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Anti-Depressive and Anti-Obesity Changes Following Either Dietary Isoflavone Treatment or Injection Treatment with the Isoflavonoid Equol: Positive Response Dependent on Animal Age and Ovarian Status in Female Long Evans Rats

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Crystal Blake

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

Anti-depressive and Anti-obesity Changes Following Either Dietary Isoflavone Treatment or

Injection Treatment with the Isoflavonoid Equol: Positive Response Dependent on

Animal Age and Ovarian Status in Female Long-Evans Rats

Crystal Blake

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Two conditions associated with ovarian depletion are increased potential for depressive episodes and increased abdominal weight gain. In five different experiments we examined the effect of soy-containing diets or equol injections on depression, serotonin levels, weight gain (BW) and white adipose tissue (WAT) deposition of female Long-Evans rats in various stages of life. Rats were intact, ovariectomized or experienced natural ovarian failure (NOF). While this paper will present each experiment, only experiment 5 is outlined here due to space limitations. From conception the rats were exposed to either a soy-rich (Phyto-600) or low-soy diet (Phyto-low). Animals experienced NOF at approximately 300 days. At 330 days-old animals underwent the Porsolt forced swim test (PFST). One month later (following 1 week of equol injections in Phyto-low fed animals) the animals were again tested in the PFST. Serum was collected before the first PFST and following the second PFST for serotonin and isoflavone analysis. This experiment demonstrated that animals fed a soy-rich diet have decreased BW and WAT compared to a low-soy diet. At the first PFST, the Phyto-low fed NOF females displayed increased immobility and lower serotonin levels compared to the Phyto-600 NOF females indicating the Phyto-low animals were more depressed than the Phyto-600 females. The second PFST demonstrated equol injection significantly increased both time mobile and serum serotonin levels in the Phyto-low fed rats suggesting that equol has antidepressant effects. This experiment demonstrated that isoflavone exposure has antiobesity-like effects. Furthermore, isoflavones (particularly equol) appear to have antidepressant potential in NOF females.

ACKNOWLEDGEMENTS

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A. Introduction:

<u>Menopause</u>

Menopause is strictly defined as the permanent cessation of menstrual periods due to the loss of ovarian follicle activity accompanied by the termination of ovarian steroid production and availability (1-5). Females are born with a finite number of ovarian follicles, which determine their reproductive lifespan; follicular depletion results in ovarian failure (termed menopause in humans) (5-7). Ovarian failure can occur as a result of natural ovarian depletion with normal aging, be chemically induced, or surgically instigated by bilateral removal of both ovaries (4,7).

Natural menopause (menopause as a result of aging) is preceded by irregular cycles first displayed between 30 to 40 years of age in humans followed by a rapid decline of fertility during the fourth decade of female life (8). This period, referred to as perimenopause, is usually associated with a gradual decline in sex hormone production over the course of several years (3,5). In the United States, menopause generally occurs between 45 and 65 years of age with an average age of 51 years (9). This age has not significantly changed over time or between ethnic groups (10). However, menopause can occur much earlier if a woman is exposed to any chemical or substance that decreases her ovarian follicle reserve (7). Early or premature menopause can be chemically induced or surgically initiated (5). Menopause is considered to be premature if it occurs before a woman is 40 years-old and early if it occurs at or before she is 45 years-old (11).

Cancer treatments such as radiation therapy or chemotherapy can chemically induce premature or early menopause (7,12). Radiation therapy can cause direct damage to the ovaries, which can result in ovarian failure (12,13). Chemotherapy influences both ovarian follicle depletion and general organ toxicity (14). Women who undergo either radiation therapy or

chemotherapy are more likely to enter menopause at a younger age (7,13,15). Ovarian depletion

by either method depends on the age of the patient, the type of agents used and dose, and the

length of exposure (7,16). Although these cancer therapies are the best known, any substance or

treatment that decreases the number of primordial follicles can also induce premature or early

ovarian failure (7).

Bilateral oophorectomy is the most abrupt form of early or premature menopause. Each

year in the United States one quarter of a million women undergo surgical menopause (bilateral

oophorectomy) (17). This procedure is accompanied by a drastic and abrupt decrease in

circulating estrogen and progesterone levels due to the loss of the ovaries (5).

Regardless of the cause, menopause (premature, early, or natural) brings changes that

affect female health and quality of life. The postmenopausal period is associated with increased

risk for certain diseases and conditions such as cardiovascular disease, osteoporosis, and

neurological disorders (18-29). These risks are further increased when menopause is premature

or early (30,31). For example, surgically induced menopause before age 45 increases the risk for

premature death and neurological problems such as dementia (5,25).

Symptoms

Menopause is associated with certain transitional symptoms, which often result in the

patient seeking medical assistance. Some of the more common symptoms addressed here

include hot flashes, weight gain, and depression.

Hot flashes are the most recognized symptom of menopause and a common reason

women seek medical attention (32). Hot flashes occur in 75-85% of postmenopausal women

and 30% of these women describe these hot flashes as severe (33-35). Hot flashes are associated

with estrogen withdrawal; however, studies have shown no significant differences in serum

estrogen levels between women who experience hot flashes and women who do not (36). While other hormones have been associated with reduction of hot flashes, the exact causes are unknown (34).

Menopause is also associated with increased body weight, body mass index (BMI), and fat content (37-39). Estrogens are known to affect energy intake, fat accumulation, distribution, and breakdown (lipolysis), and locomotive activity (energy expenditure) (40-43). Postmenopausal weight gain is usually centrally or abdominally located and associated with low estrogen levels (38,44). Unfortunately, menopausal weight gain is also associated with increased risk for both breast cancer and cardiovascular disease, causing it to be more than just an age-related inconvenience (39,45).

Finally, depression is one of the major debilitating symptoms of menopause (46). In general, depression is a disorder more commonly found in women than in men. This difference is associated with the differing estrogen levels (47). For example, the risk for depression increases during perimenopause, a time when estrogen levels are decreasing (48,49). Moreover, estrogens are known to be involved in regulating the expression and effects of behavioral hormones such as serotonin (50).

Serotonin is a chemical messenger present in the gastrointestinal tract, the blood, and other various organs (51). However, serotonin's best-known effects are in the nervous system where it functions as a behavioral neurotransmitter associated with affective disorders (51). Decreased serotonin concentrations are associated with increased depression, anxiety, and other affective disorders (52,53). Because estrogens normally affect serotonin concentrations, receptors, and function, the lack of estrogen would also have effects (50).

Hormone Replacement Therapy

Hormone replacement therapy (HRT) is the gold standard treatment for menopausal symptoms and currently is the only treatment approved by the FDA (54). Until the 2002 Women's Health Initiative (WHI), HRT consisted of either estrogen only for women without a uterus (0.625 mg/day conjugated equine estrogen) or estrogen plus progesterone for women with a uterus (0.625 mg/day conjugated equine estrogen plus 2.5 mg/day medroxyprogesterone acetate) (55). Prior to 2002, it was thought that HRT could potentially decrease the risks for cardiovascular disease, osteoporosis, and dementia (33). However, the 2002 WHI results raised questions about whether HRT should be given as a preventative treatment (19). Furthermore, the 2002 WHI raised concerns with the current HRT treatments due to the increased risk for breast cancer, heart attack, stroke, and endometrial cancer (19,26). As a result, researchers, doctors, and postmenopausal women began looking for alternative treatments to HRT for their menopausal symptoms (54,56).

However, HRT is important in women who enter menopause early or prematurely. Professional organizations such as the North American Menopause Society, The International Menopause society, and the British Menopause Society recommend the use of HRT in women who undergo premature menopause (57-59). HRT protects these women from premature coronary heart disease and death, prevents bone loss, lowers risk of fracture, reduces risk of neurological impairment, and effectively treats the climacteric symptoms of menopause (30,54).

Estrogen Receptors

The estrogen receptors are part of a family of steroid nuclear receptors (60). Steroid receptors are intracellular transcription factors, which regulate the transcription of certain genes. When ligand is present these receptors dimerize and bind to their respective hormone response elements on the DNA (60,61). Steroid receptors have several structural components in

common and two of these structures are the DNA binding domain and the ligand binding domain (60).

The first estrogen receptor, estrogen receptor alpha (ERα), was discovered in the 1950s and cloned in 1986 (62). The second estrogen receptor, estrogen receptor beta (ERβ), was discovered and cloned in 1996 (63). While the DNA binding domains for the estrogen receptors, alpha and beta, have a 96% homology, their ligand binding domains have only a 53% homology (64). This difference in the ligand binding domain indicates that there can be variation in chemical binding affinity between ERα and ERβ (64).

ERα is commonly known as the proliferative receptor and the receptor usually blamed in estrogen responsive breast cancer (65,66). On the other hand, ERβ appears to exhibit an inhibitory effect on ERα-mediated gene action though this is not ERβ's only function (67). Furthermore, it appears that the concentration and ratio of these receptors in various tissues determines the relationship between the two receptors (66,68-71).

The discovery of a second estrogen receptor changed the understanding of how estrogens work in the body and treatment possibilities. For example, these two receptors, though they are widely distributed throughout the body, do not have identical distribution. ERα is found primarily in the uterus, liver, kidney and heart. ERβ is found in the ovary, prostate, lung, GI tract, bladder, central nervous system, and bone marrow. Both receptors are co-expressed in tissues such as the mammary gland, the epididymis, thyroid, adrenal gland, bone, and certain parts of the brain (72,73).

Due to the different distributions and actions of the estrogen receptors, specialized treatments can now be aimed specifically at either of the two receptors. 17β-estradiol, the most potent naturally occurring estrogen binds to both estrogen receptors with equal affinity (74).

Other chemicals, however, can selectively bind one or the other. Selective estrogen receptor modulators (SERMs) are biochemical compounds able to agonize or antagonize estrogen receptors (75). SERMS bind the estrogen receptors with differing affinities with some compounds binding preferentially to either ERα or ERβ (74). Furthermore, the physical effects of these compounds can vary between organs of the same body (75,76).

Two of the best known SERMs are tamoxifen and raloxifene. Tamoxifen is used as a breast cancer treatment because it has antiestrogen activity in the breast (76,77). Tamoxifen has equal affinity for both estrogen receptors, reduces the risk of cancer, increases bone density, and lowers circulating cholesterol (66,78). However, tamoxifen has potential problems because it acts as an ER agonist in the uterus. As a result, tamoxifen increases both endometrial thickness and risk for endometrial cancer (66). Raloxifene is a SERM aimed at decreasing bone loss and preventing osteoporosis (76,77,79). It has a similar chemopreventive profile to tamoxifen including potential as a breast cancer treatment; however raloxifene has fewer undesirable side-effects (66,77). Unfortunately, recent studies have shown that these side-effects include increased vasomotor symptoms, increased risk for venous thromboembolic events, and increased risk of fatal stroke (79).

Alternative Treatments

As mentioned before, over 75% of postmenopausal women experience menopausal symptoms (33). However, since the 2002 WHI only 10-25% will seek help from traditional means (80). Since 2002 more women have been turning away from traditional hormone replacement therapy (exogenous estrogens) and seeking alternative and complementary sources for relief from menopausal symptoms (56,80,81). Many women appear to prefer these alternative supplements because they feel these treatments better fit their personal lifestyles, values, and

beliefs (80,82). Even women who do use traditional treatment will often also take botanical

supplements without their doctor knowing (80). Some common botanical supplements include

black cohosh, red clover, soy isoflavones, dong quai, ginseng etc.

Black cohosh is a perennial plant native to North America that has been used by

American Indians for gynecological conditions since before European arrival in the Americas

(83). Estrogenic activity has been suggested but not demonstrated, and existing data imply black

cohosh uses serotonin pathways rather than estrogenic pathways (84,87-89). Currently, this

botanical demonstrates effective treatment of menopausal symptoms and could be used safely for

six months. However, no long-term studies have been performed (80).

Red clover is a plant native to Europe and western Asia, which contains high levels of

isoflavones similar to soy. However, soy contains higher levels of daidzein and genistein while

red clover has higher levels of formononetin and biochanin A. Studies involving this compound

demonstrate no ease of menopausal symptoms in spite of the similarity to soy (80).

Dong quai is an herb native to eastern Asia and China (83). It has been used for more

than 1000 years as a spice, tonic and medicine and is the most prescribed Chinese herbal

medicine for female problems (80,83). However, there is very little data currently available on

the efficacy of this compound in treating menopausal symptoms (80). What is available suggests

that dong quai alone does not relieve menopausal symptoms (90).

Ginseng is another Asian herb popular in the American market (90). While this drug

seems to help with mood and sleep, it does not appear to treat vasomotor symptoms (90).

Furthermore, ginseng has not demonstrated any estrogenic effects (80). Similarly, other

botanicals such as valerian, ginkgo biloba, St. Johns' wort, and motherwort are used for

treatment of depression and mood symptoms and do not appear to have effects on vasomotor symptoms (80).

Use of soy isoflavones became prominent following observations that Asian women living on the isoflavone-rich Asian diet express few menopausal complaints. Whether or not this difference is due to diet, culture, or both is yet to be determined (80). Soy isoflavones will be discussed in greater detail in the phytoestrogen section. The most common isoflavones are daidzein, genistein, biochanin A, and formononetin (80).

A frequent assumption made by many women is that botanicals are safe to use because they are "natural" (80,91). However, the efficacy of these alternative medicines as viable menopausal symptom treatments are still a focus of scientific research (80,90). Currently no consensus on dosing, length of exposure or efficacy for these compounds exists (92). Long-term, randomized, placebo-controlled studies on the effects of alternative and complementary medicine in postmenopausal women have yet to be performed (80).

Polyphenols

Polyphenols are a plant-derived group of chemical substances found in berries, grapes, walnuts, peanuts, pomegranates, and other fruits and vegetables (93). Polyphenol extracts and preparations are commonly available as dietary supplements (94). The largest and best studied polyphenols are the dietary estrogen-like molecules called phytoestrogens (93).

Phytoestrogens

Phytoestrogens are non-steroidal plant-produced compounds with structural and functional similarity to 17β-estradiol (95). Phytoestrogens are able to bind the estrogen receptors however; many phytoestrogens preferentially bind ERβ (96). Phytoestrogens are divided into three categories: lignans (found in flaxseed in large quantities), coumestans (derived from

sprouting plants like alfalfa), and isoflavones (derived principally from soybeans) (66). Soy isoflavones are the most abundant phytoestrogen that occur in both rodent and human diets and the most studied (96). The most abundant concentrations of isoflavones are found in legumes and soybeans (97).

Soy isoflavone diets appear to have beneficial effects on health. Asians, who consume large daily amounts of soy isoflavones (20-50mg/day vs. 0.5-1.0mg/day in Western diets), tend to have lower rates of hormone-dependent cancers, obesity, and coronary heart disease (97,98). Like many other phytoestrogens, isoflavones can act as SERMs and have an affinity for both estrogen receptors; however, in general, isoflavones have a higher affinity for ERβ (96). The major soy isoflavones include daidzein and genistein (74,96). Rising to specific prominence has been the isoflavonoid equol, daidzein's intestinally produced metabolite. Equol has a much higher affinity than daidzein for estrogen receptor beta and a similar binding affinity to genistein (74).

Figure A

Daidzein → Equol
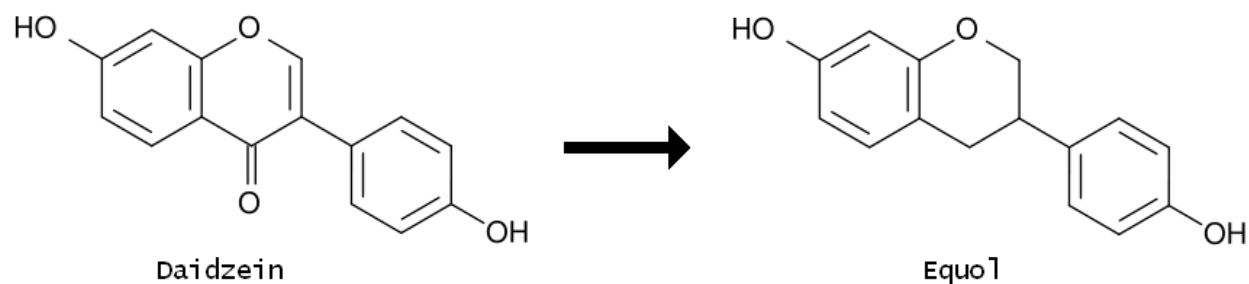
Because soy isoflavones can bind to the estrogen receptors, phytoestrogens could be a helpful alternative to HRT in treating some of the symptoms of menopause such as depression, weight gain, and vasomotor symptoms. This study will focus primarily on phytoestrogens' (particularly equol's) effects on behavior and weight gain in two animal models of menopause (ovariectomy and natural ovarian failure).

B. Materials and Methods:

General Methodology

This study reports the results of five different experiments designed to investigate the effects of isoflavone(s), administered in the diet or by injection, in female, Long-Evans rats. Animals were of varying ages and were gonadally intact, ovariectomized, or experienced natural ovarian failure (NOF). The parameters examined in this study included animal behavioral analysis, animal body weight gain, white adipose tissue deposition, and serum concentrations of serotonin and phytoestrogen isoflavones. Also compared in this study was the effectiveness of treatment timing, length, and mode of administration.

Animals and Housing

All animals tested in these experiments were female, Long-Evans rats. An in-house breeding protocol was used to generate the female offspring used in experiments 1, 2, 4, and 5. The animals used in this breeding protocol, as well as the ovariectomized females used in experiment 3 (ovariectomized at 40-45 days-old), were purchased from Charles River Laboratories (Wilmington, MA, USA), then shipped to our animal facility at approximately 50-55 days-old. Once at our facility the animals were placed on a 12-hour light/dark cycle (6am-6pm) with a light intensity of approximately 80 foot candles and were allowed free access to both food [diet treatment(s)] and tap water. All animals were housed in clear plastic cages with wire lids. Cage dimensions were 20 cm high, 24 cm wide and 40 cm long. The animals in experiment 3 and young adult animals used for breeding were housed in individual cages. The female rats tested in experiments 1, 2, 4, and 5 were initially housed with their mothers from birth until weaning at 21 days. From 21-40 days the animals were housed in groups of 3 to 4 rats per cage. From day 40 to day 60 these animals lived in paired housing and from day 60 to the

end of each experiment the females were housed in individual cages. All animals obtained from the supplier (Charles Rivers) were given at least one month to adapt to the new surroundings and diet treatments before either being mated or beginning treatment.

Mating

As stated above, the male and female animals used to produce the animals in experiments 1, 2, 4, and 5 were purchased from Charles River Laboratories when 50-55 days-old. Breeding the animals at our facilities allowed control over the animal's exposure to their individual and respective diets. The male and female animals were bred at approximately 90 days-old. Mating took place in suspended wire cages. Removable cardboard sheets were placed beneath the cages to catch feces and vaginal plugs. Each female rat was housed with a male until inseminated at which point she was exchanged for another female. Insemination was noted by the presence of vaginal plugs on the cardboard and these sheets were checked twice a day (8-9 am or 4-6 pm). All the males were fertile and each male inseminated between two and four females. One female fed the Phyto-600 diet and two females fed the Phyto-low diet did not get pregnant in the course of these experiments.

Diets

The animals obtained from Charles River laboratories were exposed prior to their arrival at our facility, to a diet (standard chow) containing approximately 200 ppm isoflavones (as previously determined by our laboratory) (99). These animals were allowed one month on their new diets (see below) before mating, treatment or testing occurred in order to decrease possible interference by previous exposure to dietary isoflavones. Once at our facility the animals were exposed to one of two diets: the Phyto-600 or the Phyto-low diets. The Phyto-600 diet is a phytoestrogen rich diet containing roughly 600 ppm soy isoflavones (Harlan Teklad Rodent Diet

8604, Madison, WI, USA). The Phyto-low diet is poor in phytoestrogen content with between 10-15 ppm soy isoflavones (Zeigler Bros., Gardner PA, USA) [catalog# 541200-12-00, Rodent PHY RDC].   All diet isoflavone concentrations were determined by our laboratory using gas chromatography and mass spectrometry (GC/MS) (99,100). The diets were balanced and matched for equal percentages of protein, carbohydrate, fat, vitamins and minerals (99).

Injections

All animals in experiments 1, 2, 4, and 5 received subcutaneous injections of either a placebo or 5.0 mg/kg body weight equol. The females on the Phyto-600 diet received placebo injections of dimethyl sulfoxide (DMSO). DMSO acts as an absorptive agent, increasing the penetration of equol through the tissues and into the circulation. It does not appear to have detrimental effects on the animals and is in fact employed for medical treatments in humans (101). The animals on the Phyto-low diet were injected with 5.0 mg/kg body weight of the soy isoflavonoid equol (in DMSO) (LC Laboratories, Woburn, MA, USA). All injection volumes were 0.1 cc and administered subcutaneously at the nape of the animal's neck. The rats were wrapped carefully in cloth to decrease animal stress and the females were returned to their cages directly following injection. All injection schedules varied among the experiments. Each schedule is listed below in the experiment protocols.

Age and Ovarian Status

The females tested in each experiment were of various ages and ovarian status. This allowed us to compare treatment effects at each of these ages and conditions. The females investigated in experiment 1 were gonadally intact. Animal testing and treatment occurred from 120 to 210 days-old. Experiment 2 animals were also intact, however, diet treatment began at conception and further treatment and testing occurred between 115-145 days-old. The animals

examined in the third experiment were ovariectomized at 50-55 days-old. Treatment and testing

occurred from 85-100 days. The females in experiment 4 began diet treatment at conception.

These animals were ovariectomized at approximately 100 days. Testing and further treatment

occurred between 150-222 days-old. The females in the fifth experiment also began dietary

treatment at conception, which continued life-long until the end of the study. Testing and further

treatment began at 295 days-old and continued until 365 days-old.

Sexual Age of Animals

The Long-Evans female rat enters puberty when approximately 40 days-old (102). This

particular breed of rat experiences decreased reproductive ability beginning at five months and

natural ovarian failure (NOF) occurs approximately between 280 to 290 days of age (103). The

timing of NOF was confirmed in experiment 5 by this lab using histological examination of

vaginal epithelial cells when the females were between 295-305 days-old. In contrast, the

average human female begins puberty when approximately 12 years old (104). In the U.S. the

average age of menopause or human ovarian failure is approximately 51 years (105,106). By

using these ranges in fertility between these two species, associations between human and rat

ages were roughly approximated to better estimate possible responses in humans. Theoretical

estimations of human responses to similar conditions were then made. The table below displays

the sexual timing, ages, and the approximate rat/human age comparisons.

Sexual Timing	Rat (days	Human (years)
Puberty	35-40	12-13
Young Adulthood	120	20-24
Mid-age	200	35-40
Post Ovarian Failure	300-360	50-55

<u>Ovariectomy</u>

Only the animals in experiment 4 were ovariectomized while at our facility. All animal surgeries were performed under the direction of the university veterinarian. Anesthesia (a 3 cc buprenex, 2 cc domitor, and 5 cc ketamine mixture) was administered intraperitoneally at 0.05cc/100g animal body weight. Anesthesia depth was measured using the toe pinch reflex. Animal body temperature was maintained with a warm water blanket during surgery and a heating pad following surgery. A single abdominal incision was made in each female and the ovaries removed. The incisions were closed with sterile surgical staples. Following surgery the animals were placed on a heating pad, injected with 0.1 cc of atipamezole, a reversal drug, and allowed to regain consciousness. Approximately 6 minutes were required to completely anesthetize animals. Once anesthetized, a reversal drug was administered and animals regained consciousness after about 10 minutes. Post-treatment painkiller was administered via drinking water for 4-5 days after surgery (20 ml acetaminophen pediatric elixir (32mg/ml) per 500 ml tap water). The animals were carefully watched for signs of infection or illness. All of the females in experiment 4 were allowed seven weeks to recover from ovariectomy before any other treatment occurred.

<u>Porsolt Forced Swim Test</u>

The Porsolt Forced Swim Test (PFST) is the standard antidepressant test used in both academic and pharmaceutical laboratories world wide to screen new drugs or compounds with potential for antidepressant activity (107,108). This test examines behavioral despair expressed in rodents following antidepressant treatment. Animal behavioral despair is expressed through animal immobility. Depressed animals assume a state and position where only very simple movements are required to keep the nose above water (i.e., floating). The PFST examines the

length of time the animal struggles when exposed to a novel environment from which it cannot escape and measures the length of time the animal spends immobile (107,109). Other parameters that can be examined by this test include swimming distance, speed, escape behaviors etc. Animals that demonstrate greater mobility, faster speed, and longer swimming distance are considered to be less depressed. These animals also tend to display more escape behaviors such as diving or inversion (positioning the head/body below the waterline, but not diving) while searching for an exit. The PFST uses a cylindrical container (diameter 19 cm, height 43 cm) which is filled to a water depth of 35.6 cm. This depth makes it difficult for animals to touch or rest their tails on the bottom of the container as well as preventing escape out of the top of the container (110). Test duration was 8 minutes. Animal behavior was recorded by a video camera located above the containers and each animal's movements were measured and analyzed using Anymaze® computer software (Stoelting Co., Wood Dale, IL, USA). Parameters examined by Anymaze® include swimming speed, total swim distance, and time spent either mobile or immobile. Escape behaviors were also observed and recorded by a trained observer who sat outside the room and manually recorded the number of times the animals either dove or inverted themselves. This observer also recorded the number of fecal boli released by each animal during the test as this is seen as an emotional response.

While the PFST is the standard antidepressant screening test there are other tests designed to measure affective disorders displayed in animals (111). These tests are designed to analyze depressive-related or anxiety-related behaviors. One of the tests, which also examine animal depressive-related behaviors by testing for immobility, is the tail suspension test first used in 1985. In this test the rat is suspended by its tail for approximately six minutes and the time the animal spends immobile is measured (112).

Anxiety-related behaviors are related to and often concurrent with depression. Some of the tests designed to examine animal anxiety include the elevated plus maze, the light/dark box, the open field test and the fear conditioning-freeze monitoring test. In the elevated plus maze, the animal is placed on a raised plus-shaped platform. Two arms of the platform are surrounded by high walls while the other two have none. The test measures the time the animal spends in the open arms of the maze compared to the closed (walled) arms. The less anxious animal will spend more time on the open arms (111,113). In the light/dark box test the animal is placed in a container with two divisions. One division is enclosed and dark while the other is clear and light. The amount of time the animal spends in the clear side is recorded. Animals expressing lower anxiety-related behaviors will spend less time in the dark side of the box (111). The open field test examines exploratory behaviors in the rat. The rat is placed in the center of a large open box and the time spent exploring the area and away from the walls of the apparatus is measured. Less anxious animals will explore more (111). Finally, the fear conditioning-freeze monitoring test is designed to gauge an animal's response to anxiety inducing stimuli. The animal is placed in a container capable of releasing one of three stimuli: sound, light, or electric shock. Animal movement is tracked and animals that are less anxious will recover from the stimuli faster and spend more time mobile (111).

Body Weight (BW), White Adipose Tissue (WAT) Deposition and Blood Collection

Animal body weights were recorded at different intervals mentioned specifically in each experiment. All animals were weighed using a Mettler 1200 balance (weight recorded in grams $\pm$ 0.1 grams) (St. Louis, MO, USA). Once injection treatments began all body weight measurements were recorded prior to the subsequent injections. Following animal sacrifice, abdominal white adipose (WAT) tissue was dissected from the base of the pelvic cavity to just

below the diaphragm and weighed on a Sartorious balance (weight recorded in milligrams $\pm$ 1.0 mg) (Brinkman Inst. Co., Westbury, NY, USA). Trunk blood was also collected and centrifuged at 2400 X g. Serum was then collected and stored at -20ºC until it was assayed for serotonin and isoflavone levels.

Serotonin ELISA

Serum serotonin concentrations were measured using ELISA kits (cat#IB89527-12-1610) obtained from Immuno Biological Laboratories (Minneapolis, MN, USA). Only experiment 3 serum was not assayed for serotonin levels due to a lack of sufficient serum volume.

Serotonin is known to be present in and affect a number of both individual organs and organ systems in the body. Most of the body's serotonin is located in various organs or blood components or regions outside of the nervous system. However, serotonin's better known effects are in the brain due to serotonin's involvement in influencing behavior (51). Decreased serotonin concentrations are associated with depression, anxiety, and other affective disorders (52). Peripheral serotonin levels appear to correlate with CNS concentrations and therefore could correlate with behavioral states (53,114-116).

Serotonin concentrations and function can be affected by estrogens, which indicate potential for other estrogen-like chemicals to affect serotonin levels and function (50). Phytoestrogens, which are able to bind estrogen receptors, could have similar effects on serotonin levels and function. Because phytoestrogens' ER binding affinities are much lower than natural 17β-estradiol these effects while similar would be less (74,96,117).

Serum Phytoestrogen Levels

Gas chromatography/mass spectrometry (GS/MS) was used to determine serum isoflavone concentrations for all samples collected as previously reported by our laboratory (99).

Specific circulating isoflavone concentrations were determined for genistein, daidzein, and the isoflavonoid equol using internal control standards as previously reported (99,118). The specific protocol for this technique was reported in greater detail in papers published by Axelson et al. and Setchell et al., in 1982 and 1997 respectively (119,120).

Treatments

Experiment 1: The objective of this experiment was to examine the effect of either diet and/or isoflavone injection on the study parameters (BW, WAT, behavior, etc.) in young adult, female rats. Half the animals in this experiment underwent a diet change during young adulthood after being exposed from conception to a phytoestrogen-low diet. The remaining animals continued on the phytoestrogen-low diet and received supplemental injections later in adulthood.

Figure B
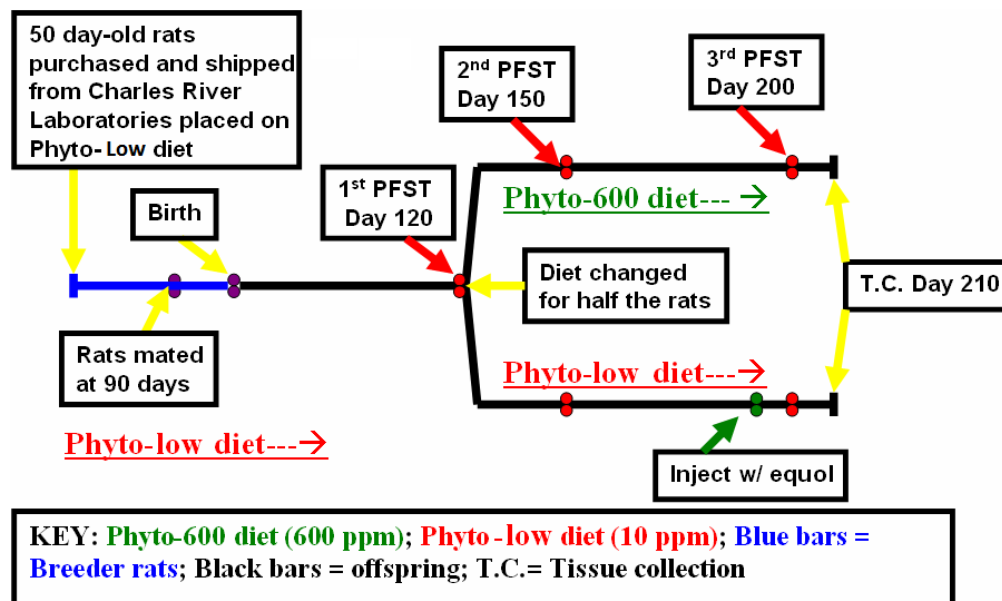
Twenty-three weight-matched, intact, female, Long-Evans rats were used in this experiment representing at least six litters. These animals were conceived on the Phyto-low diet and remained on this diet until 120 days-old. This age roughly corresponds to early twenties in human years. When the animals were 120 days-old, animals were weight-matched and twelve

rats (or approximately ½ of the total number of animals) were switched to the Phyto-600 diet. Animal body weight was measured and recorded three times a week during the course of the study.

The Porsolt Forced Swim Test (PFST) was performed at three different points during this experiment: 120 days, 150 days, and 200 days. The first PFST was administered to record baseline animal behavior prior to the diet change. The second PFST, administered at 150 days, examined changes in the expression of depressive-related behaviors as a result of the diet change. The third PFST examined the effects of equol administration on depressive-related animal behaviors in the Phyto-low fed animals. The parameters recorded for each test are as mentioned above (mobility, total swim distance, average swim speed, escape behaviors, etc.). Prior to the final PFST, the females received daily injections of either equol or DMSO (days 194-200). The animals on the Phyto-low diet received injections of 5 mg/kg (body weight) equol in DMSO and the Phyto-600 fed animals received vehicle-DMSO injections. Injections continued until the end of the experiment.

Animals were killed via decapitation at 210 days. Animal body weight, white adipose tissue weight and trunk blood were collected. Serum was tested for serotonin and isoflavone concentrations.

This experiment examines two separate situations in healthy, sexually intact adult females. In both scenarios the animals were meant to model humans raised on an isoflavone poor (Western) diet but have the ability to produce equol from daidzein if soy foods were consumed. In general, only 30-50% of the human population is able to produce equol from daidzein (121). The first part of this experiment examined dietary effects on behavior, body weight, and adipose tissue deposition in gonadally intact female rats exposed from birth to an

isoflavone poor (Western) diet. During young adulthood these female rats were switched to an isoflavone rich diet which they continued to eat until almost mid-age, which is approximately 35 years and older in humans. The second part of this experiment examined the effects on study parameters in gonadally intact female rats that remain life-long on the isoflavone poor diet and recieve equol during mid-age to model potential benefits of equol supplementation.

