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The Effects of a Resveratrol Derivative on Regulatory Behaviors and Reproductive Health in Male and Female Long-Evans Rats

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THE EFFECTS OF A RESVERATROL DERIVATIVE ON REGULATORY
BEHAVIORS AND REPRODUCTIVE HEALTH IN MALE AND
FEMALE LONG-EVANS RATS

By

Kim Fabick

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

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BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

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As chair of the candidate's graduate committee, I have read the thesis of Kim Fabick in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

THE EFFECTS OF A RESVERATROL DERIVATIVE ON REGULATORY BEHAVIORS AND REPRODUCTIVE HEALTH IN MALE AND FEMALE LONG-EVANS RATS

Kim Fabick

Department of Physiology and Developmental Biology

Master of Science

Phytoestrogens are chemicals produced by plants that act like estrogens and have the ability to bind to the mammalian estrogen receptor system. The purpose of this study is to evaluate a new phytoestrogen analog called 4-acetoxy Resveratrol. Resveratrol is a phytoestrogen that has been found in the skin of grapes. Resveratrol has been shown to be able to bind to the estrogen receptors and has a similar molecular structure as estradiol. Resveratrol has been shown to have many positive health benefits such as improving cardiovascular health, serving as a neuroprotective agent, acting as an anti-inflammatory agent, working as an anti-cancer agent, increasing sperm output, acting as an anti-aging

agent, and reducing incidence of prostatic adenocarcinoma. The challenge with using Resveratrol as an oral therapy is that it is quickly metabolized by the liver so for this study we used injections. The injections were 5mg/ Kg, 20 mg/Kg, and 90 mg/Kg of 4-acetoxy Resveratrol. We used intact 160 day old male Long-Evans rats and intact 90 day old female Long-Evans rats. The rats were given injections once a day for 21 days based on their treatment group. The animals were weighed daily and then tested in the Porsolt swim test at day 160 and 90 respectively. At the end of 21 days the rats were sacrificed and white adipose tissue, blood, brains, testis, and prostates were collected.

Administration of 4-acetoxy Resveratrol decreased weight gain but not white adipose tissue in the male rats but has no effect in the females. In the male rat administration of 4-acetoxy resveratrol the high group also decreased testosterone, 5 α -DHT, and prostate 5 α -reductase activity. The high dose of 4-acetoxy Resveratrol also caused a change in prostate histology and decreased prostate weight. 4-acetoxy Resveratrol had no effect on testis weight and only showed a slight increase in depressive-like behaviors. In the females, 4-acetoxy resveratrol had no effect on white adipose tissue deposition, estrous cycle, hypothalamus aromatase activity, or depressive-like behaviors.

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I would first like to thank Dr. Edwin Lephart for all the help and advice in working on this project. I would also like to thank Dr. Merritt Andrus for providing the compound that was used in this study. I also wish to acknowledge my other committee members, Dr. James Porter and Dr. Roy Silcox. I especially appreciate the help from the Lephart lab which includes: Crystal Blake, Jeff Hamaker, Jimmy Mitts and Tim Aucoin.

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General Introduction: Phytoestrogens are chemicals produced by plants that act like estrogens and have the ability to bind to the mammalian estrogen receptor system.

Phytoestrogens represent hundreds of molecules possessing non-steroidal, diphenolic structures found in many plants whose chemical and structural properties are similar to those of estrogens (1-4). Phytoestrogens are found in high abundance in soy food products such as soy beans, soy milk, tofu, and cereals containing soy (4). These foods contain biologically active components that promote health throughout the life cycle in humans especially during aging (5-8). Previously, investigation examining soy diets in animals have shown that phytoestrogens decrease white adipose tissue by stimulating lipolysis and inhibiting lipogenesis. It has also previously been shown, in male Long-Evans rats, that a phytoestrogen-rich diet reduces weight gain, decreases white adipose tissue deposition, and mean arterial blood pressure (9).

The purpose of this study was to evaluate a new phytoestrogen analog called 4-acetoxy Resveratrol (10). Resveratrol has been found to be a phytoestrogen because it will compete with I¹²⁵ labeled estradiol for binding to the estrogen receptor and it also has a similar molecular structure as estradiol (10). Resveratrol has been found in the skin of grapes and was originally studied to determine if it could explain the 'French paradox' and it has subsequently been found to be a phytoestrogen because it has the ability to bind to the estrogen receptor and comes from a plant (10, 11). The 'French Paradox' is the general French population consumes a high calorie high fat diet but has a lower incidence of heart related diseases or illnesses. However, it has also been previously reported that resveratrol can act as a mixed agonist/antagonist for both of the estrogen receptors, ER α and ER β (12 -15); because of this controversy, we were interested in studying it as a

phytoestrogen to see if it has similar physiological effects as other phytoestrogens previously studied in this laboratory. Recently however it has been reported that Resveratrol, like the other phytoestrogens, preferentially binds to ER β over ER α (16). Resveratrol also has the ability to act as a selective estrogen receptor modulator (SERM) with the wild type estrogen receptor (17). SERMs are a class of drugs that bind to the estrogen receptor and then elicit either agonistic or antagonistic response based on the target tissue. Finally, resveratrol has been shown to have many positive health benefits such as improving cardiovascular health, serving as a neuroprotective agent, acting as an anti-inflammatory agent, working as an anti-cancer agent, increasing sperm output, and acting as an anti-aging agent (18-24). Resveratrol is now being examined to see if it would be an effective phytoestrogen in diminishing postmenopausal symptoms (25).

Due to the results of the women's health initiative there is an interest in possibly using resveratrol to help with post menopausal symptoms instead of hormone replacement therapy. The effects of Resveratrol on reproductive physiology and behavior are currently being examined and so far Resveratrol has not shown estrogenic activity on reproductive physiology (26). However, this study is specifically interested in specifically seeing how resveratrol affects depressive-like behaviors in both intact females and intact males. The interest in males is novel because it has not been previously examined and since resveratrol is presently commercially available as a dietary supplement it is important to know what affects it has.

Currently there is one problem of using resveratrol as a commercial replacement for hormone replacement therapy which is that it is quickly metabolized (peak concentration 30 minutes after ingestion) in the liver when taken orally (27). Therefore,

to be effective orally, large doses must be taken (around 20mg/kg/day), however; in this study, a resveratrol analog was used that should be more stable in the system and therefore require lower concentrations (28). In this study injections were also used instead of oral administration.

Finally, this study was divided into two separate manuscripts in preparation for publication. In this thesis the first manuscript is the female study. In this study, high doses of resveratrol were administered and then various parameters were measured to determine the effects of giving a high dose of phytoestrogen. The parameters that were examined are weight gain, white adipose tissue deposition, estrous cycle, hypothalamus aromatase activity, and Porsolt swim test depressive-like behaviors. The second manuscript is the male study. The male study follows the female study but goes more in depth and includes prostate weight, testis weight, and histology were examined along with serum hormone levels that were quantified. Therefore, the references for both studies are cited here in combination (see below). The references were separated in the females and male investigations that follow.

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The Effects of a Resveratrol Derivative on Regulatory Behaviors and Reproductive Health in Female Long Evans Rats.

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Abstract: Phytoestrogens are the naturally occurring compounds found in plants that act like estrogens. These compounds have the ability to bind to both mammalian estrogen receptors. The phytoestrogen focused on in this study is a new phytoestrogen analog called 4-acetoxy resveratrol (4AR). Due to the women's health initiative there is an interest in using phytoestrogens to help with post menopausal symptoms; however, because of the current trend in an increase in consumption of phytoestrogens in the diet it was interesting to study the effects of a specific resveratrol analog, 4-acetoxy resveratrol, on intact females. The purpose was to determine what, if any, side effects there were when 4AR was administered subcutaneously in high doses to intact female Long-Evans rats. In this study, 4-acetoxy resveratrol was administered subcutaneously once a day for 21 consecutive days. The injections were 20 mg/Kg or 90 mg/Kg of 4AR. During the 21 day period of injections the rats were weighed daily and the estrous cycle was monitored. At the end of 21 day treatment interval the rats were sacrificed and white adipose tissue, blood, and brains were collected. Administration of 4AR in these high doses did not have the same effects as previously studied phytoestrogens. 4AR had no effect on weight gain, white adipose tissue deposition, hypothalamic aromatase activity, or depressive-like behaviors via the Porsolt forced-swim test.

Introduction: Phytoestrogens are the naturally occurring compounds found in plants that act like estrogens. These compounds have the ability to bind to both mammalian estrogen receptors. Phytoestrogens are compounds that are non-steroidal and have

diphenolic structures and processes structural properties similar to those of estrogens (1-4). The most common phytoestrogens are found in the soy plant and various soy products (4). The foods that contain phytoestrogens help promote health in humans especially during aging (5-8). Previously, soy diets have been shown to decrease white adipose tissue by stimulating lipolysis.

