AN ABSTRACT OF THE THESIS OF

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Effects of infusion of a lipid emulsion into ewes during mid- to late diestrus on serum concentrations of total cholesterol (**TC**), progesterone ($\mathbf{P_4}$), prostaglandin (**PG**) $F_{2\alpha}$ [measured as 13,14-dihydro-15-keto $PGF_{2\alpha}$ (**PGFM**)], and PGE_2 and ovulation rate were examined. In **Exp. 1**, Hampshire ewes (n = 3/treatment) were infused via jugular catheters with either 250 ml saline (**S**) or soybean emulsion (**SB**) for 5 h on each of Days 9 to 13 of the estrous cycle (estrus = Day 0). Blood samples were collected via jugular catheter at 0, 5, 6, 7, 8, and 9 h on Day 9, and 0, 5, and 9 h on Days 10 through 13. Infusion of lipid increased serum levels of P_4 and TC, which declined with time after infusion was terminated (treatment x hour interaction; P_4, $p = 0.004$; TC, $p = 0.0002$). Duration of the estrous cycle tended to be shorter ($p < 0.10$) in SB-infused ewes compared with that of S-infused ewes (16.7 ± 0.3 vs. 18.0 ± 0.6 days). In **Exp. 2**, Polypay ewes of moderate body condition were infused i.v. for 5 h on each of Days 9 through 15 of the estrous

cycle with either 200 ml SB (n=5), olive oil emulsion (**OO**; n=5) or S (n=4). Blood samples were collected every half hour on Day 9 from 0 through 8 h from initiation of infusion, and on Days 10 through 15 samples were collected at 0, 2.5, 5, 6, and 8 h from start of infusion. On Day 14 of the succeeding estrous cycle after infusion, laparotomy was performed to assess number of corpora lutea (ovulation rate) and number and diameter of follicles > 4 mm. Infusion of both SB and OO caused an increase in serum TC and P_4 (treatment x hour interaction, $p = 0.009$, $p = 0.005$, respectively). Serum PGFM concentrations were greater in lipid-infused ewes compared with controls on Days 13 through 15 (treatment x hour x day interaction, $p = 0.03$) and on Day 15 levels were highest in OO-infused ewes compared with SB and S-infused ewes ($p = 0.0005$). Infusion of OO but not S or SB into ewes on Day 9 and Days 13 through 15 stimulated production of PGE_2 during administration of lipid, whereas levels declined thereafter (treatment x hour interaction, Day 9, $p = 0.01$; Days 13 through 15, $p < 0.0001$). Mean serum concentrations of PGE_2 over time within day differed among all treatments for Days 13 through 15 ($p < 0.0001$). Duration of the estrous cycle was shortened in OO-infused ewes (16.2 ± 0.4 days) compared with that of SB and S-infused ewes (17.2 ± 0.2 and 18.0 ± 0.0 days, respectively; $p = 0.002$). Number of corpora lutea and number of follicles and diameter of follicles > 4 mm did not differ among treatment groups. These data indicate that lipid infusion stimulated increases in concentrations of TC and serum P_4 and shortened the estrous cycle.

Altered Ovarian and Uterine Function in Response to Intravascular Infusion of Long Chain Fatty Acids in Nonpregnant Ewes

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Joan M. Burke, Author

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ALTERED OVARIAN AND UTERINE FUNCTION IN RESPONSE TO INTRAVASCULAR INFUSION OF LONG CHAIN FATTY ACIDS IN NONPREGNANT EWES

REVIEW OF THE LITERATURE

REPRODUCTIVE CYCLE OF THE EWE

Estrous Cycle

Regulation of Seasonal Estrus

Sheep are seasonally polyestrus, unlike cattle and swine which are polyestrus throughout the year. In the northern hemisphere, breeding typically occurs in the fall with lambs being born in the spring. Initiation and length of the season is dependent on a number of factors such as photoperiod, nutrition, breed and latitude. All breeds of sheep maintained at higher latitudes have short, intense breeding seasons compared to those closer to the equator. Breeds that evolved near the equator have longer breeding seasons than those originating at higher latitudes. Maximal reproductive efficiency is attained when ewes are in moderate to good body condition. Poor nutrition can delay onset of the breeding season in ewes (I'Anson et al., 1991). There is evidence that when ewes have been isolated from rams for several weeks the breeding season can be initiated by their introduction (Riches and Watson, 1954; Radford and Watson, 1957). The ram

effect, as this is called, appears to occur due to pheromonal cues (Knight and Lynch, 1980).

Photoperiod has a strong influence on the reproductive cycle of the ewe as evidenced by seasonal fluctuations in estradiol negative feedback on tonic luteinizing hormone (**LH**) secretion (Legan and Karsch, 1980). Legan et al. (1977) implanted ovariectomized (**OVX**) ewes with estradiol to examine the relationship between estradiol and LH. Mean serum LH concentrations were high during the breeding season and otherwise undetectable during the normal period of anestrus. In OVX ewes without estradiol implants, mean serum LH concentrations did not change with time, suggesting that estradiol evoked a response of the hypothalamus-hypophysial axis dependent upon season of the year.

Photoperiod exerts its control over fluctuations in LH secretion through neuroendocrine mechanisms. The ewe perceives seasonal changes in duration of daylight and darkness through photoreceptors in the eye, which transmit a neural signal to the pineal gland (Turek and Campbell, 1979). The pineal gland releases melatonin, an indoleamine responsible for changes in reproductive function in a number of species of animals, which was determined in ewes (Bittman et al., 1983) and rats (Lewy et al., 1980) by pinealectomy. Melatonin is synthesized and released by the pineal gland during nighttime and is inhibited by light (Rollag and Niswender, 1976). Bittman et al. (1983) demonstrated the stimulatory effects of short days and inhibitory effects of long days on melatonin secretion by the pineal gland. Melatonin can be administered to ewes either orally, as an implant, or an injection, to simulate exposure to reduced daylight and hence, may initiate

estrous cycles in anestrous ewes (Nett and Niswender, 1982; Arendt et al., 1983). The conversion of short to long days results in a decrease in duration of nightly secretion of melatonin. This reduction in daily melatonin secretion activates a set of estradiol-sensitive catecholaminergic neurons, i.e., steroid-dependent actions, causing a decrease in LH secretion, and a set of estradiol-insensitive serotonergic neurons, i.e., steroid-independent actions, also causing a decrease in LH secretion. These steroid-dependent and steroid-independent actions of photoperiod appear to reflect changes in frequency of gonadotropin-releasing hormone (**GnRH**) pulse generator in the ovine hypothalamus (Goodman, 1988). The slow frequency of GnRH pulses in the anestrus ewe appears to be what prevents ovulation, because if frequency is artificially increased with GnRH injections, an LH surge and ovulation results (McCleod et al., 1982; Legan et al., 1985) and, if GnRH pulse frequency of cycling ewes is maintained, ovulatory cycles continue (McNatty et al., 1983).

The follicular phase increase in tonic LH secretion, which mediates the preovulatory rise in estradiol, is what drives the reproductive cycle of the ewe (McNeilly et al., 1982). During the breeding season progesterone is the primary inhibitor of LH pulse frequency. When progesterone declines at luteolysis, LH pulse frequency increases resulting in a preovulatory rise in estradiol secretion, which triggers the LH surge leading to ovulation. During anestrus, on the other hand, the inhibitory neural systems activated by exposure to long day photoperiod allow estradiol to become the primary inhibitor of LH pulse frequency. When estradiol is secreted during the estrous cycle it acts as a weak inhibitory steroid

decreasing LH pulse amplitude but not frequency; in the anestrous period estradiol becomes a potent negative feedback hormone causing a decrease in LH pulse frequency (Goodman and Karsch, 1980). Following regression of the last corpus luteum (CL) of the breeding season, LH pulse frequency does not increase, hence no rise in estradiol, no LH surge and ovulation does not occur, terminating the breeding season. The initiation of the breeding season is marked by the loss of inhibitory neuronal control of LH pulse frequency. In the absence of progesterone, pulsatile LH secretion increases, stimulating a rise in estradiol secretion, which triggers a surge of LH, and the first ovulation (Goodman, 1988). Prior to behavioral estrus that marks the beginning of the first estrous cycle of the season some ewes experience a short luteal phase caused by luteinization of follicles, decreased luteotropic support or inadequate preovulatory follicular development (Garverick and Smith, 1986).

