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TITLE:

Myths and Methodologies: Reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases

Sims S. T.[1], and Heather A.K.[2]

[1]Adams Centre for High Performance, Faculty of Health, Sport and Human Performance, University of Waikato, New Zealand

[2] Department of Physiology, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand

Correspondence to:

Stacy T. Sims, Adams Centre for High Performance, University of Waikato, 52 Miro Street, Mount Maunganui, New Zealand 3116.

E-mail: stacy.sims@waikato.ac.nz

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ABSTRACT

In recent years, the increase in scientific literature exploring sex differences has been beneficial to both the clinician and allied health science professional, although female athletes are still significantly under-represented in sport and exercise science research.

Women have faced exclusion throughout history though the complexities of sociocultural marginalisation and biomedical disinterest in women's health. These complexities have contributed to challenges of studying women and examining sex differences. One underlying complexity to methodological design may be hormonal perturbations of the menstrual cycle. The biphasic responses of estrogen and progesterone across the menstrual cycle significantly influence physiological responses, which contribute to exercise capacity and adaptation in women. Moreover, oral contraceptives (OC) add complexity through the introduction of varying concentrations of circulating exogenous estrogen and progesterone, which may moderate physiological adaptations to exercise differently than endogenous ovarian hormones. Thus, applied sport and exercise science research focusing on women remains limited, in part, by poor methodological design that does not define reproductive status. By highlighting specific differences between phases with regards to hormone perturbations and the systems that are affected, methodological inconsistencies can be reduced, thereby improving scientific design that will enable focused research of female athletes in sports science and evaluation of sex difference in exercise responses. The aims of this review are to highlight the differences between endogenous and exogenous hormone profiles across a standard 28 – 32 day menstrual cycle, with the goal to improve methodological design for studies exploring sex differences, menstrual cycle phase differences, and/or endogenous versus exogenous female sex hormones.

INTRODUCTION

Sport was traditionally created by men and for men (Hargreaves, 1994) but women are now participating, and exercising, in record numbers (Hulteen et al., 2017). Pushing cultural and physical barriers, they are training hard, achieving personal bests, and enjoying the everyday physical and social pleasures of a wide range of movement cultures. As the global popularity of sport and activity for women has increased, the need for understanding exercise physiology in the female athlete has become essential. However, this body of research has, and still does, lag behind that of men (Costello, Bieuzen, & Bleakley, 2014). Until the 1980's, it was widely assumed that the physiological responses to exercise did not truly differ between men and women. Thus, most sports science recommendations have been generalized to women- without really questioning if this direct transfer was valid. One consideration for the under-representation in sport and exercise science research are the complexities associated with the menstrual cycle. Often cited as being "too difficult" to study, due to the biphasic responses of estrogen and progesterone across the menstrual cycle, women are often excluded, or are included without consideration of ovarian hormone influences on exercise capacity and adaptations (Johnson, Greaves, & Repta, 2009). These cyclic hormone changes can affect physical and psychological potentials and ultimately influence sports performance, although effects are highly individual. Moreover, oral contraceptives (OC) add complexity through the introduction of exogenous estrogen and progesterone which leads to different circulating concentrations of the two hormones relative to endogenous hormones and, therefore, may moderate physiological adaptations to exercise differently than endogenous ovarian hormones. Research designs that effectively ignore female hormone complexity continue to contribute to the significant gap in our understanding of how to control for the sex

hormone perturbations across the natural and oral contraceptive-controlled menstrual cycles when studying women athletes.

Despite the complexity of hormone fluctuations in women, and the increasing evidence for sex hormone influence on women's athletic performance across the menstrual cycle, it is common practice for data of both women and men to be combined to increase the total "n" in sports science studies. If sex differences are acknowledged, women are often tested in the low hormone phase of the cycle (follicular phase or hormone-free week of oral contraceptive (OC) users), where it is commonly assumed that women have a physiological profile most similar to a male. These practices perpetuate assumptions that physiological responses of women are the same as men or the differences are too small for significance. In addition, women who are naturally cycling are often grouped with women on OC regimes with no plan to quantify circulating hormonal differences, or match for phases.

Why is this an issue?

