231 | 7066.6155 | 14.5 | <0.0001 |
| **TRT** | 4 | 58225.8279 | 14556.457 | 29.87 | <0.0001 |
| **Day** | 5 | 56102.1206 | 11220.4241 | 23.02 | <0.0001 |

The ANOVA of table 2 signifies highly significant differences within replication, treatment and day on Sperm Penetration Points (SPP) all exhibiting a significance of ($P <$ .0001).

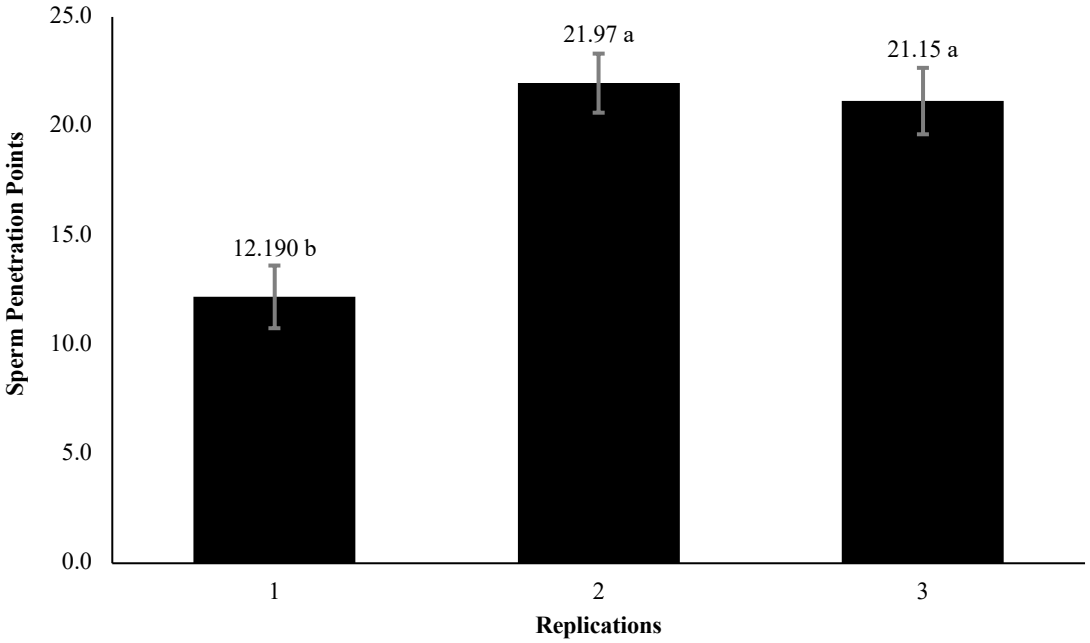



*Figure 4:* Means separation for sperm penetration points of Japanese quail eggs between the three replications.
[a,b] Means with the same letter have no significant differences ($P < 0.05$).

Figure 3 illustrates the average sperm penetration points (SPP) throughout each trial, from day one to eleven. Replication 1 at 12.190 is significantly different from replications 2 and 3 at 21.97 and 21.15 respectfully, with an a significance of ($P <$

0.0001). Figuring in quails being well into sexual maturity, this is speculated to be due to inexperienced virgin hens and cocks over sexual maturation.

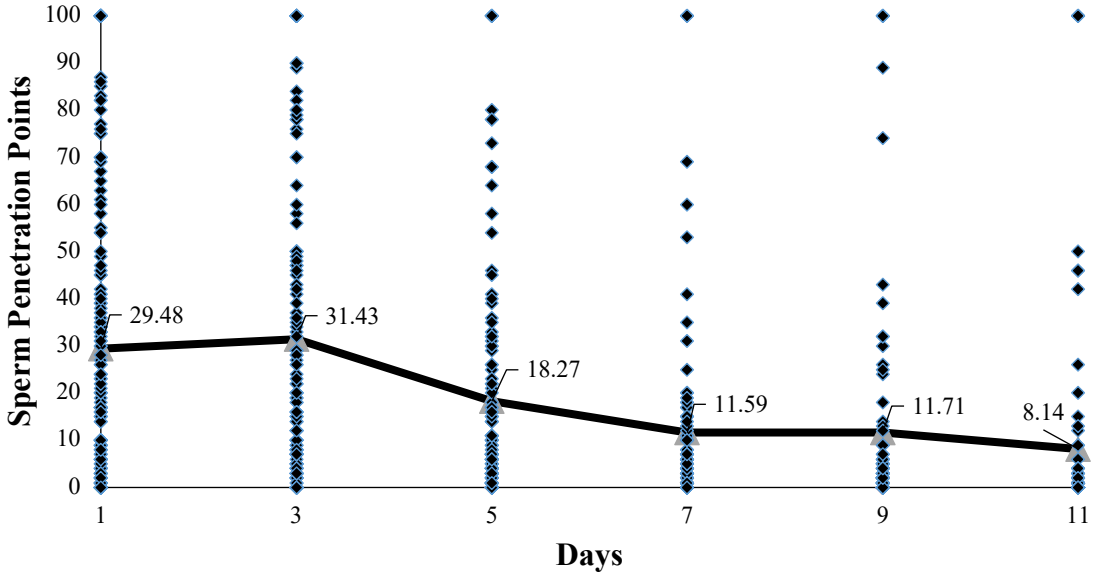



*Figure 5:* The reproductive progression of sperm penetration points of Japanese quail eggs per day of the trial averaging all three replicates. SP stays moderate through D3 and significantly drops by D5 ($P < 0.05$). Because the separation in days this graph does not represent actual trend.