Characteristics of the Estrous Cycle

Characteristics of the reproductive cycle of several species are listed in Table 1. The average length of the estrous cycle is 16.7 days in the ewe and 21 days in the cow and the sow. In the ewe, the cycle length varies with breed, stage of breeding season, and environmental stress. Estrus lasts 24 to 36 hours and is influenced by breed, age, stage of season, and presence of the male. Estrus is not observed except in the presence of the ram, and is manifested behaviorly by the willingness of the ewe to accept the male and permit copulation. Other visual

Table 1. Characteristics of the reproductive cycle of various mammalian species.

Species	Length of Cycle (d)	Follicular Phase (d)	Duration of Estrus (h)	Duration of LH surge	Hours after Peak LH	Time of Ovulation
Ewe	16-17	1-2	24-36 h	10 h	21-23	24-27 h after onset of estrus
Goat	20-21	2-3	32-40 h			30-36 h after onset of estrus
Cow	21-22	3-5	18-19 h	10 h	24	10-15 h after end of estrus
Sow	20-21	3-5	40-60 h	12-16 h	40-41	38-42 h after onset of estrus
Mare	19-25	4-8	4-8 d	10 d		1-2 d before end of estrus
Mouse	4-6	2-3	10 h		10-12	2-3 h after onset of estrus
Rat	4-5	2-3	13-15 h	2-3 h	10-11	8-10 h after onset of estrus
Guinea Pig	16-17	3-4	6-11 h			10 h after onset of estrus
Human	28	12-14	none	24-48 h	16-24	14 d prior to onset of menses

Adapted from Gibori and Miller, 1982.

indications of estrus include an edematous vulva and occasionally mucous discharge. The species listed in Table 1 are all spontaneous ovulators. Ovulation occurs 24 to 27 hours after the onset of estrus in ewes. The duration of estrus in the cow is 18 to 19 hours and ovulation occurs 10 to 12 hours after the cessation of estrus. Unlike the ewe, cows in estrus exhibit homosexual activity in the absence of the male. In the sow, estrus lasts an average of 40 to 60 hours and the ova are released 38 to 42 hours after the onset of estrus (Hafez, 1993).

Ovarian Anatomy

The mature ovary consists of the following: follicles varying in stage of development, atretic follicles (those follicles which have begun to degenerate), CL (a follicle which has ovulated with subsequent transformation of granulosa cells into lutein cells), corpora hemorrhagicum (a CL before it has fully formed), and corpus albicans (scar-like tissue of the CL of previous ovulations). The different stages of follicular development include the primary follicle (an oocyte surrounded by a single layer of cells), secondary follicle (an oocyte surrounded by multiple layers of cells including granulosa cells), tertiary follicle (continued development of secondary follicle with formation of antrum which is filled with follicular fluid), Graafian follicle (further enlargement of the tertiary follicle in preparation for ovulation, i.e. a preovulatory follicle). Cells of the maturing follicle develop into theca and granulosa cells, which are separated by the basement membrane. Theca cells are derived from ovarian interstitial tissue adjacent to the follicle and become invaded

by vascular elements, whereas the granulosa cells do not. Granulosa cells surround the oocyte and line the inside wall of the follicle (Hafez, 1993).

The CL develops after the collapse of the follicle at ovulation. The ovulated follicle becomes highly vascularized and glandular. Both granulosa and theca cells contribute to the formation of the CL and are capable of dividing and responding to LH from the blood (Hansel et al., 1973). There are two types of luteal cells, referred to as small and large luteal cells (ewes: O'Shea et al., 1979; cows: Ursely and Leymarie, 1979; Koos and Hansel, 1981). Using specific monoclonal antibodies to granulosa and thecal cell surface antigens Alila and Hansel (1984) hypothesized that the large cells and some small cells were derived from granulosa cells in the cow. The morphology of these small cells resembled that of large cells and were identified as granulosa cells that had not yet enlarged. The rest of the small cells were found to be derived from the theca interna. They suggested that as the CL ages small cells could develop into large cells. The large cells account for 3.5 to 10% of the total luteal cell population (Hansel et al., 1991). Additional cells of the CL include those associated with vascular areas, macrophages, smooth muscle cells, and fibroblasts (Rodgers et al., 1984; Farin et al., 1986).

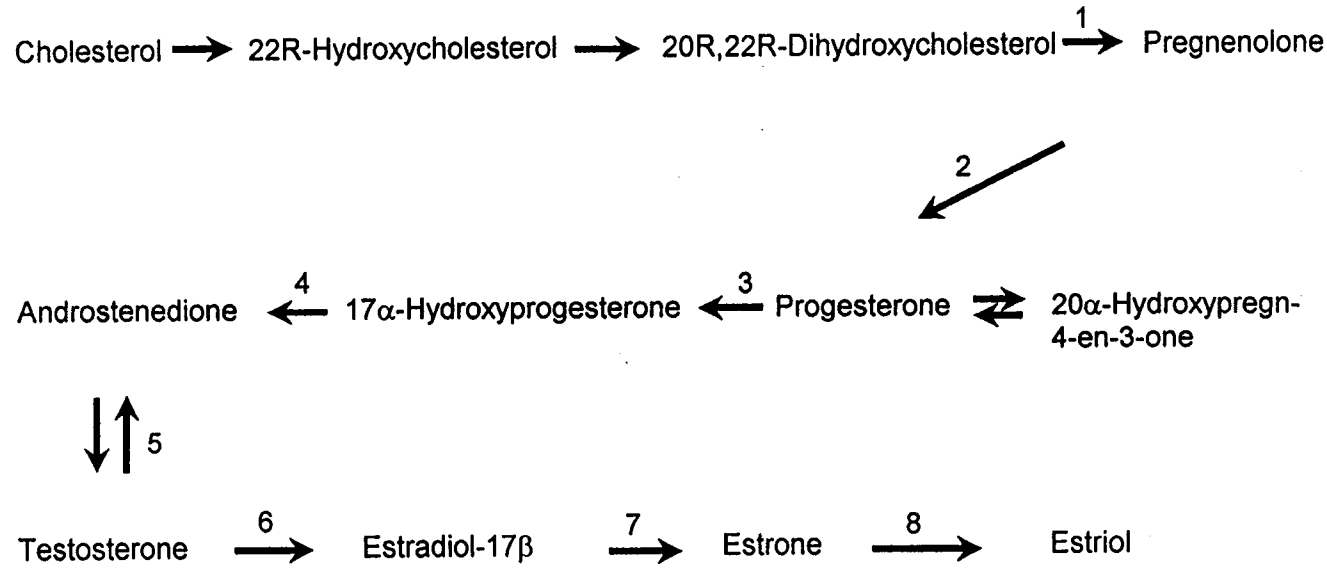
Endocrine Regulation of the Ovary

The gonadotropes of the anterior pituitary gland secrete two glycoproteins, LH and follicle-stimulating hormone (**FSH**). Luteinizing hormone consists of two glycoprotein subunits, termed α and ß; the biological activity of the individual subunits is negligible compared to the intact LH molecule. The α-subunit of ovine

LH consists of 96 amino acids and the ß-subunit 120 amino acids. Similarly, FSH consists of an α subunit that is identical to that of LH (Sairam, 1981) and a ß subunit. The amino acid sequence of the ovine ß-subunit of FSH is similar to that of human FSH, which is 118 residues long (Hadley, 1992). Secretion of these gonadotropins is regulated by GnRH that is secreted into the portal blood of the hypothalamus. A feedback loop exists between gonadotropins and follicular estradiol and luteal progesterone. During the early follicular phase estradiol exhibits a negative effect on the hypothalamus and pituitary, decreasing pituitary responsiveness to GnRH. Without this negative feedback, FSH output increases tremendously (Rozell and Keisler, 1990; Mann et al., 1992). Prior to ovulation estradiol has a positive feedback effect on gonadotropin secretion. Progesterone inhibits GnRH release, which ultimately inhibits gonadotropin secretion during the luteal phase. Follicle-stimulating hormone stimulates follicular development through granulosa cell proliferation (Hirshfield, 1991) and, in the presence of LH, stimulates estrogen production from the large ovarian follicle. High concentrations of estradiol from the follicle induces a GnRH surge which stimulates a surge of LH leading to rupture of the follicle wall and ovulation.