Understanding the physiological effects of sex hormones on other systems of the body is critical for sound scientific design. Perturbations of sex hormones across the menstrual cycle affect body water regulation (Sims, Rehrer, Bell, & Cotter, 2007, 2008; Stachenfeld, Keefe, & Palter, 2001) exercise capacity, metabolism, thermoregulatory thresholds, cognition, and sleep (Baker & Driver; Meendering, Torgrimson, Houghton, Halliwill, & Minson, 2005; Minson, Halliwill, Young, & Joyner, 2000; Tanja Oosthuyse & Andrew N. Bosch, 2010; Sharkey, Crawford, Kim, & Joffe; Sims et al., 2008; Stephenson, 1985). What often is neglected is OC use suppresses endogenous estradiol and progesterone synthesis so the

perturbations across the menstrual cycle are significantly more subtle, and exogenous OC replaces endogenous hormones with much reduced circulating levels of estrogens and progestins (Elliott-Sale et al., 2013). Furthermore, given the different half-lives of the exogenous steroids and variable impact on the endogenous hormones, the withdrawal week of OC regimes should be considered a transient hormonal profile phase (Creinin, 2002).

General effects of endogenous estrogen and progesterone

The ovaries serve the dual function of maturing and releasing eggs and synthesizing hormones. The primary sex hormones synthesized by the ovaries are estrogens *(17-β estradiol* and *estrone)* and progesterone, which act together to orchestrate the cyclic ovarian function known as the menstrual cycle. A typical menstrual cycle lasts 28-32 days (Figure 1) and consists of a follicular phase (~12-14 days, low levels of estrogens and progesterone), ovulation (~1 day, preceded by an estrogen surge) and a luteal phase (~12-14 days, high levels of estrogens and progesterone). Estrogens and progesterone can have different target organs, and normally promote different physiologic end points. However, for some actions they can act to enhance or antagonise each other's actions (Stricker et al., 2006). Estrogen secretion usually precedes progesterone secretion and primes the target tissues to respond to progesterone. Estrogens induce the synthesis of progesterone receptors, which are needed for progesterone to mediate a physiologic effect. However, progesterone accelerates the turnover of estrogen receptors in some tissues and thereby decreases responses to estrogens (Stachenfeld et al., 1999; Stachenfeld & Keefe, 2002; Stachenfeld et al., 2001; Stachenfeld, Silva, & Keefe, 2000; Stachenfeld & Taylor, 2005).

(Insert Table 1 here).

There is little doubt that estrogens and progesterone can affect female physiology. For example, estrogens affect body composition by increasing fat mass (Ziomkiewicz, Ellison, Lipson, Thune, & Jasienska, 2008), promoting water retention (Stachenfeld et al., 2001), collagen and insulin-like growth factor-1 (IGF-1) synthesis (Ho & Weissberger, 1992). Estrogens augment muscle glycogen storage capacity while also increasing free fatty acid availability and the use of oxidative pathways to use fatty acids as a fuel source (Hackney, 1990; Nicklas, Hackney, & Sharp, 1989). Ultimately, this leads to decreased carbohydrate use, or glycogen sparing (Tanja Oosthuyse & Andrew N. Bosch, 2010; T. Oosthuyse & A. N. Bosch, 2010; Oosthuyse & Bosch, 2012).The increased oxidative capacity decreases the dependence on anaerobic pathways for ATP production, and so high estrogen levels associate with lower blood lactate levels and time to exhaustion (T. Oosthuyse & A. N. Bosch, 2010). High estrogen levels also associate with enhanced gluconeogenesis, and the enhanced uptake of glucose into type I muscles (Campbell & Febbraio, 2002).