Figure 4 determines the slope of progression as SPP proceeds after male removal. Additionally this figure takes the average SPP per day throughout the three replications. Days 1 through 3 have no significant difference at 29.48 and 31.43 respectfully. Progression into day 5 at 18.27 indicates a difference of ($P < 0.0001$) from days 1 and 3. Through further progression days 7 through 11 reach a plateau at 11.59 through 8.14, with no significant differences between each other based off of ($P < 0.05$). This indicates

the population of quail could store enough semen to maintain fertility within their oviduct for at least three days. Past the three day mark, the number of hydrolysis points drop significantly and then slows in its progression from days 7 to 11. Finally, by day 11 hens were laying eggs with no SPP except for the control group.

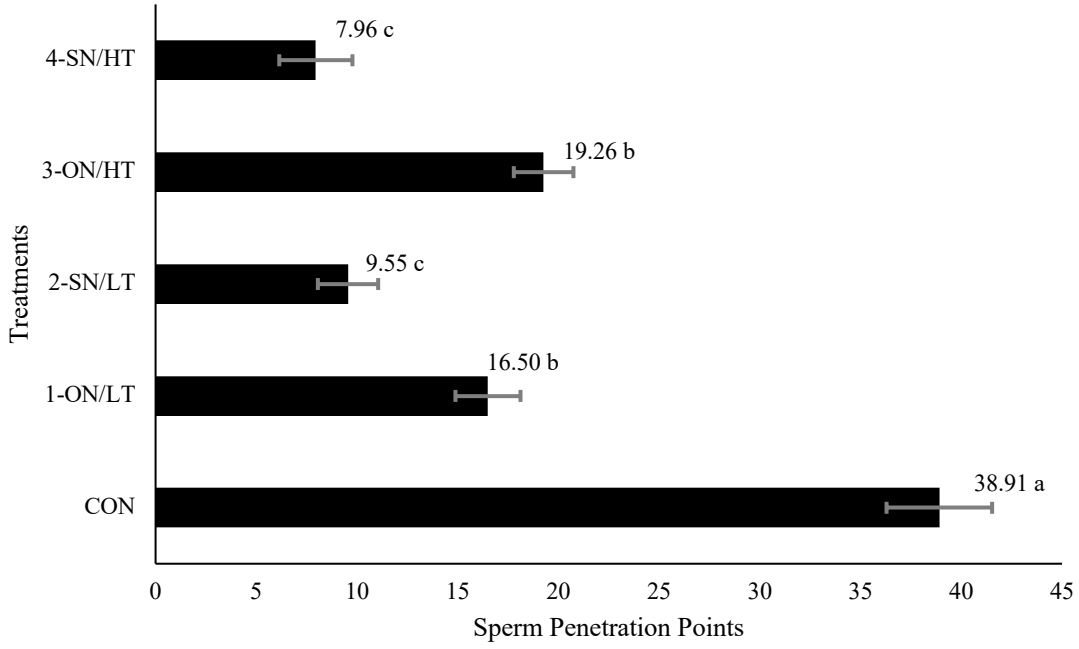



*Figure 6:* Average sperm penetration points per treatment of Japanese quail reproduction throughout the experiment.

[A,B,C] Means with the same letter have no significant differences ($p < 0.05$).

TRT 1- Optimal Nutrition/21.1-23.3°C (70-74°F)

TRT 2- Suboptimal Nutrition/21.1-23.3°C (70-74°F)

TRT 3- Optimal Nutrition/23.9-26.7°C (75-80°F)

TRT 4- Suboptimal Nutrition/23.9-26.7°C (75-80°F)

CON- Optimal variables with no male removal

Figure 5 illustrates the average sperm penetration points throughout each replication, from day one to eleven separated by treatments. As males were not separated within the control group and were able to maintain an average of 38.91 SPP per egg laid. Furthermore, the control group was significantly different from all other treatments with an significance of ($p < .0001$). Treatments 1 and 3 at average sperm penetration points of 16.5 and 19.26 respectfully had no significant difference, but were significantly lower than the control group and significantly higher than treatments 2 and 4 at 9.55 and 7.96 respectfully. Whereas, treatments 2 and 4 were not different from one another. Indicating nutrition has a more significant effect than temperature on the longevity of sperm to be able to penetrate the perivitelline layer. Figure 5 shows treatment 3, optimal nutrition/slower temperature has improved SPP compared to treatment 1 with two optimal variables. The data might suggest that Japanese quail's optimal temperature might be higher than expected.

