THE EFFECTS OF STEROID HORMONES ON *Rana pipiens*

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ABSTRACT

Male and female Rana pipiens were injected with steroid hormones for a 29 day period in order to determine the functions of these hormones in the reproductive systems and their effect on the enzyme succinate dehydrogenase in the brain. The animals were divided into four groups which received the following treatments: 17β-estradiol, 4-Pregnene-3,20-dione, 4-Androsten-17β-ol-3-one, or no steroid hormone. β-estradiol was the most lethal of these hormones.

The most significant results were seen in the female data. The larger animals had the more highly developed reproductive systems, which suggests a bimodal distribution of juveniles and adults. The hormones could only affect the sexually mature adults and had no effects on the sexually immature juveniles. There appeared to be significant correlations between body weight, ovary weight, oviduct weight, egg diameter, and egg development. Testosterone had the most significant effect on the reproductive system; it consistently stimulated egg development.

Staining for succinate dehydrogenase yielded a sexual dimorphism in the pretrigeminal nuclei. The PTN of the males stained darker than did those of the females. Estrogen decreased the amount of staining.

INTRODUCTION

The steroid hormones 17β-estradiol, progesterone, and dihydrotestosterone are directly responsible for inducing changes in the reproductive systems of amphibians. The amount of these hormones present in the bloodstream of an amphibian is regulated by a negative feedback cycle. If steroid hormone is needed, the central nervous system stimulates the hypothalamus to secrete gonadotropin releasing hormone, LH-RH. This then induces the pituitary gland to release two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH travel in the blood to the gonads, where they stimulate gametogenesis and the synthesis of steroid hormones. These hormones are secreted by Leydig cells in the male and by follicle cells in the female (Moore, 1987).

The functions of the steroid hormones once they are secreted in the amphibian are not entirely known. By injecting a steroid hormone into an amphibian and observing the changes that ensue, the functions of that hormone can be determined. Morphological effects on the reproductive systems have been observed, such as the ability of progesterone to induce ovulation in female Rana pipiens (Wright, 1961). A thumbpad is a morphological trait which appears in the male during the mating season. A thumbpad is a cluster of black epidermal spines that cover a glandular swelling on the

prepollex region. These help the male cling to the female during clasping (Lofts, 1974). Testosterone has previously been found to have a stimulatory effect on the thumbpads (Berk, 1939).

One sexual behavior which may be influenced by steroid hormones is the ability of a frog to give release calls. When clasped anterior to the hind legs by a sexually active male, a male or a sexually unreceptive female will give a release call, a clicking noise, to signal to the sexually active male to release his grasp. A sexually active female will not give a release call.

Steroid hormones may also affect the presence of succinate dehydrogenase in the brain of an amphibian. This enzyme catalyzes the oxidation of succinate to form fumarate (Kleinsmith and Kish, 1988). The presence of this enzyme in the pretrigeminal nucleus was previously found to be sexually dimorphic (Schmidt, 1982), but the role which steroid hormones play in the relative abundance of this enzyme is unknown.

MATERIALS AND METHODS

Male and female Northern Leopard frogs, Rana pipiens, were used in this experiment. A total of 51 were collected from Tuesday Lake, Morris Lake, Donut Bog, and Vernal Pond 27, located on the University of Notre Dame Environmental Research Center near Land O'Lakes, Wisconsin. Rana pipiens were used in this study because they were the most abundant frogs on the property at the time of collection. The frogs were captured using hands and nets. Once captured, they were placed into styrofoam coolers with ventilation holes.

Two days before the treatment period began, each animal was measured and weighed. Each was also examined for thumbpad development.

The frogs were randomly divided among 11 styrofoam coolers according to the hormone treatments they were to receive. Each cooler contained 4 or 5 frogs. 14 animals were treated with 4-Androsten-17β-ol-3-one (dihydrotestosterone: coolers T1, T2, and T3. This hormone functions like testosterone and will be referred to as testosterone.), 14 were treated with 4-Pregnene-3,20-dione (progesterone: coolers P1, P2, and P3). 15 were treated with β-estradiol (estrogen: coolers E1, E2, and E3), and 8 frogs underwent no treatment and served as controls. Each frog underwent hormone treatment for 29 days. The steroids were injected into the abdominal cavity. A dosage of 1μg of steroid hormone per frog per day

was used. The hormones were diluted in amphibian ringers solution (.14 g KCl, 6.5 g NaCl, .12 g $CaCl_2$, and .2 g NaHCO in 1 l distilled H_2O), so that each frog, excepting the controls, received a .1 ml injection of solution per day.

During the treatment period, the frogs were fed approximately every other day with whatever insects could be captured. These were primarily mayflies, caddisflies, and dragonflies. The habitats of the coolers were also changed approximately every 6 days, and these were maintained so as to stimulate natural conditions (water, grass, rocks, twigs, and the like) as nearly as possible.

Frogs which died later than two weeks after the treatments began were preserved in 10% buffered sugared formalin solution.

After the injections were terminated, body weights and lengths of the live animals were again measured. The frogs were also examined for thumbpad development. A qualitative measurement of release calls was also made by gently squeezing each frog just anterior to its hind legs.

All of the frogs were dissected within 48 hours of the last injection given. Benzocaine solution (10 g benzocaine in 100 ml 95% ethanol) was used to anesthetize the animals before dissection. A dosage of 2 ml of this solution per 1 l H_2O was used. The brain of each animal was saved for later examination by preserving the head in Conway's fixative.

For each female, the ovary was removed and weighed. The

oviduct was also removed and weighed. The diameter of the eggs was also measured, and the degree of egg development was noted. For each male, the weight and length of the testes were measured. Gonadosomatic indexes (GSI) were determined for each animal. The GSI is a measurement of the percent of the body weight constituted by the gonad weight. All animals were examined for the presence of tumors.

The remainder of the experiment was completed on the Notre Dame campus. Two months after the initial dissections, fourteen of the brains, from two males and two females of each of the treatment groups and two controls, were removed from the skulls. Each brain was frozen on dry ice and immersed in O.C.T. compound, a plastic which is a liquid at room temperature and a solid when frozen, in a micro test tube. The test tube was then frozen at -20° C, so that the brain and O.C.T. compound were of the same consistency. The ends of the test tube were sliced with a razor blade, so that the contents could be removed in plug form. This plug was placed on the shunt of a cryostat for sectioning.