Because of the feedback loop that exists between the gonads and the hypothalamus-hypophysial system it is appropriate to present the elements of steroidogenesis. The steroidogenic pathway is presented in Figure 1. Cholesterol, either synthesized by the cell from acetate or delivered to the cell via lipoproteins in serum (see **Cholesterol Biosynthesis**), serves as a precursor for steroid hormones (Fielding and Fielding, 1985). Cholesterol is transported to the

Figure 1. Steroidogenic Pathway



1. 20,22 Lyase
2. 3β-HSD
3. 17α-Hydroxylase
4. 17,20 Lyase
5. 17β-HSD
6. Aromatase
7. 17β-HSD
8. 16α-Hydroxylase

mitochondria by a cholesterol-binding protein. Once in the mitochondria, the side-chain cleavage complex (**SCC**), which is a mixed function oxidase system, catalyzes oxidation of carbons 20 and 22 of cholesterol. The molecule is then cleaved to pregnenolone and isocaproaldehyde. This is considered to be the rate limiting step of steroid synthesis. The cytochrome P-450_{scc} complex comprises adrenodoxin, a non-heme iron- and sulfur-containing protein, adrenodoxin reductase, an NADPH-specific flavoprotein, and a substrate-specific heme protein (Martin, 1985). Pregnenolone is dehydrogenated to progesterone by the enzyme, 3ß-hydroxysteroid dehydrogenase (**3ß-HSD**). Progesterone is hydroxylated to 17α-hydroxyprogesterone by 17α-hydroxylase, and subsequently the remaining two-carbon side chain is cleaved via 17,20 lyase to form androstenedione. Androstenedione can be dehydrogenated to form estrone (not shown) or reduced by 17ß-HSD to form testosterone. Using histochemical staining this reversible enzyme reaction has been identified in granulosa cells and found to be much more efficient than in theca cells (Hsueh et al., 1984). Aromatization of testosterone forms estradiol-17ß, the major estrogen secreted by the ovary (Matthews and van Holde, 1990).

Falck (1959) demonstrated the requirement for both theca and granulosa cells for synthesis of estradiol by autotransplanting theca interna and granulosa cells, alone or in combination, to the anterior chamber of the eye in the rat. Maximal estradiol biosynthesis was achieved when cells were autotransplanted in combination, indicating the joint action of the two cell types. A two cell type theory was proposed suggesting the participation of theca cells for the conversion of

progesterone to androgens (Short, 1962) and granulosa cells for the conversion of androstenedione to testosterone and further to estradiol (Bjersing and Carstensen, 1964; see Figure 1). Theca cells possess LH receptors, which stimulate the conversion of progesterone to androgens in response to exposure to increasing concentrations of LH. Androgens then diffuse across the basement membrane from theca to granulosa cells. Granulosa cells possess FSH receptors, which, in response to the FSH, then stimulate the conversion of androgens to estradiol (Dorrington and Armstrong, 1979).

The regulation of steroidogenesis in the CL involves the small and large luteal cells. Under *in vitro* basal conditions, large cells secrete progesterone at a higher rate than small cells (Fitz et al., 1982). Small cells possess the majority of the LH receptors. When small cells are cultured in the presence of LH (Hansel et al., 1991), dibutryl cAMP (Fitz et al., 1982), cholera toxin, and forskolin (Hoyer et al., 1984) progesterone secretion is stimulated. Levels of cAMP in small cells were elevated by LH and forskolin, whereas accumulation of cAMP in the medium occurred by stimulation of cells with LH, cholera toxin, and forskolin (Hoyer et al., 1984). Progesterone stimulation was not apparent in large luteal cells cultured with LH, dibutryl cAMP (Fitz et al., 1982), cholera toxin or forskolin (Hoyer et al., 1984). It is possible that progesterone production is maximal in large cells so that they are unresponsive to further stimulation. This indicates that steroid secretion in small, but not large cells is regulated by intracellular levels of cAMP.

Uterine prostaglandin (**PG**) $F_{2\alpha}$ can inhibit *in vitro* synthesis of luteal progesterone by preventing cholesterol utilization for steroidogenesis and

suppressing cholesterol synthesis (Pate and Condon, 1989). Oxytocin (OT) may also regulate production of progesterone. When a microdialysis system was used to study the effects of OT on luteal cells, OT caused a dose-dependent increase in progesterone release in the presence of LH (Miyamoto and Schams, 1991). In the presence of high density lipoproteins (HDL), progesterone secretion is heightened over basal levels in large luteal cell cultures, with less of an increase in small cells (Wiltbank et al., 1990).

Follicular Development

Development of the primordial follicle begins during fetal life in mammalian species. The primordial follicle consists of an oocyte arrested in prophase I of meiosis and a single layer of granulosa cells. At puberty, growth of the primordial follicle continues with various pools of these follicles being recruited during each cycle. Ultimately some are ovulated but most undergo atresia. The dominant or largest follicles synthesize and release the majority of estrogen at estrus and bind gonadotropins to granulosa cells. Ewes and sows are polyovular, unlike cows, which generally ovulate one follicle only.

Follicular growth at the antrum stage and beyond is dependent upon exposure to FSH and LH. Proliferation and differentiation of theca and granulosa cells result in the ability of follicles to respond to gonadotropins and produce estradiol, which determines which follicles become dominant and gain the LH receptors necessary for ovulation and luteinization. There are several hypotheses available concerning a variety of hormones that trigger atresia (for review, see

Hirshfield, 1991). Hormonal signals may act at the cellular level to induce death and fragmentation of granulosa cells.

The estrous cycle of the bovine is characterized by the presence of follicular waves, defined as the synchronous development of a group of follicles. Early research suggested the presence of two waves during the cycle (Rajakoski, 1960), which was later confirmed by ultrasonic monitoring of follicular development (Pierson and Ginther, 1984; 1986; 1987) or three wave cycles (Savio et al., 1988; Sirois and Fortune, 1988). Ginther et al. (1989) reported that three wave activity was associated with a longer luteal phase compared with two wave activity. Each wave includes the development of a large dominant follicle and smaller subordinate follicles. In the presence of a regressing CL the dominant follicle ovulates in response to a gonadotropin surge. If luteal regression does not occur the dominant follicle will undergo atresia. Follicular waves begin around days 2, 9, and 16 of the estrous cycle for cows with three waves and days 2 and 11 for two wave animals (Sirois and Fortune, 1988).

In comparison, early research in sheep has shown that follicles develop in two intense waves associated with changes in progesterone concentrations (Brand and de Jong, 1973). More recently, through daily laparoscopy, Noel et al. (1993) reported two waves of follicular growth during the luteal phase and one during the follicular phase. Conversely, Hay and Moor (1975) describe a continuous development of follicles through observations made at surgery or slaughter. Supporting this concept, observations made on a daily basis using transrectal ultrasonography, demonstrated a continuous development of follicles with no

evidence for follicular dominance (Schrick et al., 1993). Development of follicles did not differ between pregnant and cycling ewes until luteal regression occurred in the latter ewes, suggesting that progesterone may regulate follicular development through control of LH release.

In other species (rats, humans, pigs), the development of ovulatory-size follicles is suppressed during the luteal phase and a number of growing follicles emerge during the early follicular phase. In humans only one follicle continues to develop during the late follicular phase (Fortune, 1994).

Recruitment of follicles may be mediated by a small rise in concentrations of FSH or LH in the blood (Baird, 1978; McNatty et al., 1981; Ireland and Roche, 1987). The more dramatic the rise in FSH concentrations in the blood the more follicles are recruited (Fortune, 1994).