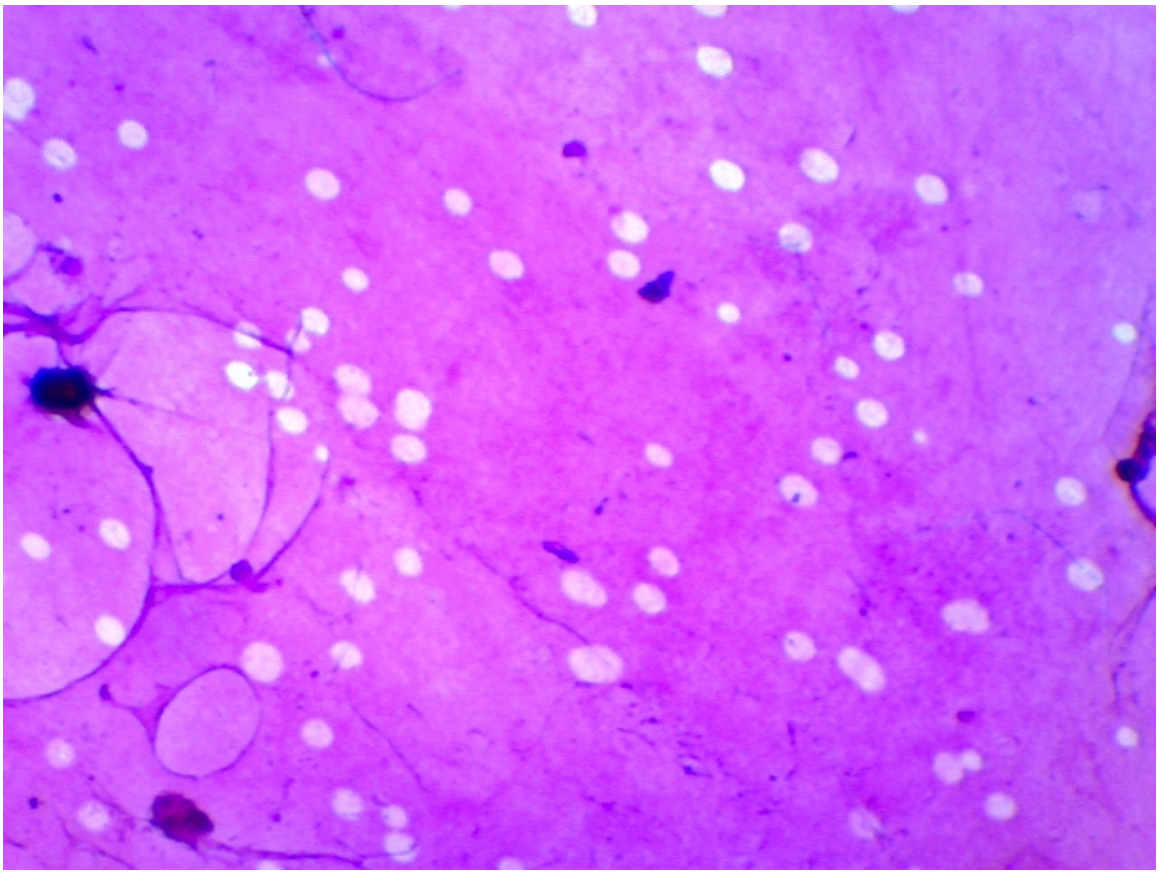


*Figure 7:* SPA slide exhibiting greater than 30 SPP around the germinal disc imprint, indicating moderate fertility.

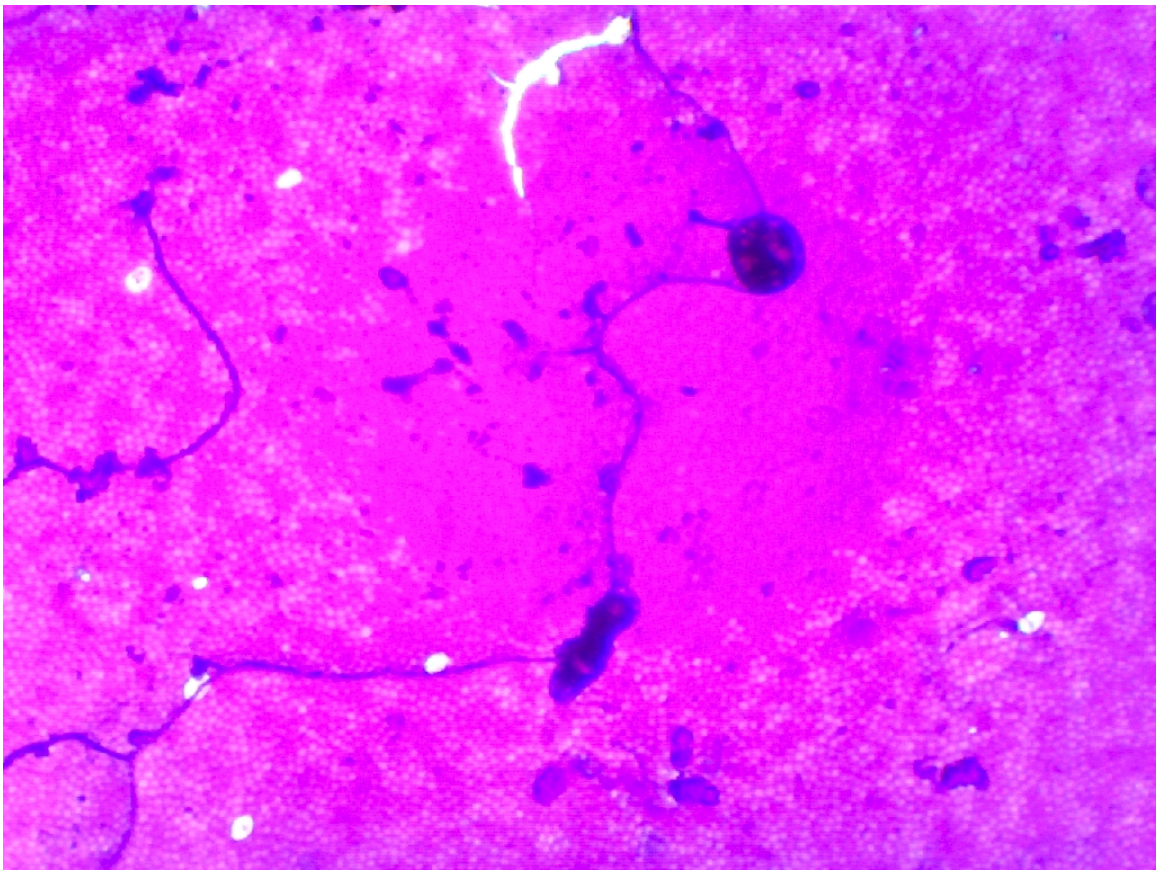


*Figure 8:* SPA slide exhibiting less than 10 SPP around the germinal disc area, exhibiting low fertility.