The phytoestrogen used in this study is a resveratrol analog called 4-acetoxy resveratrol (9). Resveratrol is a phytoestrogen that has previously been studied to explain the French Paradox. This is the observation that the French have a relatively low incidence of heart-related disease or illness in spite of eating a high calorie, high fat diet. Resveratrol has been found to be a phytoestrogen because of its ability to compete with I¹²⁵ labeled estradiol for binding to the estrogen receptor. Resveratrol has a similar molecular structure as estradiol, and has been found to bind to both estrogen receptors (9, 10). Resveratrol was thought to act as a mixed agonist/antagonist but most recently has been found to bind to the ER β receptor with a great affinity than to the ER α receptor (11-15). Resveratrol has been proven to have many health benefits such as improving cardiovascular health, serving as a neuroprotective agent, acting as an anti-inflammatory agent, working as an anti-cancer agent, and acting as an anti-aging agent (17-22). Currently, Resveratrol is being examined to see if it would be an effective phytoestrogen in diminishing post menopausal symptoms (23).

Due to the results of the women's health initiative there is an interest in using phytoestrogens to help with post menopausal symptoms; however, because of the current trend in an increase in consumption of phytoestrogens in the diet it was interesting to study the effects of a specific resveratrol analog, 4AR, on intact females (24). The

purpose was to determine what, if any side effects there are when 4AR is administered subcutaneously in high doses to intact female Long-Evans rats.

Methods: Twenty one female Long-Evans rats were ordered from Charles Rivers Laboratories (Wilmington, MA). While at the supplier they were fed a 200 ppm isoflavone diet. Once the rats arrived at our laboratory they were placed on a phytoestrogen free (10ppm) diet. The rats were allowed to habituate for two weeks. Then the rats were divided into three groups (age and body weight matched) with seven rats in each group. The rats were put into the following groups; dimethyl sulfoxide (DMSO) control, 20.0 mg/Kg, and 90.0 mg/Kg 4AR in DMSO. At the end of the two week habituation period the rats were given 0.1cc injections daily for 21 days. During this time period the rats were weighed daily. Food and water intake was monitored twice during the injection period. Before and during injection the estrous cycle was monitored for eight days. The rats were 90 days old at the time of testing. On day 21 the rats were tested in the Porsolt swim test for depressive-like behaviors. The next day they were sacrificed and blood and white adipose tissue were collected.

Porsolt Swim Test: The animals were analyzed using the Porsolt forced swim test that is a standard antidepressant test in pharmaceutical laboratories worldwide and in academic laboratories for investigating the neurobiology of depression and of antidepressant agent action (25-29). In a typical test, the rat is forced to swim vigorously; however, over time some of the animals become immobile which is a condition of behavioral despair. The total time an animal is immobile is an index of despair or depression. Other parameters include:

A. average swimming speed

- B. total time mobile (the opposite of the immobile index)
- C. number of dives the animal attempts demonstrating escape behaviors
- D. the number of boli excreted during the test

The number of boli excreted indexes the level of emotional stress, increased boli numbers during the test would indicate heightened emotional behavior. All of these parameters were recorded and quantified by AnyMaze® computer software and the total time of the Porsolt forced swim test was 360 seconds or 6 minutes.

The animals that displayed increased levels of average swim speed, mobility times, and dives along with low numbers for total time immobile and boli excreted exhibited low levels of despair or depression. Animals that exhibited behaviors opposite to the parameters above displayed increased levels of despair or depression.

Aromatase Activity: Hypothalamic aromatase was determined by dissecting out the hypothalamus and then quantifying aromatase activity via an indirect enzyme assay. The assay was a tritiated water assay in which we measure the amount of tritiated water produced during the conversion of testosterone to estradiol and it is a 1:1 ratio. So for every conversion 1 tritiated water molecule is released and thus we can use this as a way to identify the amount of aromatase activity.

Statistics: The data was analyzed by ANOVA followed by pairwise comparisons (Tukey's) and the alpha levels was set at $p < 0.05$.

Results: Throughout the course of the injections food and water intake was measured. . Figure 1 and 2 display the results of food and water intake monitoring and show no difference in intake between the three treatment groups. Figure 1 represents food and water intake at day 8 and 9 of injections. Figure 2 represents food and water intake at

day 20 and 21 of injections. Figure 3 shows the rat's weights everyday for the 21 days of injections. In this study, no differences in the rat's weights during the injection period were seen. Once the rats were sacrificed, at 90 days old, their white adipose tissue (WAT) in the abdominal cavity was dissected out and weighed. Figure 4 displays the WAT weights and shows that there was no difference in WAT amount among the treatment groups. Figure 5 shows the WAT divided by 100g of body weight; still there were no differences between the groups. The estrous cycle was monitored for 8 days before and during the injection period. Prior to the injection period all rats displayed a normal 4-day estrous cycle in all of the treatment groups. Figure 6 represents the estrous cycle of the rats during the injection period and no change in estrous cycle was seen. However, three rats in each treatment displayed abnormal estrous cycles. This is most likely due to the stress of daily injections. At the time of sacrifice the brains were also dissected out and were later used to test hypothalamic aromatase activity. Figure 7 shows the aromatase activity and again no difference in aromatase activity in the hypothalamic region was seen, even though there was a slight increase in aromatase activity in the 20mg/Kg and 90mg/Kg 4AR groups, it was not statistically significant. Figures 8-13 show the Porsolt swim test results. Each of these is an indicator for depressive-like behaviors. In each of these parameters no difference was seen in depressive-like behaviors among the treatment groups.

Discussion: Weight: Based on other studies done with phytoestrogens it was anticipated that the animals given 4AR injections would have a decrease in weight gain and white adipose tissue. However, this was not found in this study. This study found that there was no difference in weight gain or white adipose tissue. Resveratrol is a phytoestrogen

because it will bind to the estrogen receptor, but in the case of weight gain 4AR acts differently than the other phytoestrogens. Therefore, 4AR may be acting on adipose tissue by a different mechanism than other phytoestrogens. Further research needs to be conducted to determine the mechanism by which 4AR is acting on adipose tissue, if it has any action on adipose tissue.

Aromatase Activity: Aromatase is the enzyme that converts testosterone to estradiol. Aromatase activity was measured to see if 4AR would increase estrogen biosynthesis. If there was an increase in aromatase activity we would be able to conclude that there is an increase in local estradiol production. When hypothalamic aromatase activity was measured there was no difference in activity with the administration of 4AR. Based on this it is concluded that 4AR does not alter brain aromatase activity and that it does not cause any changes in hypothalamic estradiol concentrations. This is also consistent with the finding that estrous cyclicity was not interrupted with the administration of 4AR. Since, hypothalamic aromatase activity was normal it is presumed that the plasma hormone concentrations would be normal and thus would allow the rats to cycle normally.

Porsolt Test: Based on other Porsolt results when female rats were administered phytoestrogens it was expected to find a decrease in depressive-like behaviors. However, no changes in depressive-like behaviors were seen. Administration of 4AR does not appear to increase or decrease depressive-like behaviors. So unlike the other phytoestrogens 4AR might not exhibit as strong of an effect on neurotransmitter reuptake, specifically on serotonin and the 5HT1A receptor. The other consideration is that in these females the ovaries were intact, which means they were still producing their

own estradiol which could mean that they had enough circulating natural estradiol to be neuroprotective and that is why there was no difference with 4AR administration. Future studies need to be conducted using ovariectomized rats to see if once the ovaries and major source of estradiol were removed if 4AR could then exert its neuronal effects.

Conclusion: In this study 4AR, a known phytoestrogen, was given in very high doses and surprisingly seemed to have no effects on the intact female Long-Evans rats. 4AR did not have the same effects as previously studied phytoestrogens. In this study, 4AR had no effect on weight gain, WAT, aromatase activity, estrous cycle, or depressive-like behaviors. 4AR did not decrease WAT so it may be acting by another mechanism than lipolysis and it did not exhibit a decrease in depressive-like behavior so it must also be exhibiting a decrease in neuronal effect compared to other phytoestrogens. The reason could be because this study used intact females and the other studies have been done on ovariectomized females. This would change the amount of endogenous plasma hormones which could affect some of the results.

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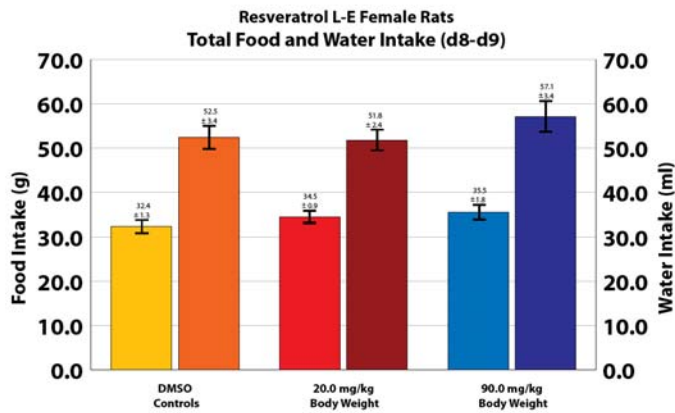


Figure 1: Food (g) and water (ml) intake was measurements at day 8 and 9 of injections. The left bars are food intake and the right bars are water intake. The yellow-orange bars are the DMSO control group. The red-maroon bars are the 20 mg/Kg 4AR group. The blue bars are the 20 mg/Kg 4AR group. No difference in intake was found.