It is not understood what factors determine which follicle(s) will become dominant. It is hypothesized that the dominant follicle indirectly causes the regression of subordinate follicles through negative feedback mechanisms. Inhibin, a peptide composed of two subunits and secreted by the follicle, along with estradiol may be involved in this process acting to suppress FSH secretion to levels that would not support the growth of subordinate follicles. Follicular fluid, which contains inhibin-like activity, inhibits FSH secretion in ovariectomized heifers (Ireland and Roche, 1983) and cycling rats (Welschen et al., 1980) and disrupts follicular development (Miller et al., 1979). Active immunization against inhibin in sheep was associated with an increase in ovulation rate (Henderson et al., 1984), suggesting suppression of some subordinant follicles did not occur. Similarly,

within 30 minutes of infusing anti-estradiol antiserum into ewes on days 10, 11, or 12 of the estrous cycle, FSH concentrations increased markedly (Pathiraja et al., 1984) illustrating estradiol's role of inhibiting FSH secretion. The dominant follicle may overcome exposure to low concentrations of FSH because of its advanced stage of development compared to subordinant follicles (Fortune, 1994). Supporting this concept, Zeleznik and Kubik (1986) reported that levels of FSH in monkeys were insufficient to recruit follicles yet adequate to maintain follicular growth once initiated. Blood flow through the follicle may be increased, supplying more FSH, and the dominant follicle may acquire LH receptors on the granulosa cells (Zeleznik, 1993).

Further development of the dominant follicle requires small but sustained increases in circulating LH, which stimulates further differentiation of the theca cell layer. An increase in estradiol production is required for further differentiation of granulosa cells. During the luteal phase, through suppression of LH accretion, progesterone indirectly inhibits estradiol secretion from the dominant follicle, initiating atresia of that follicle. This allows the next small increase in FSH to induce the next follicular recruitment (Fortune, 1994).

Follicles that undergo atresia in rats develop until the eighth or ninth generation of granulosa cells. Only follicles that are exposed to specific conditions will continue to the tenth granulosa cell generation and ovulate (Hirshfield, 1991). Similarly, in cattle the majority of changes characteristic of atresia occur within only a few granulosa cell generations near the end of follicular development. In rats atretic changes occur around the time of antrum formation (around 0.2 to 0.4 mm

follicular diameter), whereas in cattle and humans atresia occurs much later in development (15 and 20 mm, respectively). In each of these species the majority of atresia occurs just prior to the final growth to ovulatory size (Fortune, 1994).

The mechanism causing fragmentation of granulosa cells leading to the demise of the follicle is unclear. Apoptosis, or programmed cell death, may be involved. Billig et al. (1994) reported an increase in ovarian cell apoptosis (measured by DNA fragmentation) in rats treated with GnRH agonist, demonstrating a direct effect of GnRH. Treatment with FSH decreased the occurrence of apoptosis, which was partly blocked by treatment with GnRH agonist. Fragmentation of DNA was limited to the granulosa cells of preantral and antral follicles; apoptosis was not observed in granulosa cells of primary follicles or in thecal and interstitial cells. Follicular apoptosis was inhibited in rats by treatment with estrogens and stimulated by androgens (Billig et al., 1993).

Luteolysis

In the absence of factors signalling pregnancy, the cells of the CL undergo degeneration. There are numerous factors contributing to luteolysis yet the complete mechanism is unclear. Prostaglandin $F_{2\alpha}$ is the major luteolytic factor responsible for inducing luteal regression in rats and farm animals (Phariss and Wyngarden, 1969; Inskeep, 1973; Inskeep and Murdoch, 1980; McCracken et al., 1981). Prostaglandin $F_{2\alpha}$ has a venoconstrictive effect that induces hypoxia, which may result in degeneration of cells. However, it is more likely that $PGF_{2\alpha}$ acts directly on the luteal cells. In ruminants $PGF_{2\alpha}$ secreted from the uterus is

transferred directly from the utero-ovarian vein into the ovarian artery through a countercurrent mechanism of flow and then directly to the CL (McCracken, 1980). Prostaglandin $F_{2\alpha}$ stimulates the release of OT from the CL and/or posterior pituitary, which further stimulates pulsatile release of $PGF_{2\alpha}$ from the uterus (McCracken et al., 1984; Flint et al., 1990). This positive feedback loop causes pulses of circulating $PGF_{2\alpha}$ associated with luteolysis. Pulses first appear just prior to the onset of luteal regression (Zarco et al., 1988) and it appears that 5 hour-long pulses over 24 to 30 hours are required for CL regression to occur (Schramm et al., 1983). An average of 7.6 episodes of $PGF_{2\alpha}$ release occur between days 14 to 15 in nonpregnant ewes compared to 1.3 episodes during the same period in pregnant ewes (McCracken et al., 1984). During luteolysis pulses of OT and $PGF_{2\alpha}$ occur concurrently (Fairclough et al., 1980). It is not clear what initiates or terminates the positive feedback loop between OT and $PGF_{2\alpha}$.

Estradiol may be involved in initiating the loop by regulating the availability of endometrial receptors for OT thus increasing sensitivity for this neuropeptide (McCracken et al., 1981; Flint and Sheldrick, 1983). Ewes infused with estradiol-17ß into the arterial supply of an autotransplanted uterus during the late luteal phase experienced an increase in $PGF_{2\alpha}$ secretion within 90 minutes from start of infusion, compared with those ewes infused systemically, suggesting a direct effect of estradiol on the uterus (Barcikowski et al., 1974). Apparently, in order for estradiol to be effective, it must act upon a progesterone-primed uterus. When progesterone is administered to ewes early in the cycle, regression of the CL occurs prematurely (Woody et al., 1967) and endometrial concentrations of $PGF_{2\alpha}$

increases (Wilson et al., 1972), suggesting a role of progesterone in controlling the synthesis or release of $PGF_{2\alpha}$.

Receptors for $PGF_{2\alpha}$ are prevalent in large luteal cells more so than small cells (Fitz et al., 1982). Upon exposure to $PGF_{2\alpha}$, diameter of ovine large luteal cells did not change until 36 hours (Braden et al, 1988). This is beyond functional luteolysis, which is defined by a decrease in circulating progesterone (Braden et al., 1994). Prostaglandin $F_{2\alpha}$ is presumed to have a cytotoxic effect on large luteal cells as a result of a sustained induced increase in intracellular levels of calcium (Wiltbank et al., 1989a; 1992). Prostaglandin $F_{2\alpha}$ also causes activation of protein kinase C, which inhibits the secretion of progesterone (Wiltbank et al., 1989). It appears that $PGF_{2\alpha}$ activates protein kinase C in small luteal cells, which results in reduced secretion of progesterone in LH-stimulated cells (Wiltbank et al., 1989). However, $PGF_{2\alpha}$ does not appear to be luteolytic in small cells (Fitz et al., 1984), though there is a reduction in the number of small cells preceding that of large cells during $PGF_{2\alpha}$-induced luteolysis *in vivo* (Braden et al., 1988). This differential response to $PGF_{2\alpha}$ suggests that there is some other factor causing regression of small cells. Prostaglandin $F_{2\alpha}$ causes an increase in 20α-hydroxysteroid dehydrogenase activity in cultured rat granulosa cells, which acts to increase the catabolism of progesterone (Jones and Hsueh, 1981). In contrast, $PGF_{2\alpha}$ decreases the activity of other luteal enzymes such as 3ß-hydroxysteroid dehydrogenase (Dwyer and Church, 1979; Hawkins et al., 1993), cholesterol esterase, and cholesterol synthetase (Behrman et al., 1971), which would interrupt the normal function of cholesterol in steroidogenesis (Pate, 1994).

Though $PGF_{2\alpha}$ induces luteolysis it does not affect cell numbers or cell viability in mixed cultures of bovine luteal cells (Pate and Condon, 1984; Fairchild and Pate, 1987). When cows are treated with a single injection of $PGF_{2\alpha}$ on days 9 to 11 of the cycle and the CL removed within 12 hours and cultured, the luteolytic effects are reversed and the cells are maintained (Pate and Nephew, 1988). Prostaglandin $F_{2\alpha}$ may initiate the luteolytic process, but there appear to be other factors necessary to complete the process.