Progesterone also influences many physiological parameters. Progesterone increases resting heart rate (Sedlak, Shufelt, Iribarren, & Merz, 2012), basal body temperature, and ventilation (Charkoudian, Stephens, Pirkle, Kosiba, & Johnson, 1999). Although researchers have indicated that estradiol decreases body temperature by enhancing the sudo- and vaso-motor responses and heat loss, progesterone can act to increase core temperature by inducing an upward shift in the thermoregulatory set-point (Charkoudian & Johnson, 2000; Kolka & Stephenson, 1997; Sawka et al., 1989). Therefore, it is likely that the luteal phase thermoregulatory alterations are due primarily to the effect of progesterone. This increase in basal body temperature is reported to increase the subjective feeling of higher exertion or strain, decreasing athletic performance, especially in hot and/or humid environments (Janse,

Thompson, Chuter, Silk, & Thom, 2012). The effect of progesterone on augmented respiratory drive may promote maximal exercise capacity at high altitudes. Beyond these physiological effects of progesterone, what is critical to understand about progesterone is its ability to antagonise estrogenic effects (Campbell & Febbraio, 2001, 2002). For example, the enhanced carbohydrate metabolism promoted by estradiol, can be inhibited by high progesterone levels (D'Eon et al., 2002). Moreover, progesterone promotes protein catabolism, which, in turn, reducers the stimulus for muscle protein synthesis (Lamont, Lemon, & Bruot, 1987).

Hormonal Contraceptives

Hormonal contraceptives are the group of exogenous steroid hormones which suppress the secretion of gonadotropins through negative feedback inhibition. Specifically, the mechanism of action of hormonal contraceptives includes: Inhibition of ovulation by suppressing luteinizing hormone (LH) secretion from the pituitary, thickening of cervical mucus hampering the transport of sperm, and affecting the development of the endometrium. There are several delivery methods of hormonal contraceptives, each of which fall into one of two formulations: 1) the combined contraceptive contains both an estrogen and a progestin (e.g. oral contraceptives (OC), transdermal patch); or 2) a progestin-only contraceptive (e.g. mini-pill, intrauterine device, injection, implant). The progestin-only contraceptives have been minimally studied in the general population, and even less so in the athletic population. The combined OC is the most commonly researched and used in female athletes (Martin, Sale,

Cooper, & Elliott-Sale, 2017; Torstveit & Sundgot-Borgen, 2005), and thus the focus of this review.

Oral contraceptives (OCs) suppress normal menstrual cycle levels of estradiol and progesterone by inhibiting hypothalamic release of gonadotropin releasing hormone (GnRH) which, in turn, prevents pituitary secretion of follicular stimulating hormone (FSH) and LH. OCs provide pharmacological control of the reproductive cycle by consistently promoting the negative feedback loop that switches off the hypothalamus-pituitary-gonad (HPG) axis such that no endogenous estrogens or progesterone are released from the ovaries. Instead, the circulating level of both hormones is set by the concentrations supplied in the OC. Notably, while the steroid components of OCs are designed to mimic endogenous estradiol and progesterone effects in the body, the OC steroids are synthetic, and their actions differ from endogenous estradiol and progesterone in many different ways. The six main differences include: 1) The pathway of entry into the main bloodstream – orally administered OC steroids enter the hepatic circulation via the gastrointestinal tract and portal vein, however the ovarian steroids bypass hepatic circulation and enter systemic circulation via ovarian veins and the inferior vena cava- this has implications because of the steroid metabolising action of the liver; 2) Concentrations in the blood - OCs are taken once per day and enter the system in a single large dose, whereas the ovarian hormones are continuously secreted in varying amounts across the menstrual cycle; 3) The OC steroid level is constant and dependent on OC dosage and are not subjected to the negative feedback control mechanism of the hypothalamus-pituitary axis, whereas ovarian hormones are dependent on the axis allowing for their fluctuating levels; 4) The OC steroids are synthetic and have been designed to have a molecular structure that is more resistant to steroid metabolism so length of time that the OC

steroid is bioactive is longer relative to endogenous steroids; 5) The progestin associated with OC use may exert androgenic as well as anti-estrogenic activities, depending on the type and concentration of progestin; and 6) The OC steroids may have a different action on the steroid receptors than ovarian hormones, again due to structure, type, and concentration of the synthetic steroids. These differences in mechanisms of action may extend beyond the reproductive system and contraindicate the responses and actions of other bodily systems such as the cardiovascular, metabolic and renal systems.