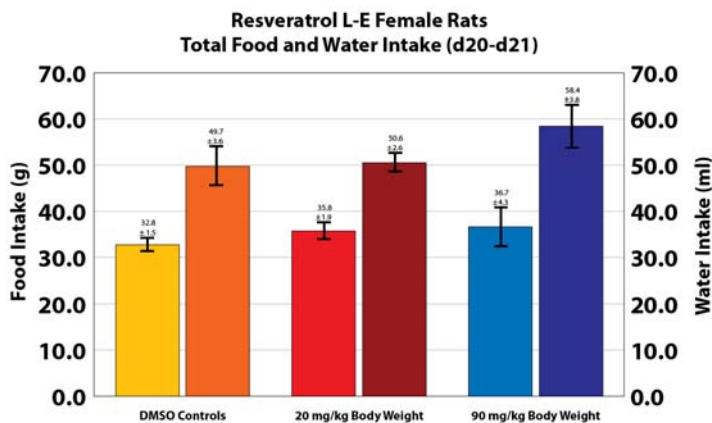


Figure 2: Food (g) and water (ml) intake was measurements at day 20 and 21 of injections. The left bars are food intake and the right bars are water intake. The yellow-orange bars are the DMSO control group. The red-maroon bars are the 20 mg/Kg 4AR group. The blue bars are the 20 mg/Kg 4AR group. No difference in intake was found.

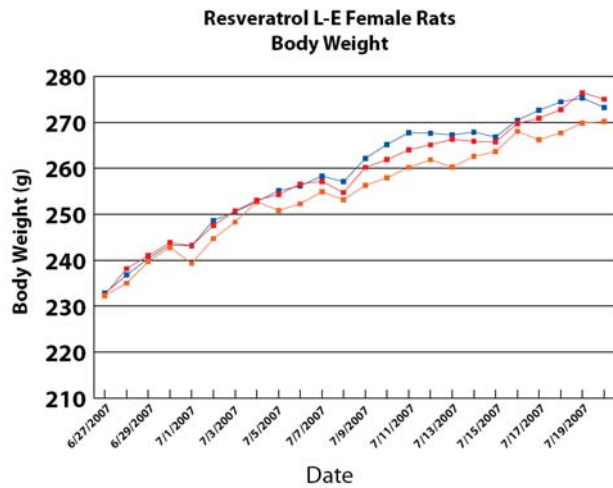


Figure 3: Daily rat weights for the 21 day injections period. The orange is the DMSO control group, the red is the 20mg/Kg 4AR group, and the blue is the 90mg/Kg 4AR group. No significant differences were found between groups.

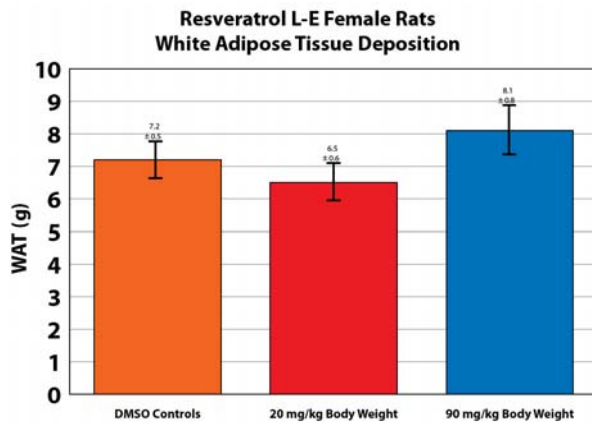


Figure 4: White adipose tissue (WAT) weight. The orange bar is the DMSO control group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference in WAT was found between the groups.

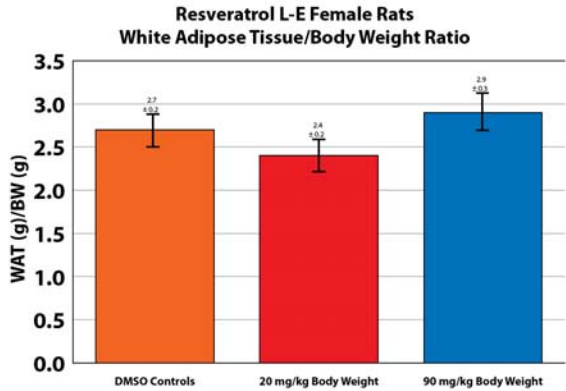


Figure 5: White Adipose Tissue/Body Weight ratio. WAT was divided by 100g of body weight. The orange bar is the DMSO control, the red bar is the 20mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference was found between the groups.

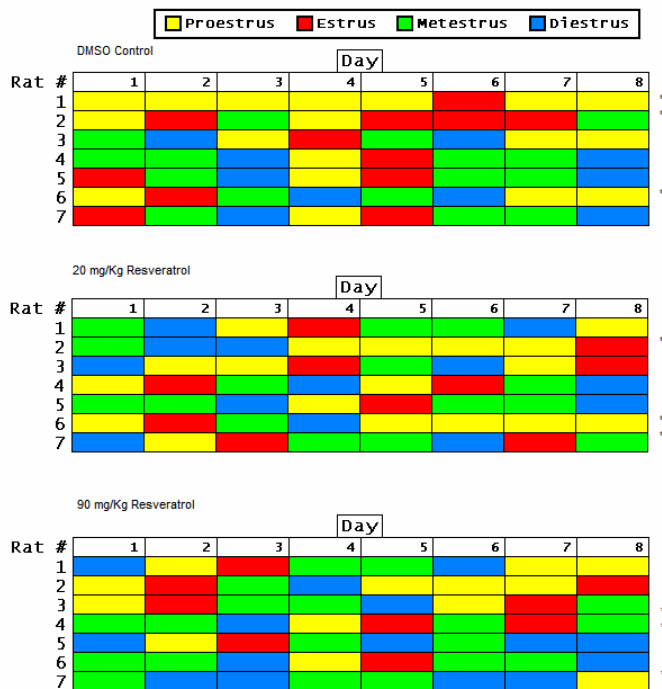


Figure 6: Estrous cycle during the injection period. The stars indicate the rats that had an abnormal estrous cycle. Before the injections all rats displayed regular 4-day estrous cycles, presumably the stress of injections caused three rats in each group to stop cycling. No significant differences in estrous cycle were found among the treatment groups.

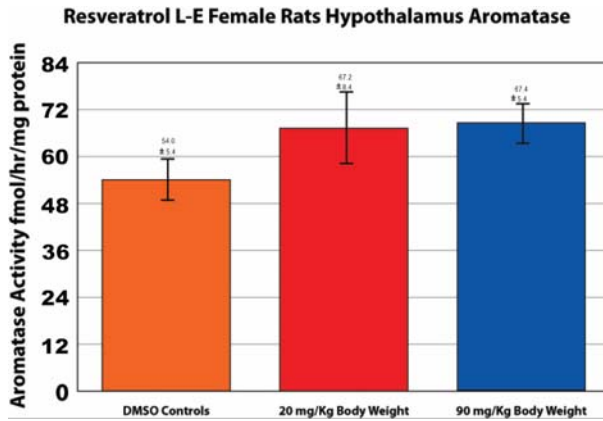


Figure 7: Hypothalamic aromatase activity. The orange bar represents the DMSO control group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference in activity was found between the groups.

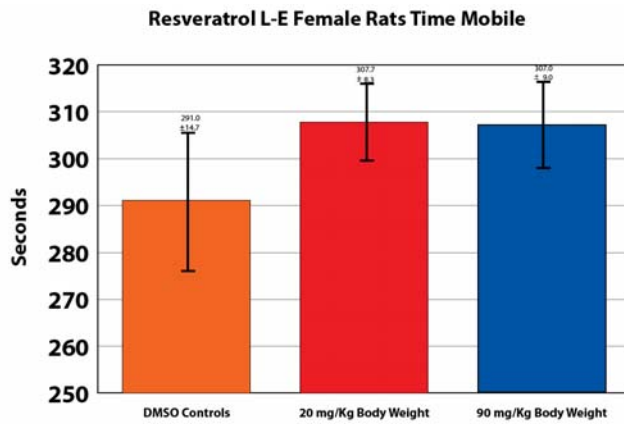


Figure 8: Time mobile in a 6 minute Porsolt swim test. The time spent mobile is an indication of little depressive-like behaviors. The orange bar is the DMSO control, the red bar is 20 mg/Kg 4AR, and the blue bar is the 90 mg/Kg 4AR group. No difference in time spent mobile was found.

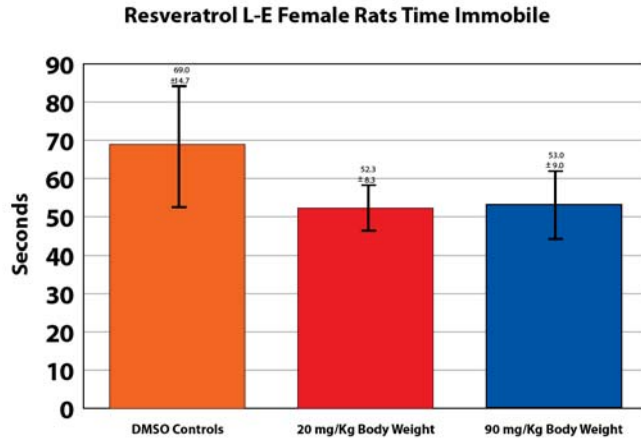


Figure 9: Time immobile in a 6 minute Porsolt swim test. The time spent immobile is an index of depressive-like behaviors. The orange bar is the DMSO control group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference in time immobile was found.