The immune system may be involved in degeneration of luteal cells. Immune cells are found throughout the ovary and fluctuate in numbers throughout the cycle (Bulmer et al., 1991). Formation of the early CL is associated with an ongoing migration of leukocytes, with macrophages, T lymphocytes, neutrophils and eosinophils found throughout the granulosa-lutein, theca-lutein and loose connective tissue (Lei et al., 1991; Wang et al., 1992b). As the bovine CL ages and luteolysis is initiated, macrophages increase in number acting to repair the tissue (Wang et al., 1992b). This occurs on day 14 in the cow just prior to the onset of luteolysis (Lobel and Levy, 1968). Cytokines such as interleukin-1 (**IL-1**), tumor necrosis factor alpha (**TNFα**), and IL-8 may act as chemoattractants for leukocytes and may enhance neutrophil adhesion to endothelial cells *in vitro* (Norman and Brannstrom, 1994). Estradiol and progesterone can modulate IL-1ß mRNA expression in human peripheral monocytes, demonstrating the importance of steroids on immune function (Polan et al., 1989). In turn, IL-1 and TNFα may promote progesterone production in the early CL and as the CL ages,

progesterone production is inhibited by TNFα, IL-1, IL-2, and interferon gamma (**IFN-gamma**) (Wang et al., 1992a).

Many luteal cells express class I and II major histocompatibility complex (**MHC**) antigens. The class I MHC molecules function to present antigens in altered self-cells to cytotoxic T cells, whereas the class II MHC molecules function to present processed antigens to T helper cells. Expression of class I MHC molecules result in the destruction of cells by invading leukocytes (Norman and Brannstrom, 1994). Interferon gamma, a T lymphocyte-derived cytokine, serves to induce the expression of class I and class II MHC antigens 25 and 370% above controls in cultured midcycle bovine luteal cells (Fairchild and Pate, 1989). The induction of class II MHC antigens by IFN-gamma was attenuated by the addition of LH to the medium. It was not determined whether the down-regulation of these antigens was due to other factors that may have changed during culture. Class II MHC expression has been shown to be down-regulated by prostaglandins (Kuby, 1992).

Interferon gamma may play a role in luteal function by enhancing prostaglandin synthesis and inhibiting luteal steroidogenesis. Bovine luteal cells from CL collected between days 10 to 14 of the estrous cycle experienced reduced production of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ by approximately 50% in the presence of IFN-gamma after 24 hours of culture (Fairchild and Pate, 1991). However, over time, production of these prostaglandins increased by 400% above controls. During this time LH-stimulated progesterone production was inhibited by treatment with IFN-gamma. This effect was not mediated by the rise in prostaglandins,

because treatment with indomethacin, a cyclooxygenase inhibitor, did not reverse the inhibition.

Interleukin-1 functions in co-stimulation of T helper cell activation, promotes B-cell maturation and clonal expansion, enhances activity of natural killer cells, and chemotactically attracts neutrophils and macrophages (Kuby, 1992). Interleukin 1 may play a role in regulation of prostaglandin synthesis in bovine luteal cells. Recombinant bovine IL-1ß increased the synthesis of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ in a dose-dependent manner, but had no effect on progesterone production in midcycle bovine luteal cells (Nothnick and Pate, 1990). When progesterone was added to the medium, prostaglandin synthesis was suppressed and IL-1ß could not overcome this effect. It was suggested that the stimulatory effect of IL-1ß on prostaglandin synthesis may be mediated by progesterone levels.

Tumor necrosis factor alpha is secreted by macrophages and induces cellular production of various cytokines (Kuby, 1992). Tumor necrosis factor alpha may play a role in modulating synthesis of luteal prostaglandins and in luteolysis. When TNF-α was added to cultured midcycle bovine luteal cells, there was a dose-dependent increase in synthesis of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but no change in progesterone production on all days of culture (Benyo and Pate, 1992). When luteal cells were cultured with TNF-α in combination with IL-1ß or IFN-gamma, $PGF_{2\alpha}$ production was 50-fold above those cells cultured with TNF-α alone. When luteal cells were cultured with TNF-α and IFN-gamma, TNF-α-stimulated production of $PGF_{2\alpha}$ was inhibited. By day 7 of culture, TNF-α inhibited LH-stimulated progesterone production. When luteal cells were cultured with TNF-α and IFN-

gamma together, cell numbers were reduced by 80%, but culture with TNF-α alone had no effect and culture with IFN-gamma reduced cell numbers by approximately one-third. Collectively, these studies suggest a role of the immune system in degeneration and removal of luteal cells to maintain cyclicity and repair ovarian tissue.

Other studies exhibited results pointing to apoptosis as the mechanism of luteal cell death. Sawyer et al. (1990) report nuclear changes in ovine luteal cells characteristic of apoptotic cells. Juengel et al. (1993) demonstrated DNA fragmentation in bovine luteal cells removed on day 19, but not days 10 or 15 of the estrous cycle. When they induced luteal regression with exogenous $PGF_{2\alpha}$ there was evidence of apoptosis in luteal cells collected between 24 and 48 hours after injection.

Ovulation

In all mammalian species ovulation is initiated by an ovulatory surge of gonadotropins, which increases blood flow to all follicles (for review, see Espey, 1994). The ovulatory follicle receives the greatest volume of blood per unit time and has more permeable capillaries than other follicles. Before ovulation, cellular layers are broken down, which includes a layer of epithelial cells at the surface, connective tissue (tunica albuginea and theca externa), theca interna, and granulosa cells which are separated from the theca interna by the basal lamina and which line the follicular fluid-filled cavity. Surface vascularity increases except at the point of ovulation, which is devoid of blood vessels. Follicular volume increases

rapidly prior to ovulation (Janson, 1975) along with follicular elasticity (Espey, 1967; Rondell, 1964). According to Espey (1994) the entire process of ovulation of the follicle is similar to an acute inflammatory action.

Ovulation occurs as a result of interaction between endocrine and neuroendocrine systems, specifically GnRH, steroids, and prostaglandins. In addition, interactions exist among neuromuscular and neurovascular systems and enzymes. A gonadotropin-induced rise of follicular prostaglandins is necessary for ovulation (Ainsworth et al., 1975; 1984; Armstrong and Zamecnik, 1975). These eicosanoids may stimulate ovarian contractions (O'Shea, 1970) and activate proteolytic enzymes (Schochet, 1916; Lipner, 1988) to digest the follicle wall. Eicosanoids may also be involved in ovulation by altering the microcirculation of the follicle. In mammals, follicular concentrations of $PGF_{2\alpha}$ and PGE_2 increase during the first several hours of the ovulatory process, peaking at about the time of rupture and declining thereafter. The concentration of PGE_2 is about twice that of $PGF_{2\alpha}$ throughout the ovulatory process (Espey, 1994). By blocking synthesis of eicosanoids using inhibitors of cyclooxygenase, research has shown a reduction of LH-induced hyperemic response in rabbits (Lee and Novy, 1978), a reduction in follicular blood flow in ewes (Murdoch et al., 1983), prevention of hCG-induced increase in vascular permeability in rats (Abisogun et al., 1988), and follicular rupture in rats (Orczyk and Behrman, 1972; Tsafriri et al., 1972). The latter event was overcome in rats by administration of PGE_2 (Tsafriri et al., 1972) and in sheep by administration of PGE_2 and $PGF_{2\alpha}$ (Murdoch et al., 1986). Other eicosanoids such as leukotrienes may be involved in the ovulatory process because their

concentration increases modestly in rat ovaries during the first several hours and these compounds have been associated with inflammatory processes (Espey, 1994).

Steroids may also be involved in the rupture of the ovulatory follicle. In the early ovulatory process estradiol synthesis is high, while progesterone synthesis is negligible. This quickly reverses so that synthesis of estradiol is low and progesterone synthesis increases (Espey, 1994). By administering aminoglutethimide and cyanoketone to rats or isoxazol to sheep (all agents that block synthesis of progesterone) follicle rupture is inhibited (rats: Lipner and Greep, 1971; Lipner and Wendelken, 1971; sheep: Murdoch et al., 1986). This is further supported by experiments in rats designed to inhibit follicle rupture with epostane, which inhibits 3ß-HSD, an enzyme involved in progesterone synthesis (Snyder et al., 1984). This inhibition was overcome by administration of progesterone.

