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Mouse Model for Combined Oral Contraceptive Administration in Diet

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BACKGROUND: Combined oral contraceptives (COCs), containing synthetic estrogen and progestin, are widely used for birth control and managing various conditions. A wide variety of COC formulations are prescribed, yet formulation-specific effects on health remain poorly understood. This study investigated the impacts of two COC formulations––low-dose ethinyl estradiol (EE) with high androgenicity progestin, levonorgestrel (LNG) and high-dose EE and low androgenicity progestin, desogestrel (DSG)––on estrous cycling and anthropometrics in a mouse model.
METHODS: Eighteen female C57Bl/6J mice (12 weeks old) were randomized into three groups: low-dose EE + LNG (2mg/kg EE, 200 mg/kg DSG), high-dose EE + DSG (5mg/kg EE, 200 mg/kg DSG) and a control group. Hormones were administered via diet over 8 weeks. Body weight, food intake and estrous cycle stages were monitored, and serum hormone levels were measured at the time of euthanasia.
RESULTS: Both COC formulations disrupted estrous cycling, with the EE + LNG group spending more time in diestrus and the EE + DSG group in metestrus, compared to the controls. No significant differences in body weight change or serum progesterone levels were observed, though serum estradiol levels were lower in both experimental groups compared to the control group.
CONCLUSION: These findings contribute to the refinement of translational rodent models for COC research and provide insight into the differential effect of the composition of different COC's.

INTRODUCTION

Over 150 million women use oral contraceptives worldwide.[1] Combined oral contraceptives (COCs) contain a synthetic estrogen and a synthetic progesterone. They are commonly prescribed for birth control as well as dysmenorrhea, acne, polycystic ovary syndrome, and endometriosis. COC formulations vary in type, dose, estrogen-to-progesterone ratio, and preparation (monophasic or multiphasic). Different formulations have variable impacts on metabolic, cardiovascular, neurological, musculoskeletal, and reproductive health, yet their overall effects on health and function remain unclear.[2-9]

Ethinyl estradiol (EE) is the most common synthetic estrogen used in COC formulations. High doses of EE (>50µg) have been largely discontinued due to increased risk of myocardial infarction and venous thromboembolism.[10] Currently, doses of EE are between 20-50µg. Ultra-low doses (<20µg) of EE are sometimes used, although they result in higher bleeding disturbances.[11] Progestins are responsible for suppressing ovulation and are classified by level of androgenicity. Second generation progestins (Levonorgestrel, Norgestrel) have high androgenic activity, whereas third generation progestins (Desogestrel, Gestodene, Norgestimate) have relatively lower androgenic activity. Second generation COCs have a greater incidence of side effects like weight gain, acne and hirsutism, which can impact adherence, while third generation progestins present a greater risk of venous thromboembolism.[12-14] Research often focuses on

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COC users versus non-users, limiting insights into formulation-specific effects.

In sports medicine, there is a great deal of interest surrounding the increased risk of anterior cruciate ligament (ACL) injury in female athletes. Multiple factors likely influence this sex-disparity, including differences in endogenous and exogenous sex hormones, from the menstrual cycle and exposure to hormonal contraceptives, respectively.[15] While some studies suggest that oral contraceptives might play a protective role in ACL injury, exactly how and why exogenous hormones reduce risk of ligament injury remains unknown.[16-18] One reason for the lack of consensus might be that studies pool users of hormonal contraceptives together, and most have not looked for a differential effect of the various formulations.[18-20] However, if the hormonal contraceptive type and dose matter to musculoskeletal tissues and risk of injury, this information would be critical to our understanding of how to personalize hormonal contraceptive choice for female athletes.

As a next step to understand the differential effect of oral contraceptive exposure to risk of ligament injury in female athletes, we developed a mouse model with voluntary oral administration of COC's. Mouse models allow for controlled studies that can lead to an understanding of the mechanistic nuances of what has been reported clinically.