Research concerned with examining the impact of steroid hormones on exercise performance has been complicated by the wide inter-and intra-individual variations of endogenous estrogen and progesterone concentrations within the natural course of the human menstrual cycle. Additionally, the inconsistency of the length of the natural menstrual cycle further complicates accurate determination and validation of exercise testing days between subjects. OCs are often used to pharmacologically generate a controlled menstrual cycle (Redman, 2004; Schaumberg, Jenkins, Janse de Jonge, Emmerton, & Skinner, 2017). Oral contraceptive use generally involves a dose of estrogen and progestin over 21 d (OC consumption phase), followed by 7 d of placebo (OC withdrawal phase). The dosage of ethinyl estradiol (EE) in the monophasic pill is constant and averages 0.03 mg/d (different brands range from 0.02 to 0.05 mg/d) (Figure 2), while in the triphasic preparations, the dosage may vary or remain constant, usually between 0.03 and 0.04 mg/d (Figure 3). The effectiveness of the combined OC regime is predominantly established as a result of the inhibition of the hypothalamic-pituitary-ovarian axis. The estrogen component of the OC inhibits FSH secretion from the pituitary, thus, FSH-dependent follicle growth is blocked. Should a dominant follicle emerge, suppression of the LH surge is prevented through the progestin component, which inhibits

ovulation (Schlaff, Lynch, Hughes, Cedars, & Smith, 2004). The progestin component alone does not seem to have a predominant effect on basal concentrations of FSH and LH but inhibits peak concentrations. Additionally, the inhibitory effects of both the estrogen and progestin components are established through synergistic interactions at the hypothalamic-pituitary level. Because the OC controls the hypothalamic-pituitary-ovarian axis through driving the negative feedback loop with constant estradiol and progestin levels, the amount of "active" circulating sex steroids is assumed to be related to the daily dosage administration. The effect of OCs on endogenous sex hormone concentration differs depending on type and brand of pill (Elliott-Sale et al., 2013), especially as both monophasic and triphasic preparations the type of progestin, dosage, potency and androgenicity can vary between brands. The variation in exogenous steroid profiles between the OC preparations should be considered when evaluating the effects of contraceptives, given that the potency, androgenicity and ratio of hormones may influence the impact of OC cycle phase on physiological responses (Table 2). Also, given the different half-lives of the exogenous steroids, researchers should also consider the withdrawal phase as a transient hormonal profile.

Oral Contraceptives: Role in experimental design

Oral contraceptives can be used as an experimental group in two ways. Firstly, they can be used to examine the effects of down-regulated estrogen and progesterone levels on performance. Secondly, they can be used to investigate the effects of the exogenous synthetic component on performance (Elliott-Sale et al., 2013). However, it is often difficult to

determine the specific role of the OC group. OC users may be used as a control group against which the effects of the menstrual cycle (MC) can be compared, as OC use results in a consistent concentration of circulating estrogen and progestin, versus the fluctuations seen in eumenorrheic females (Rickenlund et al., 2004; Sim et al., 2017). Alternatively, OC users may be tested in the first few days of the withdrawal phase, comparing to the follicular phase and/or hormone-free phase (Gordon et al., 2018; Sim et al., 2015). This comparison, however, is in appropriate because the different half-lives of the synthetic hormones may result in the withdrawal phase being more reflective of a transient hormone phase. Perhaps even more inappropriate, OC users are grouped with naturally cycling women to increase a total "n" for comparison studies (Gagnon, Crandall, & Kenny, 2013; Gagnon & Kenny, 2012).

To try and understand the impact of exogenous hormones on study design and outcomes, Schaumberg, Jenkins and colleagues' investigated the potential influence of monophasic OC on anaerobic adaptation of sprint interval training (SIT) in recreationally active women(Schaumberg, Jenkins, Janse, Emmerton, & Skinner, 2017). Forty-seven women were divided into 25 women who had been taking the same monophasic OC for at least six months (OC), and 16 eumenorrheic, normally cycling women (MC). Phase verification and ovulatory status was performed through hormone analyses and ovulation prediction testing to ensure both MC and OC women were tested in the high hormone phase (MC, mid-luteal, 6 to 8 days post-ovulation; OC, in the final two weeks of the active pill). The study found that OC use, compared to normal menstruation, dampened peak aerobic capacity and cardiac output adaptations to the SIT (Schaumberg, Jenkins, Janse, et al., 2017).