Ovulation Rate

Ovulation rate is defined as the number of follicles selected for ovulation. In sheep healthy follicles 2 mm in size are recruited, and once selection has occurred, recruitment is blocked. Ovulation rate varies among breeds of sheep due to different mechanisms of follicular recruitment (Driancourt and Cahill, 1984). For example, selection of ovulatory follicles occurred later and included smaller follicles in Booroola ewes, a prolific breed, compared to Merino ewes (Driancourt et al., 1985). In other breeds such as the Romanov, a greater number of large

follicles was available to be stimulated for ovulation compared with the less prolific Ile-de-France (Cahill et al., 1979). Similarly, morphological and functional differences in large antral follicles are apparent in Finnish Landrace compared with the less prolific Scottish Blackface or Merino x Scottish Blackface ewes (Webb et al., 1989). Differences in the pattern of follicular differentiation in Finnish Landrace ewes included an extended period of selection, fewer granulosa cells per follicle, and a reduced mitotic index in granulosa cells. To further support the phenomenon of extended period of selection, the preovulatory LH release occurred later in Finnish Landrace compared with Fingalway and Galway ewes and LH concentration was not different among breeds (Quirke et al., 1979). *In vitro* production of estradiol by follicles was similar among breeds, suggesting greater estradiol production per cell in Finnish Landrace ewes (Webb et al., 1989). McNeilly et al. (1987) attribute the high ovulation rate of Finnish Landrace cross ewes to a high incidence of estrogenicity in the large follicles, together with a low incidence of atresia following luteal regression. During the mid to late luteal phase of the cycle (days 10 to 13) plasma concentrations of progesterone were highest in Finnish Landrace ewes compared with those in the less prolific breeds.

Ovulation rate can be manipulated by a number of means. Nutritional alterations can lead to a higher rate of ovulation in farm animals and will be discussed below (see **Flushing**). Immunoneutralization of inhibin during the follicular phase of sheep and cattle results in elevated peripheral FSH concentrations and an increase in the number of follicles ovulating (O'Shea et. al, 1994). Inhibin, secreted from granulosa cells of large antral follicles (Findlay et al.,

1991), inhibits the synthesis and/or secretion of gonadotropins, preferentially FSH (Burger, 1988). It was discovered that Booroola ewes were deficient in bioactive ovarian inhibin and perhaps this contributed to an increase in ovulation rate in this breed (Cummins et al., 1983). Similarly, chronic FSH administration to ewes led to an increase in ovarian weight and ovulation rate (McNatty et al., 1993). Subcutaneous administration of anti-bovine LH antiserum to ewes on day 10 of the estrous cycle resulted in a higher ovulation rate with no differences in number of large and small follicles compared with controls (Fitzgerald et al., 1985). Plasma FSH was elevated within one day after treatment, further supporting its role in increasing ovulation rate.

Ovulation rate can also be manipulated by altering production of estradiol. By administering epostane (a 3ß-hydroxysteroid dehydrogenase inhibitor) to ewes on days 10 through 15 of the estrous cycle, ovulation rate increased through decreasing follicular steroidogenesis and not an increase in peripheral FSH concentrations (Webb et al, 1992). Pregnant mare serum gonadotropin (**PMSG**) administered at a number of stages in the reproductive cycle causes a rapid increase in estradiol production and an increase in follicle number (McCracken et al., 1971; Moor et al., 1978). Webb and Gauld (1985) suggest that PMSG acts to enhance ovulation rate by preventing follicular atresia and stimulating more follicles to develop.

Embyro-Maternal Interactions in Early Pregnancy

It is well established that 20-30% of fertilized ova in sheep die within the first few weeks of pregnancy (Edey, 1976). The greatest percentage of embryo loss occurs between days 2 and 30 of gestation which represents approximately 30% of potential lambs being born (Quinlivan et al., 1966) and constitutes a great economic loss to the sheep industry. In order to improve embryo survival, the underlying mechanisms of embryo development and elucidation of the factors that contribute to mortality must be considered.

Early Embryonic Development

Following fertilization the ovum is transported to the uterus within 66 to 72 hours and forms a blastocyst by day 6 to 7. Hatching of the blastocyst from the zona pellucida occurs on days 7 to 8. Evidence has been presented in the mouse demonstrating the requirement of PGE_2 in hatching of the blastocyst (Biggers et al., 1978). When embryos were cultured in the presence of PGE_2 agonists (7-oxa-13-prostynoic acid, medofenamic acid, indomethacin or phenidone), hatching of the blastocyst was prevented. Elongation of the blastocyst occurs between days 11 to 16, and is characterized by logarithmic growth. This rapid conceptus elongation occurs through continual hyperplasia of trophectoderm and extraembryonic endoderm. The developing embryoblast remains in the uterine horn ipsilateral to the CL (Hafez, 1993). Intrauterine migration may occur when there are multiple ovulations on the same ovary. Attachment of the conceptus to

the uterus occurs around day 16. First, a transitory attachment occurs as the trophoblast develops finger-like villi that project into the lumen of the uterine glands. These many points of attachment develop into a more complex system, where the vascularized cotyledons interlock with the placentomes of the uterine horns (Hafez, 1993).

Maternal Recognition of Pregnancy

Uterine $PGF_{2\alpha}$ controls the life span of the CL, regulating the length of the cycle in the nonpregnant ewe (McCracken et al., 1981). When $PGF_{2\alpha}$ was administered to ewes on day 4 of the cycle, premature luteal regression occurred, however when a similar dose was given to pregnant ewes on day 12 no luteolysis occurred (Inskeep et al., 1975), suggesting the presence of a substance that counteracts the luteolytic effects of $PGF_{2\alpha}$. When estradiol-17ß was given intramuscularly to ewes on two consecutive days after day 8 of the estrous cycle, premature luteal regression occurred (Stormshak et al. 1969), and when given to pregnant ewes on either days 11 and 12 or 12 and 13, luteolysis occurred in only 50% of the ewes (Kittok and Britt, 1977). In addition, if estradiol-17ß was given to hysterectomized ewes on days 11 and 12 of the estrous cycle, regression of the CL failed to occur, suggesting a role of the uterus on the action of estradiol on the CL (Stormshak et al., 1969). In the pregnant animal, the action of $PGF_{2\alpha}$ must somehow be blocked so that the CL can be maintained for the production of progesterone.

When embryonic homogenates are infused into the uterus of nonpregnant ewes by day 13, regression of the CL is blocked and progesterone continues to be secreted in quantities adequate to maintain pregnancy (Ellinwood et al., 1979). Prostaglandin E_2 may facilitate maternal recognition of pregnancy by exerting a luteotropic effect (Silvia et al., 1984). Prostaglandin E_2 has been isolated from the conceptus and the endometrium and has been shown to cause an increase in progesterone secretion *in vitro* (Fitz et al., 1984) and *in vivo* (Inskeep et al., 1975). When Pratt et al. (1977) administered PGE_2 to ewes, the estrous cycle was prolonged by 2 days. Beaver and Murdoch (1992) discovered greater concentrations of PGE_2-9-ketoreductase, an enzyme responsible for converting PGE_2 to $PGF_{2\alpha}$, in the endometrium of nonpregnant ewes compared with that of pregnant ewes on days 13 and 16 after estrus and mating, suggesting that regulation of this enzyme could be a mechanism for ensuring luteal maintenance and function during early gestation.