At first glance, the 4-day rodent estrous cycle seems quite different from the 24-to-35-day menstrual cycle in women. However, the rodent estrous cycle is commonly used to study the human menstrual cycle due to conserved reproductive functions and similar hormonal fluctuations. Proestrus and estrus, with hormonal peaks, are most similar to the ovulatory and luteal phases of the human menstrual cycle. One major hormonal difference is that estradiol only peaks once in the rodent cycle and all of the hormones peak in the same two phases (Figure 1). Further research utilizing mouse models to determine how COC formulations affect estrous cycling may inform clinical optimization and personalized treatment plans. This study aimed to assess differences to estrous cycling and anthropometrics in mice treated with (1) low levels of EE and levonorgestrel (LNG), (2) high levels of EE and desogestrel (DSG), or (3) no hormones (controls) in their diet. We hypothesized that mice exposed to both COC formulations would exhibit acyclic or suppressed estrus cycling in comparison to the control group.

METHODS

Ethical approval and animals

Animal protocols were approved by the Institutional Animal Care and Use Committee at [redacted for review]. Eighteen female C57Bl/6J mice (n=6 per group) were housed at thermoneutral conditions on a standard light cycle (12 hours light/12 hours dark) with enrichment nesting materials. Mice were 10 weeks old at acquisition. At 12 weeks, they were randomly assigned to the EE + DSG, EE + LNG, or control group (Figure 2). Animals were selected at random from acclimation housing and placed sequentially into control, EE + DSG, or EE + LNG groups until all animals were assigned. Experimental mice receiving EE + DSG or EE + LNG were housed individually due to animal facility requirements. Control animals were co-housed due to space constraints. Mice had *ad libitum* access to water and food throughout the experiment. We chose to use voluntary oral administration of COCs to increase ecological validity by mimicking clinical drug metabolism.[22] Standard chow from the institutional animal facility was available during a 5-day acclimation period, followed by an OpenStandard diet (15% kcal fat; Research Diets, New Brunswick, NJ, USA). The EE/LNG OpenStandard chow contained 2 mg/kg EE and 200 mg/kg LNG, and the EE/DSG OpenStandard chow contained 5 mg/kg EE and 200 mg/kg DSG.

Anthropometrics

Body weight was measured 4-5 times per week throughout the experiment. Food intake (g) was measured at the same time for 4 consecutive days in the last week of the experiment. Daily energy intake (g/day) was calculated as kcals based on energy density of the diet.

Estrous Cycle Staging

Vaginal cytology is an established method to determine estrous cycle stage.[23,24] Estrous cycle staging was performed 4-5 times per week throughout the experiment. The three-finger restraint technique was used to minimize stress. Vaginal lavage was performed by flushing 50µL of phosphate-buffered saline three times or until the solution was cloudy. Then, 10µL of the collected solution was plated on a glass slide and immediately viewed under a standard light microscope with a 10× objective lens. Proestrus is characterized by a predominance of nucleated epithelial cells, while estrus consists of mostly

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anucleated cornified epithelial cells. Metestrus has nucleated and anucleated epithelial cells as well as leukocytes. In diestrus, there is a predominance of leukocytes.[25]

For weeks 1-2 of the experiment, two evaluators (OR, AH) simultaneously viewed samples and reached consensus on the stage. For weeks 3-8, one evaluator (OR) viewed samples and identified estrous stages. In the case of any uncertainty, a second evaluator (AH, JC) was conferred until consensus was reached. In the case that a low cell volume was collected (<10 cells), the sample was excluded to maintain data integrity.

Euthanasia

Mice were sacrificed using standard protocols for humane euthanizing of adult mice. Carbon dioxide was administered at 3L/min for 3 minutes, as recommended by Research Animal Resources and Compliance, followed by cervicothoracic dislocation.

Serum Hormone Levels

Blood samples were collected via cardiac puncture directly following carbon dioxide euthanasia and prior to cervicothoracic dislocation. Blood samples were allowed to sit at room temperature for 20 minutes and then centrifuged at 1300 RCF for 15 minutes to isolate serum. Up to 200µl blood was collected, and up to 75µl serum was obtained. Serum samples were stored in a –80°C freezer until enzyme-linked immunoassays (ELISAs) were performed to measure serum progesterone (cat no. NBP2-60125-1, Novus Biologicals, Centennial, CO, USA) and estrogen (cat no. ab108667, Abcam Inc., Waltham, MA, USA) levels. ELISA assays were run according to manufacturers' instructions. Absorbance was measured on a microplate reader at 450nm for both progesterone and estrogen. Due to limited sample volumes, it was not feasible to run serum samples in duplicate.