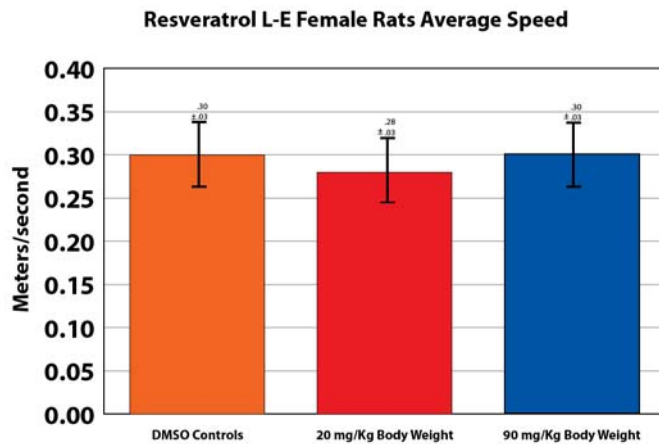


Figure 10: Average swimming speed in a 6 minute Porsolt swim test. The slower the swimming speed the more depressive-like behavior the rat is exhibiting. The orange bar is the DMSO control group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No differences in swimming speed were found.

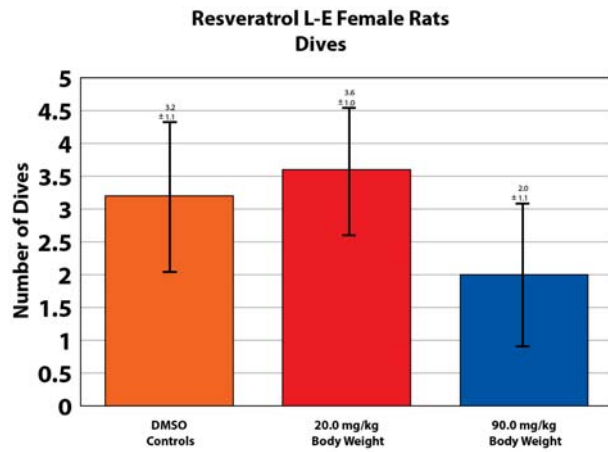


Figure 11: Dives during a 6 minute Porsolt swim test. The number of dives represents escape behavior. The orange bar is the DMSO group, the red bar is the 20 mg/Kg group, and the blue bar is the 90 mg/Kg group. No significant difference in the number of dives was found.

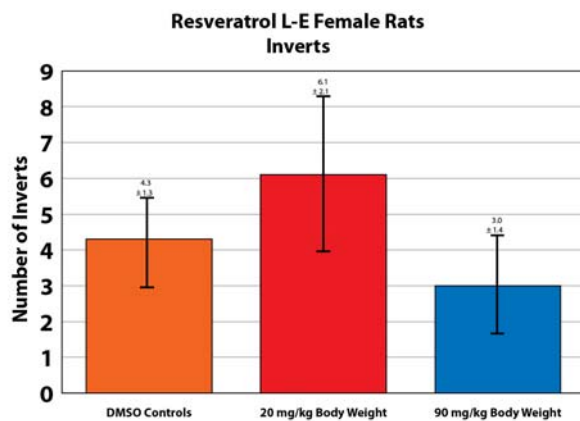


Figure 12. Inverts in a 6 minute Porsolt swim test. Inverts are partial dives and also represent escape behavior. The orange bar is the DMSO control group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant difference in the number of inverts was found.

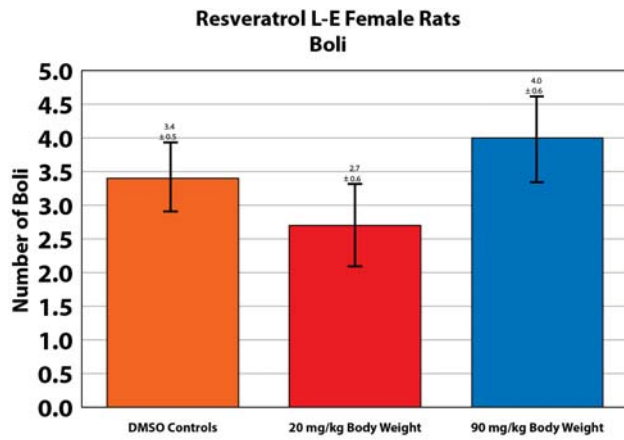


Figure 13: Number of boli released in a 6 minute Porsolt swim test. Boli released is an index of emotional stress. The orange bar is the DMSO control group, the red bar is the 20 mg/KG group, and the blue bar is the 90mg/Kg group. No difference in the number of boli was found.

The Effects of a Resveratrol Derivative on Regulatory Behaviors and Reproductive Health in Male Long-Evans Rats.

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Abstract: Resveratrol is a phytoestrogen found in the skin of grapes and has been studied to explain the French Paradox. Resveratrol has been shown to be able to bind to the estrogen receptors and mimic the effects of estradiol. Resveratrol has been shown to have many positive health benefits such as improving cardiovascular health, serving as a neuroprotective agent, acting as an anti-inflammatory agent, working as an anti-cancer agent, increasing sperm output, acting as an anti-aging agent, and reducing the incidence of prostatic adenocarcinoma. The purpose of this study was to use a resveratrol analog, 4-acetoxy resveratrol (4AR), and determine its effects in intact male Long-Evans rats on regulatory behaviors and reproductive health parameters. The problem with using Resveratrol as an oral therapy is that it is metabolized very quickly so in this study injections were used instead of oral administration. The injections were 5mg/Kg, 20 mg/Kg, and 90 mg/Kg of 4AR. The 4AR analog should be more stable than resveratrol in the system and therefore it should have a longer half-life and increased bioavailability. 140 Day-old male Long-Evans rats were used and given one injection for 21 consecutive days. The animals were weighed daily and tested in the Porsolt swim test at 160 days old. At the end of the 21 day injection period the rats were sacrificed and white adipose tissue, blood, prostate, and testis were collected. Administration of 4AR decreased weight gain but not white adipose tissue when a high dose was given, presumably because resveratrol exerts anti-androgen effects at this high dose. 4AR might accomplish this by binding estrogen receptors preferentially and decreasing lutenizing hormone (LH)

output or Leydig cell production. This may explain how 4AR decreased serum testosterone, 5 α -DHT, and prostate 5 α -reductase enzyme activity. The high dose of 4AR also caused a change in prostate histology and decreased prostate weight. 4AR had no effect on testis weight and only showed a slight (but non-significant) increase in depressive-like behaviors.

Introduction: The purpose of this study was to evaluate a new phytoestrogen analog called 4-acetoxy resveratrol (1). Phytoestrogens are non-steroidal, diphenolic chemicals found in plants that have the ability to mimic estrogen and bind to the estrogen receptors (2-5). Resveratrol is a compound found in the skin of grapes and has been studied to explain the ‘French Paradox’. The ‘French Paradox’ is the observation that the French have a low incidence of heart related disease and illness in spite of consuming a high calorie, high fat diet (6, 7). Resveratrol has been shown to be a phytoestrogen because it will compete with I¹²⁵ labeled estradiol for binding to the estrogen receptors (6). Resveratrol also acts as a selective estrogen receptor modulator (SERM) with the wild type estrogen receptor (8). SERMs are a class of drugs that bind to the estrogen receptor and then elicit either agonistic or antagonistic response based on the target tissue. Recently, resveratrol has been shown to bind to ER β with greater affinity than to ER α (9). Resveratrol has many positive health benefits such as improving cardiovascular health, serving as a neuroprotective agents, acting as an anti-cancer agent, increasing sperm output, reducing the incidence of prostatic adenocarcinomas, and acting as an anti-aging agent (10-21). Resveratrol has also been shown to sensitize prostate cancer cells to apoptosis and could therefore be used as a treatment of prostate pathologies (22).

Currently there is one problem in using resveratrol as a commercial oral therapy which is that it is quickly metabolized (peak concentration 30 minutes after ingestion) in the liver when taken orally (23). Therefore, to be effective orally, large doses must be taken (around 20mg/kg/day), however; in this study, 4AR was used and this analog should be more stable in the system and therefore require lower concentrations (1).

The purpose of this study was to determine the effects of 4AR on reproductive health in intact male Long-Evans rats, to test whether the resveratrol analog could be used to prevent and treat prostate pathologies. Since 4AR is an analog of a phytoestrogen it was also interesting to determine if the analog had any effect on depressive-like behaviors.