This experiment addressed three main questions. The first, is there a difference in study parameters based on diet in intact females? Next, do phytoestrogens have visible effects in the presence of naturally occurring estrogens? Last, does route of administration (diet versus equol injections or supplementation) have an effect on study parameters?

Experiment 2: This experiment examined the beneficial effects of lifelong phytoestrogen-rich diet exposure in intact, female rats on the study parameters (behavior, BW, WAT, etc) in early young adulthood. Approximately half of the females in this experiment were exposed lifelong to a phytoestrogen rich diet. The remaining females were exposed lifelong to diet poor in phytoestrogen content; these females received injections of equol supplement during early young adulthood.

Figure C

Phyto-600 diet--→

50 day-old rats purchased and shipped from Charles River Laboratories placed on Phyto-Low diet

Rats mated at 90 days

Birth

1st PFST Day 115

2nd PFST Day 142

T.C. Day 145

Phyto-low diet---→

Inject w/ equol

KEY: Phyto-600 diet (600 ppm); Phyto-low diet (10 ppm); Blue bars = Breeder rats; Black bars = offspring; T.C.= Tissue collection
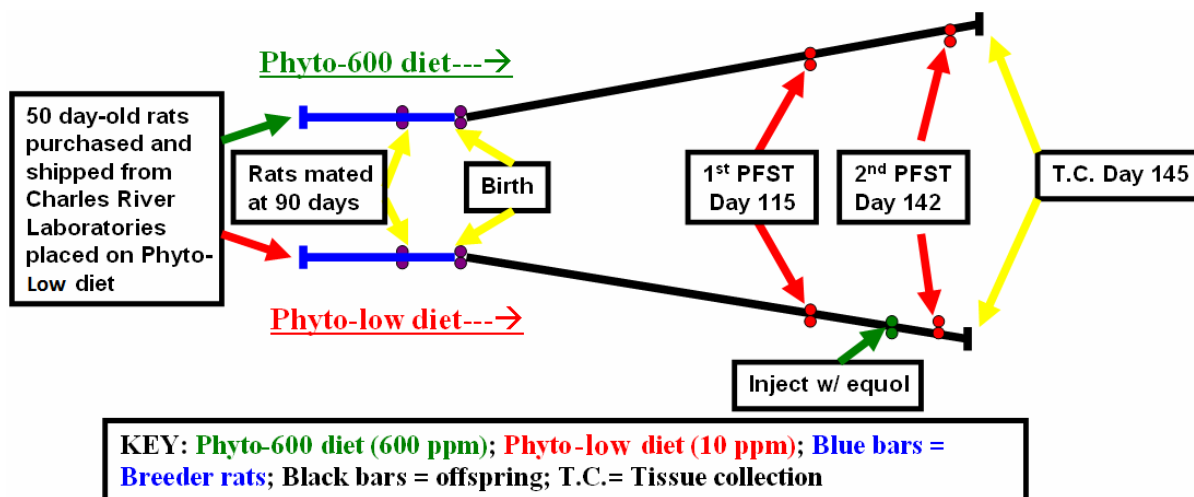
Eight females were selected from 4 litters of Phyto-low fed pups while seven females were chosen from 3 litters of Phyto-600 fed pups. Animals were taken from litters whose parents were diet matched and mated. These females continued on their parental treatment diets from conception to the end of the experiment. Animal body weight was measured three times weekly.

At two points in this experiment the animals underwent the Porsolt Forced Swim Test (PFST). The first PFST was performed when animals were 115 days-old and measured the effect of lifelong diet exposure on depressive-related behavior in young females. The second PFST occurred when animals were 142 days-old and examined any changes in behavior as a result of injected equol supplementation in the lifelong phyto-low diet animals. Behavioral parameters recorded in these tests are mentioned in the PFST section and include mobility, swim distance, swim speed, escape behaviors, etc. Before the second PFST, animals received four daily injections (days 139-142). The Phyto-low animals received injections of 5 mg/kg (body weight) equol in DMSO while the Phyto-600 animals received vehicle-DMSO injections. Injections continued until the end of the experiment.

Animals were killed at 145 days of age. Animal body weight and white adipose tissue deposition were recorded and trunk blood collected. Serum was assayed for serotonin and isoflavones levels.

This experiment examined modeled two scenarios involving healthy, sexually intact females capable of producing intestinal equol from daidzein when fed a diet rich in soy isoflavones. The first situation examined solely the effect of a phytoestrogen-high (Asian) or a phytoestrogen-low (Western) diet on study parameters in young adult females. The second

situation examined isoflavone supplementation effects (via the equol injections) on the study parameters following lifelong exposure to a Western diet.

The questions examined in this experiment are similar to those in experiment 1. First, do phytoestrogens still have a visible effect in the presence of endogenous estrogens on study parameters? Next, can equol injection successfully supplement a lifelong phytoestrogen-low diet? Does age of equol administration change equol's effects on study parameters? Finally, does length of exposure matter?

Experiment 3: The objective of this experiment was to examine the effects of diet alone on the study parameters (behavior, BW, WAT, etc) in gonadectomized, female rats that were ovariectomized shortly after puberty.

Figure D
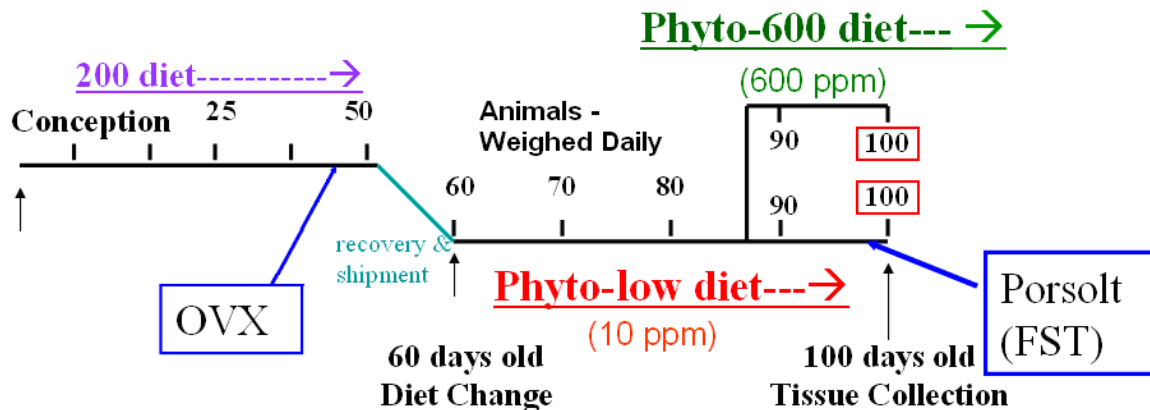
This experiment tested twenty-two ovariectomized female Long-Evans rats which were purchased from Charles River Laboratories and shipped to our facilities at 50-55 days old. Animal ovariectomy took place at the supplier when females were 40-45 days-old. While at the supplier these animals were fed a diet containing approximately 200 ppm isoflavones (standard in-house chow). Upon arrival at our facility the animals were switched to the Phyto-low diet until approximately 80-85 days. Eleven animals were then switched to the Phyto-600 diet and

fed this diet for approximately two weeks. The remaining animals continued on the Phyto-low diet. Animal body weights were recorded daily.

When animals were approximately 100 days-old the animals were tested in the Porsolt Forced Swim Test to examine the effect of diet alone in gonadectomized females. Behavioral parameters recorded in these tests were the same as previous tests and are mentioned in more detail in the PFST section.

Following the PFST the animals were sacrificed via decapitation at 100 days-old. Animal body weight and white adipose tissue deposition were recorded and trunk blood collected. Serum levels were only analyzed for isoflavone concentrations due to lack of serum volume.

In the previous studies animals were exposed to the phytoestrogen-low diet, the phytoestrogen-high diet, or both diets (see experiment 1). The animals switched to the phytoestrogen-high diet during this experiment's treatment were exposed to three diets: the supplier diet (200 ppm), the Phyto-low diet (10-15 ppm) and the Phyto-600 (600ppm) diet. While at the supplier these animals were fed a diet with 200 ppm isoflavones. Once at our facility the animals were initially fed the Phyto-low (10ppm) diet and then later fed the Phyto-600 diet. We do not believe that the supplier diet adversely affected this experiment.

This animal experiment modeled the dietary effects on young teenage girls who undergo puberty and shortly thereafter undergo menopause due to ovariectomy. This experiment examined the possibility that a modification in diet could help deal with the changes that occur as a result of premature menopause. The two main questions posed by this study were first, can a phytoestrogen diet have a positive effect on study parameters in young females who undergo

ovariectomy shortly after menarche and, second, does timing of ovariectomy play a role in female behavior and treatment response.

Experiment 4: This experiment examined the beneficial effects of diet and injection supplement on the study parameters (BW, WAT, behavior, etc.) in ovariectomized female rats. The animals in this study were all ovariectomized at an older age than in experiment 3. These females were exposed lifelong to diet treatments (i.e. from conception) and as in previous experiments the phytoestrogen-low diet animals were injected with equol supplement during mid-age.

Figure E

KEY: **Phyto-600 diet (600 ppm)**; **Phyto-low diet (10 ppm)**; **Blue bars = Breeder rats**; Black bars = offspring; T.C.= Tissue collection
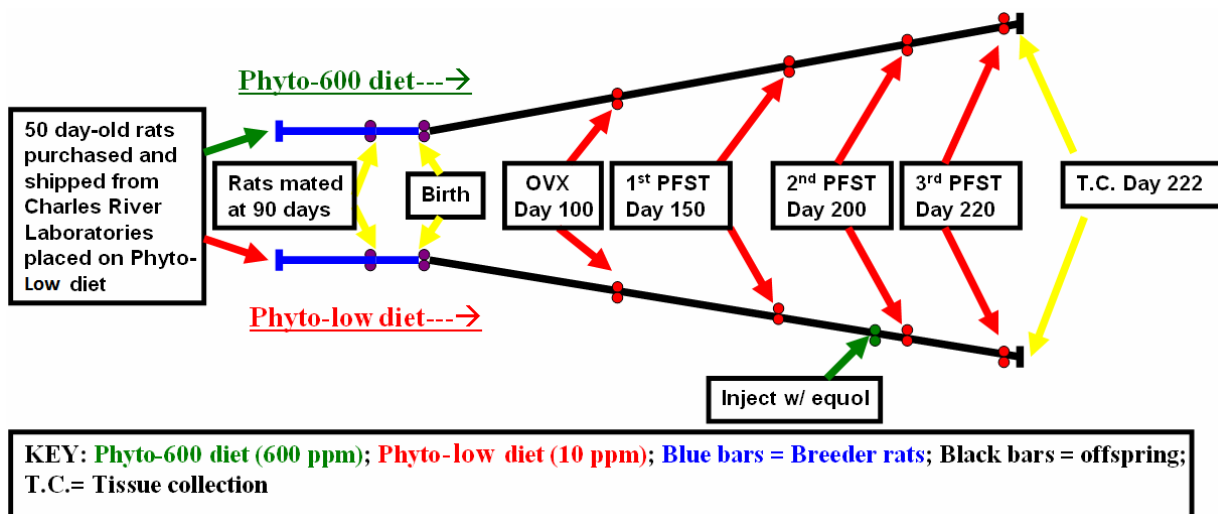
Sixteen female, Long-Evans rats were selected from male and female rats mated by diet. The pups remained life-long on the same diet as their parents. Eight of these animals came from 4 litters of pups fed the Phyto-low diet. The remaining eight animals were selected from 3 litters of pups fed the Phyto-600 diet. Animals were weighed 3 times a week throughout treatment.

The female animals were ovariectomized at approximately 100 days by our laboratory under the direction and guidance of the university veterinarian. Following ovariectomy the

animals were allowed 7 weeks to recover from surgery before other treatment occurred. Details for this procedure are located in the ovariectomy section above.

At three points in this experiment the Porsolt Forced Swim Test (PFST) was performed: 150 days, 200 days, and 220 days. The first PFST examined the effect of lifelong diet alone on behavior in young ovariectomized females. Four days prior to the second PFST equol and DMSO injections started (197-200 days). The second PFST examined the effect of injected equol on the behavior of ovariectomized animals fed the lifelong Phyto-low diet. Injections continued for the 3 weeks between the second PFST and the third PFST (Days 197-220). The third PFST then examined extended exposure equol on the behavior of ovariectomized Phyto-low animals. The second and third PFSTs also allowed us to compare short-term and long-term treatment effectiveness of the equol injections. As in experiments 1 and 2 the Phyto-low fed animals received injections of 5 mg/kg (body weight) equol in DMSO and the Phyto-600 fed animals received vehicle-DMSO injections. Injections continued until the end of the experiment.

The animals were sacrificed at 222 days via decapitation. Animal body weight and white adipose tissue deposition were recorded and trunk blood collected. Serum was collected for serotonin and isoflavone analysis.

The animals in experiment 4 model young (mid twenties) women who undergo menopause at a young age due to bilateral oophorectomy. This experiment examined the possibility that a lifelong phytoestrogen diet could affect the behavioral and weight gain symptoms which follow ovariectomy or early menopause. Short and long-term equol exposure was then examined to see if supplementation would have any effect in younger ovariectomized females. The two main questions examined here are first, does a phytoestrogen diet have a positive effect on study parameters in females who undergo ovariectomy during young adulthood

and second, is this effect different than females ovariectomized in shortly after puberty (experiment 3) or animals allowed to undergo natural ovarian failure (experiment 5).

Experiment 5: This experiment examined the effect of lifelong diet treatment exposure and equol supplementation on study parameters when females undergo natural ovarian failure (NOF). In the previous 4 experiments younger-aged animals of varying ovarian status were examined. This experiment examined the effect of lifelong diet on these parameters in NOF females and completes our examination of these effects.

Figure F
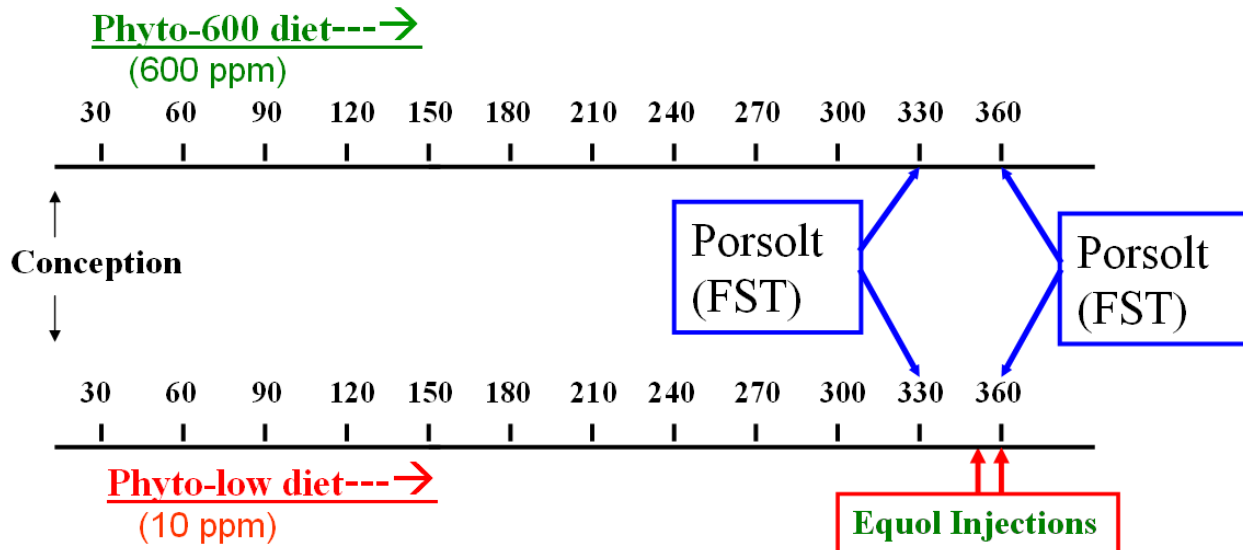
Fourteen female Long-Evans rats were used in these experiments. Six females were selected from at least 3 litters fed the Phyto-low diet. Eight animals were selected from 3 litters of animals maintained on the phyto-600 diet. As in previous experiments, the male and female breeding rats were only mated with animals fed the same diet. The offspring/pups were maintained lifelong on the same diet as their parents. The animals were weighed at selective times throughout this experiment.

The animals underwent natural ovarian failure (NOF) at approx. 300 days-old. NOF was confirmed by vaginal histological examination of epithelial cells (Days 295-305). These animals had blood collected at two times during this experiment. The initial collection occurred prior to the first Porsolt Forced Swim Test (PFST). One half the animals from each group had blood collected via tail veins to examine baseline serotonin and phytoestrogen levels. The second blood collection occurred at tissue collection.

These females were exposed to two PFST, which occurred at 330 days, and 360 days. The first PFST examined the effect of the lifelong dietary treatments on behavioral parameters between treatment groups following natural ovarian failure. The second PFST examined the effect of equol injection supplementation on the Phyto-low fed NOF animals. Injections occurred for 7 consecutive days (354-360). The Phyto-low fed animals received injections of 5 mg/kg (body weight) equol in DMSO and the Phyto-600 fed animals received vehicle-DMSO injections.

Following the second PFST, the animals were sacrificed via decapitation at approximately 365 days of age. Body weight and white adipose tissue deposition were recorded and trunk blood collected. Serum was analyzed for serotonin and phytoestrogen quantification.

These animals modeled older women either on a lifelong phytoestrogen-high (Asian) or a phytoestrogen-low (Western) diet allowed to undergo natural ovarian failure. These females were capable of producing intestinal equol from daidzein when fed a diet rich in soy isoflavones. This experiment compared two scenarios. The first scenario examined females who simply have a lifelong diet rich in phytoestrogens on the study parameters after NOF. The second scenario examined equol supplementation after NOF following lifelong exposure to a phytoestrogen poor diet to determine their influence on the study parameters.

This study examined four questions. First, does lifelong exposure to a phytoestrogen-rich diet decrease depression following natural ovarian failure? Second, is equol treatment more effective in the absence of estrogens following NOF? Third, is there a difference in treatment effectiveness on the study parameters between natural ovarian failure and ovariectomy? Finally, do phytoestrogens have the ability to affect serotonin levels after NOF?

IACUC Approval

All methods associated with animals used were approved by the Institutional Animal Care and Use Committee at Brigham Young University.

Statistical Analysis

Two-sample student t-tests with repeated measures were used to compare each of the parameters examined between the two treatment groups in each experiment. All statistics were run using the Minitab statistical software and a p-value less than 0.05 was considered significant. All results are presented as MEANS ± SEM in all of the graphs and significant differences are marked.

C. Results:

Figure G

Diet Switched = D.S.; Porsolt Forced Swim Test = PFST; Tissue Collection = T.C. Ovariectomy = OVX; Natural Ovarian Failure = NOF; Red bar represents Phyto-Low diet; Green bar = Phyto-600 diet; Black bar = supplier diet; Green box = equol injections.
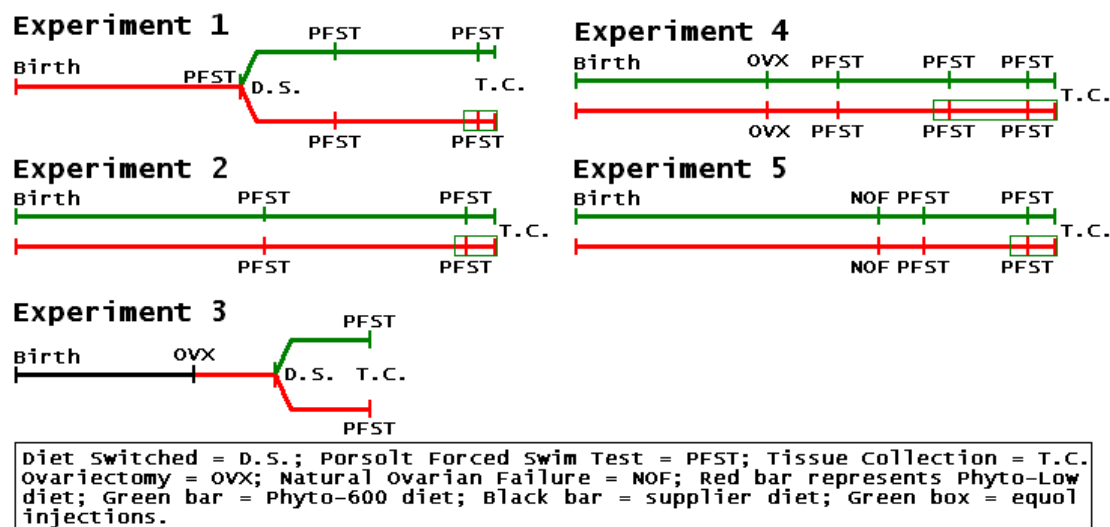
Experiment 1: Brief protocol summary – lifelong exposure from conception to the Phyto-low diet in intact females. Diet change at 120 days old (one-half of the animals were switched to Phyto-600 diet while the other half remained on the Phyto-low diet). 1st PFST at 120 days old prior to diet change; 2nd PFST at 150 days old; equol injections occurred from 196-210 days old with 3rd PFST at 200 days-old. Tissue collection occurred at age 210 days.

The effects of an isoflavone rich diet on animal body weight, white adipose tissue (WAT) deposition, behavioral parameters, and serum isoflavone and serotonin levels in intact females are described. These data examined the effects of isoflavones in the presence of naturally occurring estrogens from ovarian sources.

Body Weight and WAT

The effects of dietary isoflavones on animal body weight and the changes in animal body weight are displayed in Figure 1. Female body weight values were analyzed prior to each Porsolt forced swim test (PFST) and at tissue collection; these values are reported in Table 1. Baseline body weights at the time of the first PFST (@ 120 days old) were not significantly different between treatment groups (Figure 2A). Following diet change where half of the animals were switched to the Phyto-600 diet, the Phyto-600 fed animals experienced an immediate decrease in weight gain. When compared at the second PFST (@150 days old), the Phyto-600 animals weighed significantly less than the Phyto-low animals ($p<0.001$; Phyto-low mean = 354.0 ± 5.7 grams, Phyto-600 mean = 326.4 ± 5.4 grams; Figure 3A). The Phyto-low animals lost weight following equol injection prior to the third PFST. However, four days of equol injection were insufficient to remove the significant differences in body weight between treatment groups. The Phyto-600 animals still maintained significantly lower body weights ($p<0.001$; Phyto-low mean = 375.6 ± 7.3grams, Phyto-600 mean = 332.6 ± 6.7 grams; Figure

4A). Animal body weight at the end of the experiment (Phyto-low mean = 369.9 ± 7.3 grams,

Phyto-600 mean = 331.3 ± 6.4 grams) and white adipose tissue (WAT) deposition (Phyto-low

mean = 20.1 ± 1.7 grams, Phyto-600 mean = 13.1 ± 1.2 grams) were significantly greater in the

Phyto-low animals compared to the Phyto-600 animals ($p<0.001$ and $p<0.003$ respectively;

Figure 5A and B). When standardized to body weight, the WAT/BW ratios were also

significantly higher in the Phyto-low animals ($p<0.012$, Phyto-low mean = 0.054 ±0.004, Phyto-

600 mean = 0.039 ± 0.003; Figure 5C).

Porsolt Forced Swim Tests

There were no significant differences in baseline animal behavioral parameters in the first

PFST (Figure 2B-D). The second PFST also demonstrated no significant behavioral differences

between treatment groups (Figure 3B-D). In the third PFST, the Phyto-600 animals released

significantly more boli than the Phyto-low animals ($p<0.014$, Phyto-low mean = 3.1 ± 1.7,

Phyto-600 mean = 5.3 ± 1.2). However, there were no significant differences in animal

performance between treatment groups for any other behavioral parameters.   Notably in both

experiments 1 and 4, the immobility interval from the second PFST to the third PFST

dramatically increased.

Serum Isoflavone Levels

Serum isoflavone levels were measured with gas chromatography/mass spectrometry.

Figure 21A illustrates the isoflavone serum levels and types obtained from the 210 day-old,

intact females fed either the Phyto-600 or Phyto-low diet. Serum levels of daidzein (Phyto-low

mean = 4.8 ± 0.7 ng/ml, Phyto-600 mean = 34.9 ± 6.7 ng/ml), genistein (Phyto-low mean = 8.1 ±

0.6 ng/ml, Phyto-600 mean = 45.3 ± 8.4 ng/ml), and equol (intestinal isoflavonoid metabolite of

daidzein)(Phyto-low mean = 439.1 ± 80.5 ng/ml, Phyto-600 mean = 932.3 ± 121.4 ng/ml) were

all significantly lower in the Phyto-low animals (p<0.001-0.007). Equol was the major

circulating phytoestrogen in both treatment groups while genistein and daidzein represented

lower percentages of the total phytoestrogen content. Serum values are displayed in Table 2.

Although the Phyto-low animals received equol injections for four consecutive days, the serum

levels of equol in these animals were still less than half the levels of equol found in the animals

fed the Phyto-600 diet where natural conversion of equol took place.

Serum Serotonin Levels

Circulating serotonin levels were determined by ELISA. Serum serotonin values were

within normal ranges (as described in the vendor's assay kit values). Although there was a slight

increase in the serum serotonin concentration of the Phyto-low animals injected with equol, there

were no significant differences in serum serotonin levels between treatment groups. Notably,

these values were obtained following equol injection and tissue collection. Pre-equol injection

values for serotonin were not collected. Serum serotonin values are shown in Table 3.

Overall Results

These results demonstrate that an isoflavone rich diet decreases animal body weight gain

and WAT deposition. However, aside from increased boli excretion in the third PFST, there

were no significant behavioral differences between treatment groups. While equol injection

increased serum equol levels in the Phyto-low fed animals, these levels were still significantly

lower than the dietary values obtained by the Phyto-600 fed animals. Animal serum serotonin

levels were not significantly different between treatment groups. While an isoflavone diet does

have some positive benefits this diet does not appear to affect animal behavior when naturally

occurring estrogens are also present in intact animals.

Experiment 2: Brief protocol summary – lifelong exposure from conception to either a Phyto-low or Phyto-600 diet. 1st PFST at 115 days old. Equol injections for seven consecutive days (139-145); 2nd PFST at 142 days old. Tissue collected at 145 days old.

Experiment 2 examined the effects of lifelong diet on the study parameters in intact, young adult females. Equol effectiveness, either intestinally produced from the diet or administered via s.c. injection, in the presence of naturally occurring estrogens was tested. Length of dietary treatment exposure and age of isoflavone administration were also investigated.

Body Weight and WAT

Changes in animal body weight from 80-142 days-old are shown in Figure 6. Body weights were tested for significance at the first and second forced swim tests (PFST) and at animal sacrifice. These values, as well as the WAT values, are shown in Table 1 and are illustrated in Figures 7A, 8A and 9A. Throughout this experiment the Phyto-600 animals displayed lower average body weights than the Phyto-low animals, though this difference was not significant. WAT values also tended to be lower in the Phyto-600 animals than the Phyto-low animals; however, these differences were not significant (Figure 9B-C).

Porsolt Forced Swim Tests

During the first PFST (@115 days old), the Phyto-600 animals excreted significantly more boli than the Phyto-low animals ($p < 0.037$, Phyto-low mean $= 2.5 \pm 0.4$, Phyto-600 mean $= 3.8 \pm 0.3$). Tendencies for swimming further, faster and longer were seen in the Phyto-600 animals. However, these tendencies were not significant and no other significant behavioral differences were observed between treatment groups (see Figure 7B-D). During the second

PFST (@142 days old), following one week of equol injections, there were no significant behavioral differences between groups and no apparent trends (see Figure 8B-D).

Serum Isoflavone Levels

Isoflavone levels were determined by gas chromatography/mass spectrometry for serum obtained from 145 day-old females and are illustrated in Figure 21B. Serum levels of daidzein (Phyto-low mean = 7.0 ± 0.8 ng/ml, Phyto-600 mean = 30.5 ± 5.1 ng/ml), genistein (Phyto-low mean = 7.1 ± 1.4 ng/ml, Phyto-600 mean = 32.1 ± 11.8 ng/ml), and equol (Phyto-low mean = 345.5 ± 97.1 ng/ml, Phyto-600 mean = 753.5 ± 76.6 ng/ml) were significantly lower in the Phyto-low animals compared to the Phyto-600 animals ($p<0.001$-0.008). Although the Phyto-low fed animals were injected with equol for 4 consecutive days the serum levels of equol in these animals were still less than half the levels of equol found in animals fed the Phyto-600 diet where intestinal conversion of equol took place. Equol was the primary phytoestrogen present, while both genistein and daidzein represented smaller percentages of the total serum phytoestrogen content. Serum isoflavone values are displayed in Table 2. As seen in experiment 1, diet appears to be more effective than injection in obtaining higher serum equol levels in intact females.

Serum Serotonin Levels

Serotonin levels were measured using ELISA and are shown in Table 3. All values obtained were within the normal range described in the vendor's kit. There were no significant differences in serum serotonin concentrations between treatment groups. Like experiment 1, the equol-injected Phyto-low animals tended to have higher serotonin levels than the Phyto-600 animals, but these differences were not significant. These values were also obtained following equol administration; prior serotonin concentrations are not known.

Overall Results

The Phyto-600 fed animals tended to have lower body weights and WAT content; however, these differences were never significant. The diet change during young adulthood in Experiment 1 appears to be more effective than the lifelong dietary treatment in Experiment 2 in producing lower body weights in intact females. Behavior was also not significantly affected between treatment groups. While equol values were significantly higher in the Phyto-600 animals there were no significant differences in serotonin levels between treatment groups. Injected equol values were significantly lower than intestinally produced circulating serum values. However, neither intestinally produced nor injected equol were effective in the presence of estrogens produced from ovarian sources during this interval from birth to 145 days of age. Experiment 3: Brief protocol summary – All ovariectomized animals were fed a Phyto-low diet from 60-85 days of age after arrival at BYU. Diet change at 85 days old (one-half of the animals were fed a Phyto-600 diet while the other half remained on the Phyto-low diet). At 100 days old PFST was performed followed by tissue collection.

Experiments 1 and 2 demonstrate that isoflavones are not very effective when estrogens are also present. Experiment 3 examined the effects, on the study parameters, of isoflavone-rich or poor diets in young ovariectomized females with low estrogen levels. This experiment also introduced the possibility that timing of ovariectomy may also play a role in animal behavior and treatment response.

Body Weight and WAT

Dietary treatment lasted for approximately two weeks. Changes in animal body weight are illustrated in Figure 10 and specific values are displayed in Table 1. Throughout the experiment there were no significant differences in body weight between treatment groups.

However, the Phyto-600 animals tended to weigh less following the diet change at 85 days (Figure 10 and 11A). Animal WAT deposition was lower in Phyto-600 animals ($p<0.001$; Phyto-low mean = 16.0 ± 0.9 grams, Phyto-600 mean = 11.4 ± 0.7 grams) than the Phyto-low animals though when normalized to body weight this significance disappeared ($p<0.086$) (Figure 12B-C).

Porsolt Forced Swim Tests

There were no significant differences between treatment groups for any of the behavioral study parameters in the Porsolt forced swim test (see Figure 11B-D). This could be due to the brief duration of the treatment.

Serum Isoflavone Levels

Serum isoflavone levels were measured via gas chromatography/mass spectrometry and are illustrated in Figure 21C. In this experiment, the serum levels of daidzein (Phyto-low mean = 3.1 ± 0.3 ng/ml, Phyto-600 mean = 38.2 ± 7.0 ng/ml), genistein (Phyto-low mean = 5.3 ± 1.1 ng/ml, Phyto-600 mean = 48.5 ± 8.6 ng/ml), and equol (Phyto-low mean = 4.1 ± 0.4 ng/ml, Phyto-600 mean = 495.6 ± 33.3 ng/ml) were also significantly lower in Phyto-low fed animals than the Phyto-600 fed animals ($p<0.001$). Serum levels of genistein and daidzein represented lower percentages of the total phytoestrogen contend. Equol was the primary phytoestrogen present. Isoflavone levels of daidzein, genistein and equol were not significantly different from each other in the Phyto-low animals. Actual serum values are shown in Table 2.

Overall Results

As shown in experiment 1, switching to an isoflavone rich diet may decrease body weight and WAT deposition. However, treatment longer than two weeks may be required to obtain statistical significance in young ovariectomized females. In this study, even though there is an

absence of naturally occurring estrogens, this short-term exposure to isoflavones does not appear to affect animal behavior. This could be due to the lack of sufficient prior estrogen exposure in the animal as these females were ovariectomized shortly after puberty (45-50 days old).

Experiment 4: Brief protocol summary – lifelong exposure from conception to either a Phyto-low or Phyto-600 diet. All animals were ovariectomized at approximately 100 days old. 1st PFST at 150 days old; equol injections started four days prior to 2nd PFST at 200 days old and continued until end of experiment. 3rd PFST at 220 days old. Tissue collection at 222 days old.

This experiment tested females that were ovariectomized at an older age than the females in experiment 3. This difference examined the possibility that treatment response could be dependent on timing of ovariectomy. This experiment also tested the idea that a lifelong phytoestrogen-rich diet could positively affect performance in the study parameters of females that underwent ovariectomy during young adulthood.