Statistical Analysis

Continuous variables including weight change, serum level, and food intake were summarized with means and standard deviations. The number of stages by group was summarized using frequencies and percentages. Comparisons of continuous variables were analyzed using independent two-sample t-tests or Wilcoxon rank-sum tests if the data did not meet normality assumptions, where appropriate. To determine whether the frequency of each stage differed by group, chi-square test was used. Pair-wise comparisons between each stage were compared using chi-square test. P-values were reported with a Bonferroni adjustment to account for multiple comparisons. Statistical significance was set at $p < 0.05$.

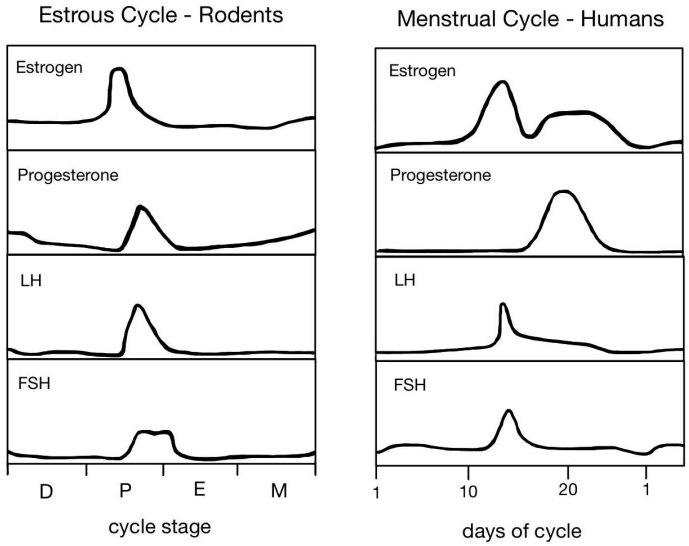

Figure 1. Comparison between the estrous cycle in rodents and the menstrual cycle in humans. Abbreviations – D: diestrus; E: estrus; FSH: follicle-stimulating hormone; LH: luteinizing hormone; M:

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metestrus; P: proestrus. Image was adapted from data in: Knobil E, Neill JD. The Physiology of Reproduction Vol. 2 (Raven, New York, 1994) [21].

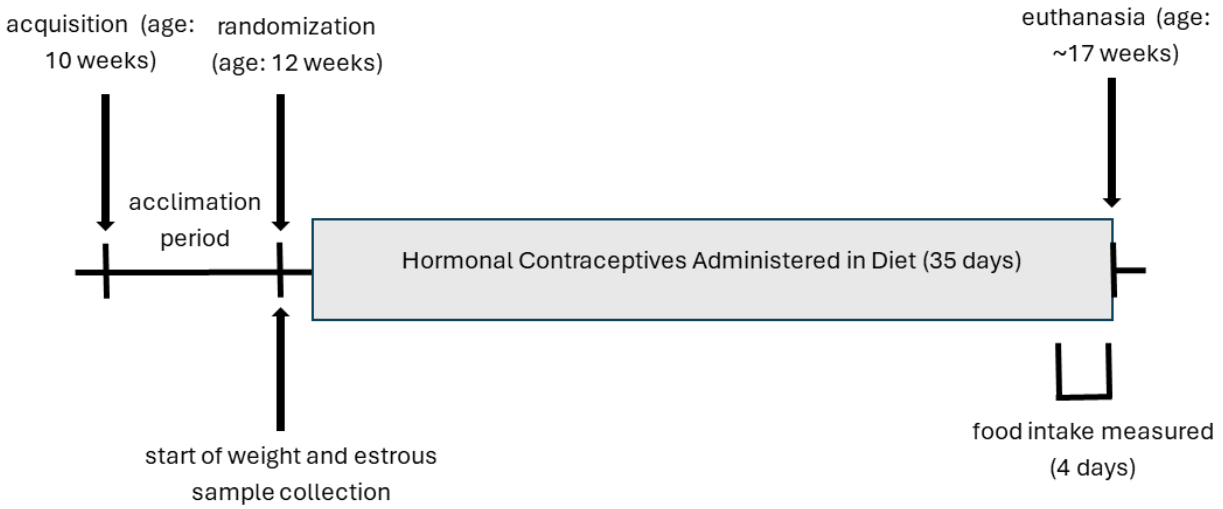

Figure 2. Experimental Timeline. 18 mice were acquired at age 10 weeks and underwent a 2-week acclimation period. Following the acclimation period, randomization into groups occurred. Starting at randomization, weight and estrous cycle samples were collected for 4-5 days/week for the entirety of the experiment. Baseline anthropometrics and estrous cycle samples were collected for 3 days following randomization and prior to starting in-diet contraceptives. Food intake was measured for mice in experimental groups for 4 days daily prior to euthanasia.