Comparison of Sexes

Upon reviewing the sport and exercise science literature, it is common for comparisons between men and women to be described as "gender" differences. Thus, gender, which fundamentally refers to social and cultural influences, is often confused with sex, referring to the biological category of influences (Krieger, 2003). The sex hormone milieu differs vastly between males and females. The predominant circulating gonadal sex steroid hormones after puberty are androgens in males and estrogens in females. Testosterone levels in males are relative consistent from day to day and do not show the fluctuations in concentration measured for estrogens and progesterone in females, and therefore across any training program. In male athletes, levels may fall if overtrained, or if taking androgen supplements. As testosterone levels will be fairly constant across a study period, exercise performance measures will also be constant across this same time frame. The constant exposure to testosterone across adult life in males is quite different to the sex hormone exposure in adult females, where the female sex hormone milieu constantly changes across the MC. Thus, for an elite female athlete, this means that the hormone milieu is changing across her training program. It stands to reason, if traditional training programs were removed from the male lens, and applied to the female, based upon her MC, to take advantage of the effects of hormone perturbations, a new methodology of training may emerge. For example, estrogen has been suggested to have an anabolic effect on muscle, and it is established that progesterone is catabolic (Lamont et al., 1987). Reis et al (Reis, Frick, & Schmidtbleicher, 1995) investigated periodisation of a resistance training program of high loads in the mid-late follicular phase, with low-frequency training in the luteal phase. The study a larger strength increase compared to the control,non-periodised training group across the whole cycle. Sung

and colleagues (Sung et al., 2014) also showed mid to late follicular-based strength training, as compared to luteal-based, increased strength, muscle volume, and cellular adaptations. Most recently, Wikstrom-Frisen et al demonstrated that high frequency periodised leg resistance training in the mid-to late follicular phase optimized strength gains, lean body mass, and power as compared to the same training in the luteal phase (Wikstrom-Frisen, Boraxbekk, & Henriksson-Larsen, 2017).

PERSPECTIVES

The days of the menstrual cycle on which to test are critical to achieve a clear picture on the effects of endogenous and exogenous hormones on exercise science and sports medicine outcomes. It is imperative the researcher identify if the hypothesis is to identify sex differences, or if the goal is to examine the effects of ovarian steroids on a specific physiological outcome. To simplify the task of determining when to test women across MC, OC, and for sex comparison, a schematic has been created in Table 3.

(Insert Table 3 here)

To compare women and men, testing women in the appropriate phase, determined by the three-step method for verification of phase (Schaumberg, Jenkins, Janse de Jonge, et al., 2017) will reduce ambiguous and combined results. Testing in the luteal phase will yield the greatest differential between sexes, yet the hormonal influence may raise additional experimental questions on the specific outcomes tested.

Looking at Figure 2, it is apparent that monophasic OC use is associated with high ethinyl estradiol and progestin until day 21, (three weeks of active pills). Due to the half-life of the exogenous hormones leading to a transient hormonal state during the placebo week (Elliott-Sale et al., 2013), it would make sense to test during the first three weeks of the monophasic pill regime, as a comparison phase of high hormone to luteal phase. OC users may also be used as a control group against which the effects of the MC can be compared, or as a means to compare men and women, as OC use results in a consistent concentration of endogenous estrogen and progesterone, thereby negating the fluctuations in hormone concentration seen in eumenorrheic females. With regard to the triphasic OC, (Figure 3) testing days range from 2-8, 8-12, 12-21, and 22-28 to cover the varying doses and concentrations of hormones. Note, however, studies should limit their sample to one type and brand of OC to minimize the large variation in hormone concentrations, which may lead to type-II errors (Elliott-Sale et al., 2013).