Body Weight and WAT

Figure 13 illustrates the changes in animal body weight from ovariectomy to tissue collection (85-222 days-old) between treatment groups. These animals underwent three PFSTs; animal body weights were tested for significance at each of these points in the experiment. This data along with the ending body weight and WAT values are displayed in Table 1. When animals were ovariectomized at approximately 100 days old the Phyto-600 animals had significantly lower body weights ($p<0.039$; Phyto-low mean = 306.5 ± 8.3 grams, Phyto-600 mean = 278.6 ± 9.0 grams). The Phyto-600 animals continued to have significantly lower body weights through the first (Phyto-low mean = 431.0 ± 12.6 grams, Phyto-600 mean = 371.5 ± 15.4 grams) and second (Phyto-low mean = 487.1 ± 20.6 grams, Phyto-600 mean = 414.6 ± 12.3 grams) forced swim tests ($p<0.01$ and $p<0.021$ respectively; Figures 14A and 15). Although, equol injections

started four days prior to the second PFST (@ 200 days of age), this did not significantly affect the Phyto-low animal body weights. Animal body weights were no longer significantly different from each other at 214 days old. The Phyto-600 animals still had lower average body weights but these values were no longer significant (Figure 16A). The Phyto-low animals displayed decreasing body weights from the start of equol injection to animal sacrifice (Figure 13). If this treatment continued longer it is possible that even the Phyto-600 animals' trend for lower body weights would have disappeared. Ending body weights, WAT and WAT/BW were not significantly different between groups. However, at this point, the Phyto-600 animals still tended to have lower values for these parameters than the Phyto-low animals (Figure 17).

Porsolt Forced Swim Tests

In the first PFST (@ 150 days of age), the Phyto-600 animals tended to swim slightly longer, further and faster and tended to dive more and excrete more boli. However, these differences were not significant (Figure 14 B-D). During the second PFST (@ 200 days old), there were no significant differences between these behavioral parameters. The only point of significance noted during the second PFST was the number of boli excreted by the Phyto-600 animals. This value was significantly greater in the Phyto-600 fed animals than the Phyto-low fed animals ($p<0.001$; Phyto-low mean = 3.9 ± 0.4, Phyto-600 mean = 6.75 ± 0.5). The third PFST (at 220 days old) had no significant differences between treatment groups for any behavioral parameters and there were no visible trends (Figure 16B-D).

Serum Isoflavone Levels

Figure 21D illustrates the serum isoflavone levels as quantified via gas chromatography/mass spectrometry. Serum levels of genistein (Phyto-low mean = $8.3 \pm$ ng/ml, Phyto-600 mean = 36.0 ± 7.8 ng/ml) and daidzein (Phyto-low mean = 8.0 ± 1.7 ng/ml, Phyto-600

mean = 40.4 ± 9.2 ng/ml) were significantly lower in the Phyto-low fed animals than in the Phyto-600 animals (p<0.005 and 0.006 respectively). However, serum levels of equol were significantly higher (at least 300 ng/ml higher) in the Phyto-low fed animals than in the Phyto-600 fed animals (p<0.002; Phyto-low = 779.2 ± 63.7 ng/ml and Phyto-600 = 415.1 ± 64.9 ng/ml). Equol was the primary phytoestrogen present in animal serum. Genistein and daidzein both represented lower percentages of total phytoestrogen content. All mean serum values are shown in Table 2.

Serum Serotonin Levels

As in all three of the previous experiments, there were no significant differences between serum serotonin levels for the Phyto-600 and the Phyto-low animals. Serotonin levels were quantified via Elisa and these values are shown in Table 3. These levels are within the normal range set by the vendor's kit.

Overall Results

In animals ovariectomized during young adulthood, lifelong isoflavone diet significantly decreased body weight gain. However, there were still no apparent behavioral effects other than increased boli excretion. Unlike previous experiments, the equol injected animals had higher equol levels than the intestinally produced equol of the Phyto-600 animals. Similar to the equol concentrations, serotonin levels were also the opposite of the previous experiments. The relationship between serum levels of serotonin and equol still needs to be further examined. Overall, ovariectomy appears to change how the diet affects the body. It appears that the later the ovariectomy, the more effective the dietary treatment.

Experiment 5: Brief protocol summary – lifelong exposure from conception to either a Phyto-low or Phyto-600 diet. All animals experienced natural ovarian failure (NOF) at approximately

300 days old. 1<u>st</u> PFST at 330 days old; equol injections started 7 days prior to 2<u>nd</u> PFST at 360 days old in the Phyto-low group only. Tissue collection at 365 days old.

Ovarian status causes animals to respond differently to isoflavone rich or poor diets. This last experiment tested the effects of lifelong exposure from conception to a phytoestrogen-rich diet on study parameters in animals that underwent natural ovarian failure (NOF). This experiment investigated the effectiveness of equol treatment in the absence of estrogens following NOF. Experiment 5 also examined equol's ability to affect serum serotonin levels. This last experiment allowed for comparisons between treatment response and ovarian status (intact, ovariectomized or NOF).

Body Weight and WAT

Animal body weight measurements were only recorded at specific points during this experiment. These major points and corresponding values are displayed in Table 1. At the first PFST (@ 330 days old), which occurred approximately a month after NOF, the Phyto-600 fed animals had significantly lower body weights (Phyto-low mean = 526.5 ± 25.9 grams, Phyto-600 mean = 469.1 ± 21.3 grams) than the Phyto-low animals ($p < 0.05$; Figure 18A). One week prior to the second PFST, the Phyto-low animals were injected with equol as mentioned previously. When measured at the second PFST (@ 360 days old), animal body weights were no longer significantly different between treatment groups (Figure 19A). However, WAT deposition was still significantly greater in the Phyto-low fed animals (Phyto-low mean = 32.1 ± 3.9 grams, Phyto-600 mean = 23.3 ± 2.7 grams) and this trend continued even when WAT was normalized to body weight WAT/BW ($p < 0.05$; Phyto-low mean = 0.063 ± 0.006, Phyto-600 mean = 0.049 ±0.005; Figure 20B and 20C).

Porsolt Forced Swim Test

In the first PFST (@ 330 days old), time immobile (Phyto-low mean = 401.5 ± 10.3 (s),

Phyto-600 mean = 345.9 ± 14.8 (s)), swimming distance (Phyto-low mean = 7.3 ± 0.8 (m),

Phyto-600 mean = 12.5 ± 1.0 (m)), and overall average speed (Phyto-low mean = 0.015 ± 0.002

(m/s), Phyto-600 mean = 0.026 ± 0.002 (m/s)) were significantly higher in the Phyto-600 fed

animals compared to the Phyto-low fed animals (p<0.005; Figure 18B-D). These animals also

dove significantly more often (p<0.005; Phyto-low mean = 0.8 ±0.4, Phyto-600 mean = 2.6 ±

0.6) indicating escape behaviors. This is the first experiment where a dramatic and significant

difference between lifelong diets occurred. Equol injection was also the most effective in this

experiment. Following equol injection, there were no significant differences observed between

treatment groups for any of the behavioral parameters in the second PFST (@ 360 days old)

(Figure 19B-D).

Serum Isoflavone Levels

Serum isoflavone levels were calculated using gas chromatography/mass spectrometry.

All isoflavone values are shown in Table 2 and the differences are illustrated in Figure 21E.

Serum levels of daidzein (Phyto-low mean = 3.1 ± 0.3 ng/ml, Phyto-600 mean = 34.2 ± 6.5

ng/ml), genistein (Phyto-low mean = 4.6 ± 0.8 ng/ml, Phyto-600 mean = 25.2 ± 5.0 ng/ml), and

equol (daidzein's intestinal metabolite)(Phyto-low mean = 4.0 ± 0.4 ng/ml, Phyto-600 mean =

321.6 ± 10.0 ng/ml) were all significantly lower in the Phyto-low animals compared to the

Phyto-600 animals prior to the first PFST (p<0.001). The concentrations of these phytoestrogens

were not significantly different from one another in the Phyto-low animals. In the Phyto-600

animals, equol formed the highest percentage of all of the phytoestrogen content. Following the

second PFST only serum levels of daidzein (Phyto-low mean = 3.1 ± 0.3 ng/ml, Phyto-600 mean

= 34.2 ± 6.5 ng/ml) and genistein (Phyto-low mean = 3.7 ± 0.5 ng/ml, Phyto-600 mean = 25.2 ±

5.0 ng/ml) were significantly lower in the Phyto-low animals compared to the Phyto-600 animals (p<0.001). Serum equol values were not significantly different between treatment groups following the second PFST. In fact, the equol values were slightly higher in the Phyto-low animals (Phyto-low = 376.3 ± 92.9 ng/ml and Phyto-600 = 321.6 ± 10.0 ng/ml). Following the second PFST, serum equol values formed the primary serum phytoestrogen for both treatment groups. Genistein and daidzein represented significantly lower percentages of total phytoestrogen content.

Serum Serotonin Levels

Serum serotonin levels were quantified using ELISA. All serotonin values are shown in Table 3 and are within the normal range stated by the vendor's kit. The Phyto-low fed animals had lower serum serotonin values prior to the first PFST than the Phyto-600 fed animals (Phyto-low = 202.0 ± 29.0 ng/ml and Phyto-600 = 338.2 ± 38.7 ng/ml). However, statistical significance could not be numerically obtained between treatment groups due to the low number of animals whose blood was drawn prior to equol injections (n=3 Phyto-low and n=4 Phyto-600). Following equol injection, the Phyto-low serotonin values were slightly higher than the Phyto-600 values (Phyto-low = 340.0 ± 44.1 ng/ml and Phyto-600 = 313.6 ± 59.7 ng/ml). Equol treatment significantly increased serum serotonin levels in the Phyto-low animals (p<0.042; see Figure 22). Even though serotonin levels were not statistically significant prior to equol administration, the Phyto-600 values were over 100 ng/ml greater (or 1.7 times higher) than the Phyto-low values. Equol injection also appears to increase the serum levels of serotonin (Figure 23).

Overall Results

After natural ovarian failure, a lifelong isoflavone rich diet is able to decrease body weight and WAT deposition as well as depressive-related behavioral parameters. Isoflavone treatment is much more effective in the absence of estrogens following normal reproductive estrogen exposure. Equol supplementation appears to compensate for a lifelong phytoestrogen-poor diet. Serum levels of the isoflavonoid equol appear to affect both animal immobility as an anti-depressive agent and increase serum serotonin levels. Overall, when comparing all five experiments reported here, isoflavone treatment (either dietary or injected) in animals that undergo NOF is the most effective on study parameters.

D. Discussion

This study demonstrated that isoflavones whether administered through the diet or via injection effectively decrease body weight and white adipose tissue deposition in females regardless of age or ovarian status. Experiments 1, 4, and 5 demonstrated dietary isoflavones decreased body weights in animals fed the isoflavone rich Phyto-600 diet. Experiments 1, 2, 4, and 5 demonstrated that equol injection also decreased body weight. Once equol injections began in the Phyto-low fed females, immediate weight loss was seen. Experiments 4 and 5 both received treatment for a period sufficient to decrease the Phyto-low fed female animal body weights until there were no significant differences between them and the Phyto-600 animals. These results are similar to those previously published in male rats (122).

Whether isoflavones were injected or consumed in the diet, the primary serum isoflavone present in each case was equol (Table 2). The resulting decrease in body weight and white adipose tissue deposition indicates that equol has potential as an anti-obesity treatment. Previous studies have demonstrated that both genistein and daidzein, when administered alone, have anti-obesity effects. Genistein appears to mimic estrogenic activity and alters food intake, body

weight, adipose tissue deposition, and lipid uptake into adipocytes by decreasing lipoprotein

lipase (LPL) activity (42,43,123-125). Daidzein also appears to decrease LPL activity, increase

lipolysis, and inhibit adipogenesis via estrogenic pathways (123,126,127).   ERβ has been shown

in previous studies to be involved in anti-obesity effects (128,129).   While equol's mechanism

of action as an anti-obesity is still unknown, equol's affinity for ERβ suggests a possible

estrogenic pathway (74,96). Conversely, unpublished data sets from our laboratory suggest that

the R-equol isomer may be responsible for the majority of the anti-obesity actions. Additionally,

a previously published abstract by our lab further supports equol's estrogenic actions because

equol's actions can be blocked by an ER antagonist (130). Tamoxifen, a SERM able to

antagonize ERs (75) is able to block the positive influence of equol in human monolayer

fibroblasts (130).

This study also demonstrated that equol decreases depressive-related behavior in females

that undergo natural ovarian failure. Before receiving equol injections the experiment 5 Phyto-

low animals demonstrated greater immobility in the PFST and decreased serum serotonin levels.

Equol administration significantly decreased the time spent immobile in the PFST and

significantly increased serum serotonin levels in these animals. This study is the first to report

equol's effectiveness as an antidepressant using the PFST. The testing of other isoflavones such

as quercitin or resveratrol in the PFST, while few in number, demonstrate that isoflavones have

potential as antidepressant treatments (131,132).

Again, while equol's mechanism of action as an antidepressant is not known, equol's

estrogenic affinity for ERβ indicates that it has the ability to affect behavioral changes in the

brain. ERβ is found in four brain regions associated with behavioral and mood disorders, the

frontal cortex, the amygdala, the hippocampus, and the hypothalamus (47,133-135). A previous

study demonstrated that following dietary isoflavone exposure in young, intact, female Long-Evans rats, equol levels in the frontal cortex, the hippocampus, the hypothalamus, and the amygdala increased demonstrating a greater ability for equol to affect these regions (136). The highest levels of equol concentration were located in the frontal cortex where equol makes up round 50% of the isoflavone concentration. Equol levels in the amygdala, hypothalamus, and hippocampus were significantly lower than the frontal cortex levels. However, in each of these regions, equol is the primary isoflavone (80-90% of total isoflavone content) (136). The location and relative concentration of equol in these brain regions indicates that equol both collects in these regions and could also initiate estrogenic actions due to the presence of ERβ.

Previous studies have demonstrated that ERβ is involved in animal expression of both anxiety and depressive-related behaviors (47,113,134,137). A study performed in ERβ knockout mice demonstrated ERβ is involved in anxiety behaviors as the knockout mice demonstrate increased anxiety (137). Others studies have shown that administering an ERβ specific agonist decreases depressive related behavior (113). These studies indicate that ERβ is important in regulating mood disorders. Additionally, because isoflavones in general demonstrate higher binding affinities for ERβ (96), these chemicals could potentially regulate mood using estrogenic pathways. Finally, unpublished data sets from our laboratory suggest that the R-equol isomer is also involved in treating anxiety and depression. The R-equol isomer is more effective than racemic equol which is more effective than the S-equol isomer in treating anxiety. R-equol is able to bind ER subtypes using molecular and biochemical mechanisms that are currently unknown

Estrogens are also known to affect behavioral hormones such as serotonin. Estrogens affect serotonin levels, as well as serotonin receptor expression, and function (50). As

mentioned above these effects are likely initiated via ERβ. In particular, the raphe nucleus of the brain which produces serotonin specifically expresses ERβ indicating that not only do estrogens potentially affect hormone levels but that isoflavones have this same potential (138). As demonstrated in Experiment 5, equol injection significantly alters serum serotonin levels. This combined with the expression of ERβ in the raphe nucleus indicates a possible pathway for equol to affect serotonin concentrations.

In humans, depression is associated with decreased serotonin levels; depressed individuals have significantly lower levels of serotonin than non-depressed individuals (114,139-141). Furthermore, the relationship between serum and CSF serotonin levels indicates potential for decreased serum serotonin concentrations to signify depression (114). One study in humans displayed that serotonin levels in non-depressed individuals were 1.6 times greater than depressed individuals (141). Another human study performed in depressed individuals demonstrated that administration of the antidepressant fluoxetine increased serum serotonin levels in depressed patient by 1.7-fold (142). Both of these results were seen in the experiment 5 females. Prior to equol injection and the first PFST, the Phyto-600 female serum serotonin values were 1.7 times greater than the Phyto-low females. The first PFST then demonstrated that the Phyto-low females expressed increased depressive-related behavior compared to the Phyto-600 females. Subsequent equol injections increased the serum serotonin levels in the Phyto-low females by 1.7-fold, similar to the results for the fluoxetine study in humans (142). This indicates that, although, in this experiment the serum values prior to the first PFST had an insufficient number of females for statistical significance, this finding can still be considered pertinent due to the similar values found in human studies. In summary, equol's ability to bind

ERβ, ERβ's locations in the brain, and the increased serum serotonin levels with decreased time immobile demonstrated in this study all indicate equol's potential as an antidepressant.

However, the use of animals with varying ovarian status in this study also demonstrated that dietary isoflavones or equol alone may have limited use as antidepressant treatments. For instance, ovarian status plays a role in equol treatment effectiveness. Equol is ineffective at decreasing depressive-related behavior in intact females or females ovariectomized shortly after puberty. Experiments 1-4 all displayed no significant differences in depressive-related behavior as measured by the PFST. Intact females have endogenous levels of estrogens that appear to hide or negate any behavioral effects either isoflavones or equol alone might have in these animals. Furthermore, the ovariectomized animals in these experiments were ovariectomized shortly after puberty and perhaps did not have sufficient estrogen exposure to have behavioral effects. Only experiment 5 showed any significant behavioral differences following lifelong endogenous estrogen exposure and natural ovarian failure. Both lifelong dietary isoflavones and equol injection treatment were able to clearly alter animal depressive-related behavior as measured by the Porsolt forced swim test. Animals that were exposed from conception to natural ovarian failure to an isoflavone rich diet displayed fewer depressive-related behaviors in the Porsolt forced swim test compared to animals on a lifelong isoflavone poor diet. Additionally, equol injection in the females on the lifelong isoflavone poor diet significantly decreased depressive-related behaviors in the Porsolt forced swim test (Figure 23). From this study it appears that while equol has potential as an antiobesity treatment regardless of ovarian status or mode of administration, as an antidepressant equol appears to be effective only following natural ovarian failure in this animal model of menopause.

Menopause/ovarian failure is a normal part of the aging process and all normal cycling females will one day undergo this condition (143). However increasing numbers of women are entering menopause prematurely due to either bilateral oophorectomy or cancer treatment (7,12). Severity of menopausal symptoms and the risk of certain diseases and conditions increase as age of menopause decreases (30). This study examined depression and obesity which are symptoms associated with menopause that affect both health and quality of life (19,46). As mentioned earlier, central obesity increases risk for many menopausal conditions such as cardiovascular disease or breast cancer (39,45),while, depression causes menopausal symptoms and associated conditions feel and appear more serious (46). Furthermore, the five experiments in this study indicate that timing and manner of ovarian failure can affect treatment response.   It appears that as an anti-obesity dietary treatment, isoflavones are effective at decreasing body weight regardless of ovarian status. However, as an antidepressant, dietary isoflavones appear to be effective only following natural menopause at which point supplemental equol while consuming a low soy diet appears to be as effective.

E. Summary:

The current study demonstrates that equol is able to decrease body weight, abdominal WAT, and depressive-related behavior in this animal model regardless of age or ovarian status. However, equol's effectiveness as an antidepressant in this model may be dependent upon the changes/timing of ovarian status with aging. This study demonstrated that equol's beneficial effects are most clearly seen in female rats that have undergone natural ovarian failure. Further studies determining equol's exact mechanisms of action and potential as an antidepressant still need to be performed.

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Tables

Table 1. Body Weight Prior to Each Porsolt Test and Tissue Collection Body Weight, WAT and WAT/BW (grams, MEAN ± SEM)_

Experiment	Diet	1st PFST	2nd PFST	3rd PFST	T.C. BW	T.C. WAT	WAT/BW
1	Phyto-low	330.4 ± 5.9	354.0 ± 5.7**	375.6 ± 7.3**	369.9 ± 80.4**	20.1 ± 1.7*	0.054 ± 0.004*
	Phyto-600	327.6 ± 5.1	326.4 ± 5.4	332.6 ± 6.7	331.3 ± 6.4	13.1 ± 1.2	0.039 ± 0.003
2	Phyto-low	318.5 ± 15.0	338.2 ± 16.5	N/A	335.1 ± 15.8	16.1 ± 2.4	0.047 ± 0.005
	Phyto-600	301.1 ± 12.9	323.2 ± 16.3		322.3 ± 15.2	11.9 ± 2.5	0.036 ± 0.006
3	Phyto-low	336.0 ± 7.3	N/A	N/A	336.0 ± 7.3	16.0 ± 0.9	0.042 ± 0.005
	Phyto-600	319.1 ± 6.8	N/A	N/A	319.1 ± 6.8	11.4 ± 0.7#	0.032 ± 0.004
4	Phyto-low	431.0 ± 12.6*	487.1 ± 20.6*	454.4 ± 56.6	453.5 ± 19.3	23.4 ± 5.1	0.049 ± 0.009
	Phyto-600	371.5 ± 15.4	414.6 ± 12.3	417.0 ± 57.8	413.6 ± 20.2	20.2 ± 2.0	0.048 ± 0.010
5	Phyto-low	526.5 ± 25.9*	507.9 ± 21.2	N/A	507.9 ± 21.2	32.1 ± 3.9#	0.063 ± 0.006#
	Phyto-600	469.1 ± 21.3	472.1 ± 19.6	N/A	472.1 ± 19.6	23.3 ± 2.7	0.049 ± 0.005

Comparing diet and/or equol treatments within a column category: ** $p<0.001$; * $p<0.01$; # $p<0.05$; PFST = Porsolt forced swim test, N/A = not assayed, T.C. = tissue collection; BW = body weight; WAT = white adipose tissue deposition, ☐ = time of equol injection.

Table 2. Serum Levels of Isoflavones, Genistein and Daidzein, and Isoflavonoid Equol (all values in ng/ml ± 1.0)

Experiment	Diet	Genistein	Daidzein	Equol	Total (G+D+E)
1	Phyto-low	8.1 ± 0.6*	4.8 ± 0.7*	439.1 ± 80.5*	452.0 ± 80.4*
	Phyto-600	45.3 ± 8.4	34.9 ± 6.7	932.3 ± 121.4	1012.5 ± 135.5
2	Phyto-low	7.1 ± 1.4*	7.0 ± 0.8*	345.5 ± 97.1*	359.5 ± 96.5*
	Phyto-600	32.1 ± 11.8	30.5 ± 5.1	753.5 ± 76.6	816.1 ± 81.0
3	Phyto-low	5.3 ± 1.1*	3.1 ± 0.3*	4.1 ± 0.4*	12.4 ± 1.4*
	Phyto-600	48.5 ± 8.6	38.2 ± 7.0	495.6 ± 33.3	582.3 ± 31.2
4	Phyto-low	8.3 ± 0.4*	8.0 ± 1.7*	779.2 ± 60.4 #	792.2 ± 63.7 #
	Phyto-600	36.0 ± 7.8	40.4 ± 9.2	415.1 ± 64.9	491.5 ± 76.3
5	Phyto-low				
	Before	4.6 ± 0.8*	3.1 ± 0.3*	4.0 ± 0.4*	11.1 ± 1.2*
	After	3.7 ± 0.5*	3.1 ± 0.3*	376.3 ± 92.9	383.3 ± 92.0
	Phyto-600				
	Before	31.4 ± 6.3	36.1 ± 7.1	339.7 ± 13.7	407.3 ± 27.0
	After	25.2 ± 5.0	34.2 ± 6.5	321.6 ± 10.0	381.0 ± 21.0

* Phyto-low value significantly lower than Phyto-600 value; # Phyto-low value significantly higher than Phyto-600 value; ** Phyto-low Before value significantly lower than After value; **Before** refers to serum measured prior to 1st Porsolt test; **After** refers to serum measured after 2nd Porsolt test.

Table 3 Serum Serotonin Levels (all values in ng/ml ± 1.0)

Experiment	Diet	Serotonin
1	Phyto-low	544.9 ± 205.9
	Phyto-600	444.5 ± 168.0
2	Phyto-low	374.0 ± 141.4
	Phyto-600	352.9 ± 133.4
3	Phyto-low	N/A
	Phyto-600	N/A
4	Phyto-low	262.9 ± 107.3
	Phyto-600	317.2 ± 129.6
5	Phyto-low	
	Before	202.0 ± 29.0**
	After	340.0 ± 44.1
	Phyto-600	
	Before	338.2 ± 38.7
	After	313.6 ± 59.7

** Phyto-low Before value significantly lower than After value; **Before** refers to serum measured prior to 1st Porsolt test; **After** refers to serum measured after 2nd Porsolt test.
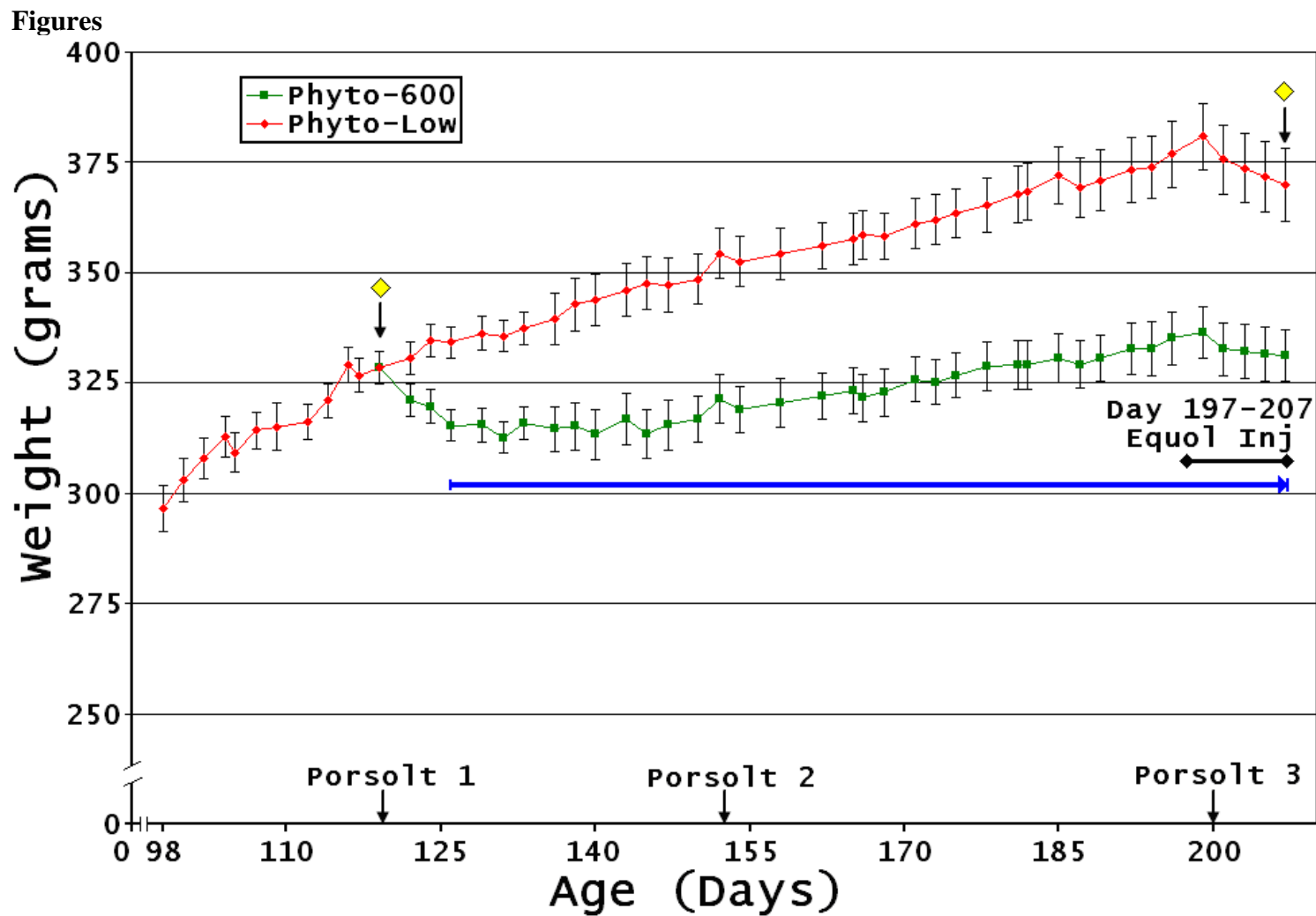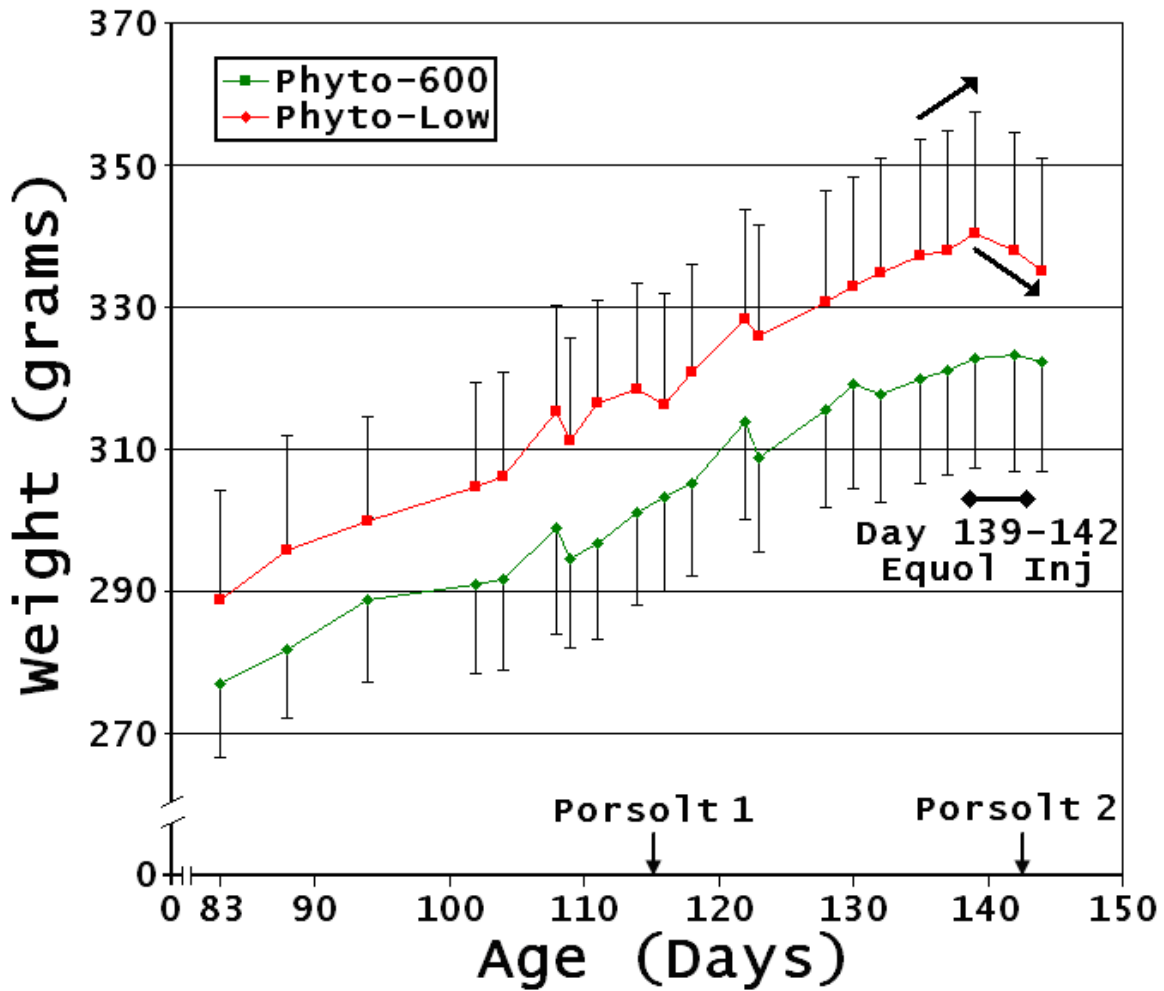
Figure 1: Experiment 1 Intact Diet-Changed Female Body Weight Changes. Up arrow change in body weight prior to equol injection was significantly different compared to down arrow, suggesting equol's anti-obesity actions. ◇ indicates diet treatment start and finish date; ▬ indicates time body weights were significantly different between treatment groups.
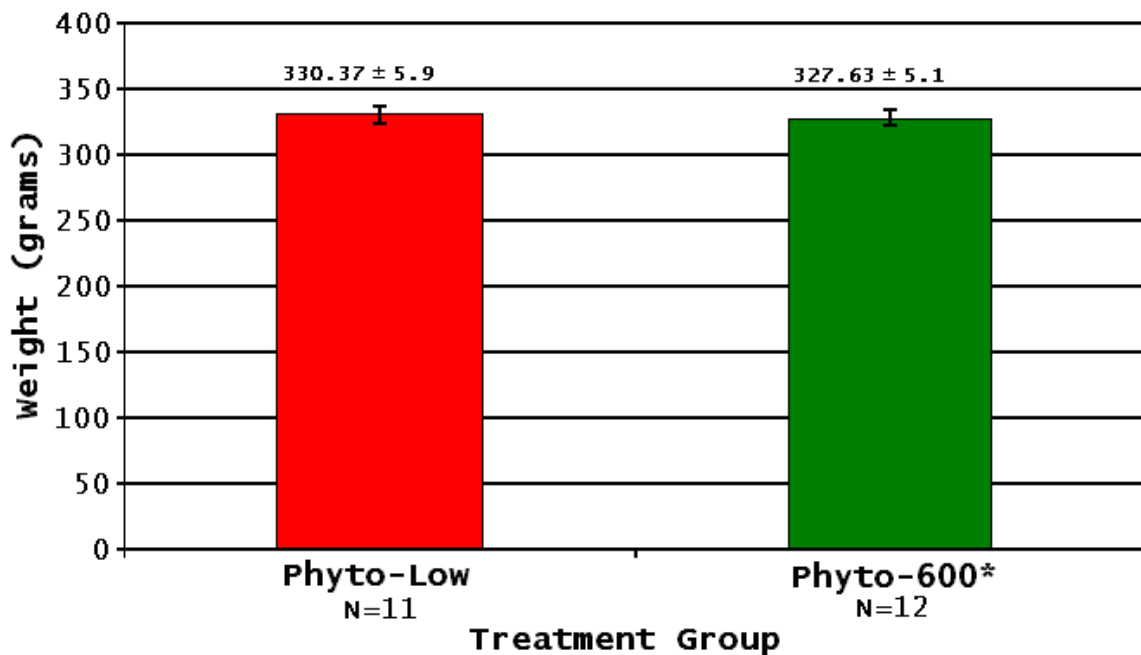
Figure 2A: Experiment 1 Intact Diet-Changed Females 1st Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Test before diet change. * indicates that diet was changed following run. There were no significant differences between treatment groups.
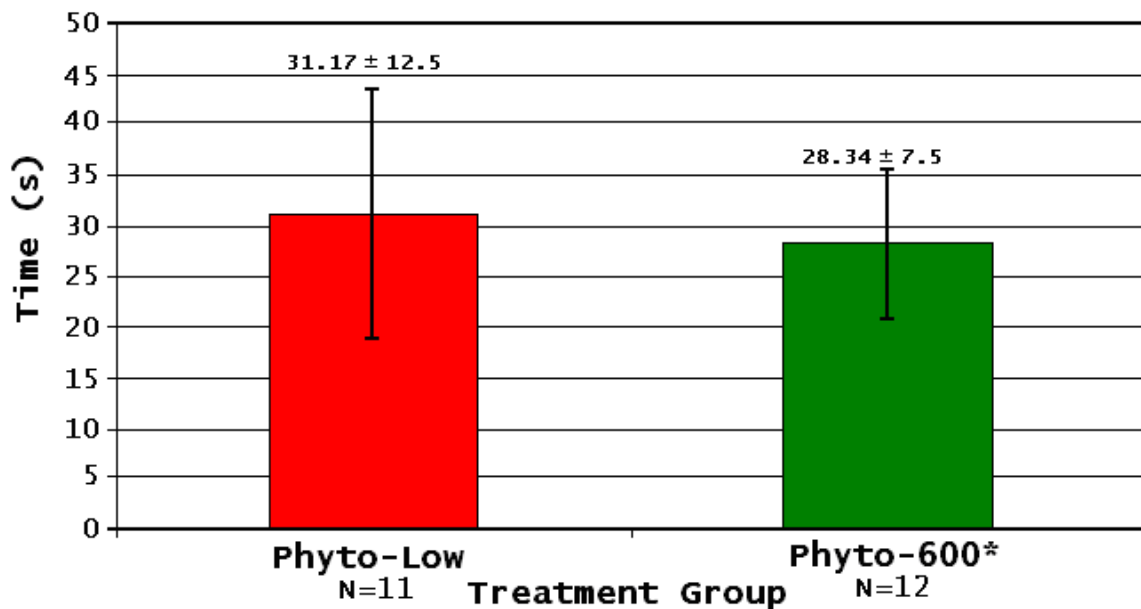
Figure 2B: Experiment 1 Intact Diet-Changed Females 1st Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). Test before diet change. * indicates that diet was changed following run. There were no significant differences between treatment groups.
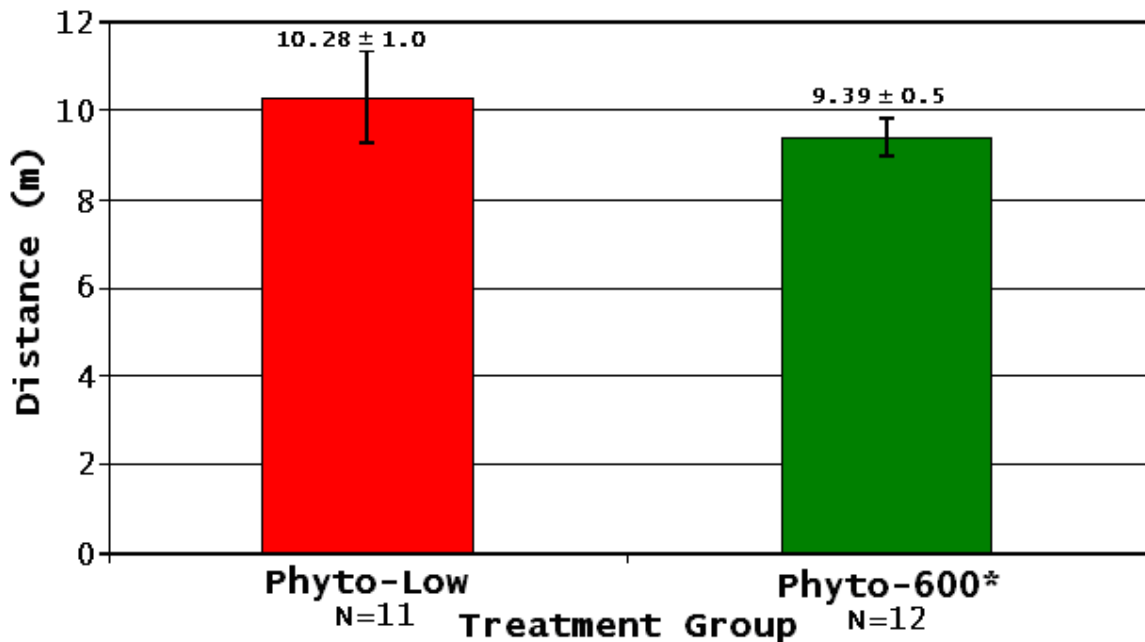
Figure 2C: Experiment 1 Intact Diet-Changed Females 1st Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). Test before diet change. * indicates that diet was changed following run. There were no significant differences between treatment groups.
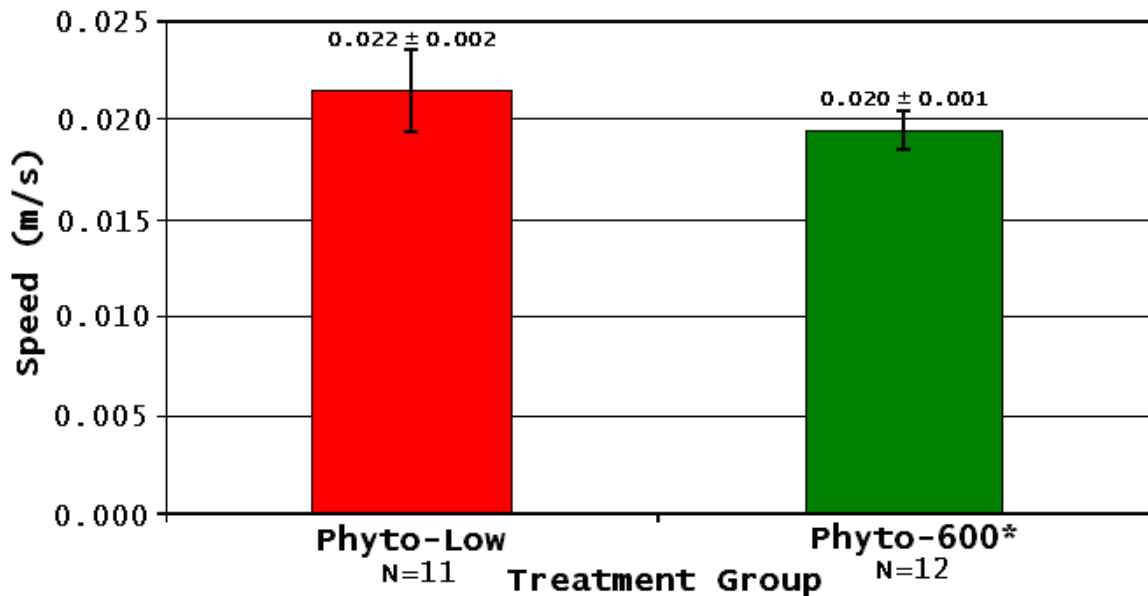
Figure 2D: Experiment 1 Intact Diet-Changed Females 1st Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). Test before diet change. * indicates that diet was changed following run. There were no significant differences between treatment groups.