Methods: Twenty male Long-Evans rats were ordered from Charles Rivers Laboratories (Wilmington, MA). While at the supplier they were feed a 200 ppm isoflavone diet. Once the rats were received they were placed on a phytoestrogen free diet (10 ppm) and they were allowed to habituate for two weeks before any testing was started after mating and the animal were approximately 140 days old. At the end of the two weeks the rats were divided into four groups with five rats per group. The groups were a dimethyl sulfoxide (DMSO) control, 5.0 mg/Kg, 20.0 mg/Kg, and 90.0 mg/Kg of 4AR in DMSO. The rats then received 0.1cc injections everyday for 21 consecutive days based on the treatment. Food and water intake was measured two separate times during injections and the rat's weight was recorded daily. At the time of testing the males were 160 days old. At day 21 of injections the rats were tested for depressive-like behaviors via the Porsolt swim test. The next day the rats were sacrificed and blood, tissue, and white adipose tissue was collected. Blood was collected to determine testosterone, estradiol, leptin, and

5 α -DHT levels in plasma. The prostate was collected and weighed and then used to quantify 5 α -reductase activity. Prostate histology was also determined by 50 μ m slices and Thionin staining. The right testis was collected and weighed to determine if resveratrol had any affect on testis size.

Porsolt Swim Test: The animals were analyzed using the Porsolt forced swim test which is a standard antidepressant test in pharmaceutical laboratories worldwide and in academic laboratories for investigating the neurobiology of depression and of antidepressant agent action (24-28). In a typical test, the rat is forced to swim vigorously; however, over time some of the animals become immobile which is a condition of behavioral despair. The total time an animal is immobile is an index of despair or depression. Other parameters include:

- A. average swimming speed
- B. total time mobile (the opposite of the immobile index)
- C. number of dives the animal attempts demonstrating escape behaviors
- D. the number of boli excreted during the test

The number of boli excreted indexes the level of emotional stress, increased boli numbers during the test indicated heightened emotional behavior. All of these parameters were recorded and quantified by AnyMaze® computer software and the total time of the Porsolt forced swim test was 360 seconds or 6 minutes.

The animals that displayed increased average swimming speed, mobility times, and dives along with low numbers for total time immobile and boli excreted exhibited low levels of despair or depression. Animals that exhibited behaviors opposite to the parameters above displayed increased levels of despair or depression.

Hormone and Enzyme Activity: In this study, radioimmunoassay (RIA) was used to determine serum leptin, estradiol, and testosterone hormone concentrations. Serum 5 α -DHT was quantified using an ELISA kit and prostate 5 α -reductase activity was a direct enzyme measurement using a TLC plate and radioactive metabolites to determine enzymatic activity.

Statistics: All data sets were tested by ANOVA followed by pair wise comparisons, where appropriate, and the alpha levels were set a $p < 0.05$.

Results: Through out the study the rats were monitored for their food and water intake. Figure 1 represents food and water intake that was measured at day 8 and 9 of the injection period. Figure 2 also represents food and water intake that was measured at day 20 and 21 of the injection period. There were no differences in food and water intake among the groups. Figure 3 displays the daily weights, at day 7 the 90mg/Kg group started to diverge and this group continued to decrease in weight throughout the study ($p < 0.01$). Once the rats were sacrificed the white adipose tissue (WAT) was dissected and weighed. Figure 4 shows WAT weight and no difference in WAT weight among the treatment groups was observed. Then the WAT was normalized to the body weight, figure 5 displays these results and no significant differences were obtained. At the time of sacrifice, blood was collected. From the serum estradiol, testosterone, DHT, and leptin levels were tested. Figure 6 shows leptin levels and no difference in concentrations were found. At sacrifice, the prostates were also collected and weighed and then sliced into tissue sections. Figure 7 shows prostate weight and shows a significant ($p < 0.01$) decrease in weight in the 90g/Kg group compared to control values. Figure 8 shows the ratio of prostate weight per 100 g of body weight. It also shows again a significant

($p < 0.01$) decrease in the 90mg/Kg group compared to controls. Figure 9 is the cross section of the prostates and shows the structural difference in the prostate cells between the DMSO group and the 90 mg/Kg group. In the 90 mg/Kg group the columnar cells had increased in size (60%) and some cells showed involutions (40%). Figure 10 shows 5 α -reductase activity in the prostate. There was a significant ($p < 0.048$) decrease in activity in the 90mg/Kg group, compared to control values. At the time of sacrifice the right testis was also removed and weighed. Figure 11 shows the right testicular weight and no significant decrease in right testicular weight was seen. Again, the testicular weight was normalized to body weight and found no significant difference in figure 12. Figure 13 shows the serum testosterone concentration. A significant ($p < 0.015$) decrease in testosterone concentration in the 90mg/Kg group was found compared to control levels. Figure 14 shows plasma 5 α -DHT concentrations. Again, a significant ($p < 0.017$) decrease in DHT concentration in the 90 mg/Kg group was seen compared to controls. Figure 15 shows serum estradiol concentration and no significant difference in concentration among the groups was seen. Figures 16-21 are the Porsolt swim test indicators for depressive-like behaviors. Figure 16 is time mobile and shows that the 90mg/Kg group spent less time mobile than the 20mg/Kg group and the 5mg/Kg group ($p < 0.052$). Figure 17 shows time immobile. Again, the 90mg/Kg group spent more time immobile than the 20mg/Kg group and the 5mg/Kg group ($p < 0.052$). However, both parameters did not reach statistical significance. Figure 18-21 display various Porsolt tests depressive-like behavior indicators and there were no differences found among the treatment groups.

Discussion: Weight: The rats were weighed daily through out the treatment interval. At day 6 the 90mg/Kg dose began to gain less weight than the other groups. This continued through out the 21 days of injections. This was consistent with what has been found in other phytoestrogens studied in male rats. Based on this it was expected to find a decrease in WAT once the animals were sacrificed. However, no difference in WAT tissue weight was seen. Normally phytoestrogens decrease weight gain by initiating lipolysis (29). However, in this study the rats weighed less but still had the same amount of WAT, therefore the decrease in weight must be due to some other mechanism than lipolysis. Serum leptin levels were tested to see if this was a possible consideration for the decrease in weight gain but there was no difference in leptin levels so this did not contribute to the weight loss. Food and water intake were monitored to determine if the decrease in weight was due to a decrease in intake. But the intake among the groups was the same so the weight loss did not come from a decrease in calorie intact. There are two possibilities as to why there was a decrease in weight gain without observing a decrease in WAT. The first is that the low testosterone levels may have simulated castration either by decreasing LH levels from the anterior pituitary or by inhibiting Leydig cell function directly. Castration has been found to reduce body weight gain; even though castrated rats achieved a higher ratio of adipose weight to body weight than noncastrated rats (30). The study by Faust et al found that castration is seen to impede body weight gain while sparing ordinary growth of adipose tissue and facilitating regrowth of adipose tissue following lipectomy. This could be one possible explanation as to why the rats lost weight but not WAT; however, our results do not directly overlap with that found by Faust. Another consideration could be that resveratrol increases locomotor activity of the

rat and has been shown to improve health on a high calorie diet (31). The study by Sinclair et al found that resveratrol improved motor function, increased insulin sensitivity, and increased hepatic mitochondrial number. They concluded that resveratrol could prevent the deleterious effects of an increase in calorie intake and shift the physiology of a high calorie intake to the physiology of a standard diet, but that this occurs without a significant reduction in body weight (31). Either of these two hypotheses could be considered in explaining the reduction in body weight without the reduction in WAT. However it seems that the reduction in testosterone may be the more likely reason a decrease in body weight without a decrease in WAT was observed in the present study. Since, the rats were eating the same diet and food intake was the same the caloric restriction effects of resveratrol do not really apply to presently obtained results. Also, the mice in the Sinclair study did not display a significant decrease in body weight and the caloric restrictions of resveratrol work by increasing activity and mitochondria so the rats' burn more energy but is not accompanied with a decrease in body weight. For these reasons it is postulated that the decrease in weight gain may be due to alterations in androgen hormones by a mechanism that is currently unknown.

Prostate: The 90 mg/Kg group showed a decrease in 5 α -reductase activity and the cross sections of the prostate found that the 90 mg/Kg group had altered cell shape, 60% of the prostates had an increase in columnar cell size, and 40% had involutions and atrophic cells. It may be that resveratrol works via an anti-androgenic action. This action has previously been shown in equol (32). In the equol study, equol was shown to also have anti-androgenic effects in the prostate and had its action by preventing the action of

DHT. It is possible that resveratrol may be working through a similar mechanism which might assist in considering the changes in body weight.

Testis: Once the animals were sacrificed the right testis was removed and then weighed. No significant decrease in testes weight among the groups was seen, even though in another study resveratrol has been shown to increase sperm output (15). It was expected to see an increase in testis size if in fact sperm output was also increased. Further tests need to be performed to determine what other effects resveratrol might have had on the testes. While not shown in this study's results, testicular histology is currently being analyzed to determine if 4AR has positive influences on sperm production.

Serum Hormone Levels: Testosterone was quantified and found that the 90 mg/Kg group showed a decrease compared to control levels. Which as previously stated may have contributed to the decrease in weight gain. Estradiol was also measured and found that there was no difference in estradiol concentrations. Finally, 5 α -DHT levels were measured and found that the 90 mg/Kg group showed a decrease in DHT concentration compared to control values. If 4AR were having anti-androgenic actions it would make sense that there is a decrease in testosterone, 5 α -reductase activity, and DHT. This would also support the recent finding that resveratrol preferentially binds to the ER β receptor because the ER β is more prevalent in the prostate (33). The information on serum hormone levels is novel and could be valuable for determining commercial levels of 4AR given as dietary supplements or as medical treatments.