Slides on which to place the brain sections were prepared in the following manner: 5.0 g Knox gelatin and .5 g chrome alum were combined in 1 l hot H_2O. This solution was mixed for 45 minutes and filtered by gravity. Slides were then dipped in this solution at room temperature and dried in the dust free atmosphere of an oven for 1 hour.

50 micron sections were made of each brain. Four slides

were made for each brain:

slide #1 row 1=cerebrum

row 2=pretrigeminal nucleus (area just posterior to optic lobes)

slide #2 row 1=medulla

Duplicates of slides #1 and #2 were made.

These slides were stored at -20 C.

The slides were stained for succinate dehydrogenase (SDH) in the following manner: They were placed in the incubation medium for 195 minutes at approximately 40°C. This incubation medium consisted of 50 ml .06M phosphate buffer (3.6 g NaH_2PO_4 and 500 ml H_2O), 20 mg nitro-blue tetrayolum, and .68 g sodium succinate. The slides were then placed in the following solutions at room temperature: 2 minutes in distilled H_2O, 2 minutes in distilled H_2O, 2 minutes in 70% ethanol, 2 minutes in 95% ethanol, 2 minutes in 100% ethanol, 2 minutes in 100% ethanol, 2 minutes in xylene, and 2 minutes in xylene. The slides were then dried and mounted with Permount.

Each slide was examined under a microscope in order to determine the degree of staining. Five sections from each brain were examined, and a value for the intensity of staining was assigned for the right and left sides of each of these brain sections.

RESULTS

The sex of each frog was not determined until each was dissected. The control group contained 6 females, 0 males, and 2 undetermined (these frogs died within the first two weeks of treatment and were not dissected). The estrogen group contained 7 females, 4 males, and 4 undetermined. The progesterone group contained 8 females, 5 males, and 1 undetermined. The testosterone group contained 6 females and 8 males. A total of 26 females, 18 males, and 7 undetermined frogs were used in this experiment.

17 of the 51 Rana pipiens used in this experiment died before the treatments concluded. This is an overall mortality rate of 33%. For all of the frogs, the mortality rate for the control group was 25% (6 survived, 2 died), 66% for estrogen (5 survived, 10 died), 29% for progesterone (10 survived, 4 died), and 7% for testosterone (13 survived, 1 died) (Appendix, Table 1). All of the frogs that died were the smaller frogs in their respective treatment groups except for C2d.7-12, E1d.7-9, E3d.7-2, and P1d.7-8. Tumors were found in 4 of the frogs. Two of these were in the estrogen group (E1B: tumors throughout the body, and E1d.7-10: one tumor on the small intestine), and two were in the testosterone group (T2E: tumors on the liver, and T3B: tumors on the kidneys).

Most of the animals grew in length and lost weight during

the experiment (Appendix, Tables 2-15). The control group gained the most length and lost the least amount of weight (Table 1).

Three of the animals exhibited a minimal amount of thumbpad development before the treatments began. Two of these were in the control group (C1d.6-17 and C2C, a female), and the other was in the progesterone group (P2A, a male). The remaining animals showed no thumbpad development. At the conclusion of the treatments, the only frog to exhibit slight thumbpad development was P2A. All of the other frogs showed no thumbpad development.

All of the surviving frogs gave release calls except for two females, one from the control group (C1B) and one from the testosterone group (T1C).

Females

The control female animals can be divided into two groups according to body weight. The ovary GSI was over 1.0% for those frogs whose body weight was greater than 20 grams. The ovary GSI was less than 1.0% for those animals which weighed less than 11 grams. The oviduct GSI was over .75% for those animals over 20 grams, while animals under 11 grams had oviducts which were too small to be removed and thus of negligible weight. The ovary weight was greater than the oviduct weight for all of the control frogs. The egg diameter measurements can also be divided into two groups. These measurements are for comparative purposes and have no units.

For those frogs over 20 grams, the egg diameter was 19 or greater, and frogs under 11 grams had egg diameters smaller than 10. Egg development also fit this pattern. Those animals greater than 20 grams had highly developed eggs; gray and/or black eggs were present. Frogs under 11 grams had poorly developed eggs; the eggs were small and cream-colored, and the oviducts were very small (Appendix, Tables 2 and 16).

Complete data for the estrogen females could only be taken for the two animals in this group which lived until the end of the experiment. These animals weighed between 10 and 16 grams, and their ovary GSIs were a little less than 1.0%. The oviduct GSI of the smaller animal was less than .5%, while those of the larger animal were over 1.0%. The oviduct weight of the smaller frog was less than its ovary weight, while the oviduct weight of the larger frog was greater than its ovary weight. The egg diameter of the smaller animal was less than that of the larger animal. Egg diameters were also measured for two of the frogs that died during the experiment. The frog with the smaller initial weight had the smaller eggs. Egg development was measured in all 6 of the females in this group. All three stages, poorly, moderately, and highly developed, were seen. The two animals that survived both had moderately developed eggs; the eggs were cream-colored, and the oviducts were large enough to be removed. The animal with the most poorly developed eggs was the one with the smallest initial body weight, and the animal with the most highly

developed eggs had the second smallest initial body weight in the group (Appendix, Tables 3 and 17).

The progesterone females can be divided into two groups according to body weight. The oviducts were too small to be removed in 5 of the 7 animals which survived. These 5 all had body weights less than 11 grams. The two animals with developed oviducts had body weights greater than 19 grams, and the oviduct GSIs were between .5% and .7%. The greatest egg diameters were found in these two animals. These eggs were highly developed, while those in the other frogs were poorly developed. The ovary GSI was between .6% and 1.0% for each animal. The ovary weights were greater than the oviduct weights for all animals in this group (Appendix, Tables 5 and 18).

The testosterone females can also be divided into two groups. For those frogs with body weights less than 12 grams, the oviduct GSIs were less than .3%, and the oviduct weight was less than the ovary weight. For those frogs with body weights greater than 18 grams, the oviduct GSIs were greater than 1.2%, and the oviduct weight was greater than the ovary weight. The ovary GSIs were between .3% and 1.2%. The larger frogs also had slightly greater egg diameters than the other frogs. All of the animals in this group had moderately or highly developed eggs (Appendix, Tables 6 and 19).