Figure 3A: Experiment 1 Intact Diet-Changed Females 2nd Porsolt Forced Swim Test Body Weight (MEAN ± SEM). 30 days after diet change. ▽ Phyto-600 animals weighed significantly less than Phyto-low animals.

Figure 3B: Experiment 1 Intact Diet-Changed Females 2nd Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). 30 days after diet change. There were no significant differences between treatment groups.

Figure 3A: Experiment 1 Intact Diet-Changed Females 2nd Porsolt Forced Swim Test Body Weight (MEAN ± SEM). 30 days after diet change. ▽ Phyto-600 animals weighed significantly less than Phyto-low animals.
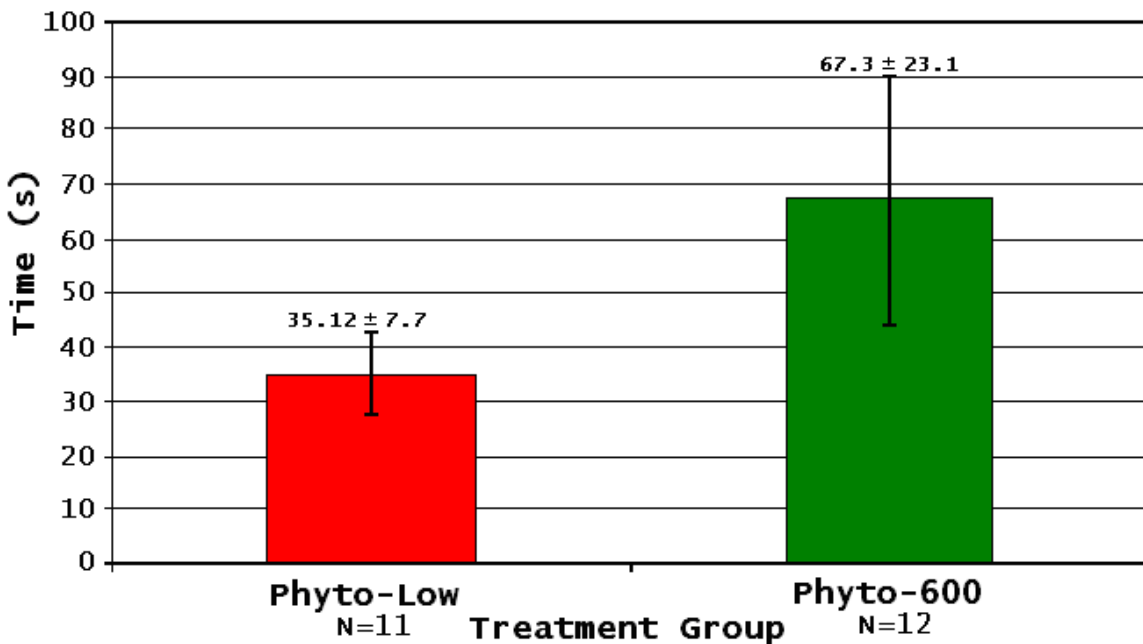
Figure 3B: Experiment 1 Intact Diet-Changed Females 2nd Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). 30 days after diet change. There were no significant differences between treatment groups.

Figure 3C: Experiment 1 Intact Diet-Changed Females 2nd Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). 30 days after diet change. There were no significant differences between treatment groups.
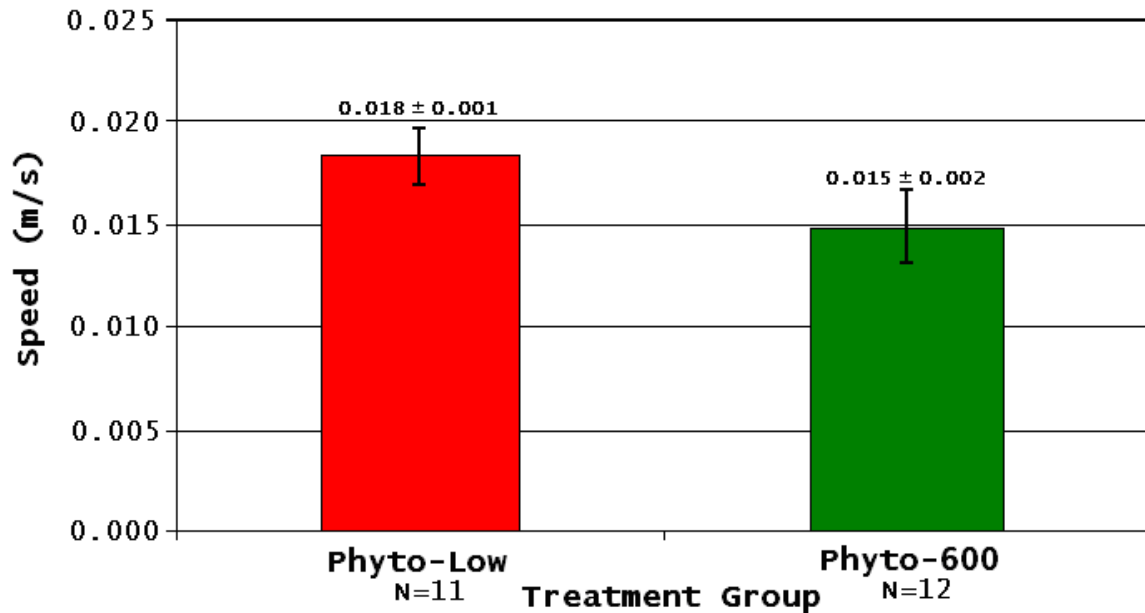
Figure 3D: Experiment 1 Intact Diet-Changed Females 2nd Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). 30 days after diet change. There were no significant differences between treatment groups.
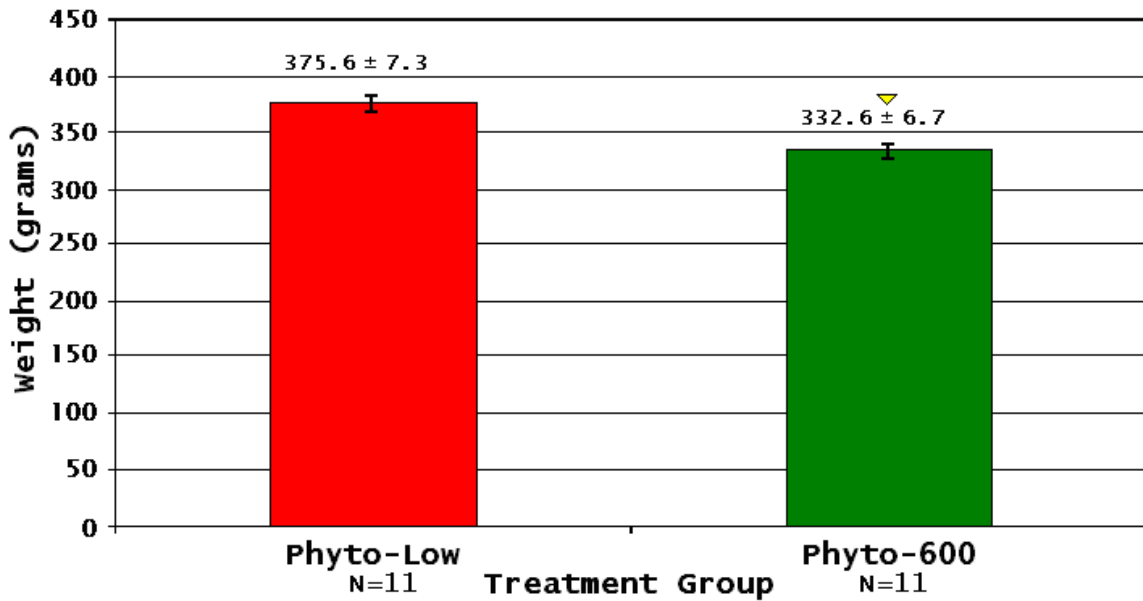
Figure 4A: Experiment 1 Intact Diet-Changed Females 3rd Porsolt Forced Swim Test Body Weight (MEAN ± SEM). 80 days after diet treatment (diet switched) and after 7 days of equol injections in the Phyto-low animals. ▽ Phyto-600 animals weighed significantly less than Phyto-low animals.
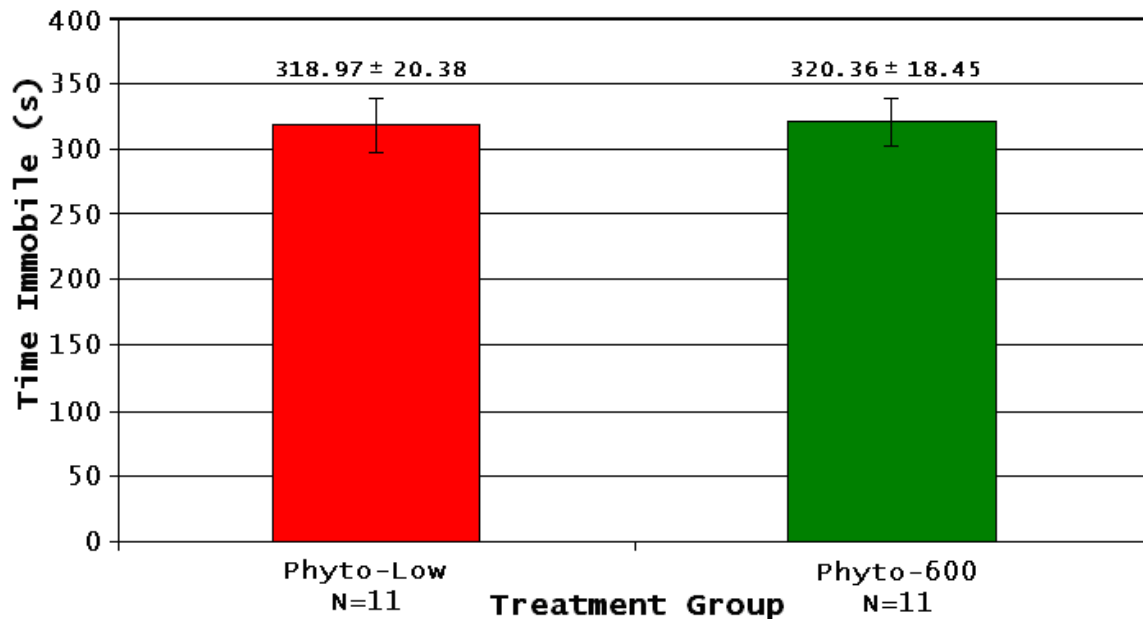
Figure 4B: Experiment 1 Intact Diet-Changed Females 3rd Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). 80 days after diet treatment (diet switched) and after 7 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
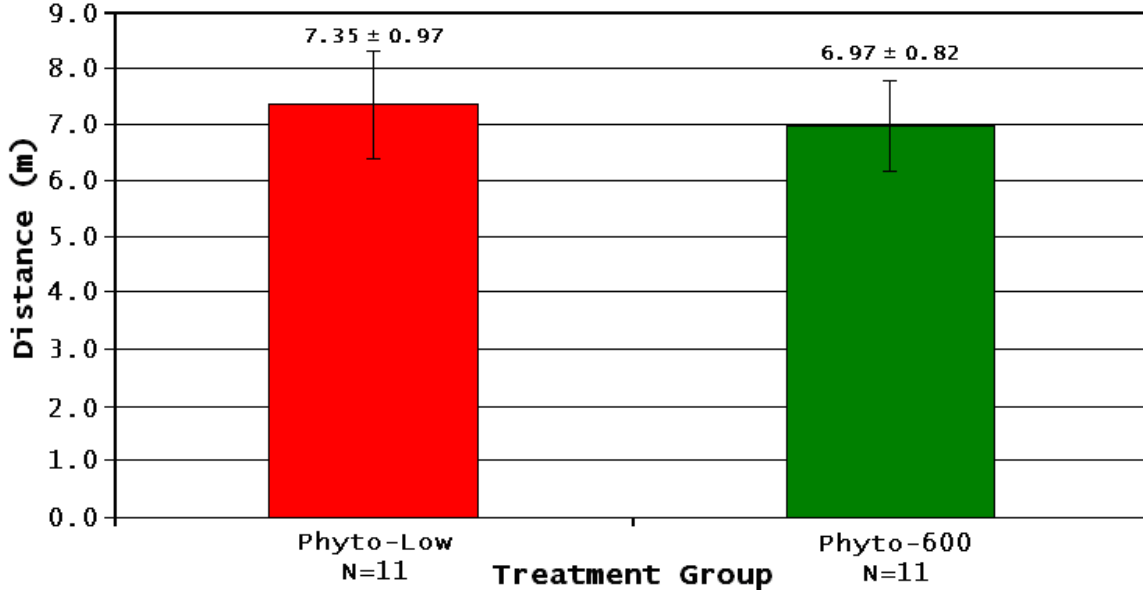
Figure 4C: Experiment 1 Intact Diet-Changed Females 3rd Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). 80 days after diet treatment (diet switched) and after 7 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
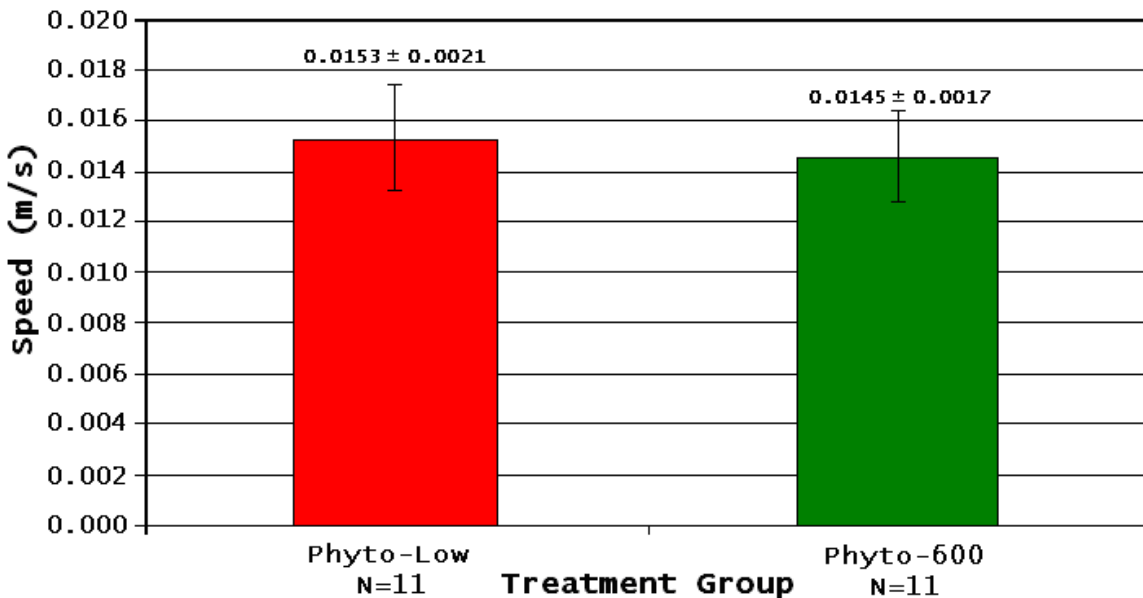
Figure 4D: Experiment 1 Intact Diet-Changed Females 3rd Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). 80 days after diet treatment (diet switched) and after 7 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
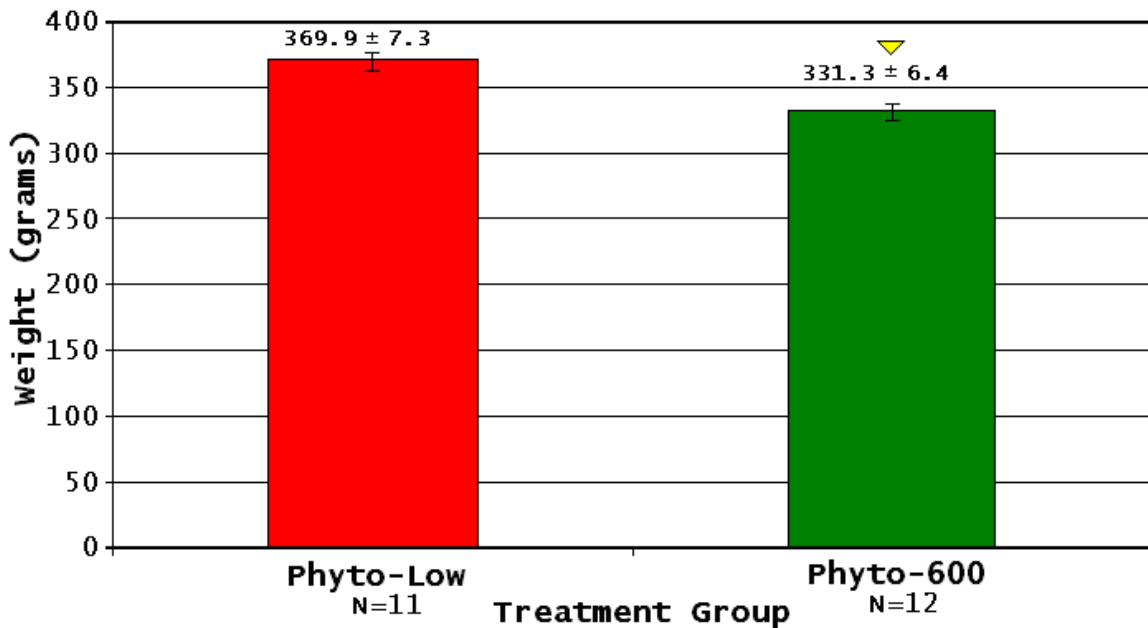
**Figure 5A**: Experiment 1 Intact Diet-Changed Females Body Weight At the End of The Experiment (MEAN ± SEM). ▽ = Phyto-600 animals weighed significantly less than Phyto-low animals.
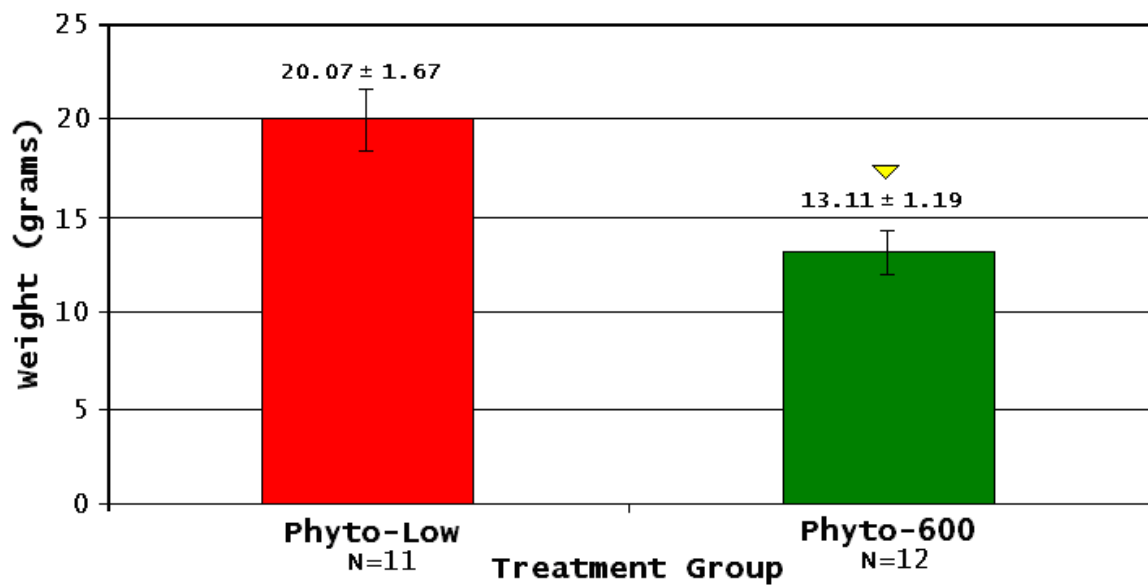
Figure 5B: Experiment 1 Intact Diet-Changed Females White Adipose Tissue (MEAN ± SEM). ▽ = Phyto-600 animals significantly lower than Phyto-low animals.
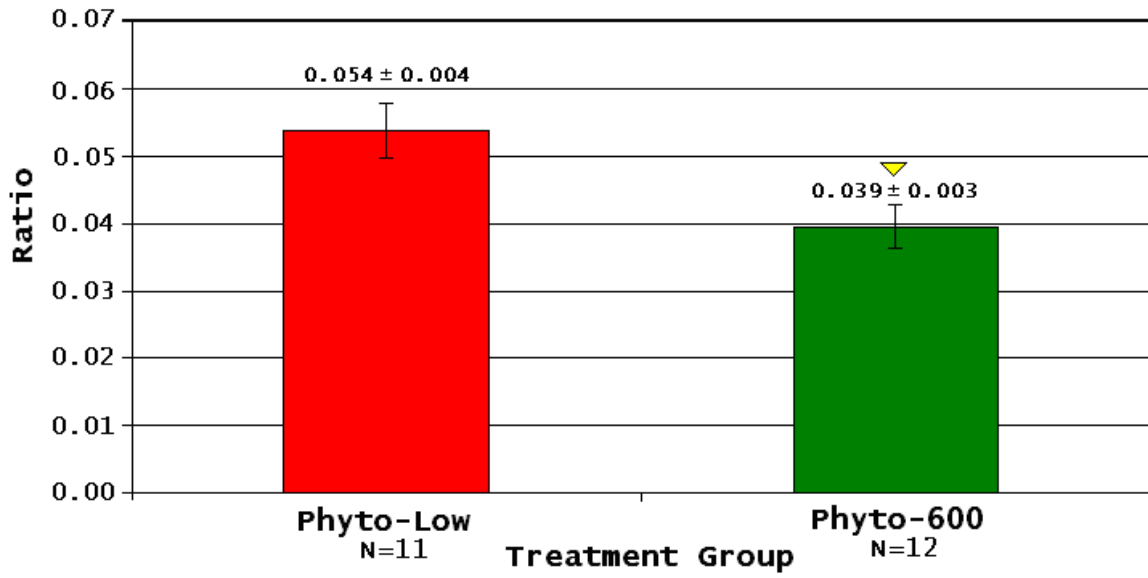
Figure 5C: Experiment 1 Intact Diet-Changed Females WAT/BW Ratio (MEAN ± SEM). WAT = White adipose tissue deposition. BW = body weight. ▽ = Phyto-600 animals significantly lower than Phyto-low animals.