Porsolt Test: Analyzing the effects of 4AR on depressive-like behaviors in the Porsolt swim test is also novel and could help with dosing of 4AR when given to patients. At 160 days of age and after 21 days of injections the rats were tested in the Porsolt swim

test. Several parameters on this test were measured to determine depressive-like behaviors. For boli, inverts, dives, and average swimming speed no significant difference was seen. These results would indicate that none of the rats displayed more depressive-like behaviors than the other rats. In time mobile and time immobile the 90 mg/Kg group was significantly different than the 5mg/Kg group and the 20 mg/Kg group. The 90 mg/Kg group spent more time immobile than the other two groups and spent less time mobile. Since time mobile is an anti-depressive-like indicator and time immobile is a depressive-like indicator these two results would indicate that the 90 mg/Kg group showed more depressive-like behaviors than the lower doses of resveratrol. However, the 90 mg/Kg group did not show significantly more depressive-like behaviors than the DMSO control group so it is possible that at low doses resveratrol decreases depression but at higher doses has no effects on depressive-like behavior. More information is needed to determine the effects of resveratrol on depressive-like behavior.

Conclusion: Based on the results of this study, administration of 4AR would decrease weight gain but not WAT when a high dose is given because resveratrol exerts, presumably, anti-androgen effects and possibly mimics castration. Resveratrol might exert its effects on the ER β receptor preferentially and has anti-androgenic effects, because a high dose of 4AR decreased testosterone, DHT, and prostate 5 α -reductase activity. The high dose of 4AR also caused a change in prostate histology and decreased prostate weight. 4AR had no effect on testis weight and only showed a slight alteration in depressive-like behaviors.

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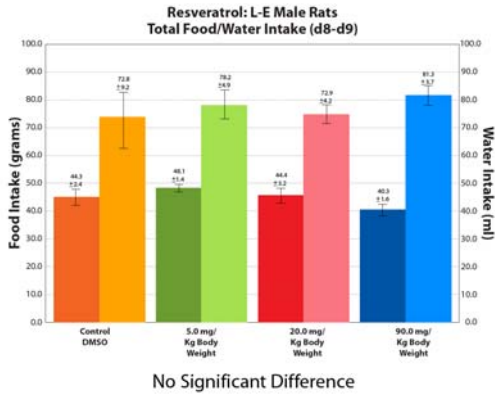


Figure 1: Food (g) and water (ml) intake was measurements at day 8 and 9 of injections. The left bars are food intake and the right bars are water intake. The yellow-orange bars are the DMSO control group. The green bars are the 5 mg/Kg 4AR group. The red-maroon bars are the 20 mg/Kg 4AR group. The blue bars are the 20 mg/Kg 4AR group. No difference in intake was found.

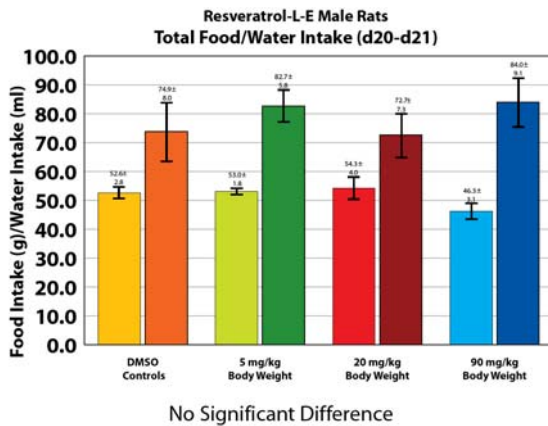


Figure 2: Food (g) and water (ml) intake was measurements at day 20 and 21 of injections. The left bars are food intake and the right bars are water intake. The yellow-orange bars are the DMSO control group. The green bars are the 5 mg/Kg 4AR group. The red-maroon bars are the 20 mg/Kg 4AR group. The blue bars are the 20 mg/Kg 4AR group. No difference in intake was found.

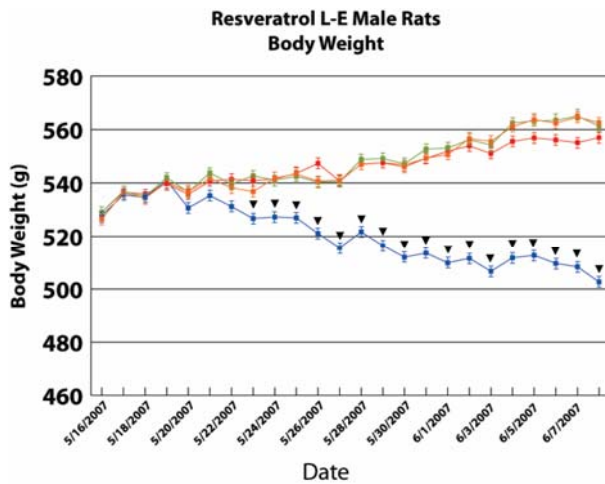


Figure 3: Daily rat weight for 21 day injection period. The orange line is the DMSO control group, the green line is the 5mg/Kg 4AR group, the red line is the 20 mg/Kg 4AR group, and the blue line is the 90mg/Kg 4AR group. The 90mg/Kg Resveratrol group showed a significant decrease in body weight starting 7 days after the injections began (significance marked by triangles).

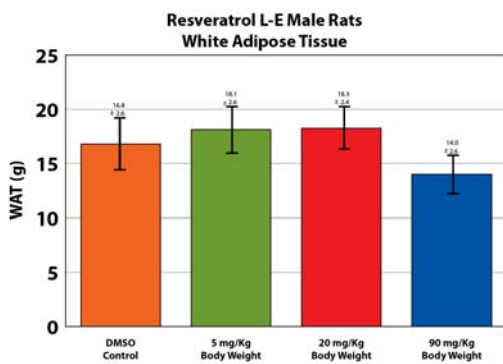


Figure 4: White adipose tissue (WAT) weight. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. There was no significant difference in WAT among the groups.

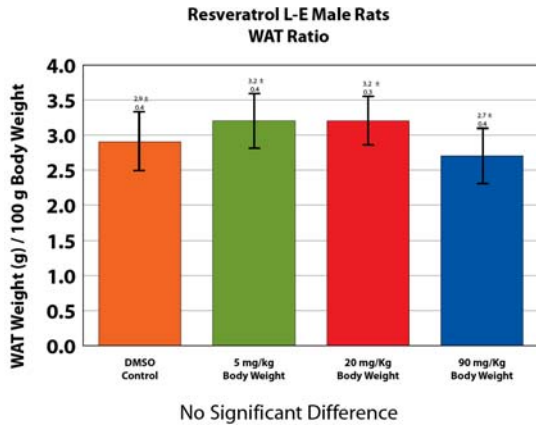


Figure 5: White adipose tissue/ Body Weight Ratio. WAT was divided by 100g of body weight. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference was found.

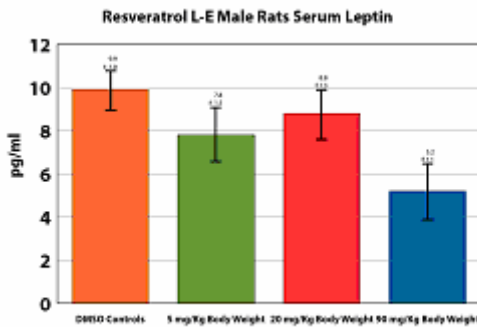


Figure 6: Serum Leptin concentration. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant differences were found.

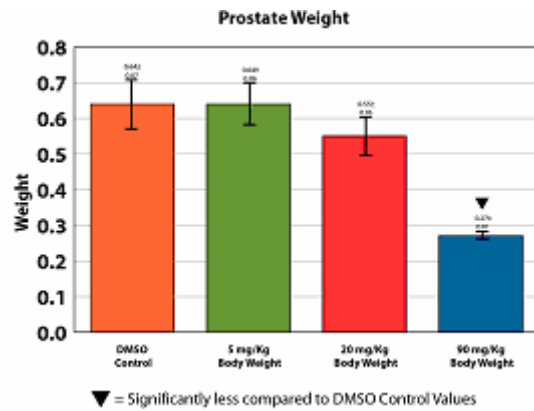


Figure 7: Prostate weight in grams. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. A significant decrease in prostate weight in the 90mg/Kg group was seen (significance marked by triangle).

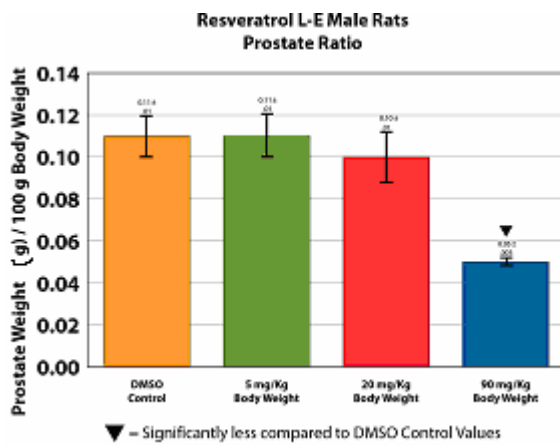


Figure 8: Prostate weight/ Body weight ratio. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. The 90mg/Kg group was significantly less than the other groups (significance marked by triangle).