Males

Complete data for estrogen males could be taken only for

the 3 animals that survived until the end of the experiment. The animal with a body weight less than 10 grams had a GSI of .14%, while those animals weighing greater than 16 grams had GSIs less than .1%. Testes length measurements were taken for all animals in this group, and they ranged from 22.5 to 42.5 (Appendix, Tables 4 and 20). These measurements are for comparative purposes and have no units.

Complete data for progesterone males was also taken only for 3 animals. The animal with a body weight less than 10 grams had a GSI of .22%, while the animals weighing greater than 15 grams had GSIs of .15% or less. Testes length measurements were taken for all animals in this group, and they ranged from 22.5 to 37 (Appendix, Tables 6 and 21).

One animal in the testosterone male group had a body weight over 23 grams, and for this animal the GSI was .08%. The remaining animals weighed less than 10 grams, and their GSIs were between .11% and .22%. The testes lengths were between 17.5 and 36.5 (Appendix, Tables 8 and 22).

Succinate Dehydrogenase

Before they were stained, the brain sections were a yellow color from the Conway's fixative. During the incubation period, the sections gradually lost their yellow cast and turned white; only then did staining become apparent.

The pretrigeminal nucleus (PTN) sections were the most intensely stained of the three brain areas which were stained. No staining was apparent in the cerebrum sections, and a very

small amount was observed in the medulla sections.

The majority of the PTN sections were classified as either lightly or moderately stained. The only section to exhibit no staining was the left side of a section of a progesterone treated male's brain (P3C). The only brain sections that were heavily stained were those of another progesterone treated male (P2A).

For all animals, the estrogen group exhibited a lesser degree of staining than did the other three groups (Table 4). The control, progesterone, and testosterone groups were similar in their intensity of staining.

For the females, the control brains were the most highly stained, followed by the progesterone, estrogen, and testosterone groups. For the males, the testosterone group was the most highly stained, followed by the progesterone and estrogen groups. The male brains as a whole were stained slightly more than the female brains.

DISCUSSION

Females

For the female control group, there is a statistically significant positive correlation between the ovary weights and the body weights of the animals after the treatment period (Appendix, Table 16). There is also a significant positive correlation between the oviduct weights and body weights in this group. The correlation between ovary weight and oviduct weight is not as significant but still merits mention. This suggests that as the eggs mature and grow larger in the ovaries, the oviducts also grow larger. One factor which increases the weight of the oviducts is the formation of secretory granules in glands in the walls of the oviducts. These granules are precursors of oviducal jelly. This jelly coats the eggs as they pass through the oviduct and causes them to stick together in a mass after they are released from the female (Thornton and Evennett, 1969).

Egg diameter is also significantly correlated with body weight in the control group (Figure 1). As expected, egg diameter increases as ovary weight increases. Egg development is also positively correlated with body weight, but the correlation between egg development and ovary weight is not as strong. This is because the eggs in an ovary may be highly developed but may not be abundant. The weight of the ovary may not be a good indication of the maturity of the eggs in

that ovary. The correlation between egg diameter and egg development is notable; highly developed (black and gray) eggs tend to be the largest.

Of all of the female treatment groups, the estrogen group data is the least conclusive, since complete data was taken for only two animals. A positive correlation exists between the ovary weights and body weights of the animals (Appendix, Table 17). The oviduct weights also positively correlated with the body weights. This strong correlation also exists between ovary weight and oviduct weight. Egg diameter and egg development are also correlated with body weight (Figure 1). Like the control group, egg diameter increases as ovary weight increases. Egg development and ovary weight correlate more weakly for the same reason as for the control group. Egg development and egg diameter are correlated, but not very strongly.

A positive correlation also exists between ovary weight and body weight in the progesterone female group (Appendix, Table 18). A very strong correlation exists between oviduct weight and body weight in this group. The correlation betwen ovary weight and oviduct weight is likewise significant. Egg development is more highly correlated with body weight than is egg diameter (Figure 1). Egg development and egg diameter are also significantly correlated with ovary weight. The correlation between egg development and egg diameter is not very strong.

For the female testosterone group, oviduct weight is correlated more strongly with body weight than is ovary weight (Appendix, Table 19). A positive correlation exists between ovary weight and oviduct weight. Egg diameter is correlated with body weight (Figure 1). However, egg development is not correlated with body weight. Egg development is also not significantly correlated with ovary weight. This again illustrates that highly developed eggs may be present in low numbers, or poorly developed eggs may be present in high numbers. The correlation between egg diameter and ovary weight is not very strong but notable. There is not a significant correlation between egg diameter and egg development in this treatment group.

The correlations between measurements within each female treatment group are similar throughout the four groups. The only great differences are found in the testosterone group when egg development is one of the variables compared. There is no correlation between egg development and body weight, ovary weight, or egg diameter in the testosterone group; these correlations are significant in the other three groups. However, the testosterone treated females with highly developed eggs were those with the highest ovary GSIs (Appendix, Table 19). This is consistent with the findings of Licht (1983). Licht found that egg diameter and ovary GSI increase as testosterone levels increase. Since none of the testosterone treated females in this study had poorly

developed eggs, testosterone may induce egg development. The testosterone treated females had more highly developed eggs as a group than did the other three groups (Table 2). T tests indicate that this difference is not statistically significant, but it still merits mention and should be studied further.

In the female control, estrogen, and progesterone groups, the larger frogs were the most sexually mature. This suggests a bimodal distribution of juveniles and adults. Since the reproductive systems of the juveniles are not yet fully developed, they may not yet have the capability to respond to steroid hormones. Thus the hormones are unable to induce any changes in the juveniles. Only the reproductive systems of the adults are fully developed and capable of responding to the hormones. The data suggests that the body weight of juveniles is 12 g or less, and the body weight of adults is 16 g or greater (Figure 1). The frogs reach sexual maturity at some body weight between these two values.