Figure 6: Experiment 2 Intact Lifelong Diet Female Body Weight Changes. Up arrow change in body weight prior to equol injection was significantly different compared to down arrow, suggesting equol's anti-obesity actions.
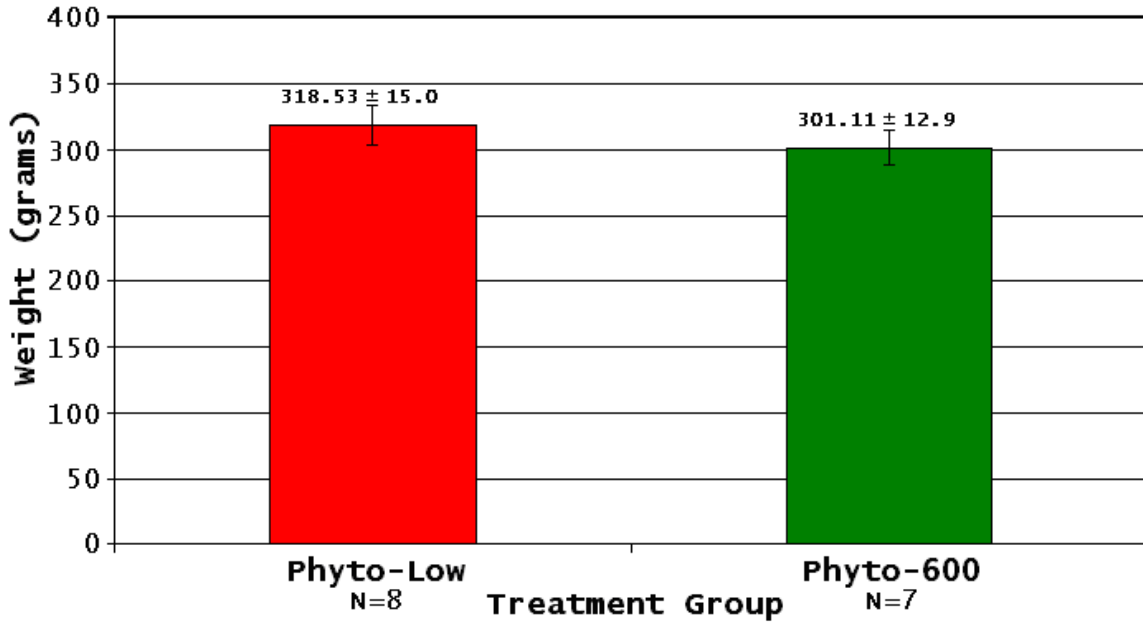
Figure 7A: Experiment 2 Intact Lifelong Diet Females 1st Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Animals age 115 days. There were no significant differences between treatment groups.
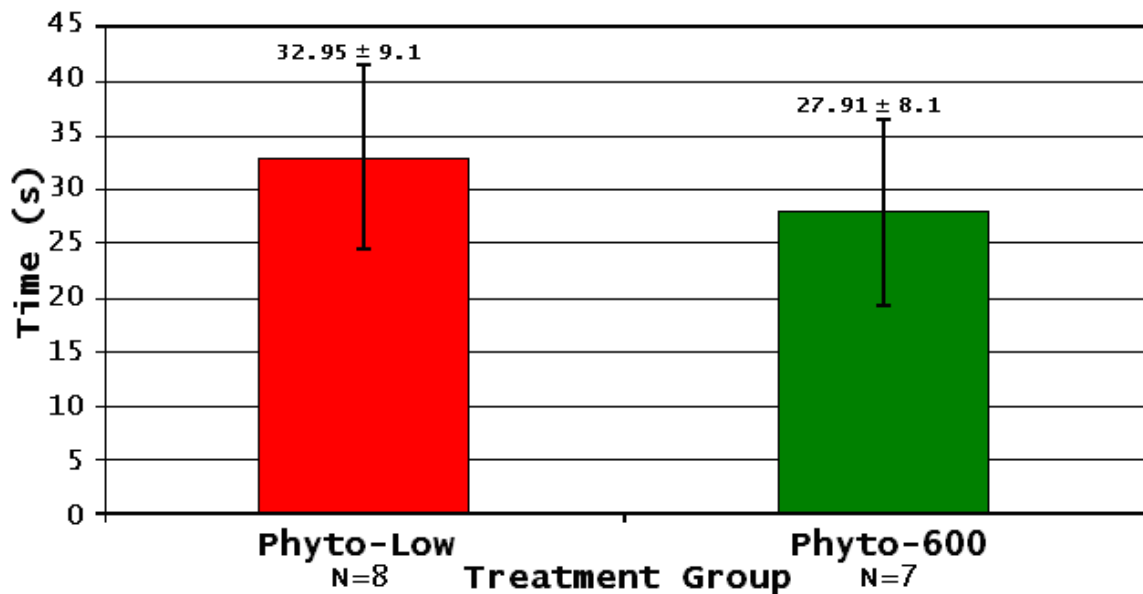
Figure 7B: Experiment 2 Intact Lifelong Diet Females 1st Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). Animals age 115 days. There were no significant differences between treatment groups.
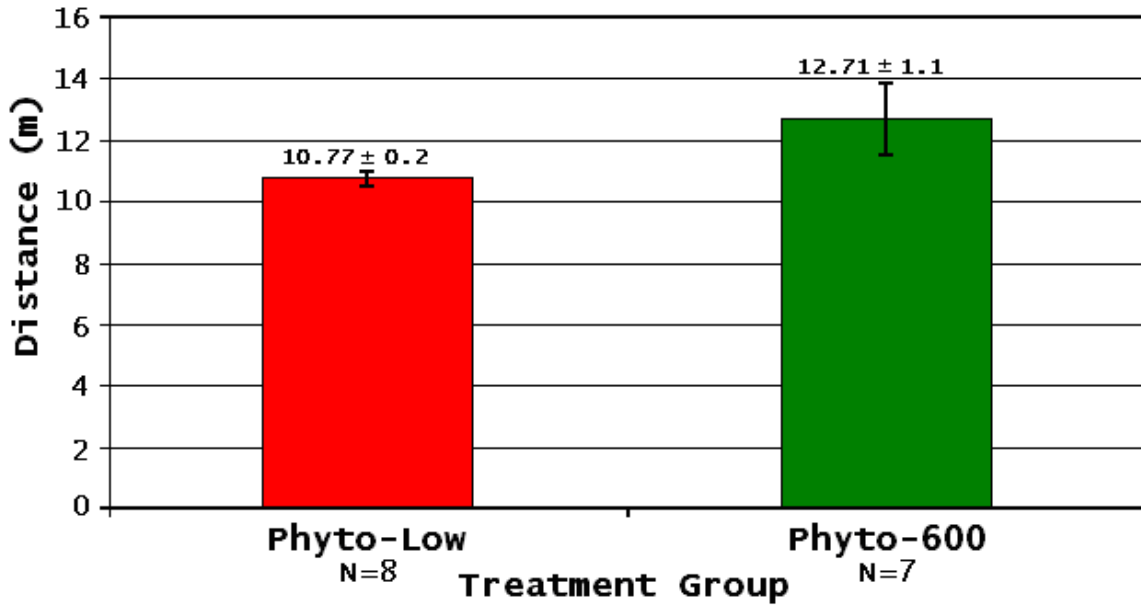
Figure 7C: Experiment 2 Intact Lifelong Diet Females 1st Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). Animals age 115 days. There were no significant differences between treatment groups.
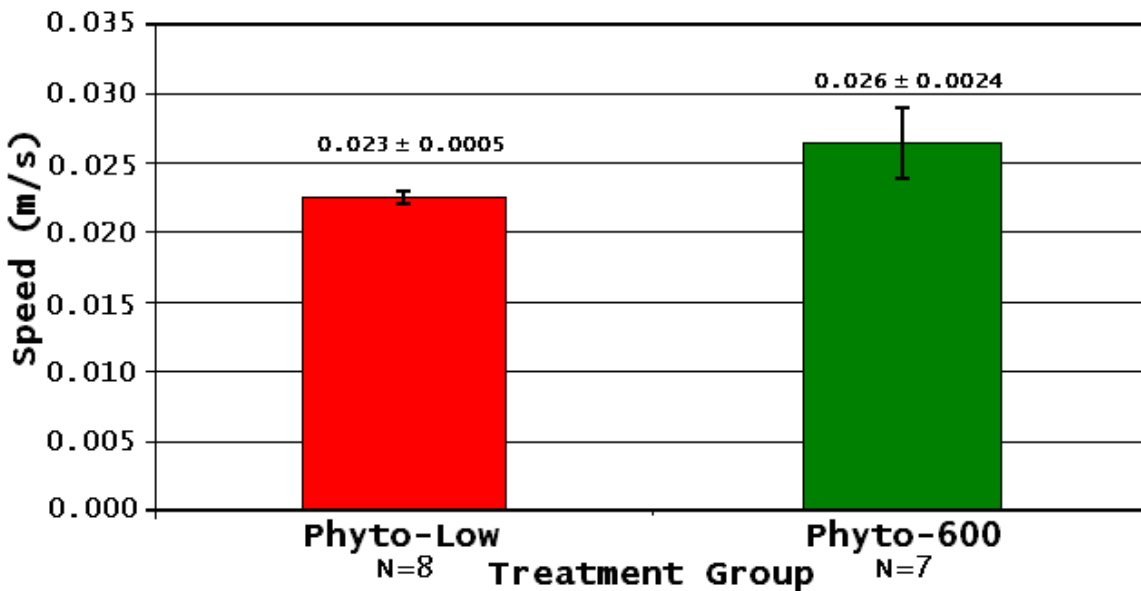
Figure 7D: Experiment 2 Intact Lifelong Diet Females 1st Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). Animals age 115 days. There were no significant differences between treatment groups.
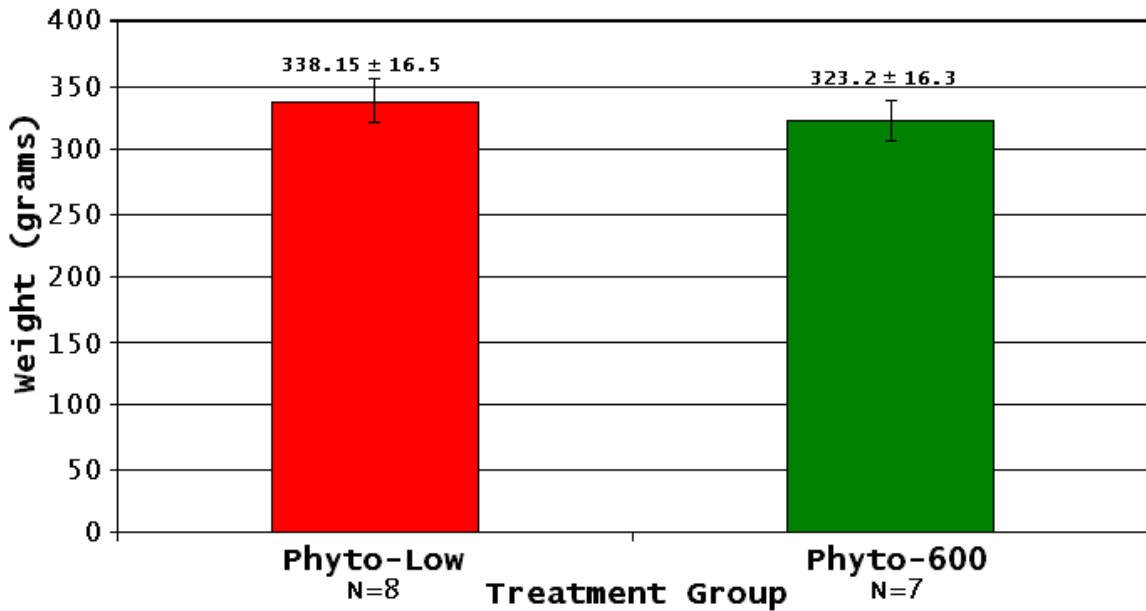
Figure 8A: Experiment 2 Intact Lifelong Diet Females 2nd Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Animals age 142 days and following 4 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
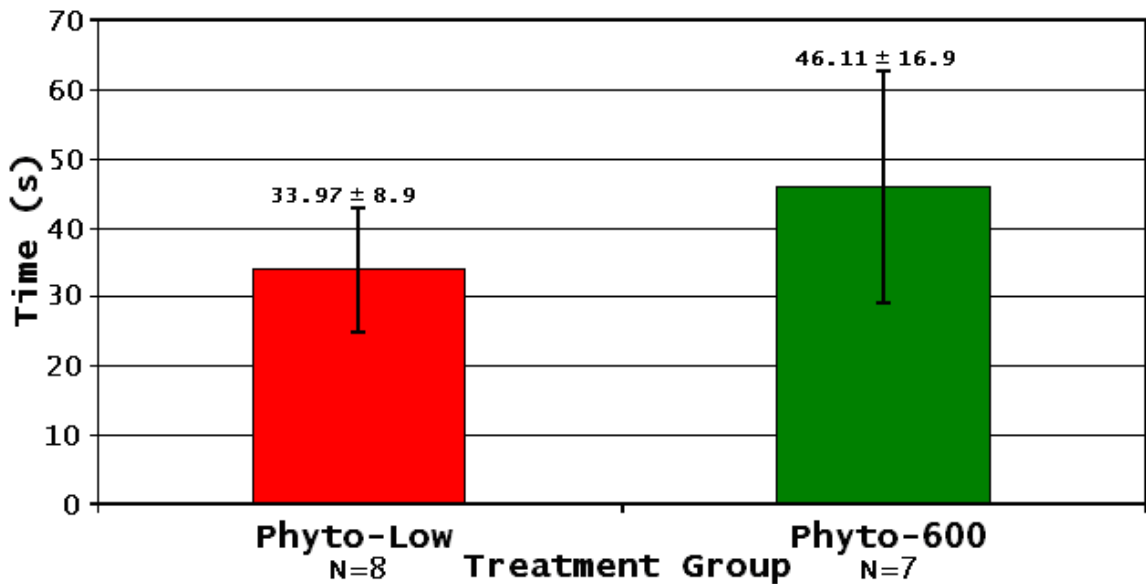
Figure 8B: Experiment 2 Intact Lifelong Diet Females 2nd Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). Animals age 142 days and following 4 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
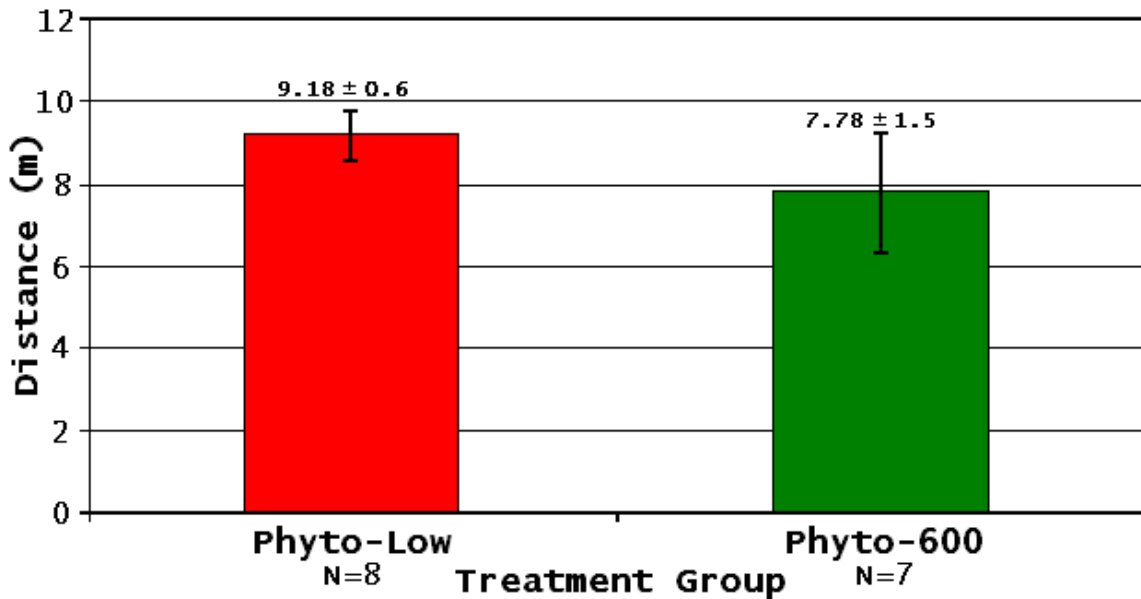
Figure 8C: Experiment 2 Intact Lifelong Diet Females 2nd Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). Animals age 142 days and following 4 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
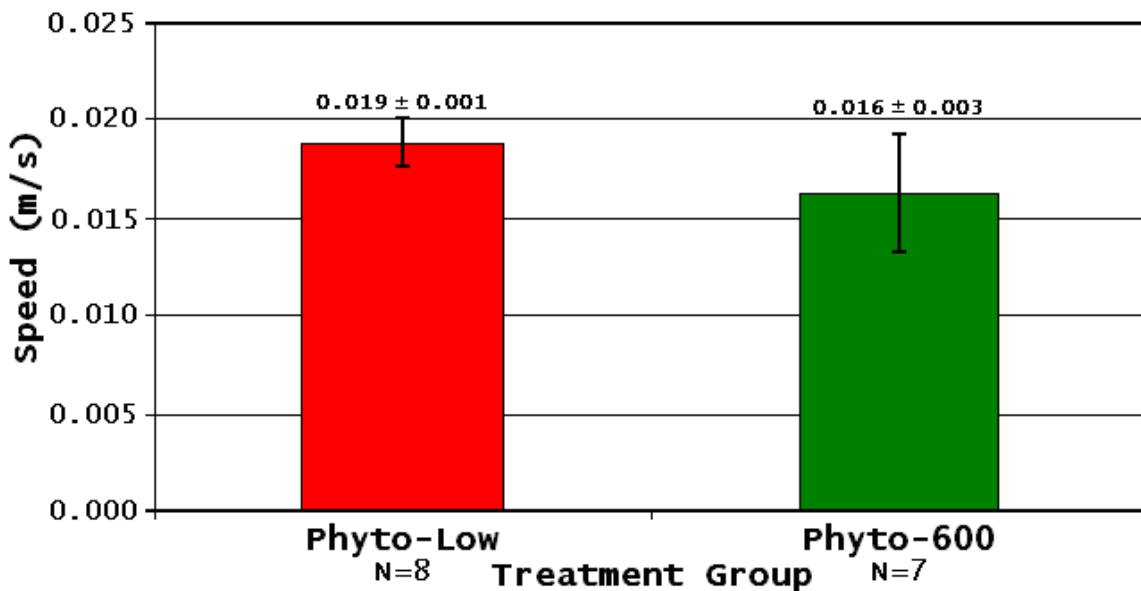
Figure 8D: Experiment 2 Intact Lifelong Diet Females 2nd Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). Animals age 142 days and following 4 days of equol injections in the Phyto-low animals. There were no significant differences between treatment groups.
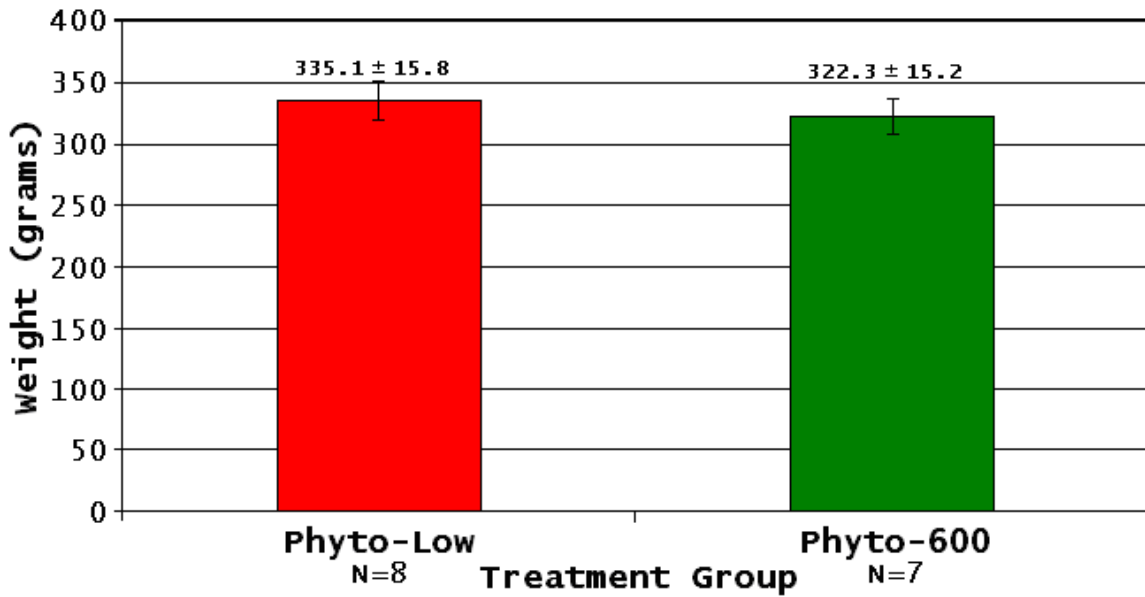
Figure 9A: Experiment 2 Intact Lifelong Diet Females Body Weight At the End of the Experiment (MEAN ± SEM). There were no significant differences between treatment groups.
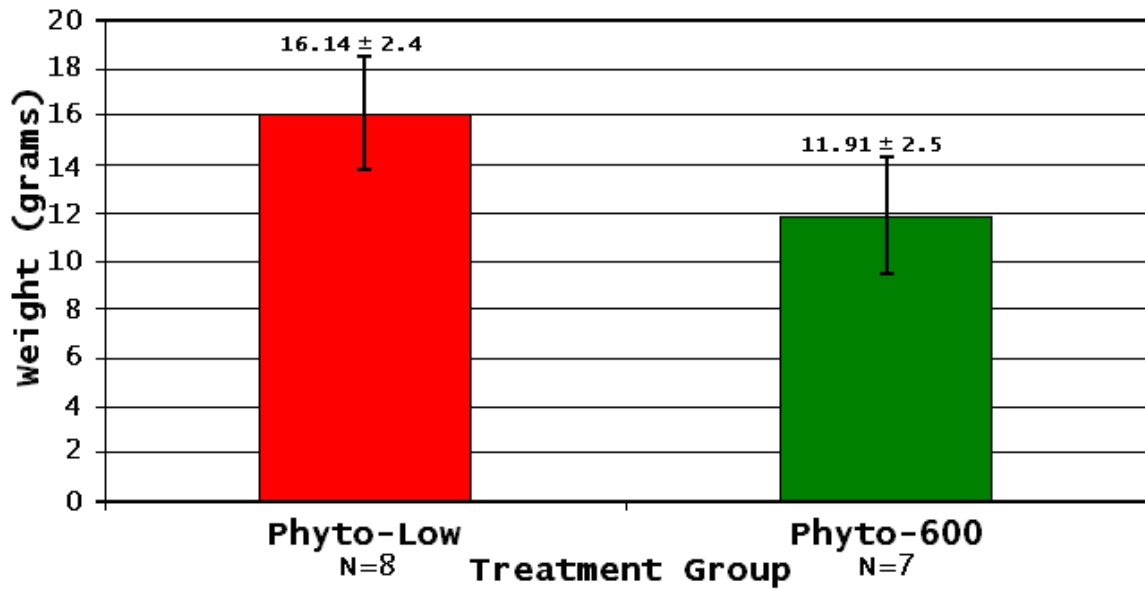
Figure 9B: Experiment 2 Intact Lifelong Diet Females White Adipose Tissue (MEAN ± SEM). There were no significant differences between treatment groups.
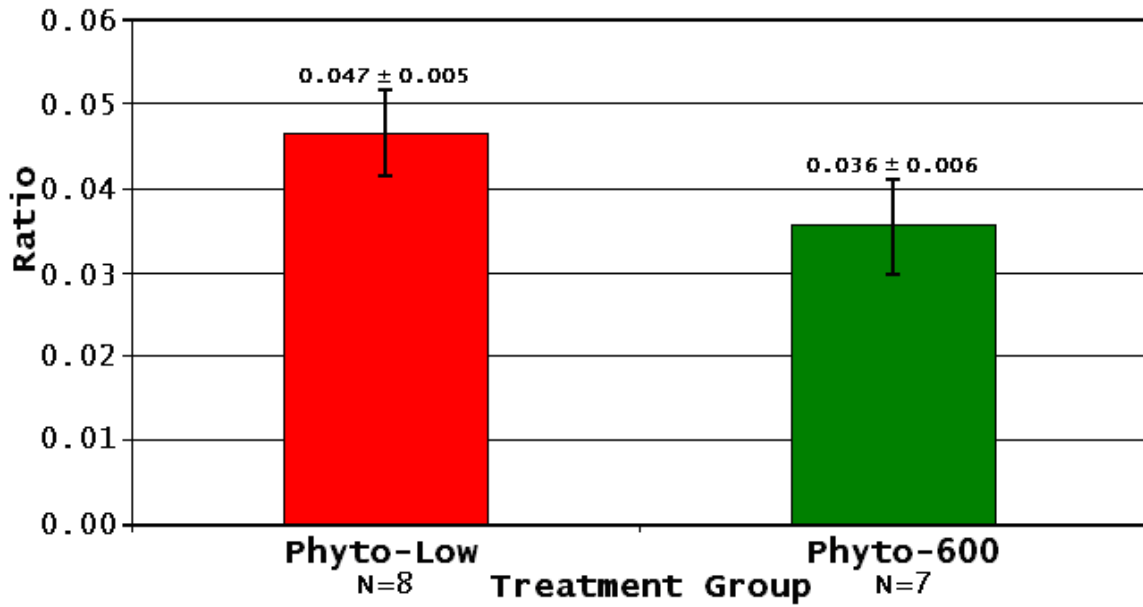
Figure 9C: Experiment 2 Intact Lifelong Diet Females BW/WAT Ratio (MEAN ± SEM). There were no significant differences between treatment groups.
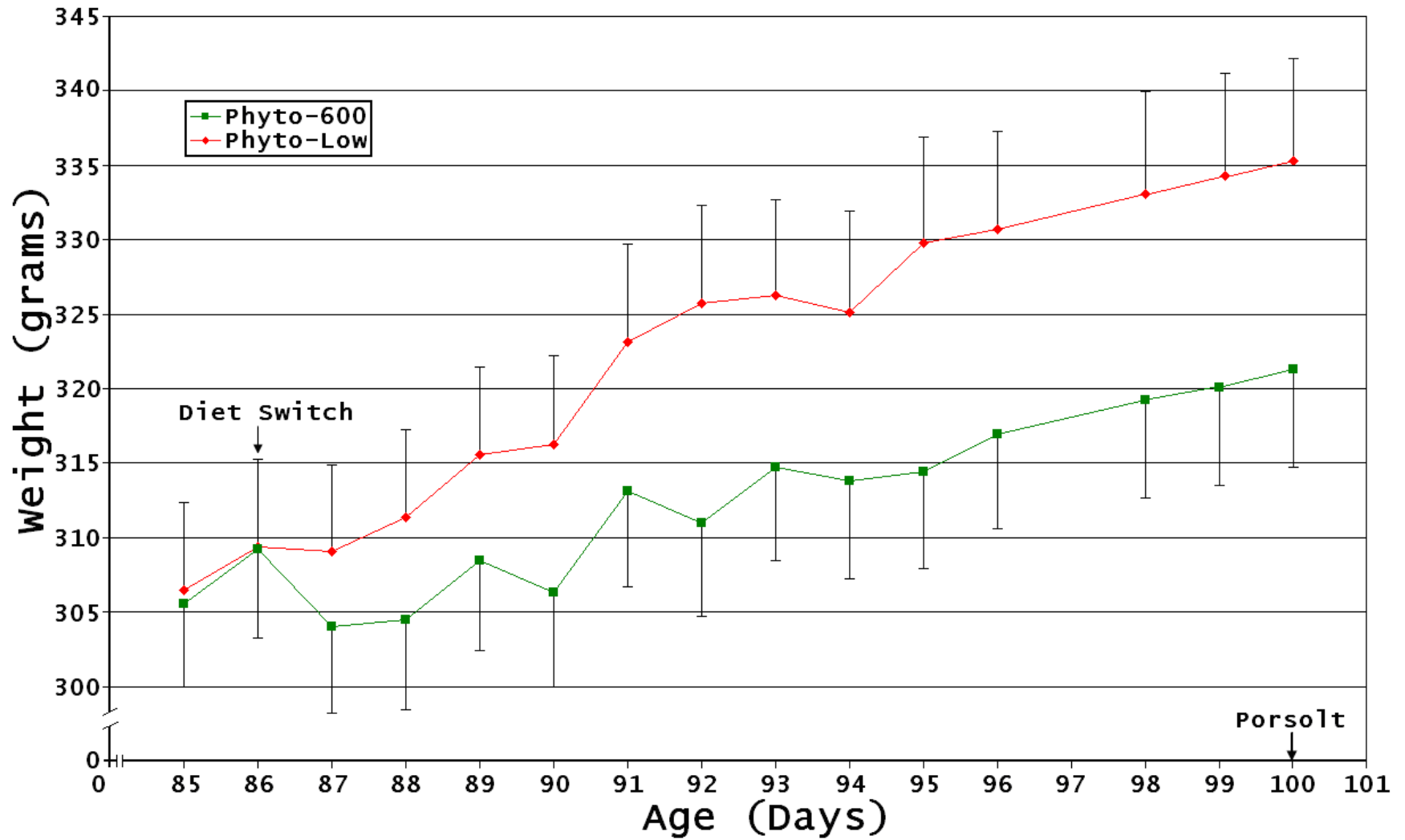
Figure 10: Experiment 3 Ovariectomized Diet-Changed Female Body Weight Changes.
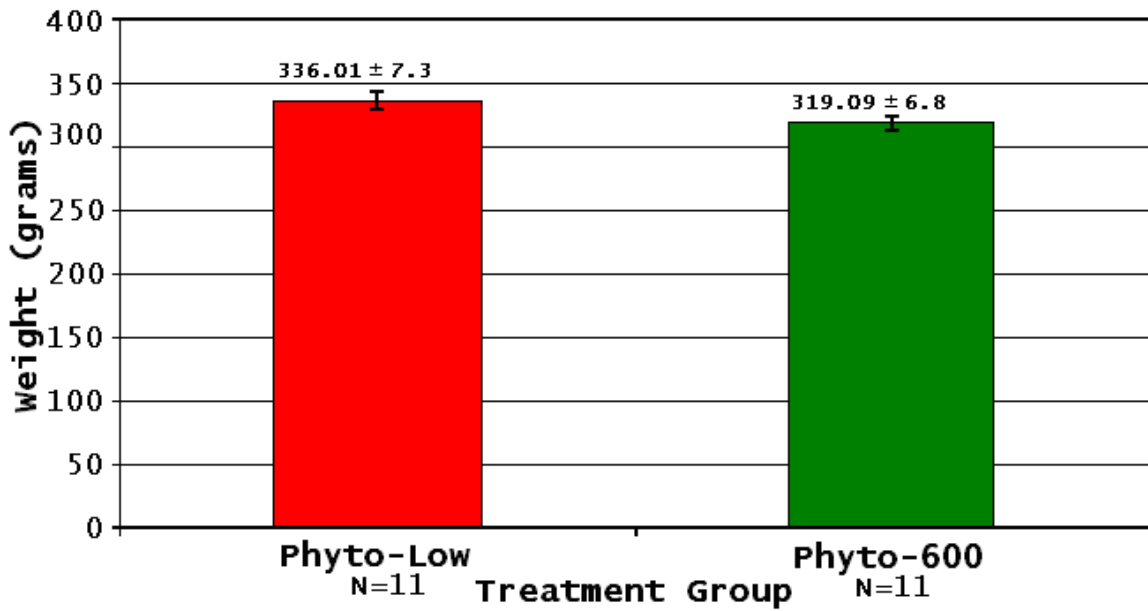
Figure 11A: Experiment 3 Ovariectomized Females Porsolt Forced Swim Test Body Weight (MEAN ± SEM). There were no significant differences between treatment groups.
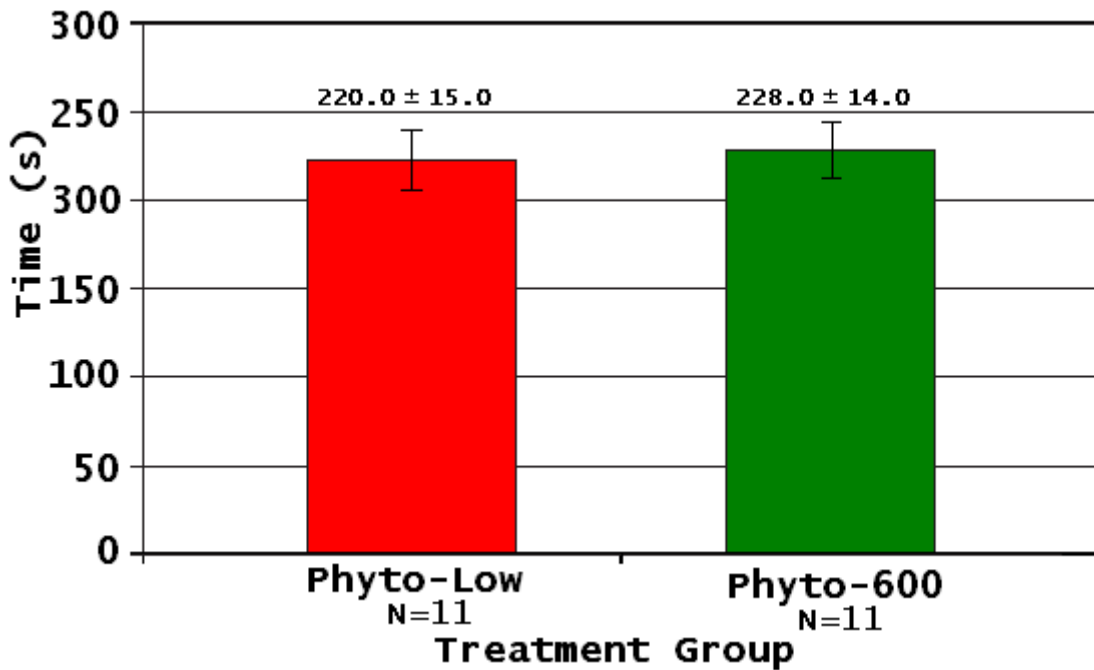
Figure 11B: Experiment 3 Ovariectomized Females Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). There were no significant differences between treatment groups.
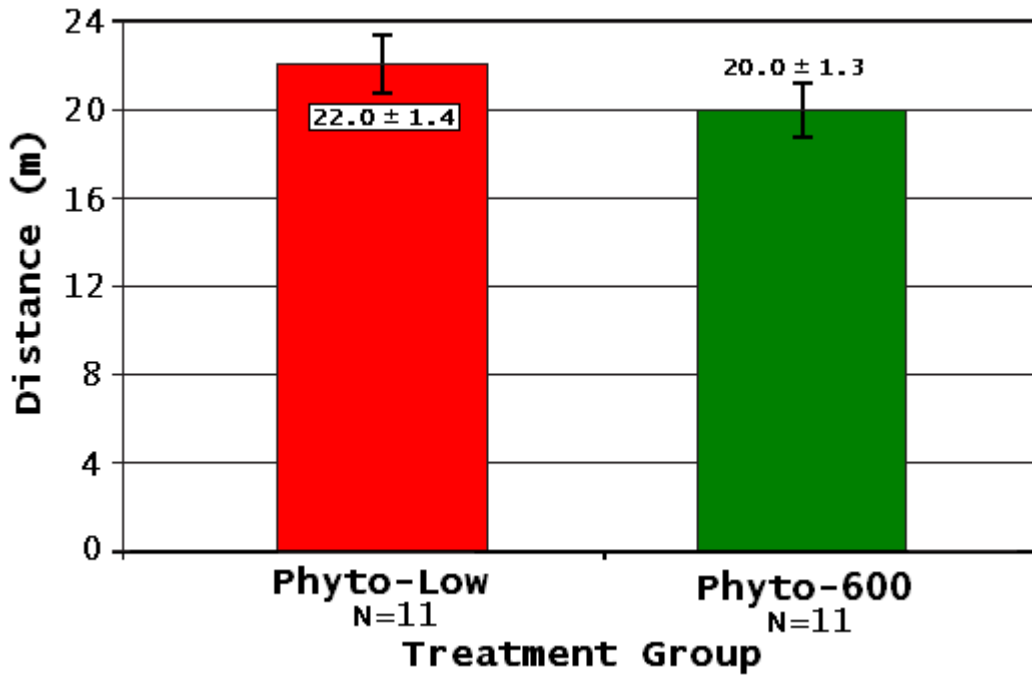
Figure 11C: Experiment 3 Ovariectomized Females Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). There were no significant differences between treatment groups.
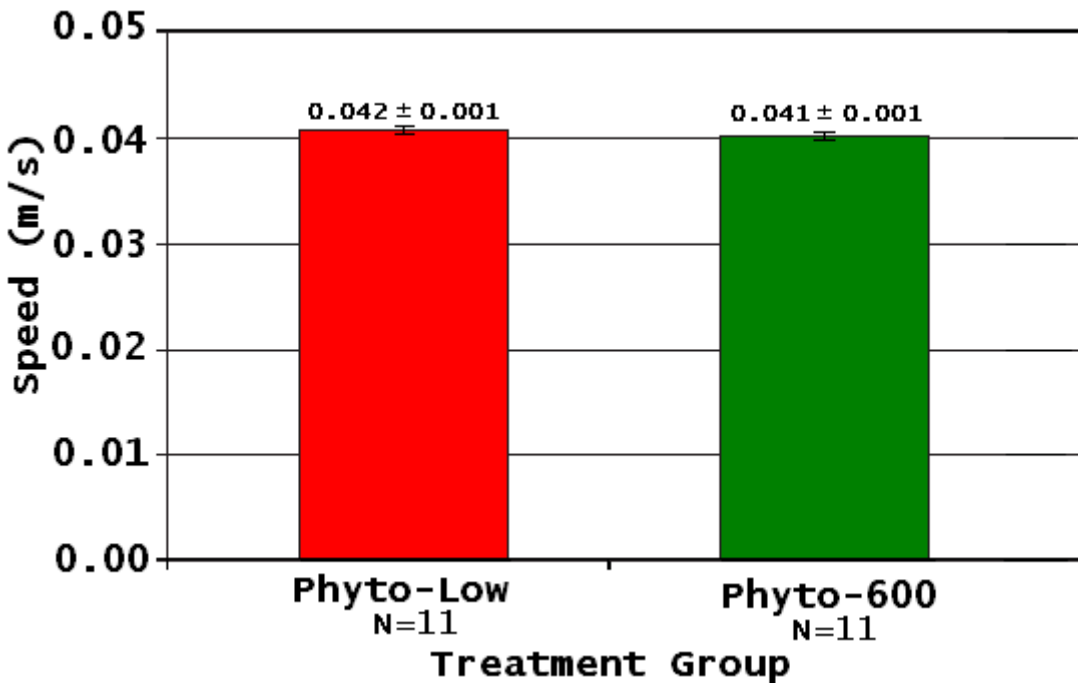
Figure 11D: Experiment 3 Ovariectomized Females Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). There were no significant differences between treatment groups.
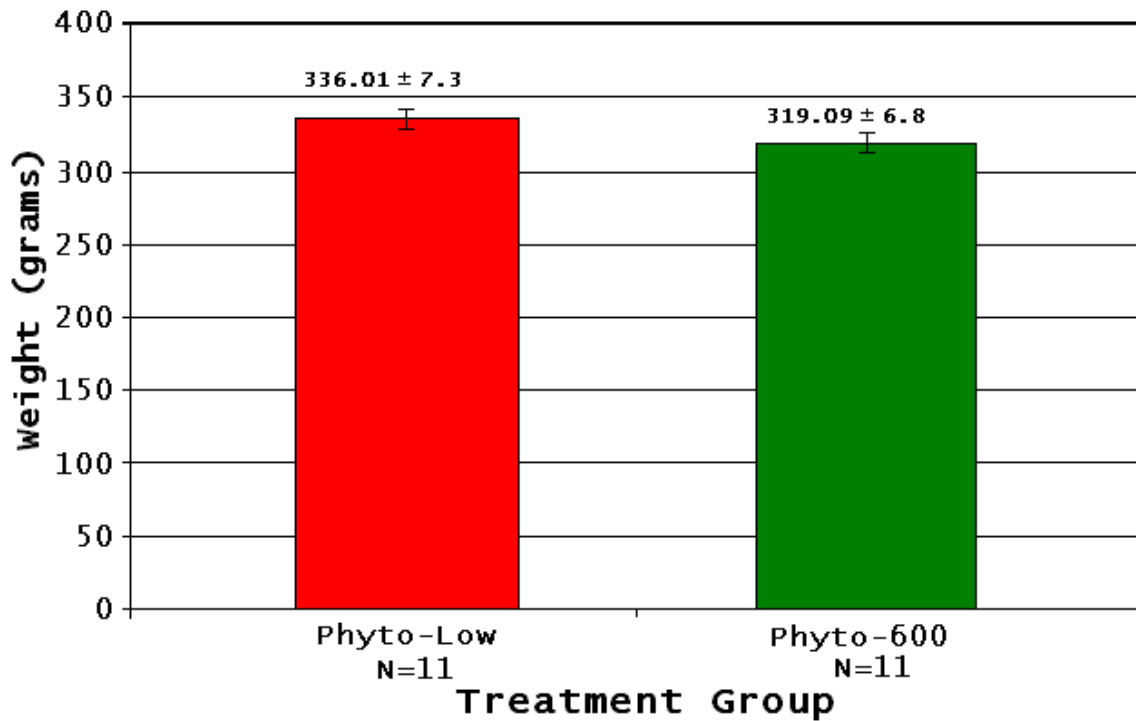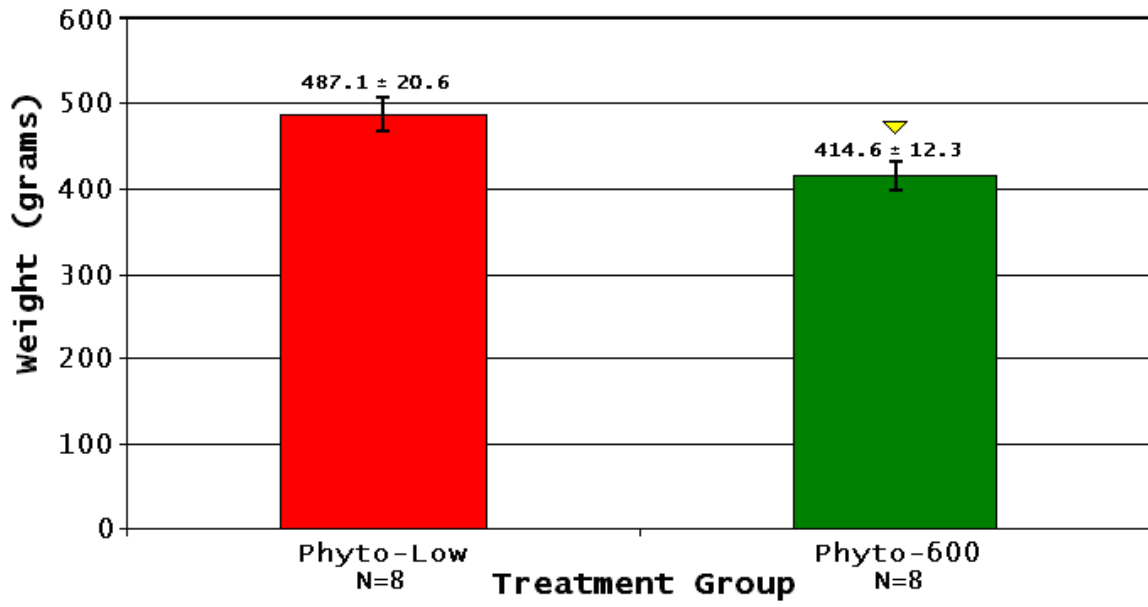
Figure 12A: Experiment 3 Ovariectomized Females Body Weight At the End of the Experiment (MEAN ± SEM). There were no significant differences between treatment groups.
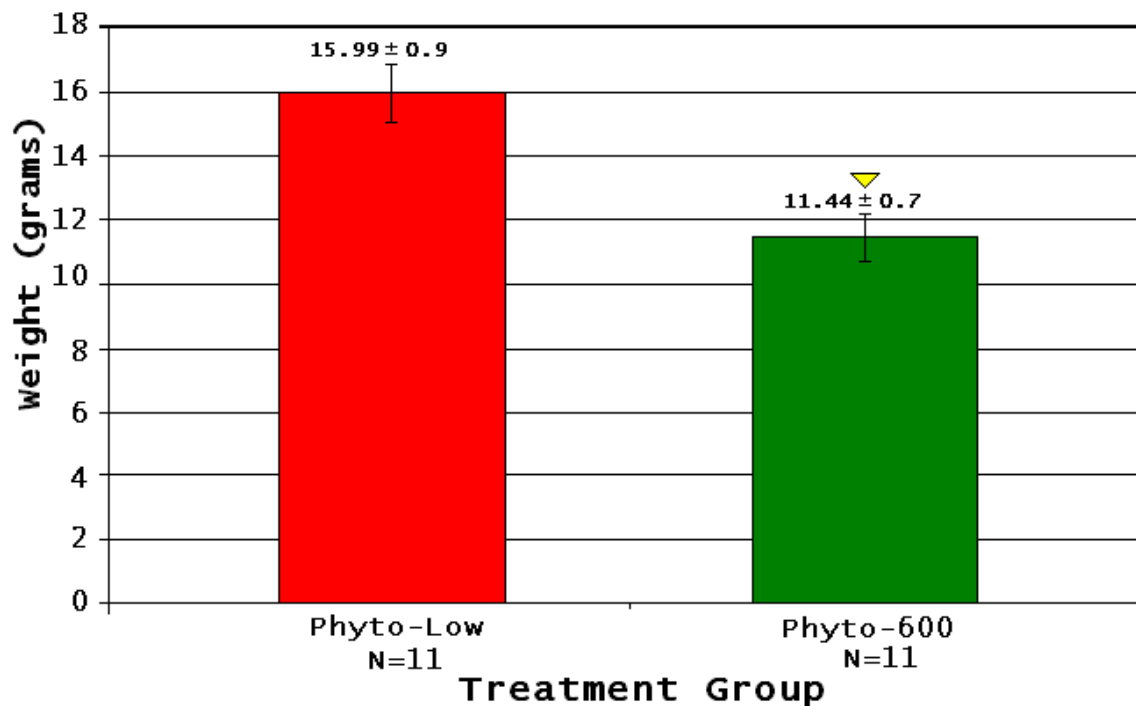
Figure 12B: Experiment 3 Ovariectomized Females White Adipose Tissue (MEAN ± SEM). ▽ Phyto-600 animals WAT significantly lower than Phyto-low animals.
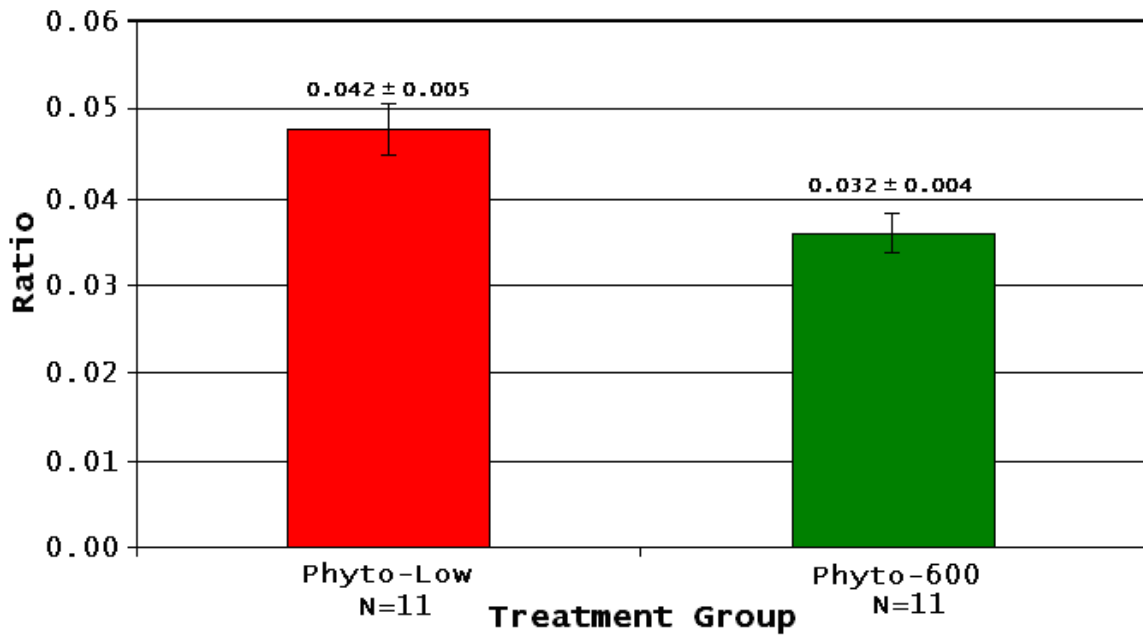
Figure 12C: Experiment 3 Ovariectomized Females White Adipose Tissue to Body Weight Ratio (MEAN ± SEM). There were no significant differences between treatment groups.
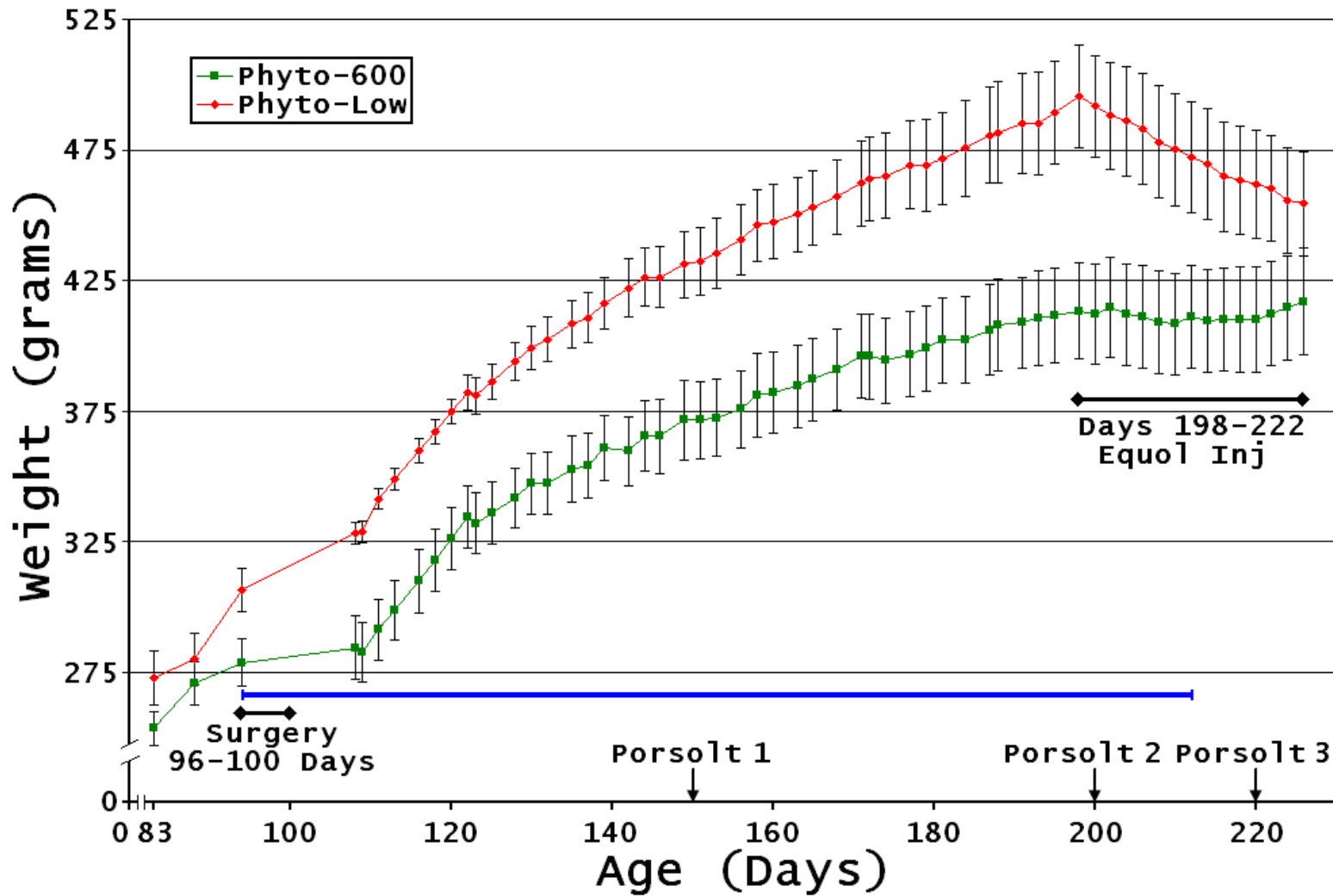
Figure 13: Experiment 4 Ovariectomized Lifelong Diet Female Body Weight Changes. Up arrow change in body weight prior to equol injection was significantly different compared to down arrow, suggesting equol's anti-obesity actions. ▬ indicates time body weights were significantly different between treatment groups.
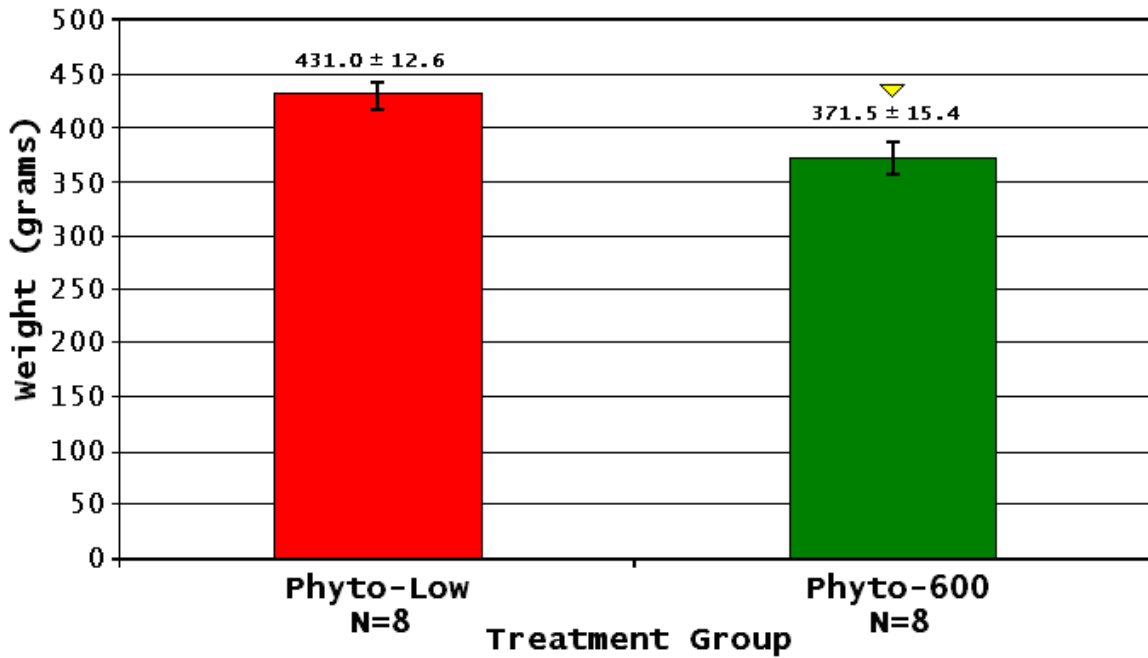
Figure 14A: Experiment 4 Ovariectomized Females 1st Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Animals age 150 days. ▽ Phyto-600 animals weighed significantly less than Phyto-low animals.
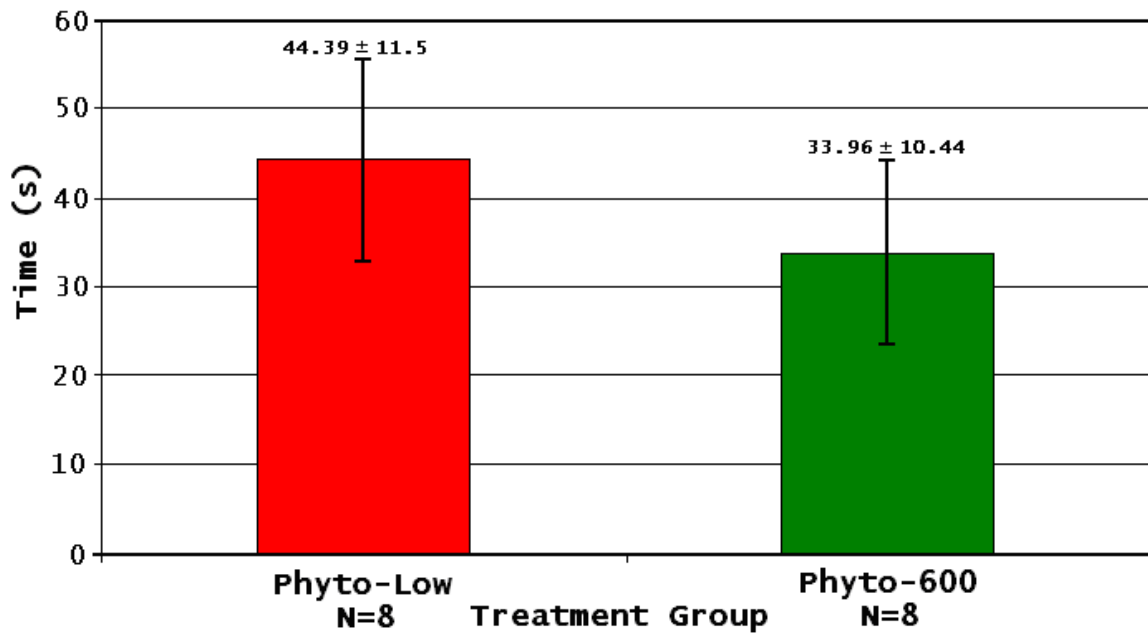
Figure 14B: Experiment 4 Ovariectomized Females 1st Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). Animals age 150 days. There were no significant differences between treatment groups.
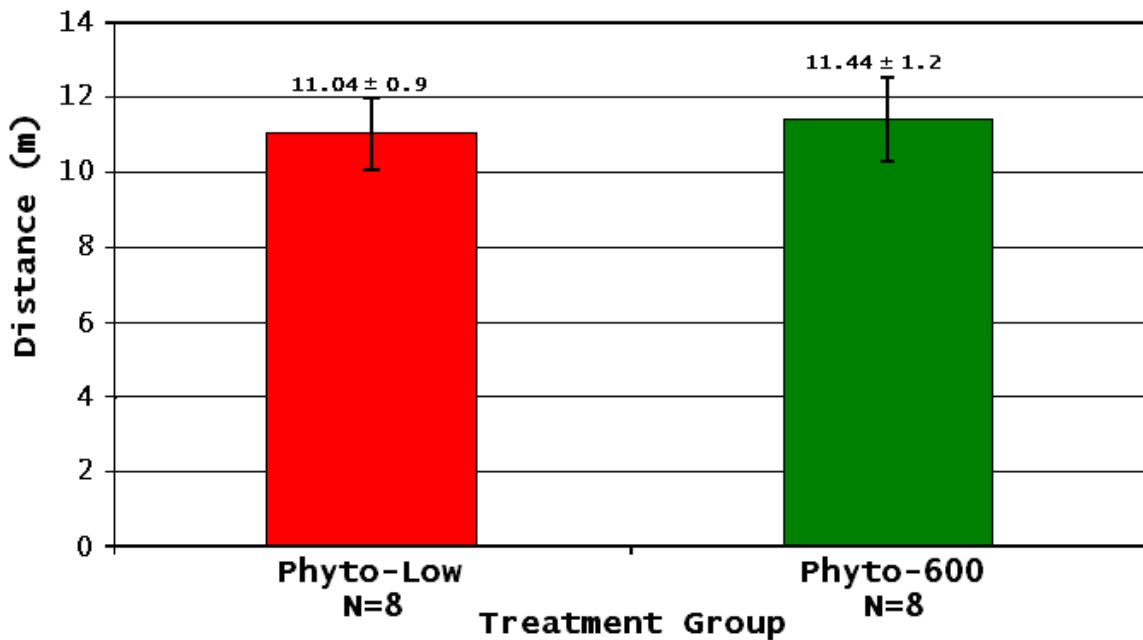
Figure 14C: Experiment 4 Ovariectomized Females 1st Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). Animals age 150 days. There were no significant differences between treatment groups.
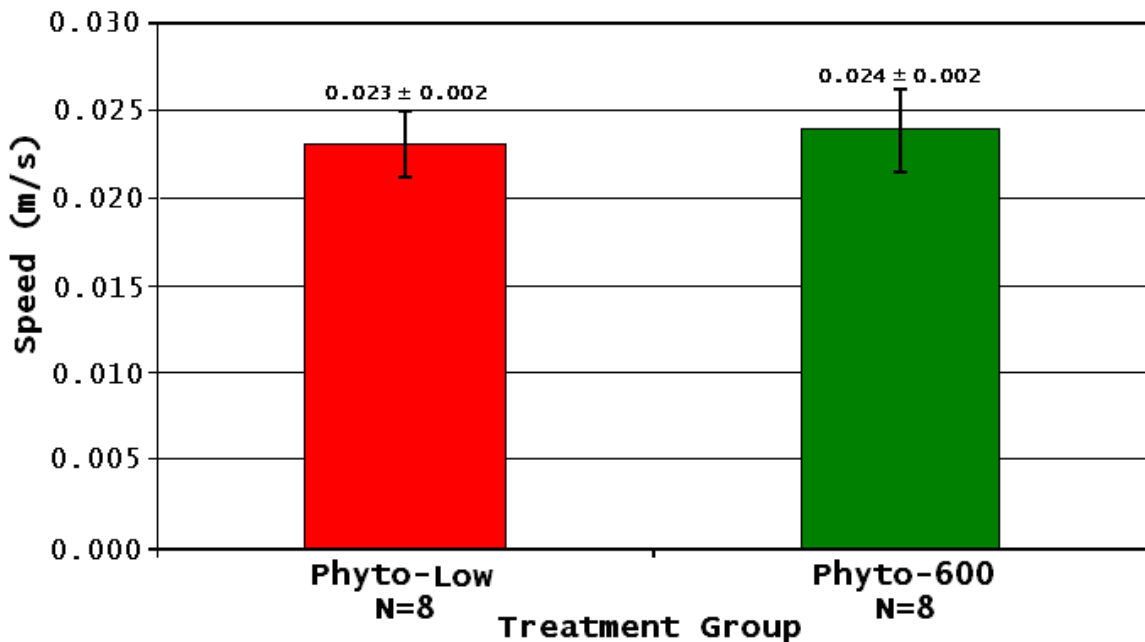
Figure 14D: Experiment 4 Ovariectomized Females 1st Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). Animals age 150 days. There were no significant differences between treatment groups.