RESULTS

Anthropometrics

All mice gained body mass over the course of the experiment. The average changes in body mass for the control, EE + DSG, and EE + LNG groups were 2.05 ± 0.44g, 1.92 ± 0.68g and 1.35 ± 2.91g, respectively. There was no significant difference in body mass change between groups ($p = 0.455$). The EE + LNG group had an increasingly larger average mass than the EE + DSG group as the experiment progressed, though the difference was not significant. Complete body mass data is available in Figure 3.

Food Intake and Hormonal Doses

In the last week of the experiment, the EE + LNG group ate an average of 1.04 ± 0.20g of food per day, and the EE + DSG group ate an average of 1.02 ± 0.20g of food per day. Given the EE + LNG food contained 2mg/kg EE and 200mg/kg of LNG, the mice had an approximate daily dose of 2µg EE and 0.2mg LNG. The EE + DSG food contained 5mg/kg EE and 200mg/kg DSG, so the EE + DSG mice had an approximate daily dose of 5µg EE and 0.2mg DSG.

Two mice in the EE + DSG group initially lost body mass following experimental diet implementation. On days 8 and 11 of the experiment, the two mice were supplemented with one pellet of normal chow. Both mice showed immediate preference for the normal chow compared to the experimental chow, though they continued to eat the experimental chow throughout the experiment and regained the lost weight.

Estrous Cycle Staging

Representative images of each stage are shown in Figure 4A. The average frequencies of estrous cycle stages per group, including intermediate stages (i.e. metestrus/ diestrus), are shown in Figure 4B. Overall, the control group spent more time in proestrus and estrus than both experimental groups (Proestrus: control = 37 days, EE + LNG = 13 days, EE + DSG = 11 days; Estrus: control = 32 days, EE + LNG = 13, EE + DSG = 8). The experimental

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groups spent more time in diestrus than the control group (Diestrus: EE + LNG = 113 days, EE + DSG = 84 days, control = 57 days). Interestingly, the EE + DSG group had a higher frequency of days in metestrus than the EE + LNG group (Metestrus: EE + DSG = 98 days, EE + LNG = 58 days). The total frequency of stages was significantly different across groups ($p < 0.001$).

Serum Hormone Levels

There were no significant differences in serum hormone levels among the three groups. The average serum progesterone levels for the control, low EE + LNG and high EE + DSG were 0.30 ± 0.10ng/mL, 0.35 ± 0.22ng/mL and 0.22 ± 0.05ng/mL, respectively ($p=0.24$). The average serum estradiol levels for the control, low EE + LNG and high EE + DSG were 26.7 ± 10.1pg/mL, 14.2 ± 11.2pg/mL and 14.7 ± 2.8pg/mL, respectively ($p=0.089$).

Behavioral Observation

While it was not a primary outcome of this study, behavioral differences between experimental groups were qualitatively observed. When restrained, the low EE + LNG group frequently tried to escape restraint and more readily squeaked. In comparison, the high EE + DSG group often froze during restraint and rarely squeaked.