This bimodal distribution is demonstrated by examining the maturity of the eggs. The progesterone group is most consistent with the control group. In both groups, the small animals (less than 11 grams) had poorly developed eggs, and the large animals (greater than 20 grams for the controls and greater than 19 grams for the progesterone animals) had highly developed eggs (Appendix, Tables 2, 5, 16, and 18). This implies that progesterone had no effect on egg development in

the juvenile animals. Progesterone could not induce maturation of eggs in animals that were not yet normally sexually mature. The small animals (those less than 10 grams) in the estrogen group were also sexually immature and had poorly developed eggs (Appendix, Tables 3 and 17). Those animals over 10 grams had moderately, and in one case highly, developed eggs. The divisions within this group are not as defined as those in the previously discussed groups. This may be due to an effect of the hormone. For instance, normal egg development may have been inhibited by estrogen. E1d.7-9 had an initial body weight of 33.07 grams but only moderately developed eggs. Another factor that interfered with normal egg development in this group was the high mortality rate. The full effects of the hormone could not be determined in the animals that died prior to the completion of the treatment period. The animal in this group which died the earliest of those that were dissected (E3d,6-28) had the most highly developed eggs; this supports the hypothesis that estrogen inhibits egg development.

The female testosterone data, on the other hand, is inconclusive as to whether large or small animals are more sexually mature (Appendix, Tables 7 and 19). None of the animals, regardless of weight, had poorly developed eggs. Thus this data indicates that none of these animals were juveniles.

T test results reveal that there is no significant

difference between the female control group and the female treatment groups for the ovary GSI (Table 2 shows mean values). This means that none of the treatments had any significant effect on ovary weight. Likewise, there is no significant difference between the control group and the treatment group for the oviduct GSI. The progesterone group is the closest to a significant difference, mainly because most of the oviducts of this group were very small (Table 18). As discussed earlier, progesterone was previously found to induce jelly release (Thornton and Evennett, 1969), which would increase the size and weight of the oviducts. The results of this experiment suggest that progesterone does not stimulate jelly release, since the oviducts are small. But the animals with these small oviducts also have small body weights (Appendix, Table 5), and thus may not have reached sexual maturity and cannot be affected by progesterone. Wright (1961) concluded that progesterone is also needed for the maturation and ovulation of the oocyte. But in this experiment, progesterone did not induce more egg maturation than was found in the control group.

Males

For the male estrogen treatment group, testes weight is not strongly correlated to body weight after treatment (Appendix, Table 20). Testes length, however, is highly correlated to body weight. Testes weight and testes length are also strongly correlated. This indicates that the shape

of each testis is approximately the same, and the surface area of each testis is proportional to its weight.

In the male progesterone group, testes length is strongly correlated to body weight (Appendix, Table 21), but testes weight is not correlated to body weight. Testes weight and testes length are also not significantly correlated. This suggests that the testes in this group had various shapes.

Testes weight is also not strongly correlated with body weight in the testosterone group (Appendix, Table 22). Likewise, testes length is not strongly correlated with body weight. There is no correlation between testes weight and testes length in this group.

One difficulty encountered in measuring testes weights was that the scale was accurate only to centigrams. All of the testes weighed fell within a range of 3 centigrams. A scale that measured milligrams or smaller units would have produced more accurate results. It is difficult to determine significant correlations when such a small range of measurement is used. The only significant correlation for all of the groups is that between testes length and body weight. The larger animals have the larger testes, regardless of which treatment they received.

A Kruskal Wallis test on the three treatment groups for the GSI yielded a result which indicates that there is no significant difference between the three groups (Table 3). Thus none of the hormones had a significant effect on the

testes (as far as can be seen in a change in size) compared to the effects of any other hormone. Thus it cannot be concluded from these measurements that any one steroid hormone induces spermatogenesis any more than the other hormones. Basu and Nandi (1965) concluded that testosterone inhibits spermatogenesis in Rana pipiens. These results could be better analyzed if a control was used. T tests on testes length for the different groups led to the conclusion that there are no statistically significant differences between the groups.

A more thorough examination of the male reproductive system may yield more conclusive results as to the different effects of the three hormones. Since testosterone seemed to induce egg development in the female, it may also alter sperm development in the male. It cannot be concluded that the hormones had no effects on the male unless all parameters are examined.

Unlike the female data, no pattern is evident in the male data to indicate which animals are most sexually mature. Since no control was used, it is impossible to determine if a pattern normally exists, and the hormones skewed this pattern, or if no pattern normally exists. The smaller animals in all of the treatment groups tended to have higher GSIs than did the larger animals. The small animals are more likely to be younger and thus less sexually mature. Thus sexual maturity in males is not necessarily indicated by the

GSI, and testes size cannot be predicted from body weight.

The testes of the male can convert progesterone to testosterone (Dale and Dorfman, 1967). Thus it is impossible to determine whether effects on the progesterone treated males are due to the action of progesterone or testosterone. The mean GSI of the progesterone group is very close to that of the testosterone group; this suggests either that progesterone was converted to testosterone, or that progesterone and testosterone have similar effects on the testes.

All animals

Ideally, male and female controls would have been used in this experiment. However, sex determination was difficult until the animals were dissected, so at the beginning of the experiment the frogs were randomly distributed among the groups. Unfortunately the control group contained no males, so the male data cannot be compared to untreated animals.

The steroid hormone dihydrotestosterone (DHT) was used instead of testosterone in this experiment, because both male and female Rana pipiens can convert testosterone to estrogen. But they cannot convert DHT to estrogen. DHT functions like testosterone, so by using DHT, the effects produced can be conclusively attributed to the actions of testosterone.

The mortality rate was the highest for the estrogen treated group, followed by the progesterone group, the control group, and the testosterone group. One possible cause of

death is the steroid hormone itself. An ideal dosage is one great enough to induce a morphological change in the animal, but small enough so as not to be lethal. Since the dosage of each steroid hormone was the same, the ideal dosage of each individual hormone may not have been used. Estrogen is a known carcinogen and was the most lethal of the hormones. The presence of tumors in two of the estrogen treated frogs supports its carcinogenic ability. The tumors found in two of the testosterone treated frogs may indicate that testosterone is also carcinogenic, or these tumors may have been benign, as is suggested by the low mortality rate of this group. The first six frogs to die during the experiment were not dissected; thus it is not known whether or not tumors were present in these frogs. In all of the groups, most of the animals which died were the ones with the smallest initial body weights and lengths (Appendix, Table 1). Since all of the animals received the same dosage, the smaller animals received higher dosages in proportion to their body weights. Thus these animals were more apt to receive lethal dosages. Also, the frequency of deaths increased toward the end of the experiment. 8 animals died during the first 3 weeks of the experiment, and 9 died during the final week of the experiment. This indicates that the hormones may have side effects which become more lethal with time until the body can no longer counteract them.