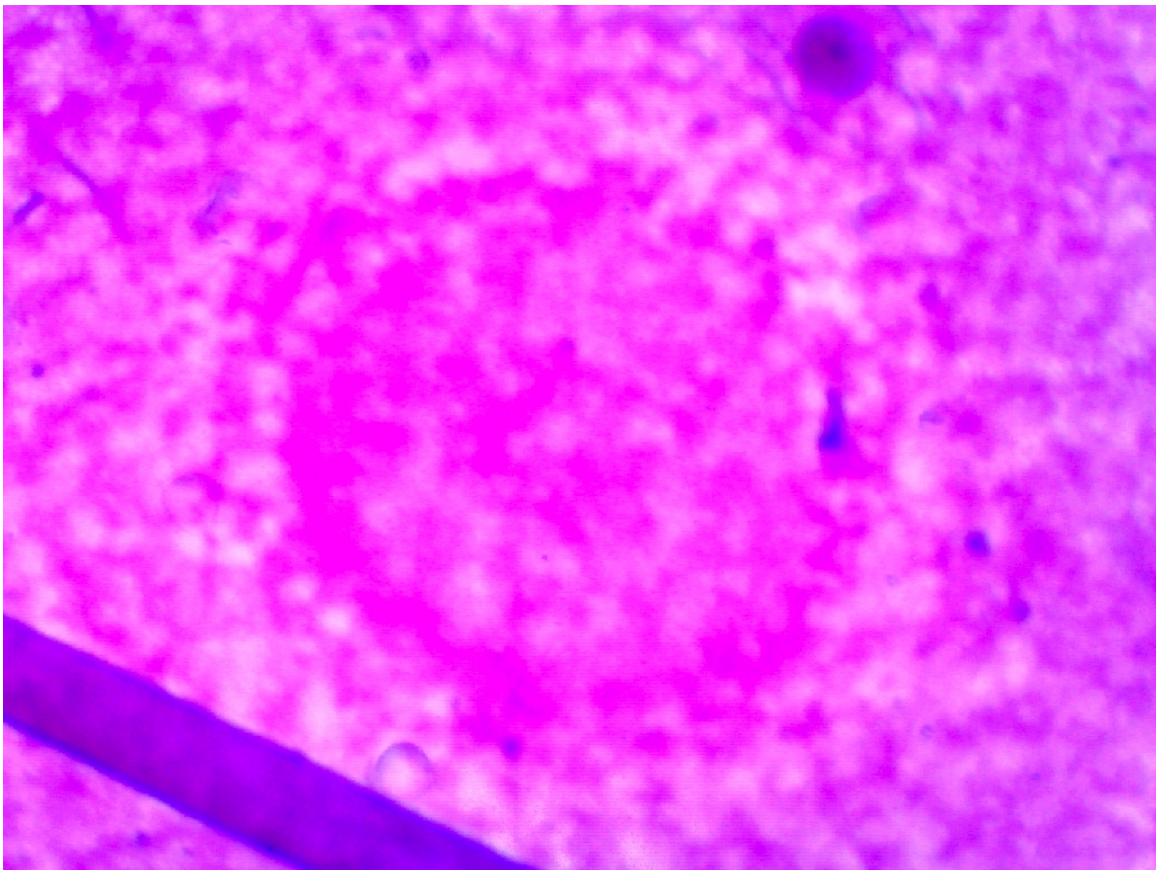


Figure 9: SPA slide exhibiting no sperm penetration around the germinal disc imprint, indicating no fertility.

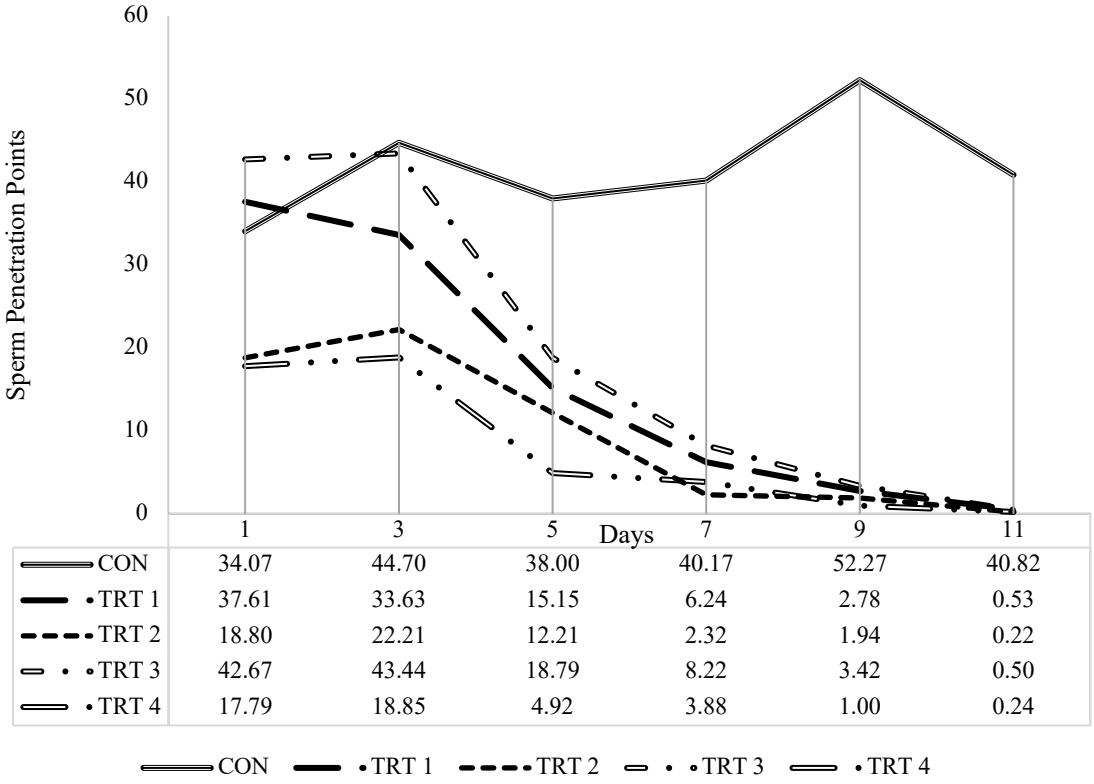
Figures 6-8 were included to aid the reader in their understanding of the author's research. The pictures have been zoomed in on the germinal disc area due to the cameras range of focus, and do not represent the entire observation site. Figure 6 exemplifies sperm penetration greater than 30. Figure 7 exhibits less than 10 penetration sites. Finally, figure 8 provides only a germinal disc imprint, indicating no fertility. Within chickens the known minimum of 30 SPP per 1.35mm$^2$ of the germinal disc will maintain greater than 95% fertility (Bramwell, et al., 1995). In Japanese quail it has been found

that 3 sperm cells trapped on $mm^2$ of the perivitelline layer above the germinal disc will maintain 95% fertility (Santos et al., 2013). Finally, resent studies found in Chinese painted quail (*Coturnix chinensis*) could maintain 95% fertility at approximately 75 penetration points around the germinal disc (Ramachandran et al., 2019).