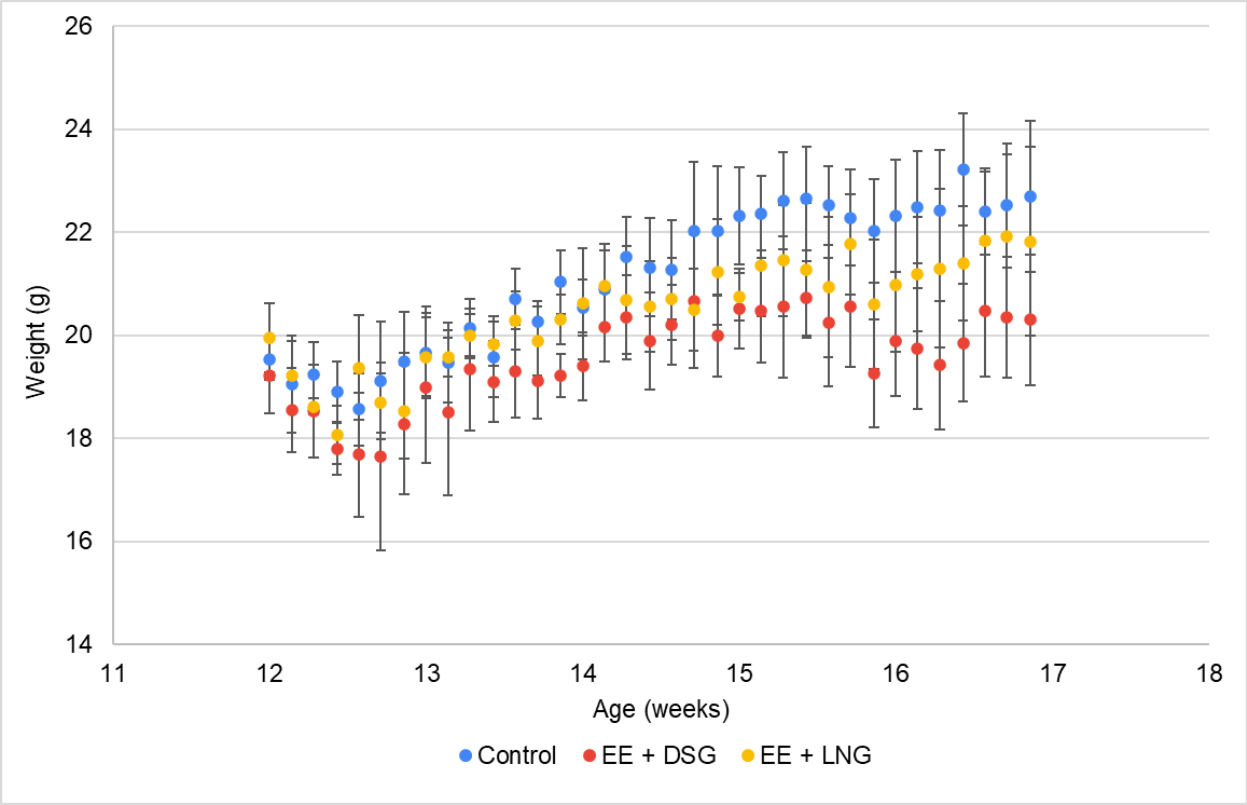

Figure 3. Average weight by hormonal group. Abbreviations – DSG: desogestrel; EE: ethinyl estradiol; LNG: levonorgestrel. N=6 per group.

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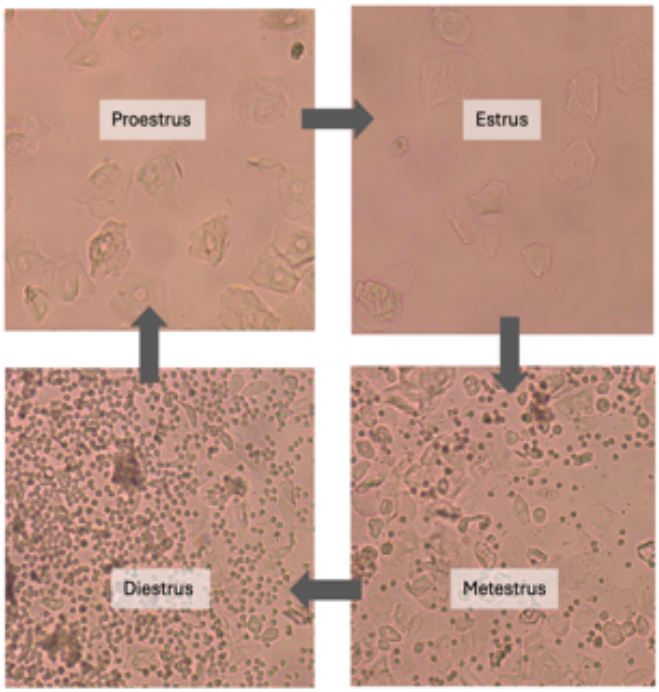
A