Figure 15: Experiment 4 Ovariectomized Females 2nd Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Animals age 200 days and following 4 days of equol injections in the Phyto-low females. ▽ Phyto-600 animals weighed significantly less than Phyto-low animals.
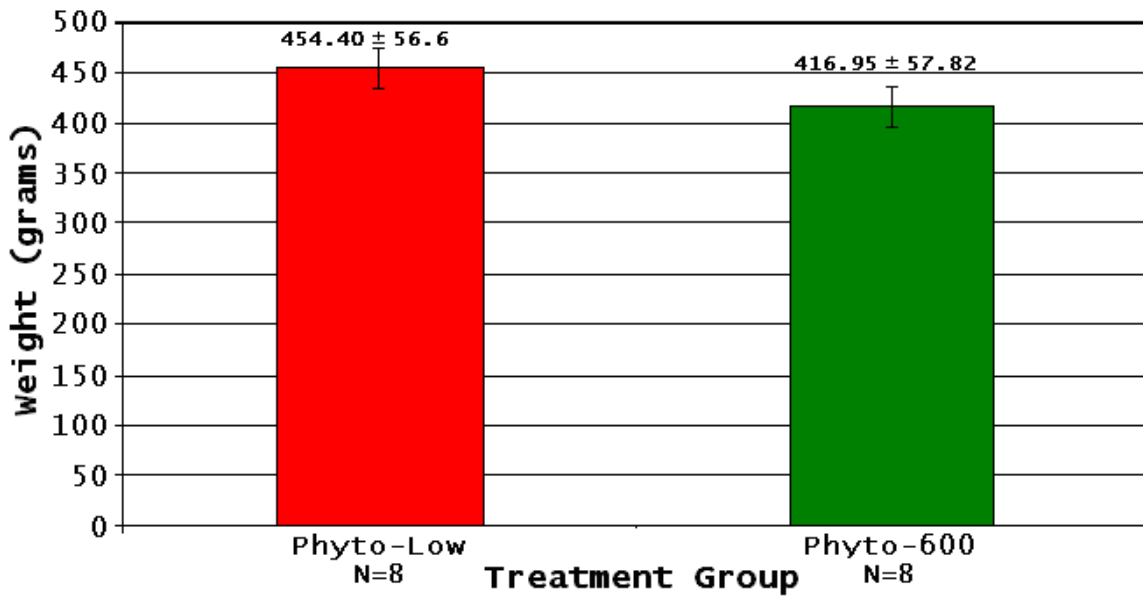
Figure 16A: Experiment 4 Ovariectomized Females 3rd Porsolt Forced Swim Test (MEAN ± SEM). Animals age 220 days and following 24 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
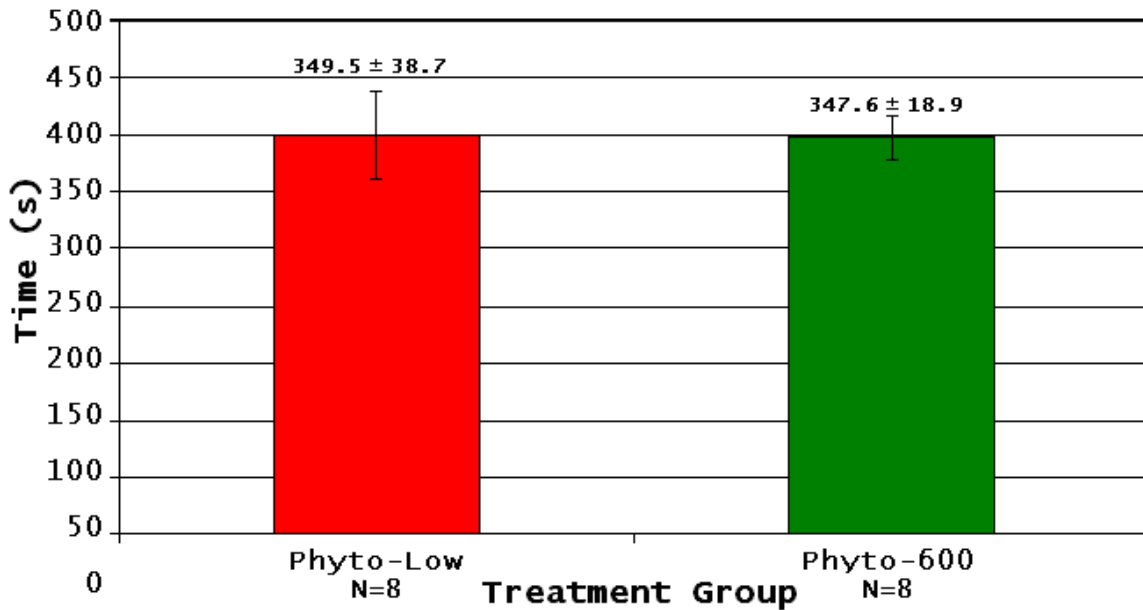
Figure 16B: Experiment 4 Ovariectomized Females 3rd Porsolt Forced Swim Test (MEAN ± SEM). Animals age 220 days and following 24 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
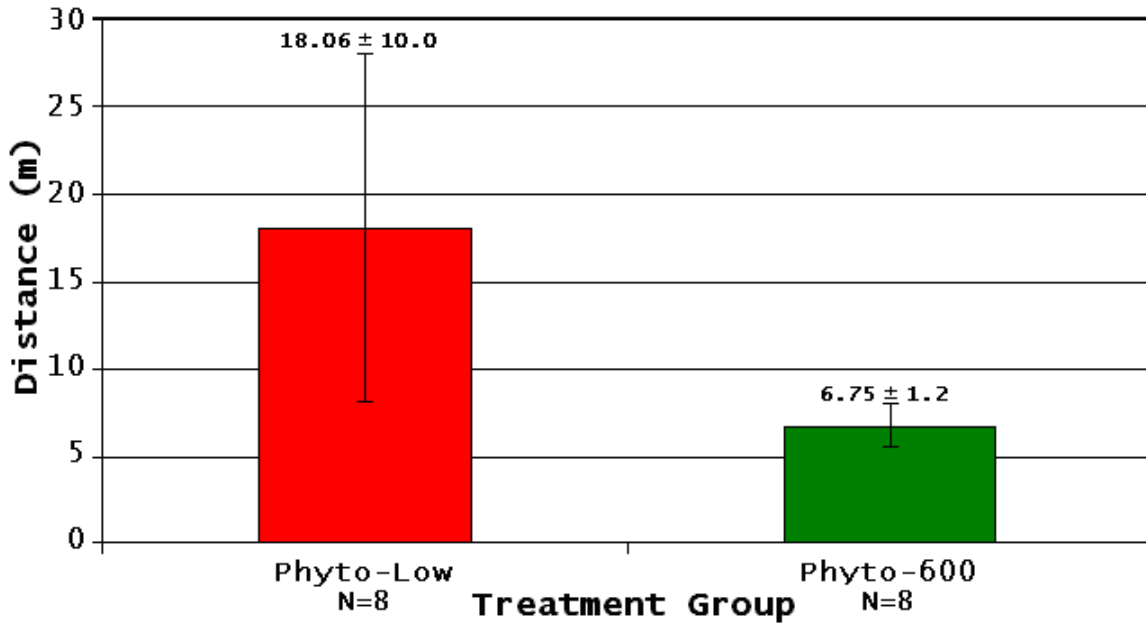
Figure 16C: Experiment 4 Ovariectomized Females 3rd Porsolt Forced Swim Test (MEAN ± SEM). Animals age 220 days and following 24 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
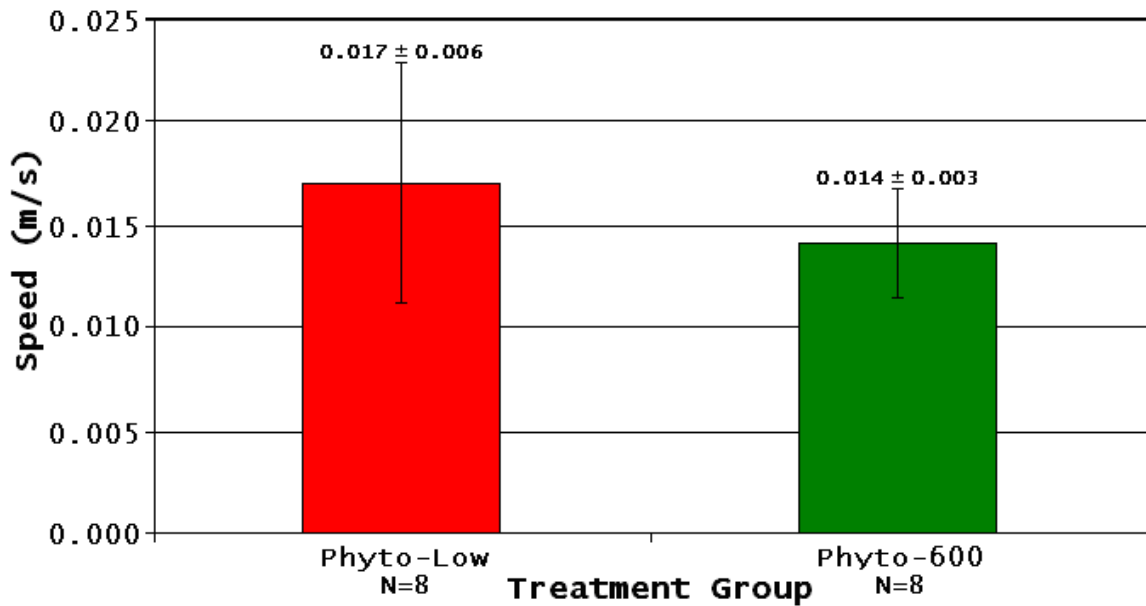
Figure 16D: Experiment 4 Ovariectomized Females 3rd Porsolt Forced Swim Test (MEAN ± SEM). Animals age 220 days and following 24 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
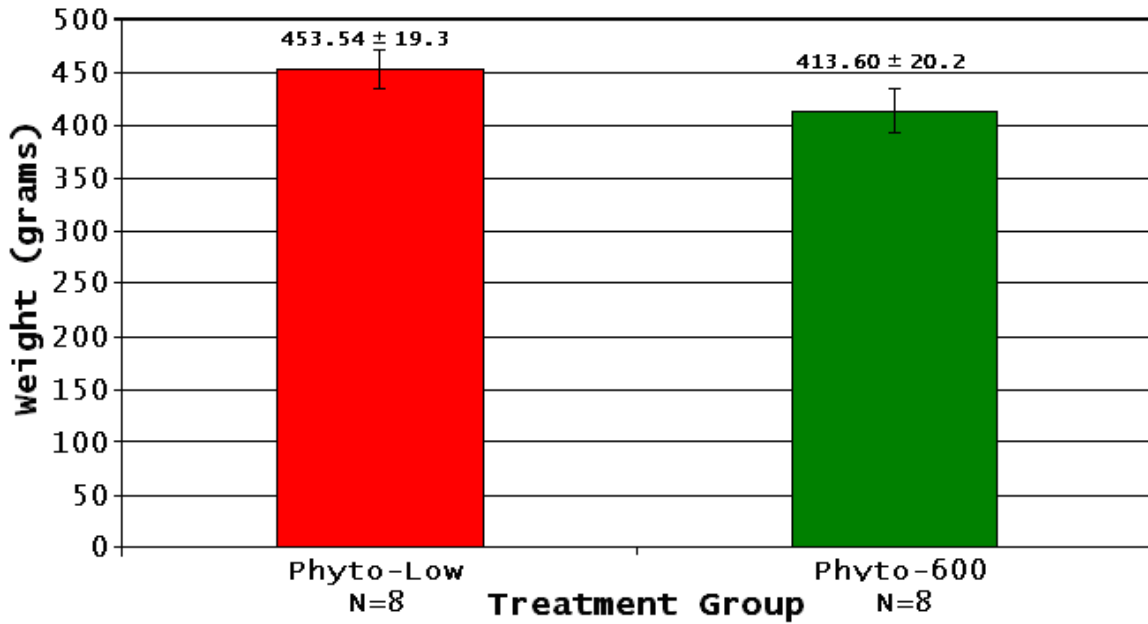
Figure 17A: Experiment 4 Ovariectomized Females Body Weight At the End of the Experiment (MEAN ± SEM). There were no significant differences between treatment groups.
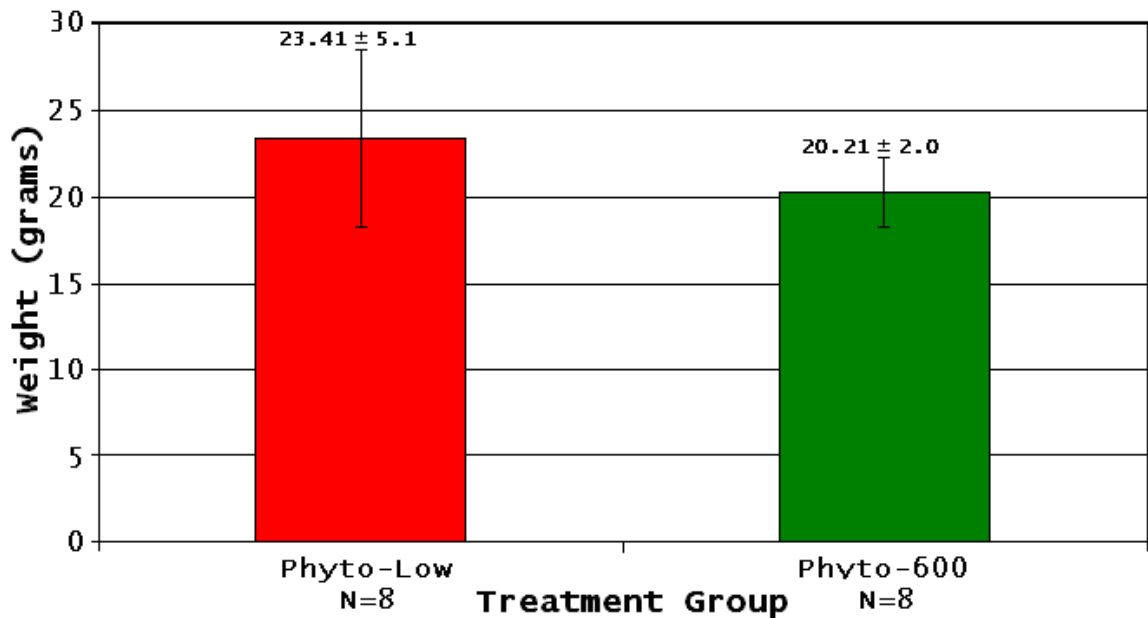
Figure 17B: Experiment 4 Ovariectomized Females White Adipose Tissue (MEAN ± SEM). There were no significant differences between treatment groups.
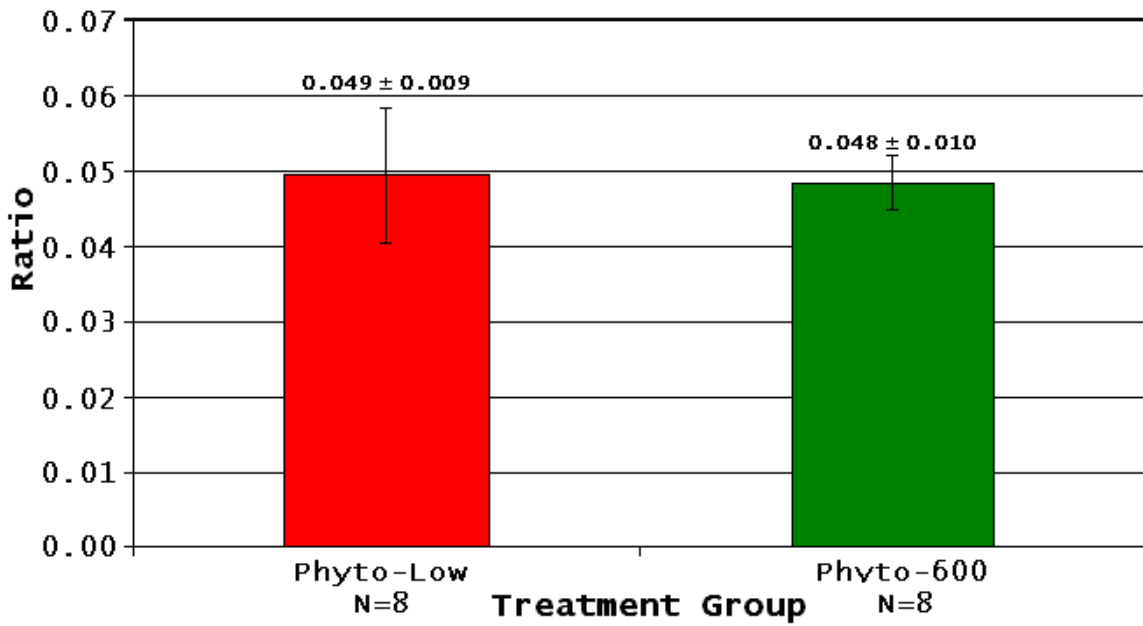
Figure 17C: Experiment 4 Ovariectomized Females White Adipose Tissue to Body Weight Ratio (MEAN ± SEM). There were no significant differences between treatment groups.
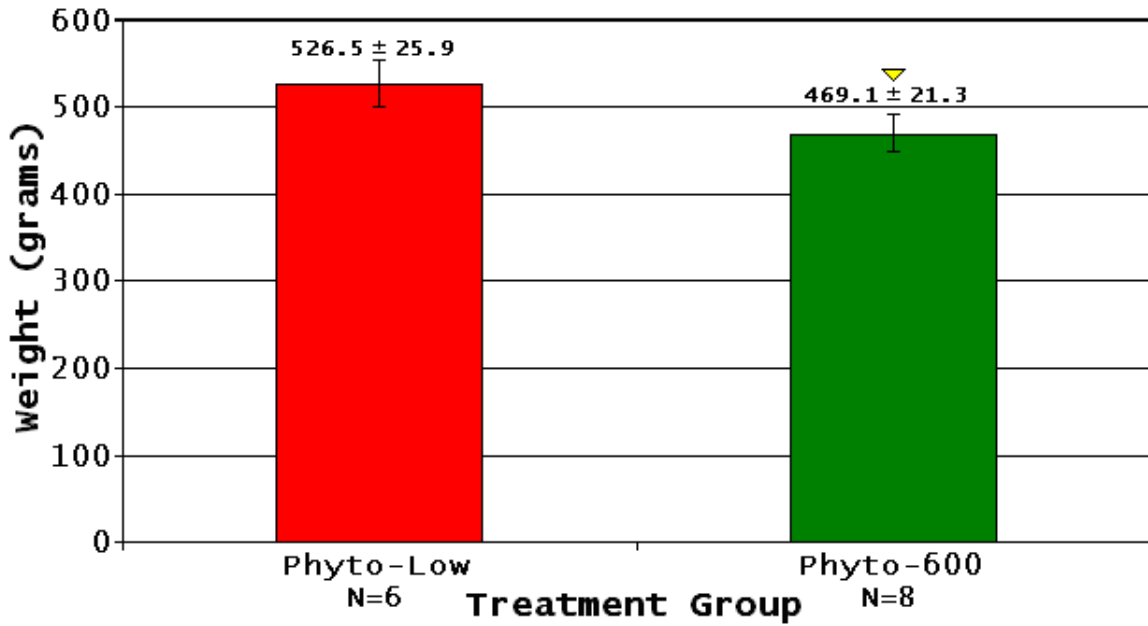
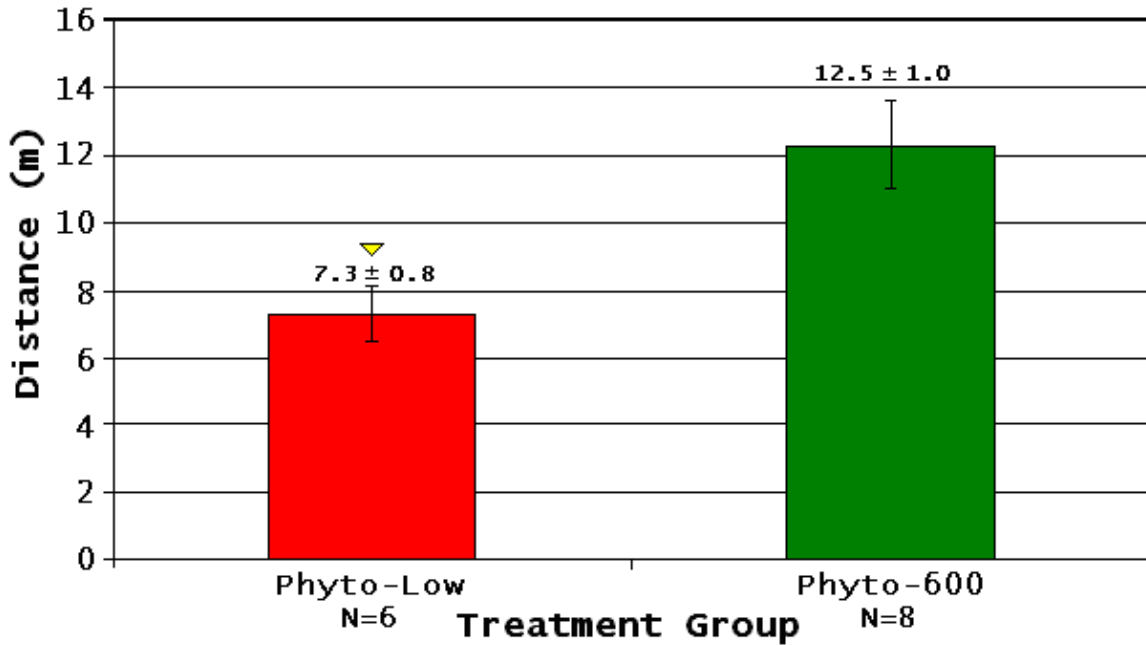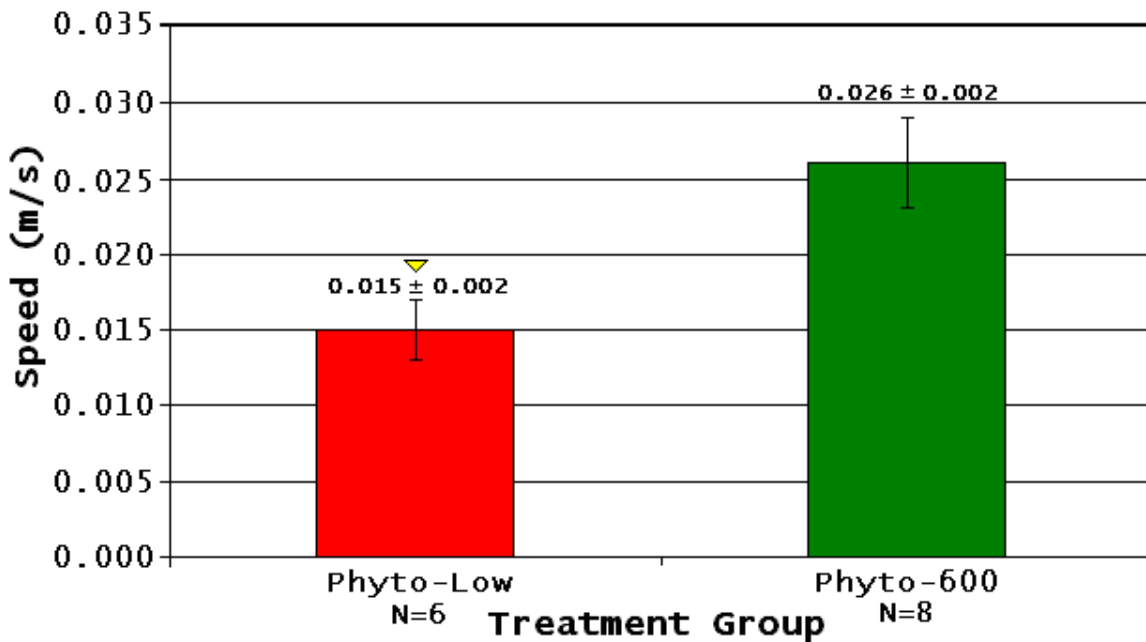**Figure 18A**: Experiment 5 Natural Ovarian Failure Females 1st Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Animals age 330 days. ▽ Phyto-600 animals weighed significantly less than Phyto-low animals.
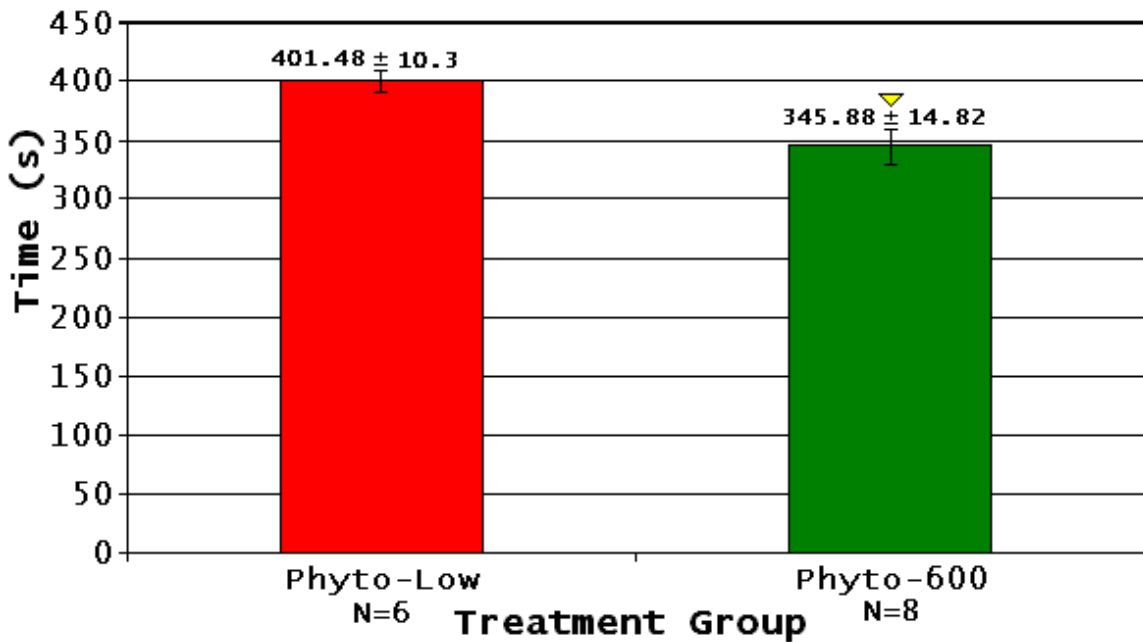
Figure 18B: Experiment 5 Natural Ovarian Failure Females 1st Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). Animals age 330 days. ▽ Phyto-600 animals spent significantly less time immobile than Phyto-low animals.