Table 3.
*ANOVA on the variable of day nested into the treatment on reproductive parameters of sperm penetration points on Japanese quail.*

| **Source** | **DF** | **Type III SS** | **Mean Square** | **F Value** | **Pr > F** |
|---|---|---|---|---|---|
| **TRT(Day)** | 29 | 226101.794 | 7796.6136 | 15.55 | < 0.0001 |

The ANOVA in table 3 signifies the significant difference between day nested within treatment. The high significance (P < .0001) clarifies the interaction which is explained in the figure 9.




| | 1 | 3 | 5 | 7 | 9 | 11 |
|---|---|---|---|---|---|---|
| CON | 34.07 | 44.70 | 38.00 | 40.17 | 52.27 | 40.82 |
| TRT 1 | 37.61 | 33.63 | 15.15 | 6.24 | 2.78 | 0.53 |
| TRT 2 | 18.80 | 22.21 | 12.21 | 2.32 | 1.94 | 0.22 |
| TRT 3 | 42.67 | 43.44 | 18.79 | 8.22 | 3.42 | 0.50 |
| TRT 4 | 17.79 | 18.85 | 4.92 | 3.88 | 1.00 | 0.24 |

*Figure 10:* Average SPP for treatment, per day of Japanese quail reproduction through the experiment. SP stays moderate through D3 and significantly drops by D5. Because the separation in days, this graph does not represent actual trend.

The complex relationship between the longevity of SPP per treatment by day is best illustrated by the line graph of figure 9. This figure sums the initial average SPP per trial and tracks the progression per day. It is again confirmed that this population of quail can store an efficient amount of semen to where SPP count will not drop significantly for three days. Thereafter, SPP begins to decrease to full infertility by day 11. Furthermore, treatments 1, 3, and the control all on optimal nutrition, started off with higher SPP,

which carried on throughout each day of the experiment. Inferring even more so, that nutrition is a crucial factor to sperm penetration. Treatment 3 was subjected to higher temperatures and was fed optimally, yet, preformed visibly better than treatment 1 with optimal nutrition and temperature although not significantly. Treatment 4 was subjected to both higher temperatures and suboptimal nutrition had the lowest average SPP. Yet, it was not significantly different from treatment 2, which also had suboptimal nutrition. Again signifying nutrition being a more important variable to SPP compared to mild heat stress.

Table 4.

*Reproductive correlation between replication, Treatment, Day, and Sperm Penetration Points of Japanese quail.*

| | **Replication** | **TRT** | **Day** | **SPP** |
|---|---|---|---|---|
| Rep | - | - | - | - |
| | - | - | - | - |
| TRT | -0.02161 | - | - | - |
| | 0.5187 | - | - | - |
| Day | -0.03709 | -0.00864 | - | - |
| | 0.268 | 0.7963 | - | - |
| SPP | 0.12018 | -0.2043 | -0.38 | - |
| | 0.0003 | <.0001 | <.0001 | - |

The top number of the correlation table represents the association between variables. The bottom number of the correlation table represents the significance.

The correlation analysis on table 4 was analyzed using the Davis correlation coefficients. Replication has a negligible positive correlation to SPP (0.12018). This slight positive correlation is explained as the first replication had significantly lower SPP's that the other to (Figure 4). With this knowledge, the data suggests that sperm penetration increased as the quail became more experienced breeders. Treatment has a negative correlation to SPP (-0.2043) indicating a low negative correlation. Going form the control to treatment four, negative variables were added, explaining the negative correlation. The effect of day on SPP shows a moderate negative association (-3.8); as the days advance since male removal increase, SPP decreases.

**Percent Hatch**

The following data was analyzed from the percent hatch calculations conducted during the trial. Days 2, 4, 6, 8, 10, and 12 were set for incubation of eggs laid. The 13$^{th}$ day had no fertile eggs, so no eggs were set for incubation on day 14.

Table 5.

*ANOVA on the variables of replication, treatment, and day on percent hatch of Japanese quail.*

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| **Replication** | 2 | 864.88218 | 432.44109 | 0.96 | 0.387 |
| **TRT** | 4 | 24914.147 | 6228.53676 | 13.82 | <0.0001 |
| **Day** | 5 | 23112.2396 | 4622.44792 | 10.26 | <0.0001 |
| **Rep(TRT)** | 8 | 1204.4395 | 150.55494 | 0.33 | 0.9506 |

The ANOVA of table 5 exhibits highly significant differences within treatment and day on percent hatch. No significance lies within the replication at (*P* 0.387) or treatment nested within replication (*P* 0.9506). Treatment and day show significance both exhibiting (*P* <.0001). The following figures, tables and paragraphs analyze where these differences lay.