Besides the hormones, the availability of food may

account for mortality. The frogs may eat more in the wild than they were able to in captivity. Since 4 or 5 animals were in each cooler, competition for food could also be a factor. This may also account for the mortality of the smaller animals; the larger frogs may outcompete the smaller for the available food. The stress of being in captivity is another factor contributing to mortality. These two factors account for the mortality rate of the control group (25%), and may also wholly account for deaths in the progesterone and testosterone groups, since these mortality rates, 7% and 29% respectively, are low if the control mortality is taken as normal. As discussed above, the high mortality rate for estrogen (67%) indicates that the hormone itself is a significant factor.

Most of the animals lost weight during the experiment (Table 1), indicating that they ate less either because of a low food availability or because of the stress of the treatments and captivity. T test results indicate that there is a significant difference in the change in body weight of the testosterone treated animals compared with the controls. The difference between the estrogen group and the controls, and between the progesterone group and the controls, is not statistically significant. The data suggests that the stress of receiving injections played a role in growth and weight loss, since the treated animals grew less and lost more weight than did the control frogs. All of the groups but estrogen

had a mean increase in body length, indicating that growth did not cease although weight gain did. The data indicates that the estrogen treated frogs decreased in length, which is theoretically impossible. The most plausable reason for this is error in measurement, which was most likely to occur due to inexperience in measuring prior to the treatment period. The changes in lengths of the estrogen treated frogs were the only measurements to differ significantly statistically from the control group's length measurements.

One problem encountered in measuring and weighing the frogs was that each was not individually tagged. Thus it was difficult to keep track of a single frog and match its initial measurements with its final measurements. This problem was kept to a minimum by keeping only 4 or 5 animals in each cooler, and by making sure these animals had a wide range of body lengths and weights.

Testosterone has formerly been found to have a stimulatory effect on the thumbpads (Berk, 1939), but in this study the only animal to show any thumbpad development after the treatment period was a male in the progesterone group (P2A). This animal also showed this slight development prior to treatment, which suggests that the hormone was not responsible for it. Two control animals, one of which was a female, also showed slight thumbpad development prior to treatment. Since a female was determined to have slight thumbpads, this suggests that the coloring of the digits may

have been the animal's normal coloring. In other words, they were not thumbpads. This may also apply to the other two animals. The dosage of the hormones, particularly testosterone, may not have been high enough to induce development. Stress may again account for the lack of development. Another possibility is that the mating season had already occured. Thus the thumbpads had already formed and regressed and could not be induced to develop again.

According to the release call data, the only sexually receptive females were one from the control group (C1B) and one from the testosterone group (T1C). All of the other animals were either males or sexually unreceptive females. However, the data for egg diameter and development (Appendix, Tables 16 and 19) suggest that C1B and T1C were not sexually receptive, because their eggs were poorly and moderately developed, respectively. A sexually receptive female should have highly developed eggs. The lack of a release call can be attributed to experimental error. These frogs may not have been squeezed firmly enough.

One reason for the lack of conclusive results between the treatment groups is the stress the frogs experienced during the treatment period. When frogs are stressed, a natural reaction could be to turn off their reproductive systems. The environment of captivity was one cause of this stress. The frogs were in more crowded conditions than they are normally, and they did not have the freedom to get as much exercise as

they would normally. Another cause of stress was the injections themselves. This stress could be reduced if the hormones were administered by absorption through the skin instead of by injection. But by this method the dosage cannot be as closely regulated.

Another reason for the lack of conclusive results is that the mating season had already occured when the experiment was begun. Thus the hormones did not affect the animals as strongly as they might if administered prior to the mating season. The reproductive systems may have been unreceptive to steroid hormones, since the changes that accompany the mating season had recently occured. The experiment may have been more successful if it was conducted in the spring, prior to the mating season, rather than during the summer (June and July). Evidence which supports the hypothesis that the mating season had passed is found in the number of males and females that were captured for this study. 51% were female, 35% were male, and 14% were of undetermined sex. After the mating season, males leave the breeding site earlier than do females. However, this data may also suggest that males are more difficult to capture than females. The relatively low number of highly developed eggs found also suggests that the breeding season already occured.

Ideally, the data for this experiment would have been taken only for those animals which lived for the duration of the treatment period. Some data, such as egg diameters and

testes lengths, was taken for animals which died prior to the completion of the treatments. Since these animals had different treatment periods, the results are not highly conclusive when compared to those animals that did not die. As much data was collected as possible since the number of animals in each treatment group was relatively low. A more thorough experiment would include more animals in each treatment group.

Succinate Dehydrogenase

The pretrigeminal nucleus (PTN) sections of the frog brains stained more intensely than did the cerebrum and medulla sections. This suggests that there are more mitochondria present in the PTN than in the other two areas, since the tricarboxylic acid cycle occurs in the mitochondria, and SDH is an enzyme in that pathway.

Schmidt (1982) found a sexual dimorphism in SDH staining in the PTN of the American toad. The results of this study indicate that this sexual dimorphism is also present in Rana pipiens. The mean value for male staining is higher than that for female staining (Table 4). Although this difference is not statistically significant, it still warrants mention. It suggests that males have more mitochondria in the PTN than do females. Schmidt also suggested that the PTN is involved in vocalizations during the mating season and thus would be more active in the male. The results of this experiment concur with this hypothesis.

If males and females are considered together, the estrogen group is the only group to show a difference from the controls in the amount of staining. It is lower in the estrogen group than it is in the other three groups. This suggests that estrogen has an effect on the amount of SDH in the PTN. This effect could be inhibitory: estrogen may directly or indirectly inhibit the ability of succinate to bind to the enzyme, or it may block the formation of SDH. The effect may also be to degrade the enzyme once it is present. This may be done directly by estrogen or by a pathway which estrogen initiates.

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Table 1. Mean percent change in body weight and body length for all animals.

Treatment Group	Percent Change In Body Weight	Percent Change In Body Length
control	-3.6+/-9.1	5.4+/-2.3
estrogen	-13+/-3.2	-5.5+/-3.9
progesterone	-12+/-2.2	3.8+/-1.4
testosterone	-17+/-3.2	3.7+/-1.2

Table 2. Mean Values For Female Data. The measurements for egg diameter are for comparative purposes and have no units. All weights are in grams. GSI=gonadosomatic index. Egg development: 1=poorly, 2=moderately, 3=highly.