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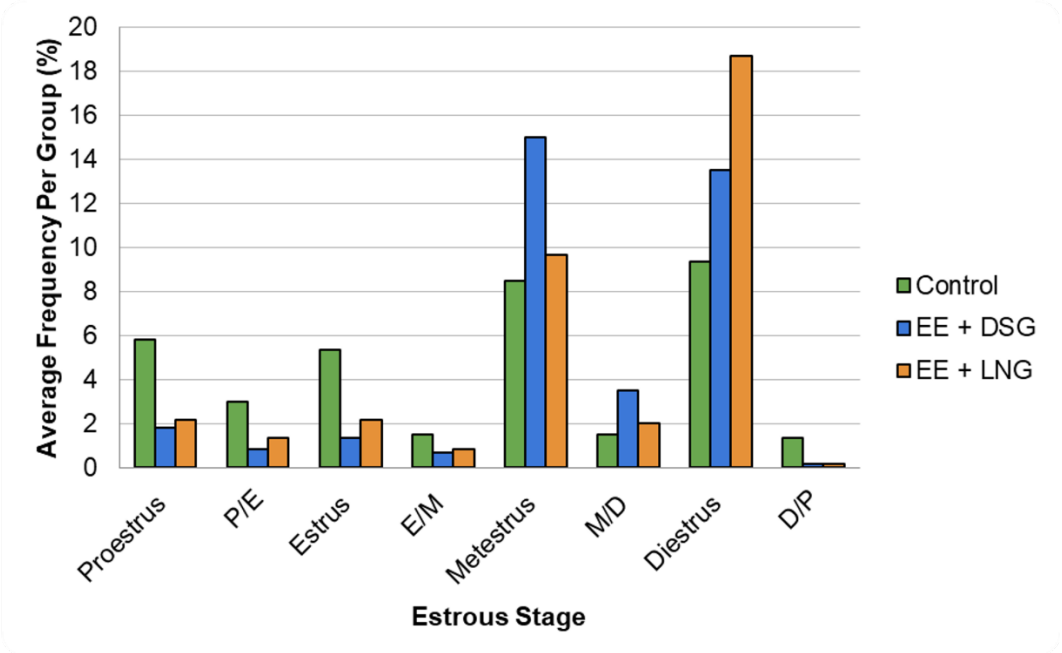