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Table 1. Production Rate of Sex Steroids in Women at Different Stages of the Menstrual Cycle

SEX STEROIDS (in mg or ug per 24h)	**DAILY PRODUCTION RATE**		
	Early Follicular	*Preovulatory*	*Mid-Luteal*
Progesterone (mg)	1	4	25
17 α-Hydroxyprogesterone (mg)	0.5	4	4
Dehydroepiandroesterone (mg)	7	7	7
Androstenedion (mg)	2.6	4.7	3.4
Testosterone (ug)	144	171	126
Estrone (ug)	50	350	250
Estradiol (ug)	36	380	250

Source: Stricker et al, 2006

Table 2. Progestins of combined OCPs: generations and secondary effects

1. **Norethindrone**: 1st generation, low progestational and slight estrogenic activity. Less androgenic than 2nd gen, more androgenic than newer (levonorgestrel, norgestrel). Increases HDL, lowers LDL
2. **Norethindrone Acetate**: 1st gen, low progestational slight estrogenic activities. Less androgenic than 2nd gen, more androgenic than newer (levonorgestrel, norgestrel).
3. **Ethynodiol Diacetate**: 1st gen. medium progestational , little estrogenic and androgenic activity. Paired with high-dose estrogen combinations
4. **Levonorgestrel**: 2nd gen, most widely prescribed worldwide. High progestational and androgenic. Negative effect on serum lipoproteins. (emergency contraceptive, extended cycle brands)
5. **Norgestrel**: 2nd gen. high progestational and androgenic; strong anti-estrogen effects
6. **Desogestrel**: 3rd gen. high progestational selectivity, minimizing androgenic effects and estrogenic activity. Less negative effect on metabolism, weight gain
7. **Norgestimate**: 3rd gen. high progestational activity, slight estrogenic effects, less androgenic. Minimal effect on lipoproteins, CHO metabolism
8. **Drospirenone**: 4th gen. Suppresses secretion of AVP. Aldosterone; side effect of elevated K+ (e.g. YAZ)

Table 3. Chart to determine what day of Menstrual Cycle (MC) or Oral Contraceptive (OC) phase to test women for sex and/or phase comparisons

Women versus Men	**MC versus Monophasic OC**	**MC versus Triphasic OC**
Control: Monophasic OC, Day 1-21	Low Hormone: Day 2-5 (mc) vs Day 22-23 (oc)	Low Hormone: Day 2-5 (mc) vs Day 22-23 (oc)
Sex Differences: Naturally cycling women, in both high and low hormone phases, verified with 3-step method	High Hormone: Day 23-26 (mc) vs Day 3-6^{+} (oc)	High Hormone: Day 23 -26 (mc) vs Day 9-11 OR 17- 19 (oc)** ** depending on elevated hormone interaction and system
Further investigation warranted across all categories: Comparisons to IUD, Injection, Mini-pill		

Figure 1. Reference range data, by day, in normal cycling women ((Stricker et al., 2006))