Treatment Group	Control	Estrogen	Progesterone	Testosterone
Body Weight After Treatment	17.88 +/-4.01	13.20 +/-2.33	12.93 +/-2.21	13.26 +/-2.10
Ovary Weight	.28 +/-.12	.12 +/-.02	.10 +/-.02	.11 +/-.02
Ovary GSI	1.2 +/-.37	.88 +/-.04	.77 +/-.06	.84 +/-.11
Oviduct Weight	.14 +/-.07	.11 +/-.07	.04 +/-.03	.10 +/-.05
Oviduct GSI	.50 +/-.24	.74 +/-.37	.18 +/-.12	.55 +/-.26
Egg Diameter	16 +/-3.7	11 +/-2.6	11 +/-.96	11 +/-.65
Egg Development	2 +/-.45	2 +/-.26	1.5 +/-.33	2.3 +/-.21

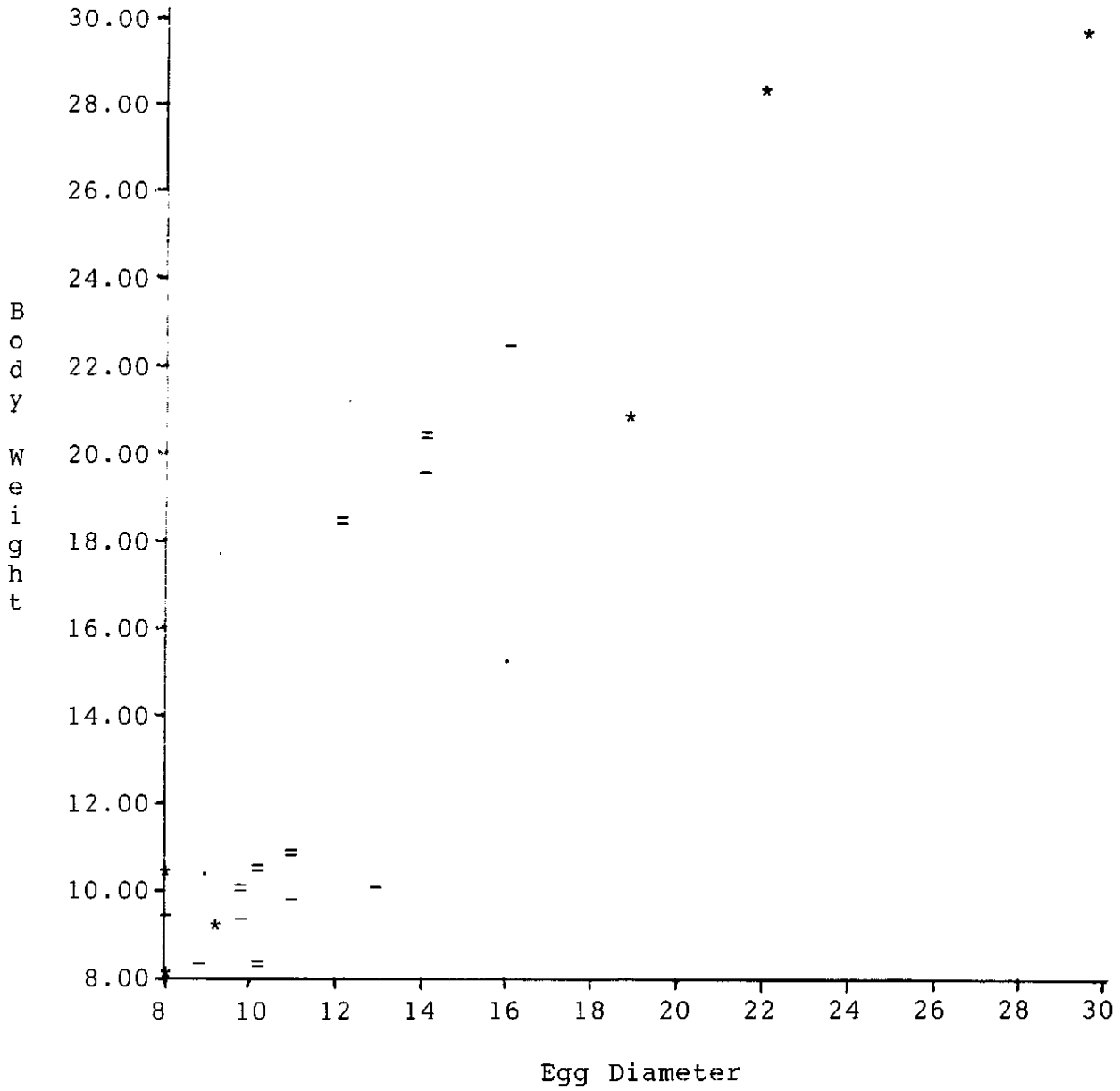

Figure 1. Female Frogs. Relation between body weight in grams after treatment and egg diameter. The measurements for egg diameter are for comparitive purposes and have no units. Pearson's correlations are as follows: Control (*) r=.97, Estrogen (.) r=1, Progesterone (-) r=.86, and Testosterone (=) r=.94. Those animals with the greatest body weights were the most sexually mature.

Table 3. Mean Values For Male Data. The measurements for testes length are for comparative purposes and have no units. All weights are in grams. GSI=gonadosomatic index.

Treatment Group	Body Weight After Treatment	Testes Length	Testes Weight	GSI*
estrogen	14.83+/-4.00	32.4+/-4.4	.01+/-.003	.10+/-.02
progesterone	14.76+/-3.06	31.2+/-2.8	.02+/-.006	.15+/-.04
testosterone	10.09+/-2.33	25.8+/-2.5	.01+/-.002	.14+/-.02

*A Kruskal Wallis test indicates that there are no significant differences between the three groups (r=.39).

Table 4. Succinate Dehydrogenase Staining. Mean values for amount of staining observed in the pretrigeminal nucleus of Rana pipiens brains. 0=no staining, 1=light staining, 2=moderate staining, 3=heavy staining.