Figure 18C: Experiment 5 Natural Ovarian Failure Females 1st Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). Animals age 330 days. ▽ Phyto-low animals swam a significantly shorter distance than Phyto-600 animals.

Figure 18D: Experiment 5 Natural Ovarian Failure Females 1st Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). Animals age 330 days. ▽ Phyto-low animals swam significantly slower than Phyto-600 animals.
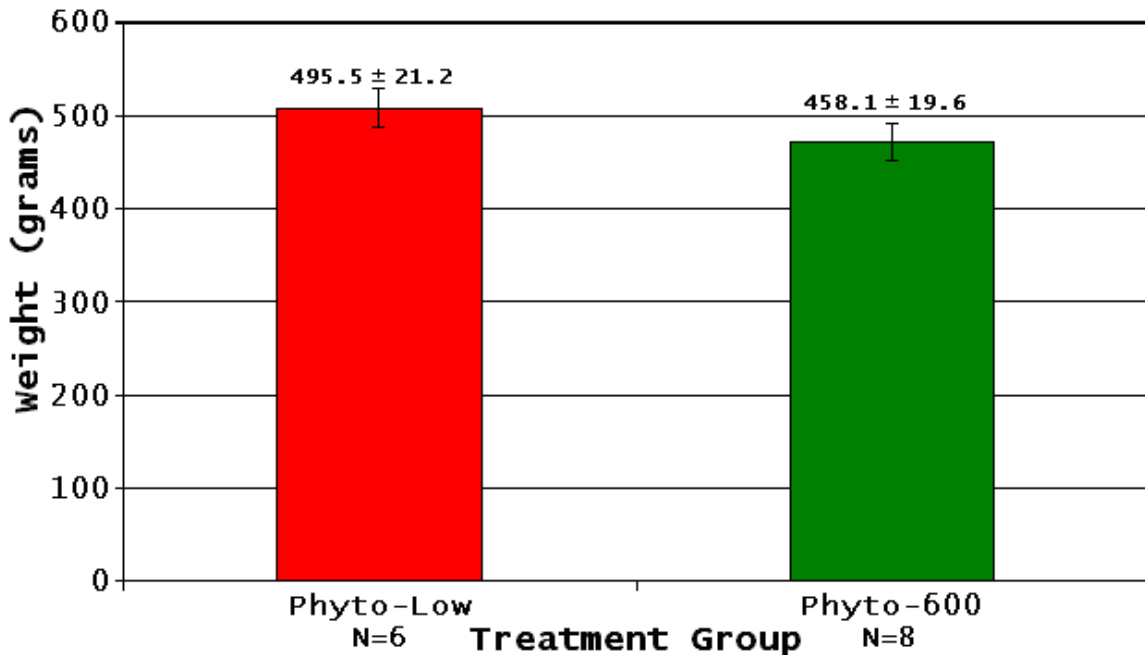
Figure 19A: Experiment 5 Natural Ovarian Failure Females 2nd Porsolt Forced Swim Test Body Weight (MEAN ± SEM). Animals age 360 days and following 7 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
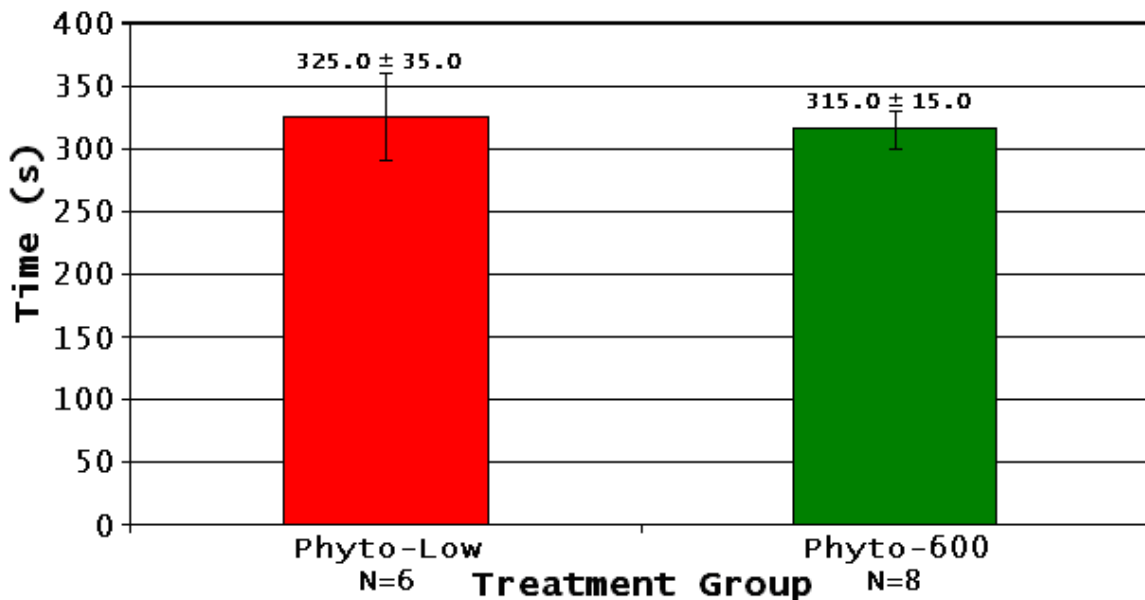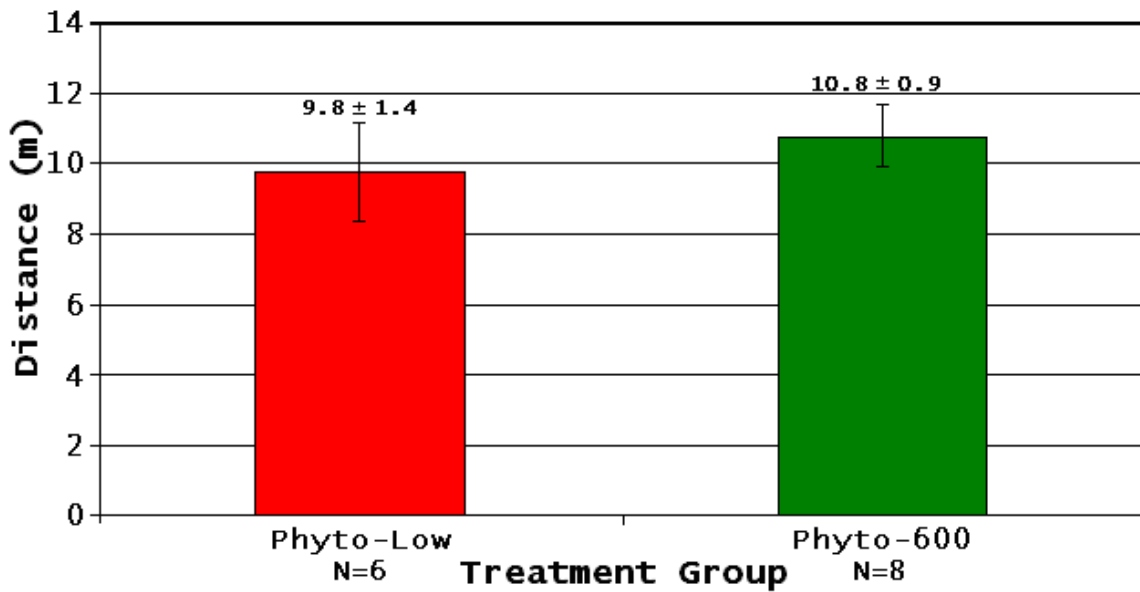

Figure 19B: Experiment 5 Natural Ovarian Failure Females 2nd Porsolt Forced Swim Test Time Immobile (MEAN ± SEM). Animals age 360 days and following 7 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
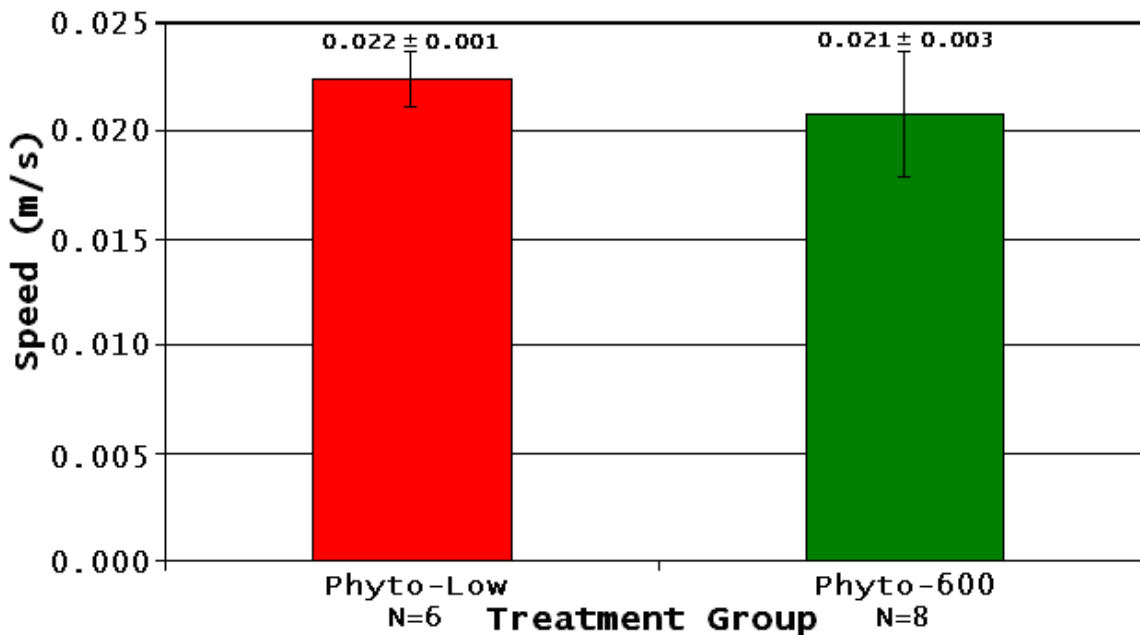
Figure 19C: Experiment 5 Natural Ovarian Failure Females 2nd Porsolt Forced Swim Test Total Distance Travelled (MEAN ± SEM). Animals age 360 days and following 7 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.