Another factor that may be responsible for CL maintenance is ovine trophoblast protein I (**oTP-I**), also known as IFN-tau. It is believed that oTP-I inhibits the episodic release of $PGF_{2\alpha}$ by the uterus (Godkin et al., 1984) or inhibits the rise in concentration of uterine OT receptors (Flint et al., 1991), preventing the development of endometrial responsiveness to OT-induced uterine production of $PGF_{2\alpha}$. *In vitro* secretion of oTP-I by the conceptus occurs between days 13 to 21 of gestation (Godkin et al., 1982) and *in vivo* secretion occurs between days 14 and 24 (Kazemi et al., 1988). A similar protein has been isolated in the cow, bovine trophoblast protein-I (**bTP-I**), which is also secreted by conceptuses *in vitro*

between days 15 to 25 of pregnancy (Bartol et al., 1985; Helmer et al., 1987). When infused into the uterus of nonpregnant ewes or cows, oTP-I or bTP-I can extend the duration of the estrous cycle by inhibiting endometrial secretion of $PGF_{2\alpha}$ (Knickerbocker et al., 1986; Thatcher et al., 1989; Roberts et al., 1990). These trophoblast proteins are related to IFN-α, which is a cytokine responsible for interfering with viral replication and regulation of the immune response. When recombinant bovine IFN-α was infused into the uterus of nonpregnant cows from days 15.5 to 21, the cycle was prolonged by 4 days and concentrations of serum progesterone did not diminish as in control cows (Plante et al., 1988). Similar actions occurred when this recombinant protein was injected intramuscularly around the time of maternal recognition of pregnancy (Plante et al., 1988), suggesting that this protein acts systemically as well as locally. When ewes were given intramuscular injections of recombinant bovine IFN-α between days 12 and 18 after breeding, embryo survival and pregnancy rates were greater than in control ewes (Nephew et al., 1990; Schalue-Francis et al., 1991). The possibility that oTP-I and bTP-I are acting directly on the CL to inhibit the luteolytic effect of $PGF_{2\alpha}$ must not be dismissed.

Factors Affecting Embryo Survival and Mechanisms Causing Loss

The greatest proportion of embryo loss occurs during early gestation. Some of this loss may be due to genetic abnormalities (lethal genes) and represents a favorable means of preventing abnormal animals from being born (Bishop, 1966). Causes of the remaining decrease in embryo survival may be due

to asynchrony between embryo and ewe, so that a failure to produce sufficient signals between conceptus and mother at the right time occurs (Wilmut and Sales, 1981; Heap et al., 1986). The embryo either fails to prevent luteolysis or a fatal modification occurs in development due to an inappropriate uterine environment.

Embryonic survival in pubertal animals is generally considered to be lower than mature animals and pregnancy rate from first estrous mating is lower than in multi-estrous animals (MacPherson et al., 1977; Byerly et al., 1987). Staigmiller et al. (1993) attributes this to an unfavorable uterine environment in heifers, where pregnancy rate at pubertal estrus was only 24% of heifers at third estrus; both groups of animals received oocytes of equivalent quality from mature cows on day 7 post-estrus by nonsurgical transfer. In contrast, embryos from either first- or second-estrous gilts transferred to first- or third-estrous recipients demonstrated that embryonic mortality in pubertal gilts was due to a deficiency of the oocyte rather than to an abnormal intrauterine environment in gilts (Archibong et al., 1992). Similarly, Koenig and Stormshak (1993) confirmed through cytogenetic evaluation that ova from first estrous gilts were defective compared with those of third-estrous gilts. Embryo mortality did not differ between first- and third-estrous recipients, however, survival of embryos from second-estrous donor gilts was greater (84.9%) than that of embryos transferred from first-estrous gilts (63.8%) (Archibong et al., 1992).

Differences in embryo survival rate have also been reported between ewes with single versus multiple ovulations. Edey (1976) uncovered greater losses in twin than single ovulators, because when one embryo of twins dies, the pregnancy

continues, but the second embryo is still at risk. Gunn and Doney (1975) found, inconclusively, a greater loss in ova shed singly than of ova shed as multiples; however, this may have been confounded with the effect of body condition, because ewes in poor body condition had more single than multiple ovulations.

If transportation of the ova to the uterus is accelerated or delayed, hormonal imbalances may occur resulting in early embryonic death. If signals from the conceptus are deficient due to its abnormally small size, it may not be able to counter the effects of luteolysis, thus regression of the CL occurs and pregnancy terminates (Wilmut et al., 1985).

Progesterone is vital for embryo survival and an insufficiency can lead to embryonic loss (Parr et al., 1982; Ashworth et al., 1987). Fluctuations in systemic progesterone due to various stress factors, including poor nutrition and climate, may be partly responsible for embryo mortality. If progesterone concentrations are inadequate, embryo survival is hindered. When systemic concentrations of progesterone were reduced by the administration of a progesterone inhibitor (4,5-epoxy-17-hydroxy-4,17,dimethyl-3-oxoandrostane-2-carbonitrile), pregnancy and embryo survival rates were reduced (Ashworth et al., 1987). Changes in nutrition after breeding can cause fluctuations in progesterone concentrations as discussed below.

NUTRITION AND REPRODUCTION INTERACTIONS

Flushing

Flushing of Ewes

Flushing of ewes around the time of breeding has long been practiced to increase ovulation rate, resulting in more lambs born per ewe. The term *flushing* refers to an elevated level of nutrient intake to increase body weight and condition prior to and during breeding. A response to flushing generally occurs in flocks with sub-optimal ovulation rates, which can occur for a number of reasons, including poor body condition and nutrition and high production demands (i.e., high lambing rate). Studies on the effects of flushing date back to Haepe (1899), Marshall (1908), Marshall and Potts (1921) and Clark (1934). The response to flushing is variable, dependent on a variety of factors including, but not limited to condition of the ewe prior to breeding, genetic differences, and duration of the flushing period (Gunn et al., 1972; Cumming et al., 1975; Rattray et al., 1981; Rhind et al., 1984). Generally, ewes in poor condition respond better to flushing because ewes in good condition already have optimal ovulation rates (Allen and Lamming, 1961; Coop, 1966a; Gunn et al., 1972). A positive relationship has been found between the duration of flushing and ovulation rate; maximal response occurs in those ewes flushed for a minimum of 30 to 60 days, with no further increase in ovulation rate thereafter (Allen and Lamming, 1961; Bellows et al., 1963a).

Genetics influence the effects of flushing prior to breeding. Ovulation rate in crossbreeds such as the Finnish Landrace (x Dorset x Rambouillet) are not influenced by level of nutrition before breeding as are many other breeds (Rhind and Schanbacher, 1991). Effects of ewe body condition or level of feeding on number of follicles greater than 4 mm and number of CL were not different in these crossbred ewes compared with their purebred counterparts. Ovulation rate remained high, despite differences in body condition or level of feed intake before mating. Results of this study contrast with those of other studies conducted to examine effects of body condition on ovulation rate, including that of Scottish Blackface ewes, in which ovulation rate was dependent on ewe body condition (McNeilly et al., 1987). The authors suggested that because the numbers of ova shed by the ewes were almost equal to the number of large estrogenic follicles, most of the estrogenic follicles present at luteal regression developed to the point of ovulation.

Mechanisms of Flushing

There are at least three possible biological pathways that could result in a flushing response. First, flushing may be explained by a direct effect of supplemental energy exerted on the ovary. Increasing level of feeding in ruminants results in an increase in production of short chain fatty acids by the rumen, including propionate, which is the major gluconeogenic precursor of the ruminant animal. Hence, elevated dietary energy can lead to in an increase in production of ATP. There is a series of biochemical events leading to this, initiated by an

influx of glucose precursors. Gluconeogenic precursors can be transported through the bloodstream to reach target tissues. Upon entering the cell, these molecules can be converted to pyruvate via glycolysis, which then can enter the citric acid cycle to generate ATP through the electron transport chain in the mitochondria. More energy available for cell maintenance functions, as well as production functions, such as cell division, may result in enhanced follicular growth.

A second theory explaining how flushing can lead to an increase in ovulation rate is that dietary energy can influence hypothalamic regulation of pituitary gonadotropin secretion (Coop, 1966b). Concentrations of the gonadotropins LH and FSH are reduced in the undernourished ewe due to reduced GnRH secretion (Foster et al., 1989). Also, ewes in moderate versus low body condition had increased LH and slightly higher FSH secretion during the follicular phase (Rhind and Schanbacher, 1991). It is possible that when ewes are flushed from a sub-maintenance to a maintenance level, GnRH secretion increases, allowing greater secretion of LH and FSH. Hence, follicle development would be stimulated and ovulation rate enhanced. Brien et al. (1976) noted higher plasma concentrations of FSH and elevated ovulation rate in lupin-supplemented compared to unsupplemented ewes 5 days, but not 2 days, prior to estrus. Haresign (1981) found no differences in pituitary or plasma LH concentrations at estrus, yet increased ovulation rate in ewes fed 200% maintenance compared with 100% maintenance was observed. Conversely, Rhind and Schanbacher (1991) studied the effects of feeding *ad libitum* versus maintenance level diet and reported lower mean LH concentration in ewes on the elevated intake during the luteal phase.