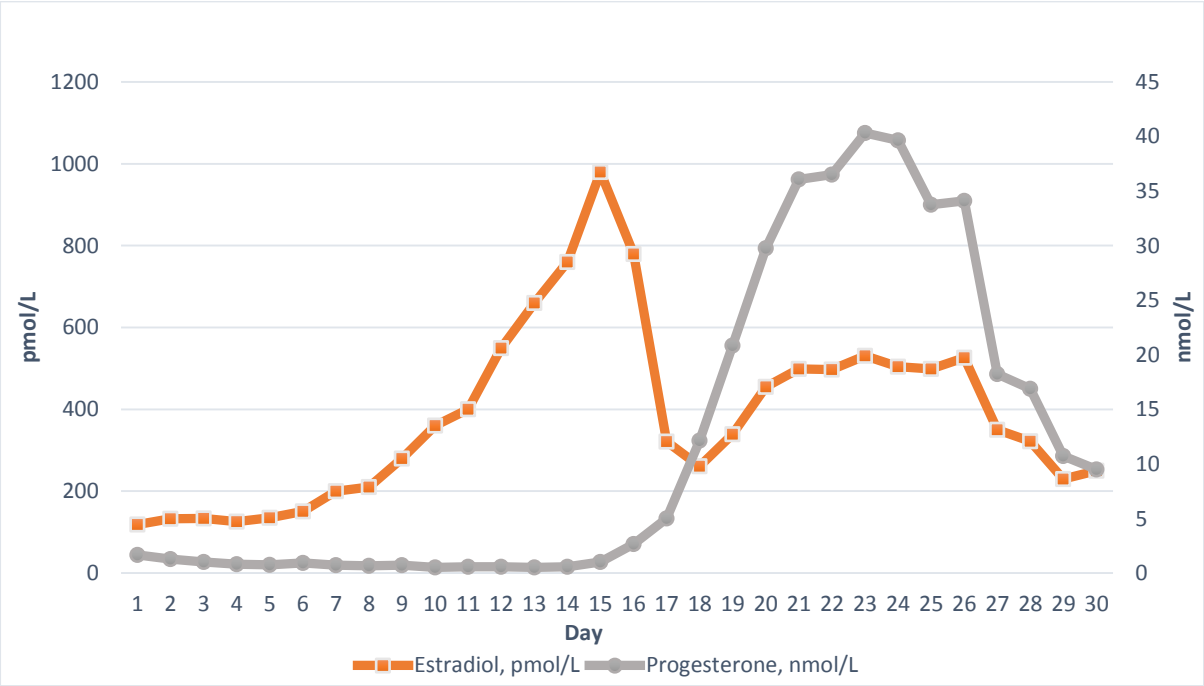

Figure 2. Daily exogenous hormone dosage, ug, Monophasic Oral Contraceptive (data derived from Wyeth Pharmaceuticals, Alesse-28)

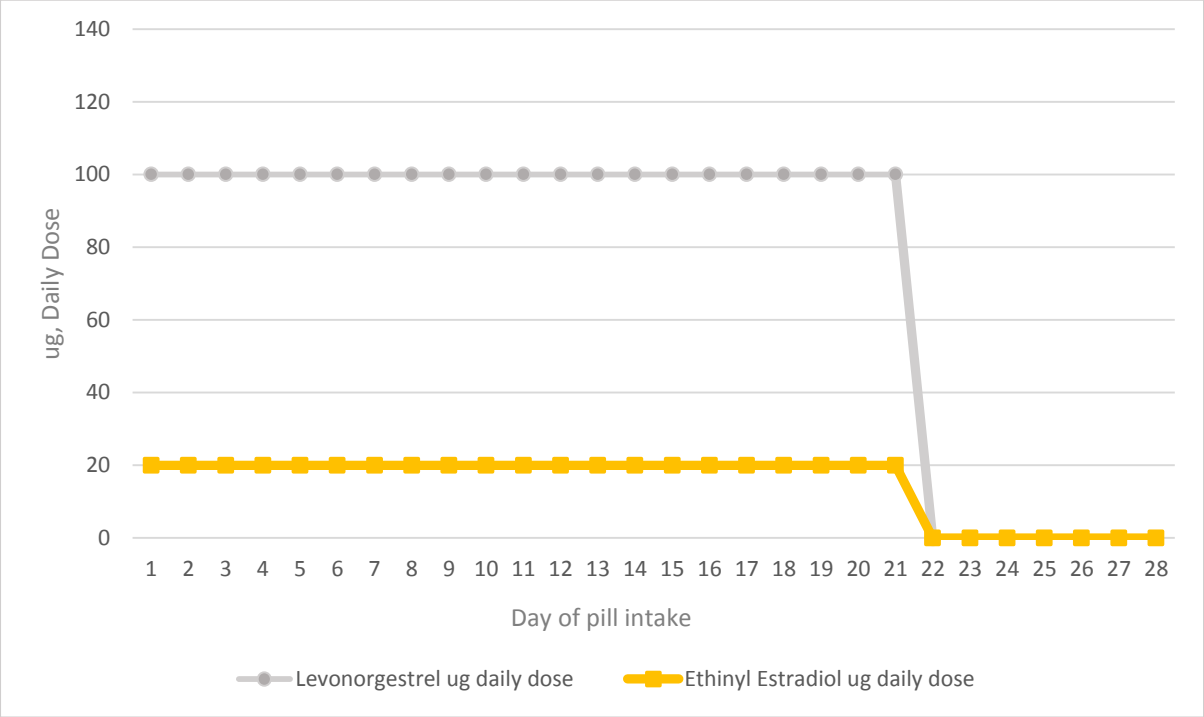

Figure 3. Daily exogenous hormone dosage, ug, Triphasic Oral Contraceptive

(data derived from Berlex Laboratories, Tri-Levlen 28)