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C	P	P/E	E	E/M	M	M/D	D	D/P
Control 1	7, 0.2 [0.07, 0.33]	0 [0, 0]	2, 0.06 [-0.02, 0.13]	3, 0.09 [-0.01, 0.18]	7, 0.2 [0.07, 0.33]	2, 0.05 [-0.02, 0.13]	12, 0.34 [0.19, 0.5]	2, 0.05 [-0.02, 0.13]
Control 2	6, 0.17 [0.05, 0.29]	6, 0.17 [0.05, 0.29]	3, 0.08 [-0.01, 0.17]	2, 0.06 [-0.02, 0.13]	7, 0.2 [0.07, 0.32]	1, 0.03 [-0.03, 0.08]	10, 0.28 [0.13, 0.42]	1, 0.03 [-0.03, 0.08]
Control 3	4, 0.11 [0.01, 0.21]	4, 0.11 [0.01, 0.21]	8, 0.22 [0.09, 0.36]	1, 0.03 [-0.03, 0.08]	8, 0.22 [0.09, 0.36]	1, 0.03 [-0.03, 0.08]	9, 0.25 [0.11, 0.39]	1, 0.03 [-0.03, 0.08]
Control 4	4, 0.11 [0.01, 0.21]	2, 0.05 [-0.02, 0.13]	10, 0.27 [0.13, 0.41]	1, 0.03 [-0.03, 0.08]	8, 0.22 [0.09, 0.35]	1, 0.03 [-0.03, 0.08]	9, 0.24 [0.11, 0.38]	2, 0.05 [-0.02, 0.13]
Control 5	6, 0.16 [0.04, 0.28]	3, 0.08 [-0.01, 0.17]	6, 0.16 [0.04, 0.28]	2, 0.05 [-0.02, 0.13]	12, 0.32 [0.17, 0.48]	2, 0.05 [-0.02, 0.13]	6, 0.16 [0.04, 0.28]	0 [0, 0]
Control 6	8, 0.22 [0.08, 0.35]	3, 0.08 [-0.01, 0.17]	3, 0.08 [-0.01, 0.17]	0 [0, 0]	9, 0.24 [0.11, 0.38]	2, 0.05 [-0.02, 0.13]	10, 0.27 [0.13, 0.41]	2, 0.05 [-0.02, 0.13]
EE/DSG 1	2, 0.05 [-0.02, 0.13]	1, 0.03 [-0.03, 0.08]	2, 0.05 [-0.02, 0.13]	0 [0, 0]	13, 0.35 [0.2, 0.51]	2, 0.05 [-0.02, 0.13]	16, 0.43 [0.27, 0.59]	1, 0.03 [-0.03, 0.08]
EE/DSG 2	3, 0.08 [-0.01, 0.17]	1, 0.03 [-0.03, 0.08]	0 [0, 0]	1, 0.03 [-0.03, 0.08]	15, 0.39 [0.24, 0.55]	6, 0.16 [0.04, 0.27]	12, 0.32 [0.17, 0.46]	0 [0, 0]
EE/DSG 3	3, 0.08 [-0.01, 0.17]	1, 0.03 [-0.03, 0.08]	3, 0.08 [-0.01, 0.17]	2, 0.05 [-0.02, 0.12]	14, 0.39 [0.22, 0.52]	1, 0.03 [-0.03, 0.08]	14, 0.37 [0.22, 0.52]	0 [0, 0]
EE/DSG 4	1, 0.03 [-0.03, 0.08]	1, 0.03 [-0.03, 0.08]	1, 0.03 [-0.03, 0.08]	0 [0, 0]	23, 0.64 [0.28, 0.8]	2, 0.06 [-0.02, 0.13]	8, 0.22 [0.09, 0.36]	0 [0, 0]
EE/DSG 5	2, 0.06 [-0.02, 0.13]	1, 0.03 [-0.03, 0.08]	0 [0, 0]	0 [0, 0]	9, 0.26 [0.11, 0.4]	8, 0.23 [0.09, 0.37]	15, 0.43 [0.27, 0.59]	0 [0, 0]
EE/DSG 6	0 [0,0]	0 [0, 0]	2, 0.05 [-0.02, 0.13]	1, 0.03 [-0.03, 0.08]	16, 0.43 [0.27, 0.59]	2, 0.06 [-0.02, 0.13]	16, 0.43 [0.27, 0.59]	0 [0, 0]
EE/LNG 1	1, 0.03 [-0.03, 0.08]	4, 0.11 [0.01, 0.2]	5, 0.13 [0.02, 0.24]	0 [0, 0]	16, 0.42 [0.26, 0.58]	1, 0.03 [-0.03, 0.08]	10, 0.26 [0.12, 0.40]	1, 0.03 [-0.03, 0.08]
EE/LNG 2	2, 0.05 [-0.02, 0.13]	2, 0.05 [-0.02, 0.13]	2, 0.05 [-0.02, 0.13]	2, 0.05 [-0.02, 0.13]	10, 0.27 [0.13, 0.41]	5, 0.14 [0.03, 0.25]	14, 0.38 [0.22, 0.54]	0 [0, 0]

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EE/LNG 3	2, 0.05 [-0.02, 0.12]	1, 0.03 [-0.03, 0.08]	0 [0, 0]	1, 0.03 [-0.03, 0.08]	12, 0.32 [0.17, 0.46]	2, 0.05 [-0.02, 0.12]	20, 0.53 [0.37, 0.69]	0 [0, 0]
EE/LNG 4	2, 0.06 [-0.02, 0.14]	1, 0.03 [-0.03, 0.09]	3, 0.09 [-0.01, 0.19]	0 [0, 0]	9, 0.26 [0.12, 0.41]	0 [0, 0]	19, 0.56 [0.39, 0.73]	0 [0, 0]
EE/LNG 5	3, 0.08 [-0.01, 0.17]	0 [0, 0]	0 [0, 0]	1, 0.03 [-0.03, 0.08]	6, 0.16 [0.04, 0.28]	2, 0.05 [-0.02, 0.13]	25, 0.68 [0.53, 0.83]	0 [0, 0]
EE/LNG 6	3, 0.08 [-0.01, 0.17]	0 [0, 0]	3 [-0.01, 0.17]	1, 0.03 [-0.03, 0.08]	5, 0.13 [0.02, 0.24]	2, 0.05 [-0.02, 0.12]	24, 0.63 [0.48, 0.79]	0 [0, 0]