Treatment Group	Value In All Animals	Value In Females#	Value In Males*
control	1.6+/-.2	1.6+/-.2	-
estrogen	1.38+/-.11	1.45+/-.15	1.3+/-.2
progesterone	1.68+/-.43	1.5+/-.4	1.85+/-.95
testosterone	1.65+/-.19	1.4+/-.3	1.9+/-.1

#Mean value for all females=1.49+/-.11

*Mean value for all males=1.68+/-.28

APPENDIX

Individual Animal Data

Table 1. Mortality During the Experiment. The first treatment day was June 13 and the last was July 11. Weights are in grams and lengths are in centimeters. C=control, E=estrogen, P=progesterone, T=testosterone.

Date	Animal	Body Weight Prior To Treatment	Body Length Prior To Treatment
6-17	C1	6.66	4.6
6-24	E2	8.13	4.5
6-25	P3	6.40	4.4
6-25	E3	8.40	4.4
6-26	E3	9.23	4.6
6-28	E2	7.82	4.8
6-28	E3	10.46	4.6
7-2	E3	16.26	5.9
7-6	E2	8.85	5.0
7-8	T3	6.88	4.4
7-8	P1	13.35	5.5
7-9	E1	33.07	7.4
7-9	P1	7.69	4.8
7-10	E1	8.17	4.6
7-11	E1	11.43	4.7
7-11	P1	9.91	4.8
7-12	C2	14.21	5.3

Table 2. Control. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	% Change In Body Weight
C1A	32.04	28.60	-10.70
C1B	10.73	8.25	-23.10
C1C	12.16	9.30	-23.50
C2A	7.67	10.50	36.90
C2B	30.81	29.61	-3.89
C2C	20.46	21.03	2.79

Table 3. Estrogen Females. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	%Change In Body Weight
E1B	13.57	10.87	-19.90
E1d.7-9	33.07	-	-
E2B	16.22	15.53	-4.25
E2d.7-6	8.85	-	-
E3d.6-28	10.46	-	-
E3d.7-2	16.26	-	-

Table 4. Estrogen Males. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	%Change In Body Weight
E1A	9.29	7.32	-21.20
E1d.7-10	8.17	-	-
E1d.7-11	11.43	-	-
E2A	18.31	16.20	-11.50
E3A	23.18	20.98	-9.49

Table 5. Progesterone Females. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	% Change In Body Weight
P1A	25.28	22.81	-9.77
P1d.7-11	9.91	-	-
P2B	10.91	9.53	-12.60
P2C	22.45	19.98	-11.00
P2D	9.74	8.57	-12.00
P2E	12.03	9.90	-17.70
P3A	10.66	9.60	-9.94
P3B	12.86	10.15	-21.10

Table 6. Progesterone Males. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	% Change In Body Weight
P1B	11.33	9.13	-19.40
P1d.7-8	13.35	-	-
P1d.7-9	7.69	-	-
P2A	18.89	19.65	4.02
P3C	17.23	15.49	-10.10

Table 7. Testosterone Females. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	% Change In Body Weight
T1A	10.45	11.04	5.65
T1C	14.43	10.64	-26.30
T2A	21.47	18.67	-13.00
T2C	23.20	20.76	-10.50
T3A	10.48	8.26	-21.20
T3D	12.90	10.19	-21.00

Table 8. Testosterone Males. Body weight in grams prior to and after treatment and percent change in body weight.

Animal	Body Weight Prior To Treatment	Body Weight After Treatment	% Change In Body Weight
T1B	11.21	9.14	-18.50
T1D	10.43	9.16	-12.20
T2B	9.05	7.03	-22.30
T2D	9.48	6.81	-28.20
T2E	9.15	7.17	-21.60
T3B	23.23	23.88	2.80
T3d.7-8	6.88	-	-

Table 9. Control. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
C1A	7.3	7.4	1.4
C1B	4.7	5.0	6.4
C1C	4.9	5.1	4.1
C2A	4.4	5.1	16
C2B	7.2	7.3	1.4
C2C	6.4	6.6	3.1

Table 10. Estrogen Females. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
E1B	6.3	5.0	-21
E1d.7-9	7.4	-	-
E2B	6.3	6.1	-3.2
E2d.7-6	5.0	-	-
E3d.6-28	4.6	-	-
E3d.7-2	5.9	-	-

Table 11. Estrogen Males. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
E1A	4.8	4.8	0.0
E1d.7-10	4.6	-	-
E1d.7-11	4.7	-	-
E2A	6.4	6.4	0.0
E3A	6.5	6.3	-3.1

Table 12. Progesterone Females. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
P1A	7.0	7.5	7.1
P1d.7-11	4.8	-	-
P2B	4.9	4.9	0.0
P2C	6.9	6.5	-5.8
P2D	4.7	4.9	4.3
P2E	5.2	5.5	5.8
P3A	5.1	5.5	7.8
P3B	5.5	5.5	0.0

Table 13. Progesterone Males. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
P1B	5.0	5.4	8.0
P1d.7-8	5.5	-	-
P1d.7-9	4.8	-	-
P2A	6.0	6.4	6.7
P3C	5.6	5.8	3.6

Table 14. Testosterone Females. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
T1A	4.9	5.2	6.1
T1C	5.6	5.4	-3.6
T2A	6.2	6.9	11
T2C	6.7	7.1	6.0
T3A	5.2	5.1	-1.9
T3D	5.4	5.4	0.0

Table 15. Testosterone Males. Body length in centimeters prior to and after treatment and percent change in body length.

Animal	Body Length Prior To Treatment	Body Length After Treatment	% Change In Body Length
T1B	5.1	5.3	3.9
T1D	4.8	5.2	8.3
T2B	4.3	4.6	7.0
T2D	4.6	4.8	4.3
T2E	4.5	4.6	2.2
T3B	6.1	6.5	6.6
T3C	5.0	4.9	-2.0
T3d.7-8	4.4	-	-

Table 16. Control Female Data. The measurements for egg diameter are for comparative purposes and have no units. All weights are in grams. A value of 0.0 for oviduct weight indicates that the oviducts were thin threads and too small to be removed. GSI=gonadosomatic index. Egg development: 1=poorly, 2=moderately, 3=highly.