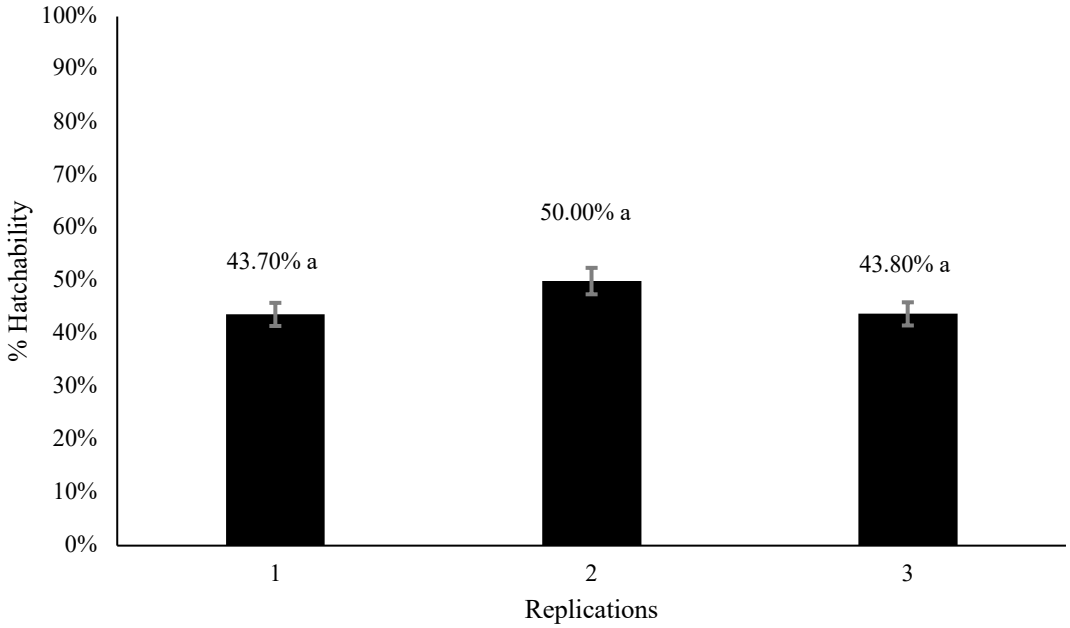



*Figure 11:* The overall average percent hatch of Japanese quail between replications.
[A] Means with the same letter have no significant differences (*P* < 0.05).

Figure 10 illustrates the differences of overall percent hatchability between replications. Between the three replicates there was no significant difference Rep one at 43.7%, Rep two at 50%, and Rep three at 43.8%. As describe by the ANOVA of table 5,

all represented means were within 10% of each other confirming the insignificance between the replications on percent hatch.

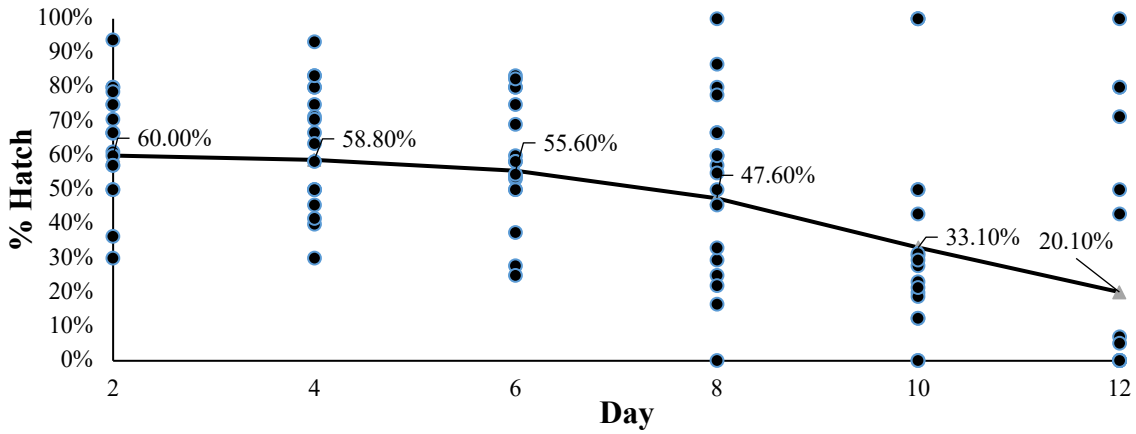



*Figure 12:* The reproductive progression of overall average percent hatch of Japanese quail per day of the trial. Because the separation in days, this graph does not represent actual trend.

Figure 11 determines the slope of progression as percent hatch drops after male removal. Additionally, this figure takes the average SPP per day throughout the three replications. Days 2 through 8 (60%-47.6%) had no significant difference(*P* 0.0834), indicating the quail's ability to maintain an adequately hatchable egg for eight days after male removal. After day eight significant reduction in hatchability occur each consecutive day. As compared to SPP, where the significant drop happened by day 5. Finally by day 14 the only eggs that hatched were from the control group. Day 12 exhibits 20.1% hatch, coming mostly from the control group as well as treatment 3 with one chick hatching.