Figure 19D: Experiment 5 Natural Ovarian Failure Females 2nd Porsolt Forced Swim Test Overall Average Speed (MEAN ± SEM). Animals age 360 days and following 7 days of equol injections in the Phyto-low females. There were no significant differences between treatment groups.
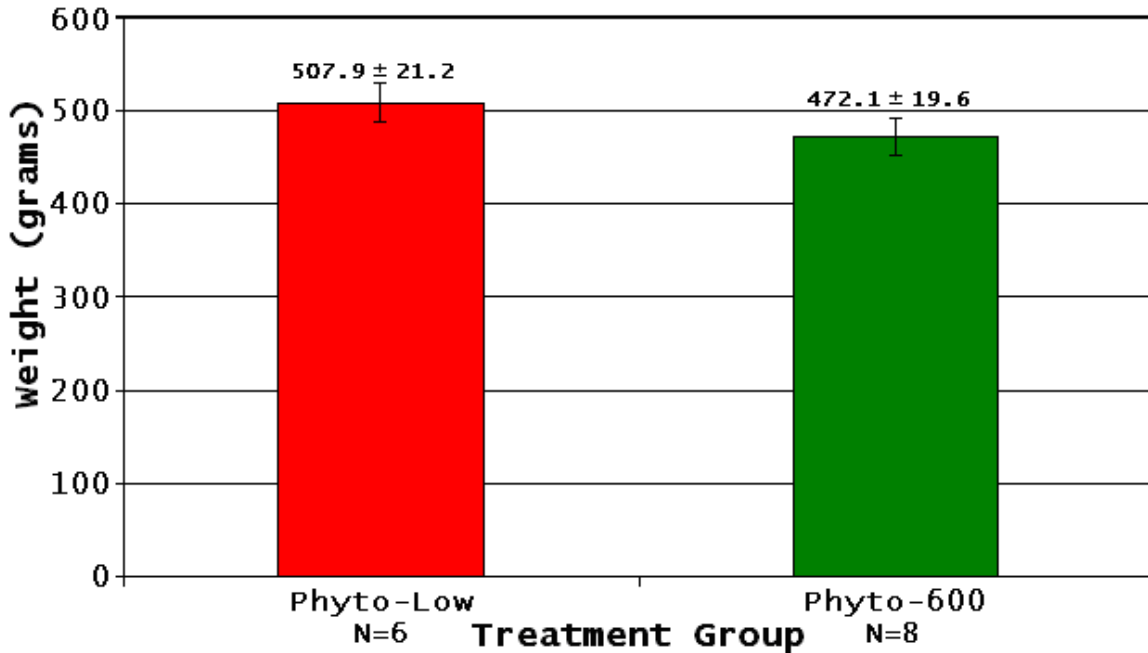
Figure 20A: Experiment 5 Natural Ovarian Failure Females At the End of the Experiment Body Weight (MEAN ± SEM).
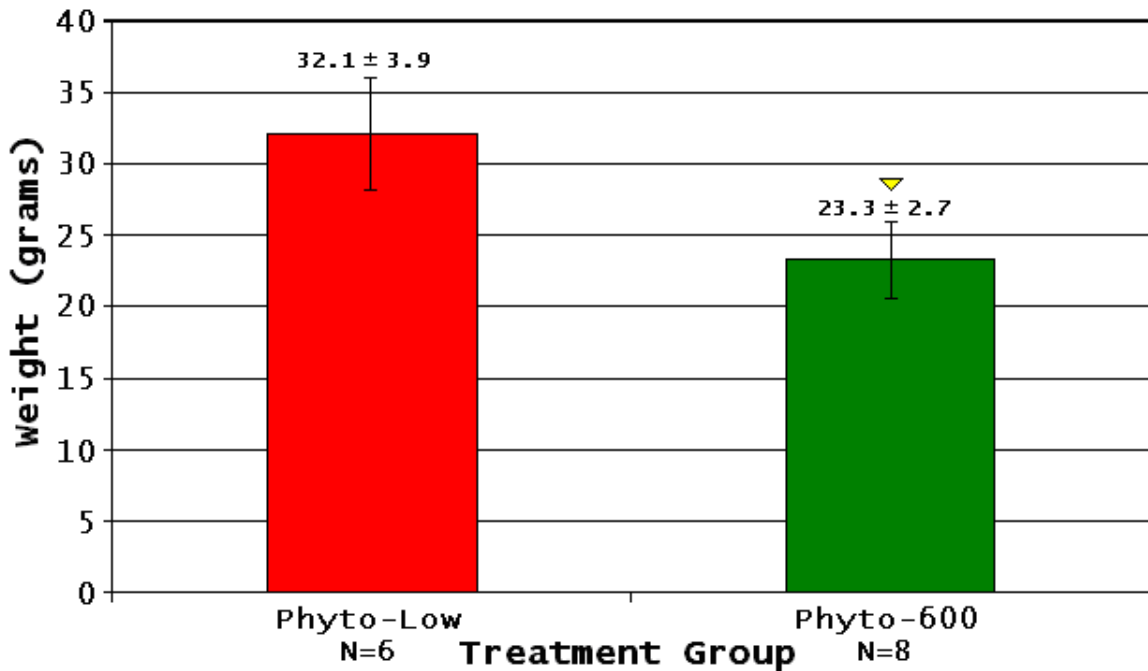

Figure 20B: Experiment 5 Natural Ovarian Failure Females White Adipose Tissue (MEAN ± SEM). ▽ Phyto-600 animals had significantly less white adipose tissue than Phyto-low animals.
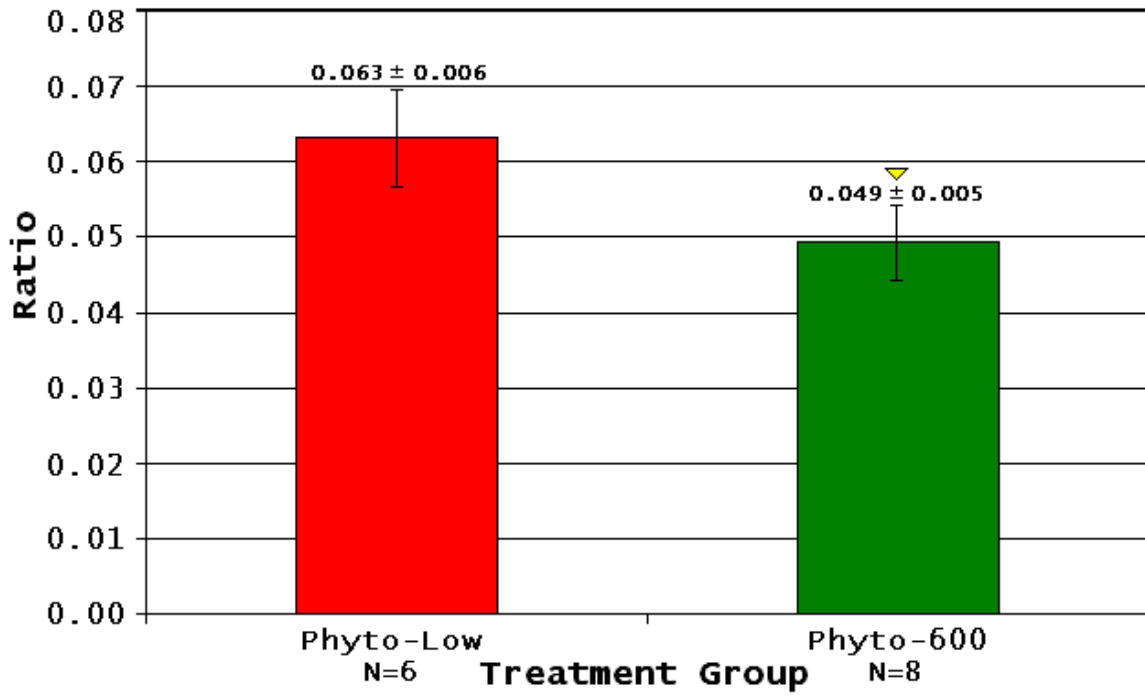
Figure 20C: Experiment 5 Natural Ovarian Failure Females White Adipose Tissue to Body Weight Ratio (MEAN ± SEM). ▽ Phyto-600 animals had a significantly lower white adipose tissue to body weight ratio than Phyto-low animals.
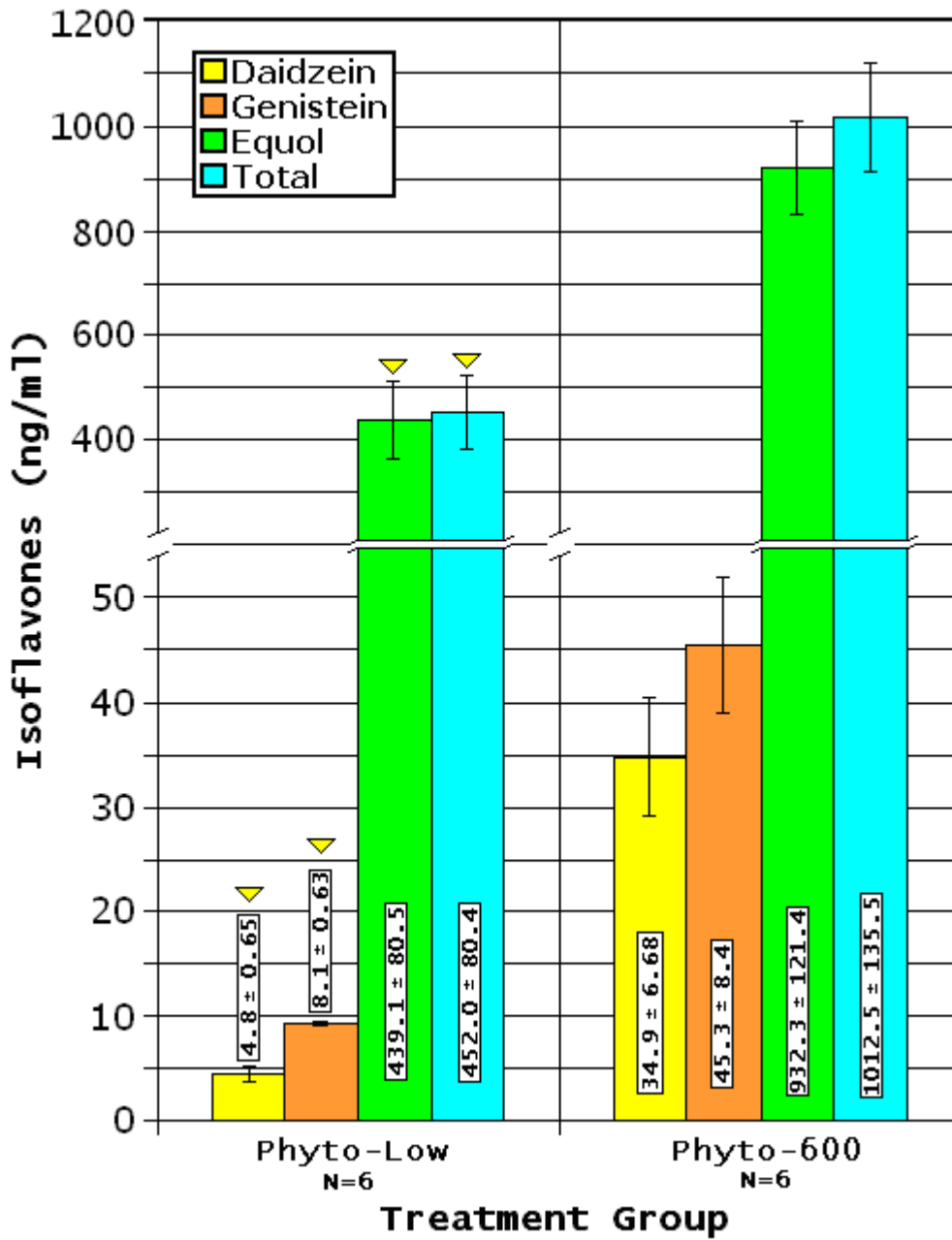
Figure 21A: Experiment 1 Serum Isoflavone Levels (ng/ml ± 1.0). ▽ Phyto-low values were significantly lower than Phyto-600 values.
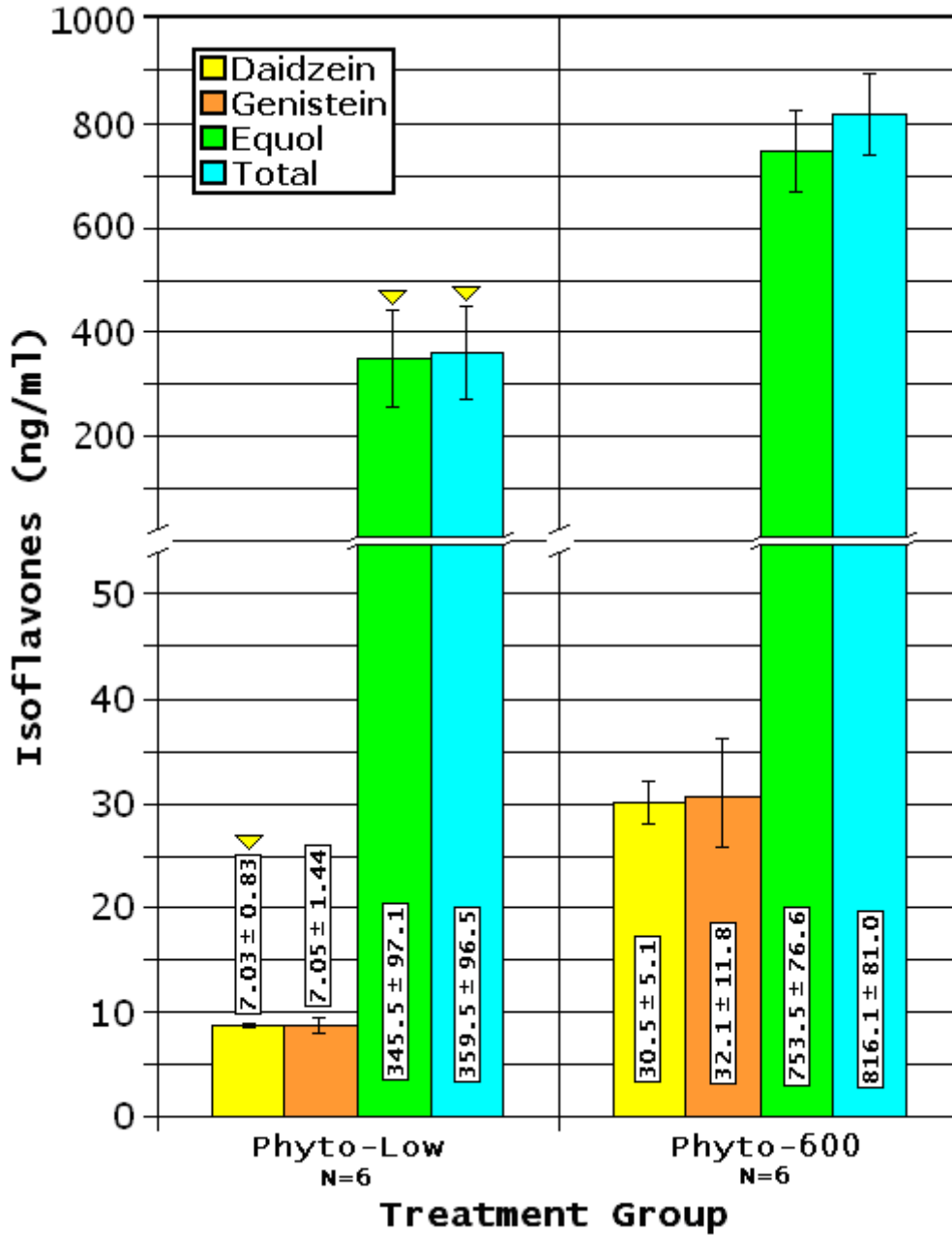
Figure 21B: Experiment 2 Serum Isoflavone Levels (ng/ml ± 1.0). ▽ Phyto-low values were significantly lower than Phyto-600 values.
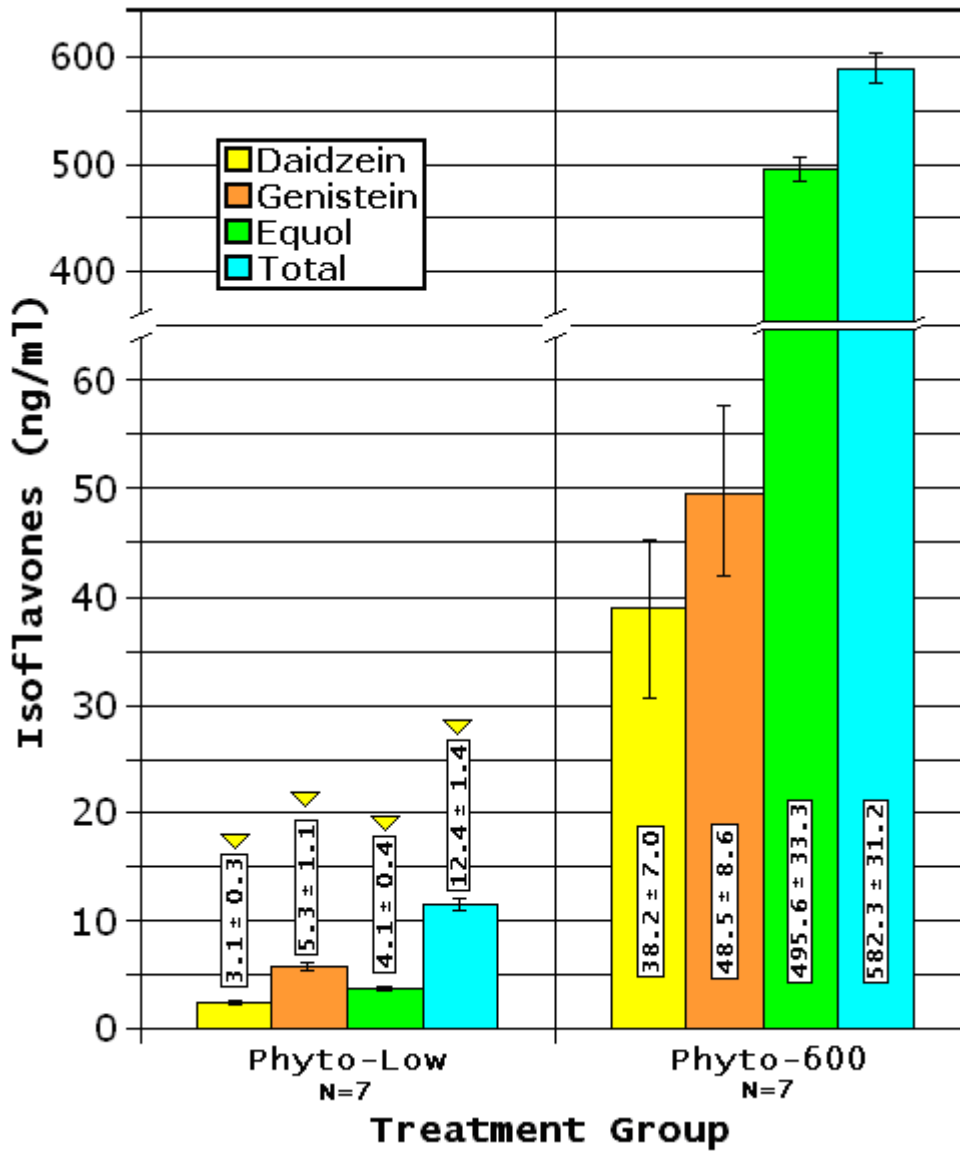
Figure 21C: Experiment 3 Serum Isoflavone Levels (ng/ml ± 1.0). ▽ Phyto-low values were significantly lower than Phyto-600 values.

Figure 21D: Experiment 4 Serum Isoflavone Levels (ng/ml ± 1.0). ▽ Phyto-low values were significantly lower than Phyto-600 values. ▽ Phyto-600 values were significantly lower than Phyto-low values.

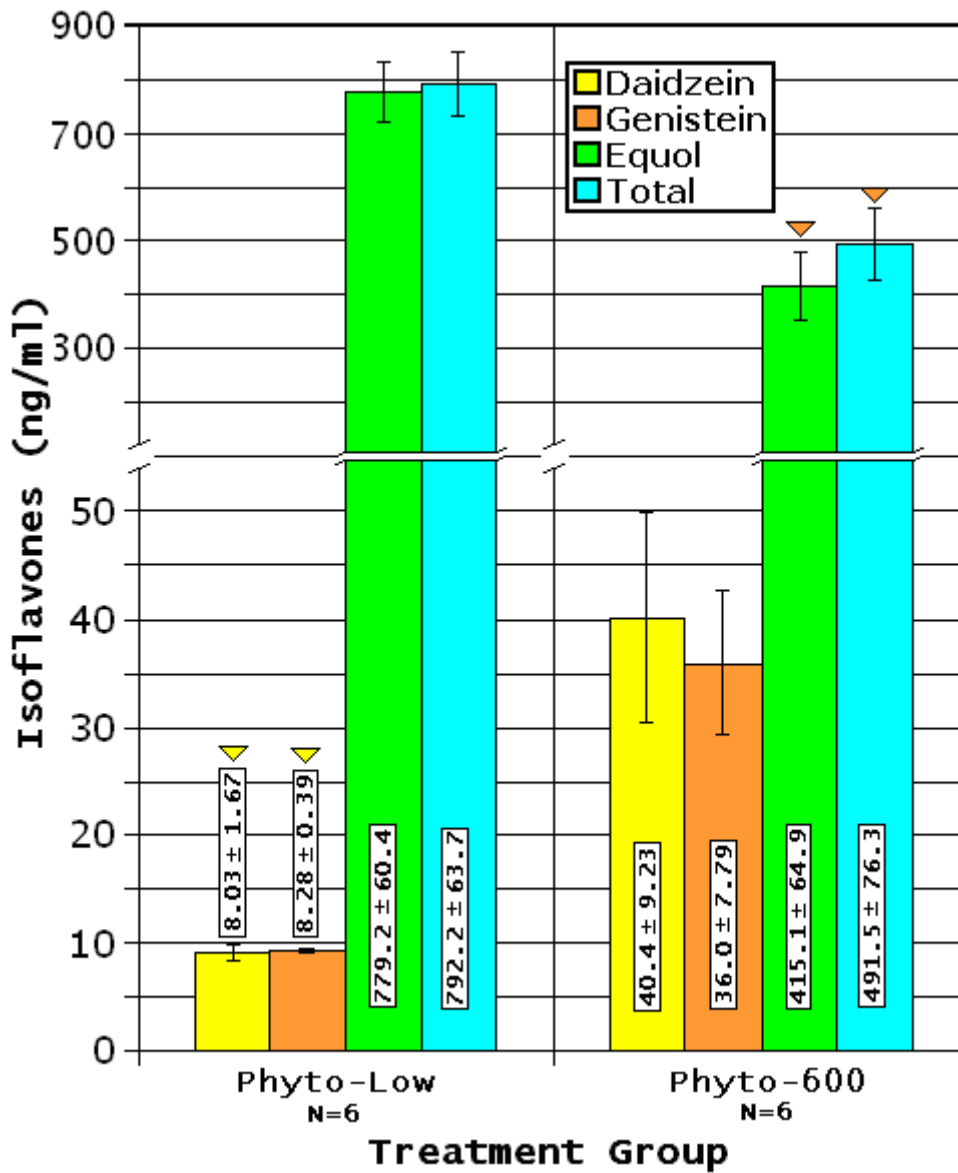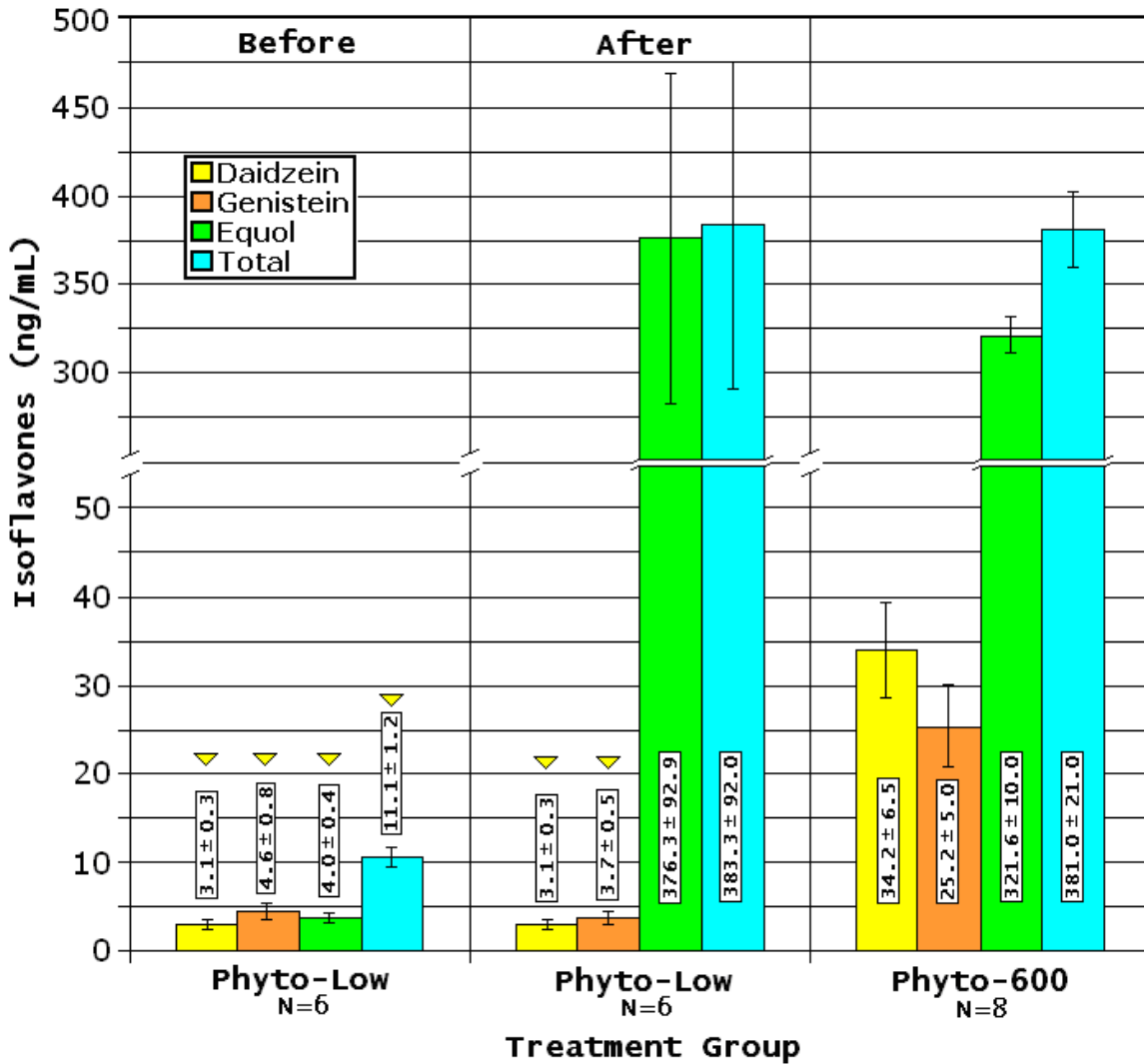**Figure 21E**: Experiment 5 Serum Isoflavone Levels (ng/ml ± 1.0). ▽ Phyto-low values were significantly lower than Phyto-600 values. **Before** refers to serum drawn prior to 1st Porsolt test, **After** refers to serum measured after 2nd Porsolt test.
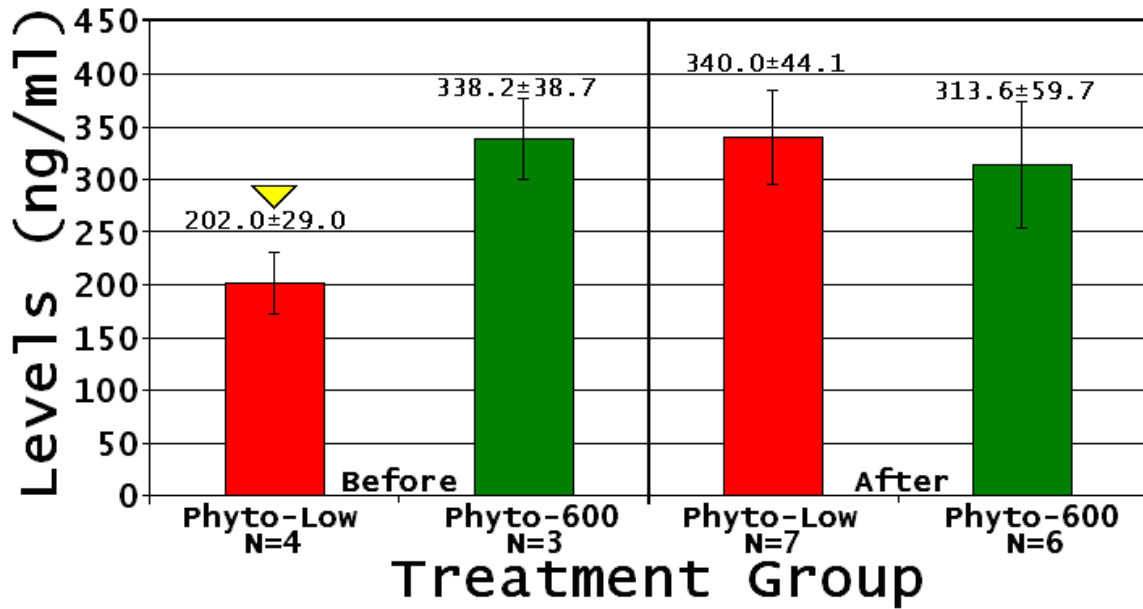
Figure 22: Experiment 5 Natural Ovarian Failure Serotonin Levels (ng/ml ± 1.0). ▽ Phyto-low **Before** values were significantly lower than Phyto-low After values. **Before** refers to serum drawn prior to 1st Porsolt test, **After** refers to serum measured after 2nd Porsolt test.
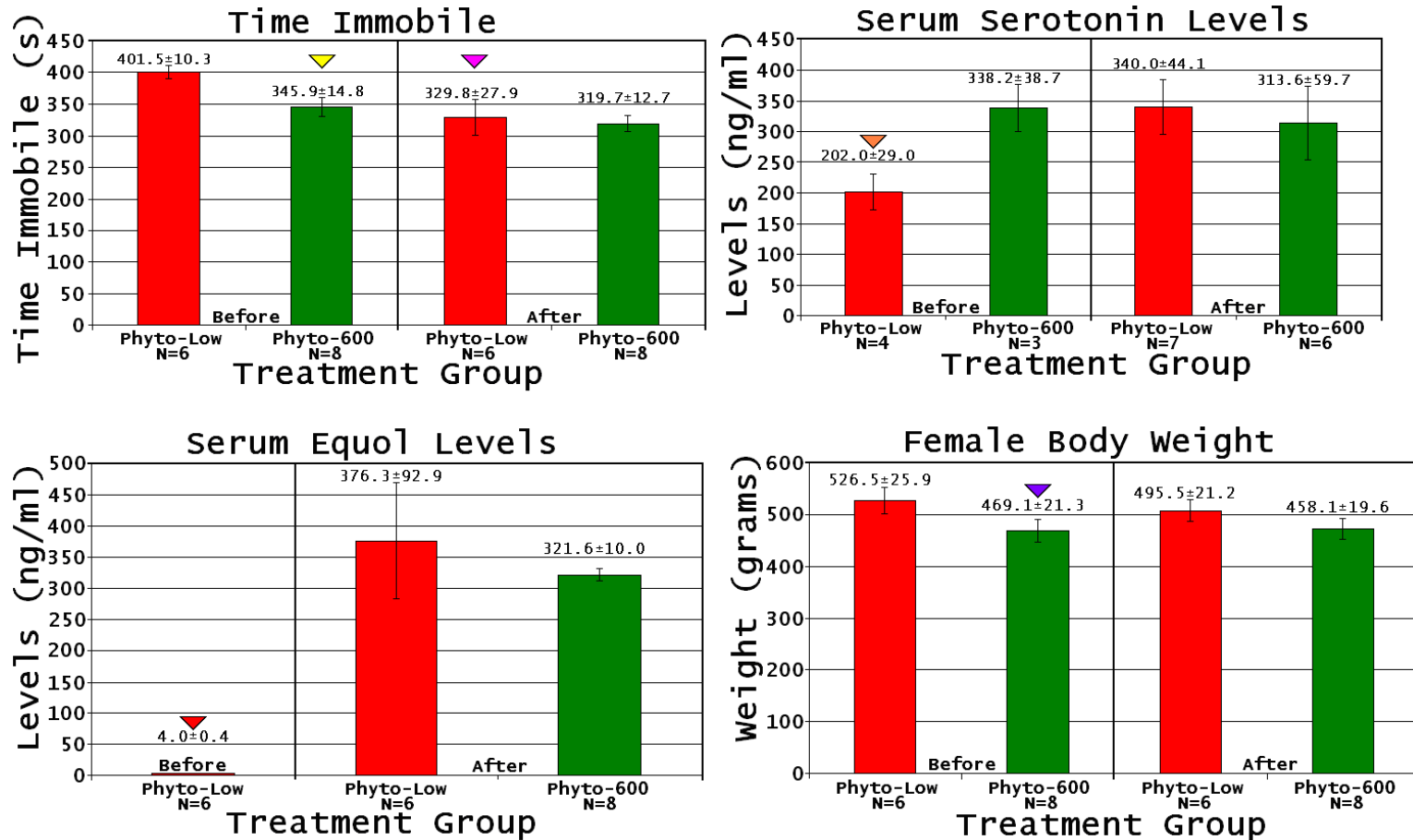
Figure 23: Experiment 5 Natural Ovarian Failure Females Summary Serotonin Values, Equol Values, Time Immobile and Body Weight. ▽ Phyto-600 animals spent significantly less time immobile than Phyto-low animals. ▽ Phyto-low **After** values were significantly lower than Phyto-low **Before** values., ▽ Phyto-low **After** values were significantly higher than Phyto-low **Before** values., ▽ Phyto-low **Before** values were significantly lower than Phyto-low **After** values., ▽ Phyto-600 animals weighed significantly less than Phyto-low animals, **Before** refers to serum drawn prior to 1st Porsolt test, **After** refers to serum measured after 2nd Porsolt test.

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Academic Training
 Chandler Gilbert Community College, Chandler, Arizona
 1999-2001, Associates of General Studies received May 2001

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 2001-2003, B.S. Zoology emphasis Human Biology received December 2003

 Brigham Young University, Provo, Utah
 2006-2008, M.S. in Physiology and Developmental Biology received April 2008

 Brigham Young University, Provo, Utah
 2008-Present, Ph.D. in Physiology and Developmental Biology, expected August 2010

Teaching and Research Experience
 Instructor, Brigham Young University
 Department of Physiology and Developmental Biology, 2010
 Taught first half of the semester, PDBIO205 Human Biology
 Guest instructor, PDBIO 305 Human Physiology

 Teaching Assistant, Brigham Young University
 Department of Physiology and Developmental Biology, 2006-2010
 Worked as a teaching assistant for PDBIO205 Human Biology, PDBIO305 Human
 Physiology, and PDBIO325 Tissue Biology.

 Research Assistant, Brigham Young University
 Department of Physiology and Developmental Biology, 2007-2010
 Performed experiments with various phytoestrogen treatments on Long-Evans rats with
 Professors Lephart and Porter

Employment
 2006-present Teaching assistant, Research assistant, Brigham Young University
 2006 Math/Science Tutor, Chandler-Gilbert Learning Center
 2004 Math/Science Tutor, Chandler-Gilbert Learning Center
 2000-2001 Math/Science Tutor, Chandler-Gilbert Learning Center

Activities
 2008-2010 Brigham Young University Graduate Student Society Council Member
 2004-2006 Served as a full-time missionary in the Taiwan, Taichung Mission
  Mandarin speaking for the Church of Jesus Christ of Latter-day Saints
 2001 Vice President of LDSSA at Chandler-Gilbert Community College
 2000 Council Member of LDSSA at Chandler-Gilbert Community College

Awards and Distinctions
 2009-2010 Graduate Research Fellowship Award
 2009 Research Presentation Award

2006-2010	Graduate Research Assistantship, Funded by Edwin D. Lephart, USDA Grant
2006-2010	Graduate Teaching Assistantship, Funded by Brigham Young University, Department of Physiology and Developmental Biology
2000-2001	Phi Theta Kappa member.

Articles and Abstracts

<u>Published Material</u>:

K. Fabick, **C. Blake**, J.P. Porter, K.D.R. Setchell, E.D. Lephart. "Positive Benefits of Consuming Soy-Derived Isoflavones on Body Weight Gain, Adipose Tissue Deposition and Preliminary Cardiovascular Parameters Examined in a Ovariectomized Rat Model." *Experimental Biology 2007*. 34 (2007): W 135.

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