Animal	Ovary Weight@	Ovary GSI	Oviduct Weight#	Oviduct GSI	Egg Diameter^	Egg Development*
C1A	.55	1.9	.41	1.4	22	3
C1B	.02	.24	0.0	0.0	8	1
C1C	.06	.65	0.0	0.0	9	1
C2A	.06	.57	0.0	0.0	8	1
C2B	.76	2.6	.23	.78	30	3
C2C	.25	1.2	.17	.81	19	3

@Pearson's correlation test indicates a strong correlation between ovary weight and body weight (r=.96). Ovary weight is also correlated with oviduct weight (r=.83).

#An r value of .93 indicates that oviduct weight is correlated with body weight.

^Egg diameter is correlated with body weight (r=.97) and ovary weight (r=.97) as well as with egg development (r=.92).

*An r value of .95 suggests that egg development is correlated with body weight, and an r value of .85 indicates that egg development is also correlated to ovary weight.

Table 17. Estrogen Female Data. The measurements for egg diameter are for comparative purposes and have no units. All weights are in grams. GSI=gonadosomatic index. Egg development: 1=poorly, 2=moderately, 3=highly.

Animal	Ovary Weight@	Ovary GSI	Oviduct Weight#	Oviduct GSI	Egg Diameter^	Egg Development*
E1B	.10	.92	.04	.37	9	2
E1d.7-9	-	-	-	-	15	2
E2B	.13	.84	.17	1.1	16	2
E2d.7-6	-	-	-	-	5	1
E3d.6-28	-	-	-	-	-	3
E3d.7-2	-	-	-	-	-	2

@Pearson's correlation tests indicate strong correlations between ovary weight and body weight (r=1) and between ovary weight and oviduct weight (r=1).

#Oviduct weight is correlated with body weight (r=1).

^Egg diameter is correlated with body weight (r=1) and ovary weight (r=1). Egg diameter is more weakly correlated with egg development (r=.80).

*An r value of .95 indicates that egg development is correlated to body weight. Egg development correlates more weakly with ovary weight (r=.85).

Table 18. Progesterone Female Data. The measurements for egg diameter are for comparitive purposes and have no units. All weights are in grams. A value of 0.0 for oviduct weight indicates that the oviducts were thin threads and too small to be removed. GSI=gonadosomatic index. Egg development: 1=poorly, 2=moderately, 3=highly.

Animal	Ovary Weight@	Ovary GSI	Oviduct Weight#	Oviduct GSI	Egg Diameter^	Egg Development*
P1A	.18	.79	.16	.70	16	3
P1d.7-11	-	-	-	-	10	1
P2B	.07	.73	0.0	0.0	10	1
P2C	.19	.95	.11	.55	14	3
P2D	.05	.58	0.0	0.0	9	1
P2E	.06	.61	0.0	0.0	11	1
P3A	.07	.73	0.0	0.0	8	1
P3B	.10	.99	0.0	0.0	13	1

@Pearson's correlation tests indicate a strong correlation between ovary weight and body weight (r=.96) and between ovary weight and oviduct weight (r=.93).

#Oviduct weight is strongly correlated to body weight (r=.99).

^The correlation between egg diameter and body weight is an r value of .86, and that between egg diameter and ovary weight is an r value of .87. An r value of .82 indicates some correlation between egg diameter and egg development.

*Egg development is highly correlated to body weight (r=.99) and to ovary weight (r=.96).

Table 19. Testosterone Female Data. The measurements for egg diameter are for comparative purposes and have no units. All weights are in grams. GSI=gonadosmatic index. Egg development: 1=poorly, 2=moderately, 3=highly.

Animal	Ovary Weight@	Ovary GSI	Oviduct Weight#	Oviduct GSI	Egg Diameter^	Egg Development*
T1A	.04	.36	.01	.09	11	2
T1C	.08	.75	.01	.09	10	2
T2A	.17	.91	.28	1.5	12	2
T2C	.19	.92	.24	1.2	14	3
T3A	.10	1.2	.01	.12	10	3
T3D	.09	.88	.03	.29	10	2

@Pearson's tests indicate correlations between ovary weight and body weight (r=.87) and between ovary weight and oviduct weight (r=.92).

#Oviduct weight is strongly correlated to body weight (r=.96).

^Egg diameter is correlated to body weight (r=.94) and more weakly to ovary weight (r=.78). A significant correlation does not exist between egg diameter and egg development (r=.40).

*Egg development is not correlated to body weight (r=.19). Development is also not significantly correlated to ovary weight (r=.45).

Table 20. Estrogen Male Data. The measurements for testes length are for comparitive purposes and have no units. Weight is in grams and is the weight of both testes combined. GSI=gonadosomatic index.

Animal	Mean Testes Length#	Testes Weight*	GSI
E1A	22.5	.01	.14
E1d.7-10	25.0	-	-
E1d.7-11	43.5	-	-
E2A	28.5	.01	.06
E3A	42.5	.02	.09

#Testes length is strongly correlated to body weight as indicated by a Pearson's r value of .92. Testes length is also correlated to testes weight (r=.96).

*Testes weight is weakly correlated to body weight (r=.77).

Table 21. Progesterone Male Data. The measurements for testes length are for comparitive purposes and have no units. Weight is in grams and is the weight of both testes combined. GSI=gonadosomatic index.

Animal	Mean Testes Length#	Testes Weight*	GSI
P1B	34.0	.02	.22
P1d.7-8	22.5	-	-
P1d.7-9	26.5	-	-
P2A	37.0	.03	.15
P3C	36.0	.01	.06

#Pearson's tests indicate a strong correlation between testes length and body weight (r=1) and no significant correlation between testes length and testes weight (r=.33).

*Testes weight is not significantly correlated to body weight (r=.39).

Table 22. Testosterone Male Data. The measurements for testes length are for comparative purposes and have no units. Weight is in grams and is the weight of both testes combined. GSI=gonadosomatic index.

Animal	Mean Testes Length#	Testes Weight*	GSI
T1B	17.5	.02	.22
T1D	35.0	.01	.11
T2B	21.0	.01	.14
T2D	20.0	.01	.15
T2E	25.5	.01	.14
T3B	36.5	.02	.08
T3C	29.0	.01	.13
T3d.7-8	22.0	-	-

#Pearson's tests indicate no strong correlation between testes length and body weight (r=.63) and no correlation between testes length and testes weight (r=.059).

*Testes weight is not strongly correlated to body weight (r=.71).