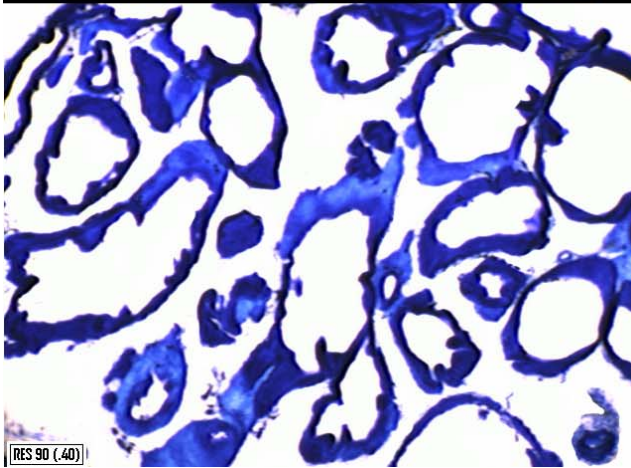
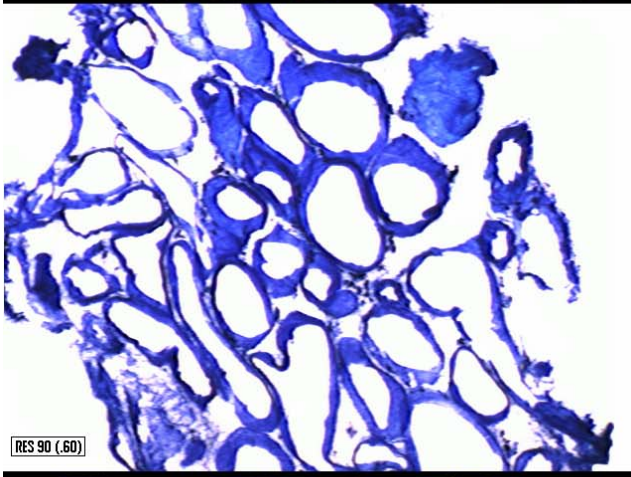
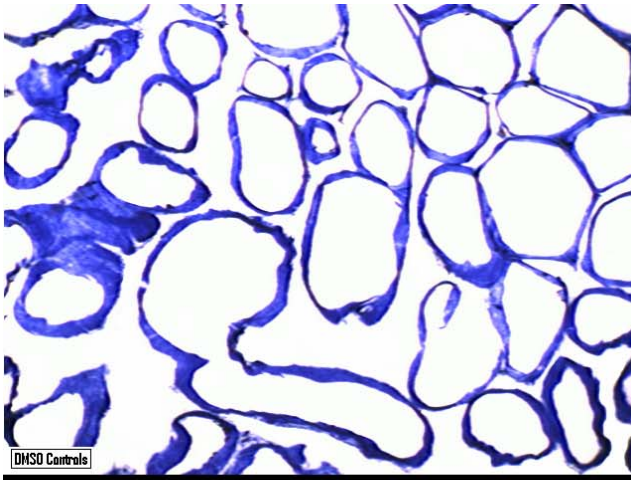


Figure 9: Prostate histology. These are 50 μm cross sections of rat prostates. In the 90 mg/Kg group 40% of the prostates had altered cell shape compared to controls. The other treatment group's results are not displayed graphically because the results are similar to the control.

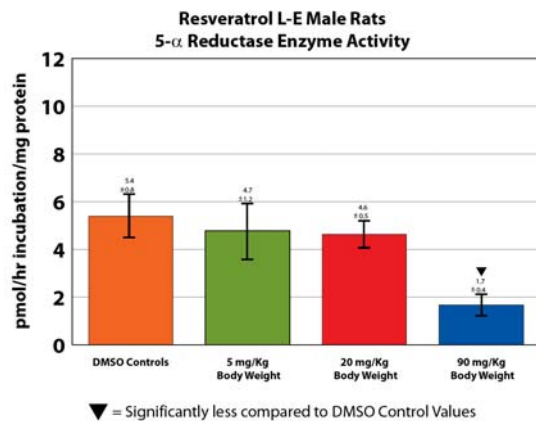


Figure 10: Prostate 5 α -reductase activity. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. The 90mg/Kg group showed a decrease in activity compared to the other treatment groups (significance marked by triangle).

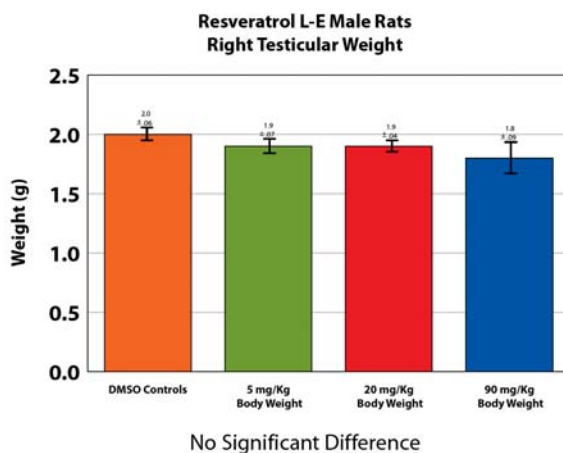


Figure 11: The right testis weight. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant difference in testis weight among the treatment groups.

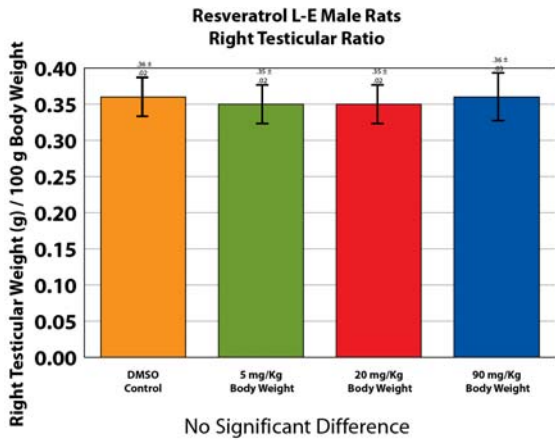


Figure 12: The right testis weight/ body weight ratio. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference was found.

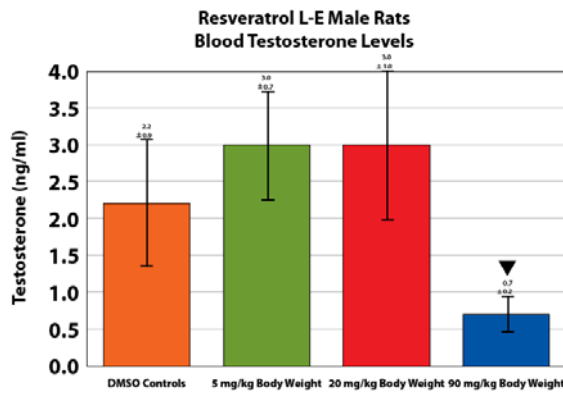


Figure 13: Serum testosterone levels. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. The 90mg/Kg group showed a decreased amount of testosterone (significance marked by triangle).

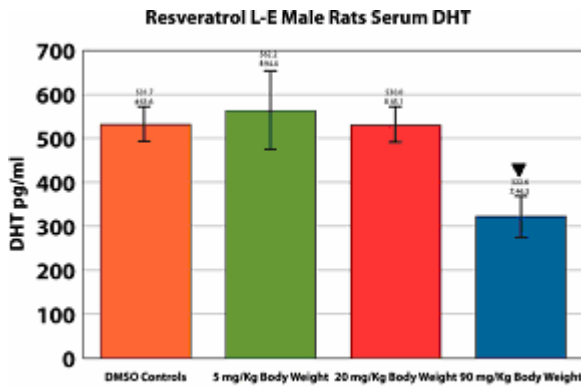


Figure 14: Serum DHT levels. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. The 90mg/Kg group showed a significant decrease compared to all other groups (significance marked by triangle).

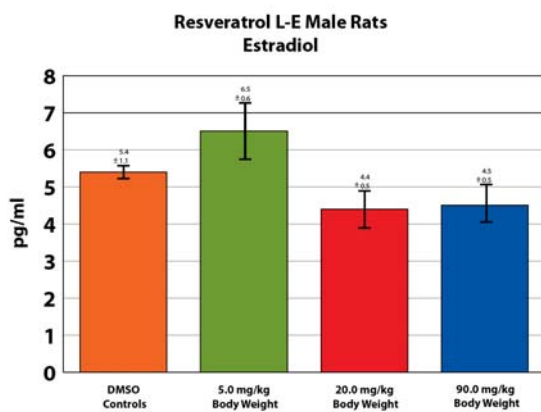


Figure 15: Serum Estradiol levels. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant difference in estradiol concentration was found between the treatment groups.