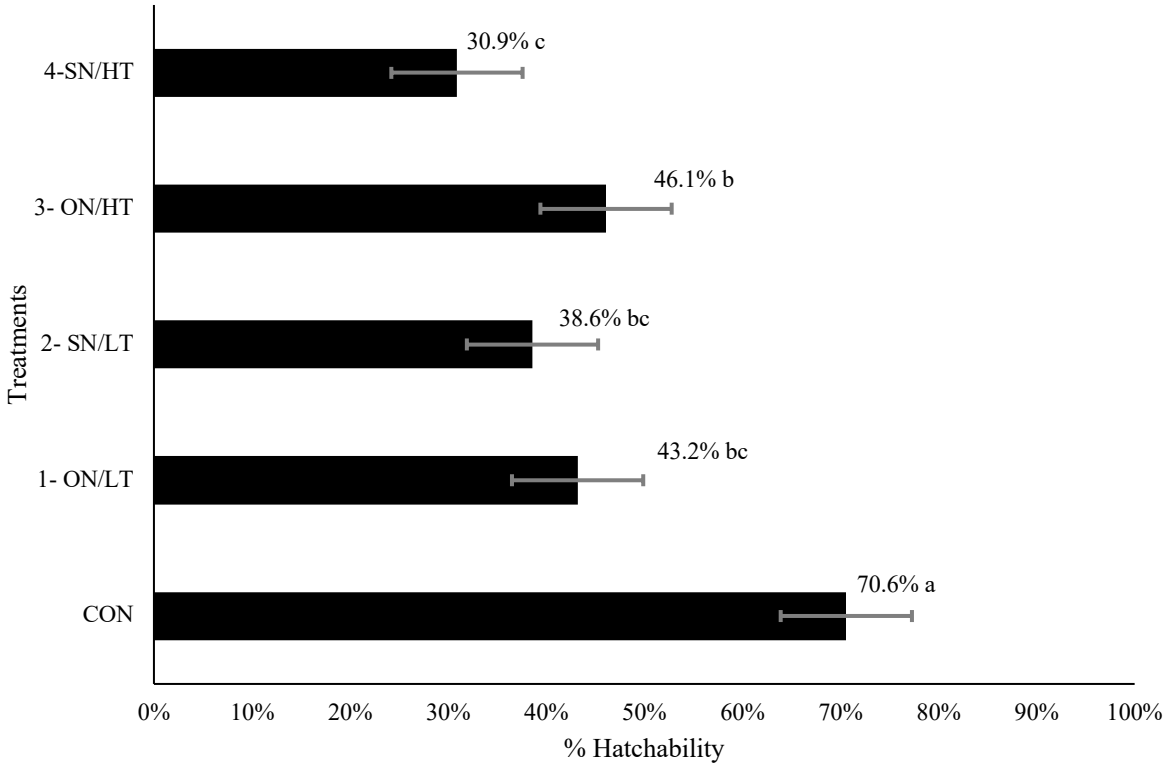



*Figure 13:* Average percent hatch per treatment of Japanese Quail.

[A,B,C] Means with the same letter have no significant differences ($p < 0.05$).

TRT 1- Optimal Nutrition/21.1-23.3°C (70-74°F)

TRT 2- Suboptimal Nutrition/21.1-23.3°C (70-74°F)

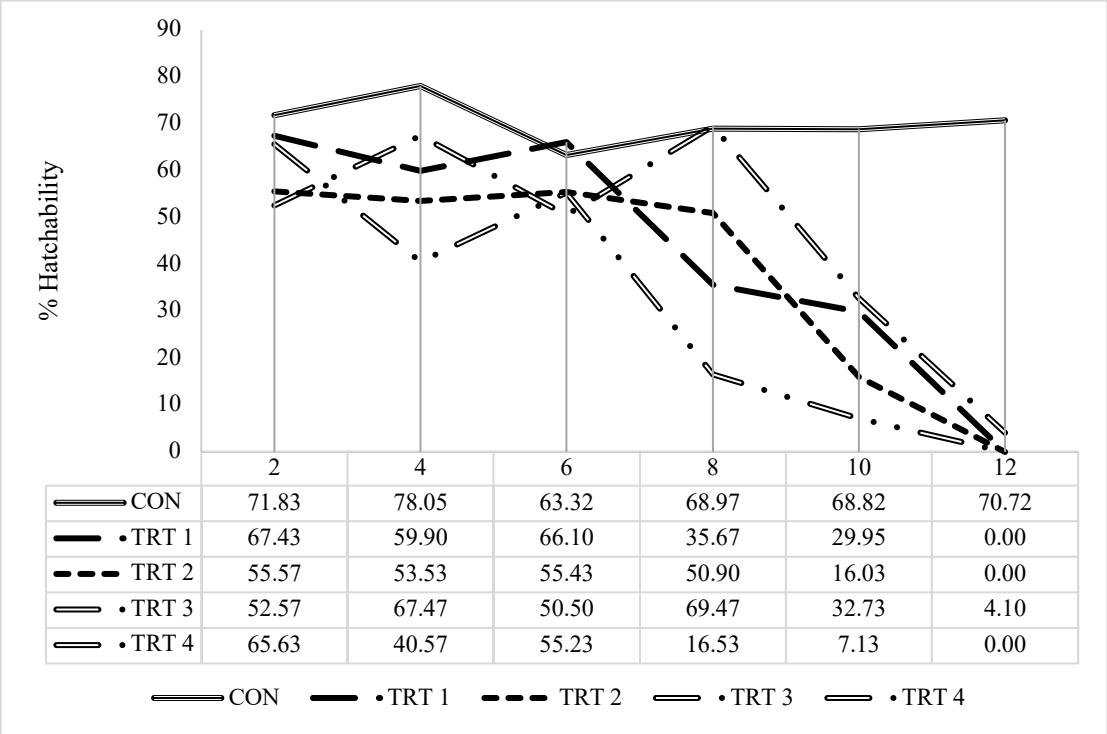
TRT 3- Optimal Nutrition/23.9-26.7°C (75-80°F)

TRT 4- Suboptimal Nutrition/23.9-26.7°C (75-80°F)

CON- Optimal variables with no male removal

Figure 12 illustrates the average percent hatch throughout each replication, from day one to twelve separated by treatments. Males were not separated with the control group, thus were able to maintain an average of a 70% hatch. The control maintains an average percent hatch of 70.6% closely corresponding to industry standards of 75% (Shanawany, 1994).This creates a significant difference between treatments and

control. Treatments 1, 2, and 4 at 43.2%, 38.6%, and 30.9% respectfully; and had no significant difference between any other treatments except the control ($P < 0.0001$). The only significant difference besides the control happened between 3 at 46.1% and 4 30.9% with an significance value of ($p < 0.0335$).




| | 2 | 4 | 6 | 8 | 10 | 12 |
|---|---|---|---|---|---|---|
| CON | 71.83 | 78.05 | 63.32 | 68.97 | 68.82 | 70.72 |
| • TRT 1 | 67.43 | 59.90 | 66.10 | 35.67 | 29.95 | 0.00 |
| TRT 2 | 55.57 | 53.53 | 55.43 | 50.90 | 16.03 | 0.00 |
| • TRT 3 | 52.57 | 67.47 | 50.50 | 69.47 | 32.73 | 4.10 |
| • TRT 4 | 65.63 | 40.57 | 55.23 | 16.53 | 7.13 | 0.00 |

*Figure 14:* The progression of percent hatch by day separated with treatments. Because the separation in days, this graph does not represent actual trend.