Figure 4. Estrous cycle stages. (A) Representative images of each stage of the estrous cycle. (B) Average frequency of stages by hormonal group. Abbreviations – D/P: diestrus/proestrus; E/M: estrus/metestrus; M/D: metestrus/diestrus; P/E: proestrus/estrus. (C) Count, Frequency (Count/Total Count Per Mouse) [95% Confidence Interval] of days spent in estrous cycle stages for all animals. Abbreviations – EE/DSG #: P: Proestrus; P/E: Proestrus/Estrus; E: Estrus; E/M: Estrus/Metestrus; M: Metestrus; M/D: Metestrus/Diestrus; D: Diestrus; D/P: Diestrus/Proestrus. N=6 per group.

DISCUSSION

This study evaluated the effects of dietary COCs on the estrous cycle. Specifically, we assessed differential impacts of a highly estrogenic, lowly androgenic formulation (EE + DSG) and a lowly estrogenic, highly androgenic formulation (EE + LNG) on the estrous cycle when administered in diet. Both formulations disrupted normal estrous cycling, although phase changes occurred in both groups. Notably, the EE + LNG group spent more time in diestrus compared to the EE + DSG group, while the EE + DSG group spent more time in metestrus compared to the EE + LNG group. The time spent in proestrus and estrus did not differ between experimental groups.

Previous research has shown that COCs interrupt estrous cycling in rodents, yet optimal doses for translational research remain unclear. Using 4µg EE and 550µg of norgestrel in diet, Fuller et al. found mice arrested in diestrus.[26] Comparatively, Schuh et al. treated mice with 0.01875µg EE/20g mouse and 0.75µg LNG/20g through oral gavage and found suppressed, irregular cycles with less time spent in proestrus and estrus compared to untreated controls.[27] This study also found suppressed, irregular cycles and less time in estrus with applied treatment of 2µg EE and 200µg LNG or 5µg EE and 200µg DSG compared to controls. Further research is needed to assess ovulation suppression at various doses to best approximate minimum effective dose in various metabolic profiles.

Women taking combination monophasic oral contraceptives experience fluctuations in endogenous and exogenous ethinyl estradiol and progestin throughout the pill cycle.[19] If oral contraceptives play a protective role in ACL injury as some studies suggest, determining translationally relevant doses by COC formulation in rodents may provide valuable insight into how exogenous hormone combinations differentially impact musculoskeletal tissue in women. This data could be critical in understanding secondary effects of COCs and inform personalized contraceptive choice for female athletes. This study adds to the body of literature by identifying phase-specific disruptions caused by different hormonal formulations which may inform translationally relevant dosages across formulations. It also emphasizes the importance of analyzing COC data by progestin type and dose.

Limitations of this study include a lack of generalizability due to small numbers (n=6 per group), an inability to track exact food intake and

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hormone doses, lack of blinding for estrous cycle staging, and the individual housing of experimental mice due to facility requirements for hazardous substances. Our findings highlight the strengths and limitations of using an in-diet hormone delivery model. Compared to oral gavage, an in-diet approach allowed for voluntary administration, mimicking clinical COC use and first-pass metabolism, while reducing daily workload for research personnel. However, we observed two EE + DSG mice preferentially eat normal chow over the supplemented diet, suggesting the need to optimize diet palatability. Moreover, more precise methods to measure food intake would improve estimates of daily hormonal consumption. Finally, ELISA assays are reported to be less reliable than mass spectrometry for quantification of sex hormones.

Future research is needed to elucidate the biological mechanisms through which EE and progestin interact, as well as their combined impacts on growth, estrous cycling and behavior. Additional research on effects of synthetic and endogenous estrogen may also contribute to a greater understanding of differential impacts of various COC formulations and doses to further inform and refine translational relevance of rodent models.

CONCLUSION

These findings contribute to the refinement of translational rodent models for COC research and provide insight into the differential effect of the composition of different COC's.

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Conflict of Interest Statement

EKC received support for the present manuscript from The 2018 Scott Nadler PASSOR Musculoskeletal Research Grant from the Foundation for Physical Medicine and Rehabilitation

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