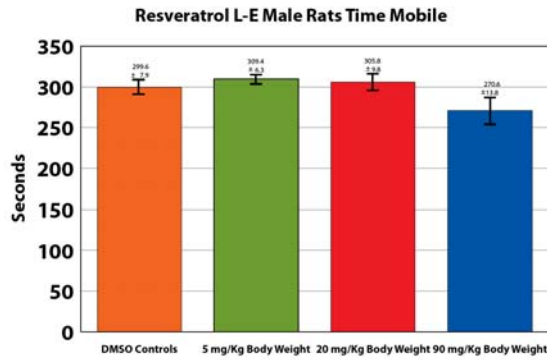


Figure 16: Time mobile in a 6 minute Porsolt swim test. Time mobile is an anti-depressive like index. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. The 90 mg/Kg group spent less time mobile than the 5mg/Kg and the 20 mg/Kg group; however this difference did not reach significance.

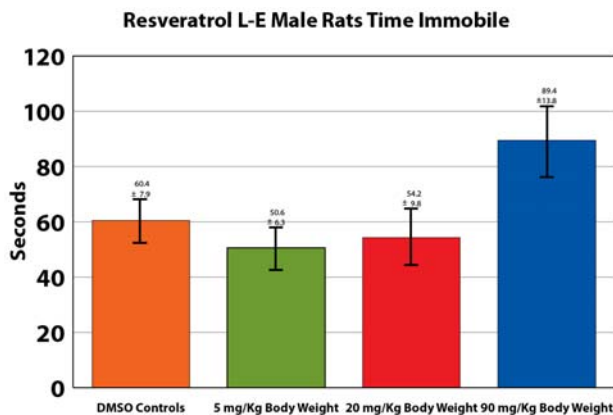


Figure 17: Time immobile in a 6 minute Porsolt swim test. Time immobile is a depressive-like indicator. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. The 90 mg/Kg group spent more time immobile than the 5mg/Kg group and the 20mg/Kg group; however this difference did not reach significance.

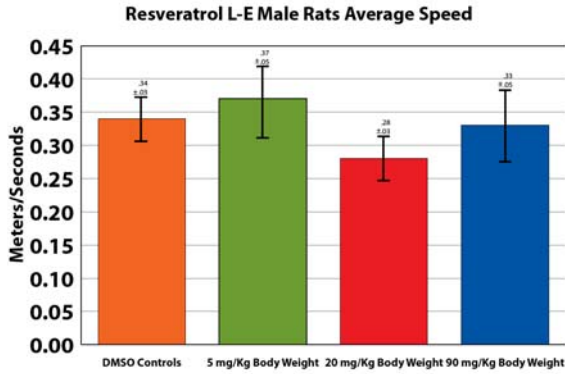


Figure 18: The average swimming speed during a 6 minute Porsolt swim test. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No difference in swimming speed among the groups.

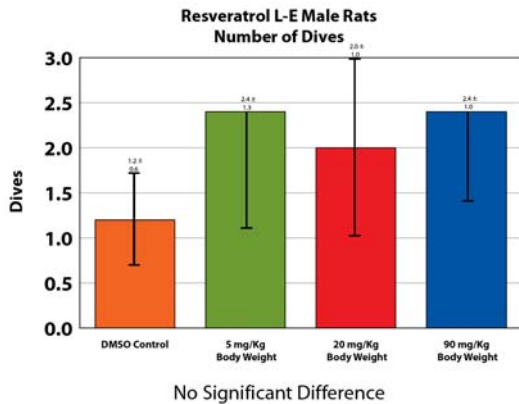


Figure 19: The number of dives during a 6 minute Porsolt swim test. Diving represents escape behavior and is therefore an anti-depressive indicator. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant difference was found.

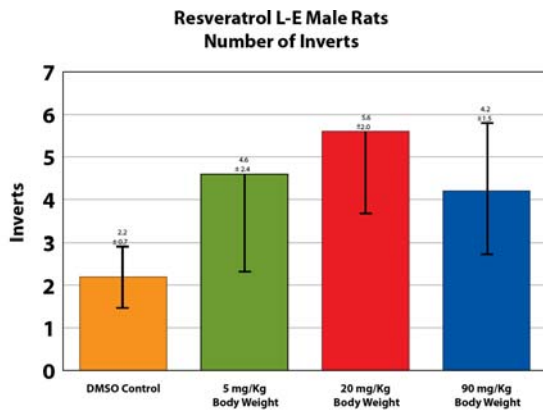


Figure 20: The number of inverts during a 6 minute Porsolt swim test. Inverts are partial dives and are thus an anti-depressive indicator. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant difference between groups was found.

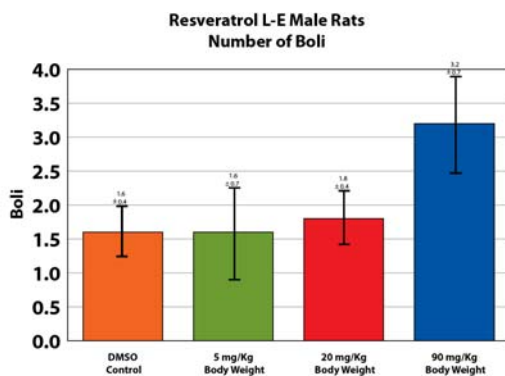


Figure 21: The number of boli released during a 6 minute Porsolt swim test. The number of boli released is an indicator of emotional stress. The orange bar is the DMSO control group, the green bar is the 5 mg/Kg 4AR group, the red bar is the 20 mg/Kg 4AR group, and the blue bar is the 90 mg/Kg 4AR group. No significant difference between groups was found ($p < 0.181$).

General summary: This study examined intact male and female Long-Evans rats with 4AR, a resveratrol analog, administration for 21 consecutive days. The injections were given subcutaneously at the nape of the neck. The animals were then monitored for regulatory behaviors and reproductive health was examined to determine the effects of administration of 4AR.

The females were 90 days old at the time of sacrifice and were divided into three groups: DMSO control, 20mg/kg, and 90 mg/kg 4AR. Surprisingly, even at these high doses the females showed no alterations in the parameters studied and 4AR did not have the same effects as previously studied phytoestrogens. 4AR did not have the same effects as previously studied phytoestrogens. The administration of 4AR had no effect on weight gain, WAT, aromatase activity, estrous cycle, or depressive-like behaviors. Therefore, in the intact females 4AR was inactive on the parameters measured. One possible explanation for this could be that the other studies with phytoestrogens have been done on ovariectomized females that have lower levels of endogenous hormones. If 4AR was administered to ovariectomized females it is possible that some effects would be seen because of the lower levels of endogenous estrogens.

The males were 160 days old at the time of sacrifice and they were divided into four groups: DMSO control, 5mg/Kg, 20 mg/Kg, and 90 mg/Kg 4AR. Administration of 4AR was shown to decrease weight gain but not WAT in a high dose thus 4AR exerts anti-androgen effects and mimics castration. The high dose of 4AR also caused a change in prostate histology and decreased prostate weight but had no effect on testis weight. The 90mg/Kg dose of 4AR decreased testosterone, DHT, and 5 α -reductase activity which shows that 4AR exerts its effects on the ER β receptor preferentially and has anti-

androgenic effects. 4AR only showed a slight increased effect on depressive-like behaviors.

Further research is required to determine the influence of 4AR on regulatory behaviors and reproductive health, since this body of work only represents preliminary data sets that suggest that resveratrol analogs are safe even at very high pharmacological doses.

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Curriculum Vitae

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Academic Training

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Teaching and Research Experience

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Department of Physiology and Developmental Biology, 2007
Performed experiments with various phytoestrogen treatments on Long-Evans rats with Professors Lephart and Porter

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Department of Physiology and Developmental Biology, 2006-2008
Teaching assistant in Human Physiology.

Teaching Assistant, Brigham Young University
Department of Neuroscience, 2007-2008
Teaching assistant in Introduction to Neuroscience

Awards and Distinctions

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Conference Presentation

Positive Benefits of Consuming Soy-Derived Isoflavones on Body Weight Gain, Adipose Tissue Deposition, and Preliminary Cardiovascular Parameters Examined in an Ovariectomized Rat Model, Poster presented at Experimental Biology Annual Meeting 2007, Washington D.C. April, 2007

Abstracts

K. Fabick, C. Blake, J.P. Porter, K.D.R. Setchell, E.D. Lephart. 2007. **Positive Benefits of Consuming Soy-Derived Isoflavones on Body Weight Gain,**

Adipose Tissue Deposition, and Preliminary Cardiovascular Parameters Examined in an Ovariectomized Rat Model. Annual Mtg. Experimental Biology 2007, Washington D.C., Apr. 28-May 2, 2007.

C. Blake, K. Fabick, E.D. Lephart. 2007. **Long-Evans Newborn Male Rats, Prenatally-Treated with Phytoestrogen Display Variations in Body Weight and External Genital Development.** Annual Mtg. American Association of Pharmaceutical Scientist, San Diego, CA, Nov. 11-15, 2007.

Publications (In Progress)

The Effects of a Resveratrol Analog on Regulatory Behaviors and Reproductive Health in Female Long-Evans Rats.

The Effects of a Resveratrol Analog on Regulatory Behaviors and Reproductive Health in Male Long-Evans Rats.

The Effects of Prenatally Administered Phytoestrogens on the Reproductive and Behavioral Development of Long-Evans Rats.