The complex relationship between how long hens can lay hatchable eggs per treatment by day is best illustrated by the line graph of figure 13. This figure sums the initial average percent hatch per trial, and tracks the progression per day. Additionally this graph confirms the quails ability to lay moderately hatchable eggs until 8 days after

male removal within treatments (excluding the control). The graph indicates this population of quail had the ability to maintain an average of 70% hatch if males were not removed and under optimal conditions.

Table 6.

*Reproductive correlation between replications, treatment, day, and percent hatch of Japanese quail.*

| | **Replication** | **TRT** | **Day** | **% Hatch** |
|---|---|---|---|---|
| Rep | - | - | - | - |
| | - | - | - | - |
| TRT | -0.01296 | - | - | - |
| | 0.8946 | - | - | - |
| Day | -0.00338 | -0.00308 | - | - |
| | 0.9725 | 0.9749 | - | - |
| % Hatch | 0.02669 | -0.45187 | -0.48075 | - |
| | 0.785 | <.0001 | <.0001 | - |

The top number of the correlation table represents the association between variables. The bottom number of the correlation table represents the significant difference.

The correlation analysis demonstrates its results on table 6 and were analyzed using the Davis correlation coefficients. The second two replications were slightly higher than the first, although not significantly. Which explains why there is a positive negligible association between replication and hatch (0.02669). Finally, there is equally moderate negative correlation between percent hatch on treatment (-0.45187) and day (-0.48075). Going form the control to treatment four, negative variables were added,

explaining the negative correlation. As the day progressed further form the absence of males percent hatch decreased.

**Correlation between Sperm Penetration and Percent Hatch**

The following data is analyzed through a correlation between the sperm penetration and percent hatch calculations. With the type of data collection within the trial, a correlation analysis was essential to better understand overall reproductive efficacy.

Table 7.

*Reproductive correlation between treatment, day, sperm penetration points, and percent hatch of Japanese quail.*

| | **TRT** | **Day** | **SPP** | **% Hatch** |
|---|---|---|---|---|
| TRT | -<br>- | -<br>- | -<br>- | -<br>- |
| Day | 0<br>1 | -<br>- | -<br>- | -<br>- |
| SPP | -0.43268<br>0.0169 | -0.65871<br><.0001 | -<br>- | -<br>- |
| % Hatch | -0.53795<br>0.0022 | -0.5058<br>0.0044 | 0.76404<br><.0001 | -<br>- |

The top number of the correlation table represents the association between variables. The bottom number of the correlation table represents the significant difference.

Table 7 sums up the understanding of how sperm penetration and percent hatch relate and were analyzed using the Davis correlation coefficients. Treatment and day have no correlation. Treatment has a moderate negative correlation on SPP (-0.43268). Treatment has a substantial negative correlation on percent hatch (-0.53795). Treatment of SPP and percent hatch exhibit the increase in suboptimal variables as the treatment number advances. Day also has a substantial negative correlation on SPP and percent hatch by (-0.65871) and (-0.5058) respectfully. As the days proceed after male removal both SPP and percent hatch decrease. Finally SPP and percent hatch have a very strong positive correlation of (0.76404). Meaning as SPP increases percent hatch increases.

# Chapter 5

## Summary and Conclusion

From the study, the average SPP of the control maintained a steady average of 38.91 sperm penetration points, with a steady percent hatch of 70.6%. The data suggests that for this population of Japanese quail, a flock that averages approximately 40 penetration sites per germinal disc will give eggs that have a 70% hatch success. These findings are comparable to the results of Bramwell et al, (1995), Santos et al, (2013), and Ramachandran et al., (2019). More research could be conducted looking specifically at sperm penetration without the addition of variables. It was determined that optimal nutrition had a significant effect on sperm penetration points of the perivitelline layer around the germinal disc. Heat stress did not have as much effect as hypothesized, although, we did not tale internal body temperatures. Although this may suggest that Japanese quail's thermoneutral zone may be misunderstood. This even could mean that under optimal nutrition, this population of quail were more adapt to handle higher temperatures. Trials specifically looking into nutritional and temperature variables could be conducted. In addition trials that look into the thermoneutral zone of quail might be needed. Figure 6 for SPP per treatment mimicked the figure 11 of percent hatch per treatment. Nutrition's effect on percent hatch was noticeable on figure 11 but was not significant. The results suggest this population of quail could store enough semen in their oviduct for up to 3 days to produce enough hydrolysis points to maintain fertility.

However, the same quail hens could lay hatchable eggs for up to 8 days before significantly dropping. If this holds true indefinitely, anything less 11 penetration holes around the germinal disc will significantly drop the hatch rate of this population of Japanese quail. Separating the males from the females has very little application into production. The purpose was moreover to look into physiology of longevity of semen within the oviduct and how that could be interoperated into production. Where, the nutrition and heat stress can be applied into production. Quail eating a diet low in protein and calcium have decreased reproductive performance. Where, reproductive performance is not as significantly affected by what is currently understood as mild heat stress. Additional studies are justified based on the data provided by this research and should be conducted to increase our understanding of reproductive monitoring. Breeder facilities and hatcheries alike could benefit with the predictive knowledge that these types of trials hold.

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## VITA

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