This trend continued into the follicular phase, where the mean LH pulse amplitude was significantly lower in ewes fed *ad libitum*. Similarly, gilts fed a medium level of nutrition had greater serum concentrations of LH than those fed a low or high level of nutrition 30 days prior to and after breeding (Dyck et al., 1980). Estienne et al. (1989) reported the acute effects of an increase in energy and found when ovariectomized ewes were given an infusion of lipid emulsion (20% Intralipid®) over a 10 hour period, there was no change in serum concentrations of LH compared with saline-infused ewes.

A third mechanism of flushing can be explained by the hepatic steroid metabolizing enzyme or mixed function oxidase (**MFO**) theory. A monooxygenase degrades steroids, such as progesterone and estrogens in the liver by incorporating one oxygen atom into the steroid, the other atom being reduced to water utilizing two hydrogen atoms from the reduced form of nicotinamide adenine dinucleotide phosphate (**NADPH**) (Matthews and van Holde, 1990). The steroid becomes hydroxylated and the NADPH, which is associated with a cytochrome, becomes oxidized. An increased level of feeding or elevated dietary protein leads to an increase in blood circulating to the liver (Bensadoun and Reid, 1962), which is the main site of steroid catabolism (Bedford et al., 1974); hence an increase in clearance rate of steroids is hypothesized (Dziuk, 1982; Thomas et al., 1984; Thomas et al., 1987). Concomitantly, a decrease in steroids is associated with an increase in gonadotropins (Thomas et al., 1984). This theory is supported by the fact that when phenobarbital, an effective inducer of MFO, and which is hydroxylated by cytochrome P450, was administered to nonpregnant cyclic ewes,

ovulation rate increased to 0.24 ova per ewe (Thomas et al., 1984). The MFO theory does not explain the lower mean LH concentration in *ad libitum* fed ewes in the study by Rhind and Schanbacher (1991) or greater serum concentrations of LH in gilts in the study by Dyck et al. (1980).

In each of these theories, follicular growth is stimulated, demonstrated by a greater number of follicles of larger class sizes (El-Sheikh et al., 1955; Lucy et al., 1990). These follicles have the potential to grow and develop to the point of ovulation. Therefore, feeding increased level of nutrition prior to breeding elevates the upper limit of ova shed, resulting in a higher lambing potential.

Flushing of Gilts

Flushing has also been practiced in swine. The effects seem to be limited to gilts rather than sows, because when sows are given supplemental energy prior to mating, there is no increase in ovulation rate (Zimmerman, 1966; Myers and Speer, 1973). When provided with supplemental energy above the maintenance requirements for 8 to 14 days prior to estrus, ovulation rate increased as determined by an increase of 2.2 to 3.6 CL (Self et al., 1955; Schultz et al., 1966; Edey et al., 1972; Aherne and Kirkwood, 1985; Cox et al., 1987).

Flowers et al. (1989) examined plasma concentrations of FSH and LH prior to estrus in flushed gilts compared to controls. Plasma concentrations of FSH were greater in flushed gilts between -5 and -3 days prior to estrus, however, there were no differences in plasma concentrations of LH between treatments. Pulses of LH per 6 hours were greater between -4 and -2 days prior to estrus in gilts

supplemented with energy. Similarly, Cox et al. (1987) reported an increase in number of pulses of LH in flushed gilts. The authors suggested that, as with ewes, flushing can stimulate follicular growth by elevating the secretion of gonadotropins. Flowers et al. (1989) also examined plasma concentrations of insulin and found that it tended to be greater in flushed gilts throughout the flushing period. The effect of flushing on gonadotropin secretion may by mediated by changes in insulin secretion.

It was suggested that the mechanism by which feeding influenced ovulation rate may have been by recruitment of more follicles during the flushing period prior to mating or by decreased follicular atresia, providing larger numbers of preovulatory follicles (Cox et al., 1987).

Post-mating Flushing

Extremes of nutrition after mating can cause an increase in embryo mortality. A low plane of nutrition after breeding decreases embryo survival probably due to a direct effect of undernutrition (Parr et al., 1982). A higher plane of nutrition after mating has been reported to cause a higher rate of embryo mortality in sheep (El-Sheikh et al., 1955; Cumming et al., 1975; Robertson and Hinch, 1990), goats (Mani et al., 1992), pigs (Self et al., 1955; Kirkwood and Thacker, 1988) and cattle (Robinson, 1986).

An inverse relationship has been found between level of nutrition and progesterone in gilts (Dyck et al., 1980), ewes (Parr et al., 1982; 1987) and cattle (Jordan and Swanson, 1979). Reduced survival rate may be due to increased

clearance rate of progesterone as discussed earlier or a lower secretion rate. When ewes were fed twice their maintenance requirement, plasma progesterone declined compared with that of ewes fed a maintenance diet (Parr et al., 1987). When a subset of these ewes were administered progesterone, embryo survival increased compared to those overfed ewes not receiving progesterone. Progesterone treatment did not affect embryo survival in ewes fed at maintenance or 25% maintenance. Diskin and Niswender (1989) noted little differences in embryo survival rate in ewes treated with exogenous progesterone from days 7 through 50 of gestation compared with untreated ewes. Ewes were fed 110% of NRC (1985) requirements, which may not have been significantly different from ewes fed maintenance level in the study by Parr et al. (1987).

Because of reduced embryo survival in animals that are overfed after mating, it has been suggested that by decreasing feed intake at this time, embryo survival may increase. However, when embryo survival is initially high in a group of animals, a reduction in feed intake may have no benefit. Pharazyn et al. (1991) found no change in embryo survival between gilts fed a low and high energy diet post-mating, and embryo survival rate was high in these particular gilts. Fertility was also high (>75% embryo survival) in the ewes utilized by Diskin and Niswender (1989), that failed to respond to progesterone treatment with increased survival of embryos.

There may be a critical time period during which nutrition affects embryo survival. Dyck and Strain (1983) reported that embryo survival decreased in gilts

when there was an increase in feed intake between days 0 through 10, but not days 11 through 20 of gestation.

Condition at mating contributed to nutrition-related embryo loss (Gunn et al., 1972). Ewes in poor condition at mating were more likely to have a higher rate of embryo loss. On the other hand, Rhind et al (1984) reported no differences in embryo mortality due to condition of ewe, although ovulation rates were higher in ewes of superior condition.

The variable effects of a higher plane of nutrition after breeding may be attributed to differences in breed. Dyck (1991) reported no effect of diet on embryo survival in crossbred gilts. Similarly, Cumming et al. (1975) demonstrated that crossbred ewes had lower rates of embryo mortality on an increased level of nutrition compared with Merinos. Conversely, Schultz et al. (1966) observed a decrease in embryo survival in crossbred gilts receiving a lower level of nutrition for 25 to 27 days post-mating.

LIPIDS

Lipids are a group of chemically diverse compounds that possess nonpolar structures, giving them low solubility in water. Fat, as it is often called, is comprised of one to three fatty acids esterified to glycerol. The three-fatty acid form, or triacylglycerol, is derived from the diet or mobilized from adipocyte storage. Lipids function as structural elements in cell membranes, provide energy for cell functions, and serve as precursors for steroidogenesis and eicosanoid synthesis. Dietary lipids represent a high energy substance capable of providing

37 kJ/g through oxidation compared to only 17 kJ/g for carbohydrates and proteins (Matthews and van Holde, 1990).

Fatty acids can be classified by chain length, which ranges from 1 to 35 carbons (C) or by their degree of hydrogenation, ranging from completely saturated to polyunsaturated with several double bonds present. Very short fatty acids, or volatile fatty acids, produced in the rumen by microbes include acetic acid (2-C), propionic acid (3-C), and butyric acid (4-C). The number of double bonds and their location is important in the metabolism of the fatty acid because there are specific enzymes that act to anabolize or catabolize the fatty acid at specific sites on the molecule (Byers and Schelling, 1988).

Lipid Metabolism

Fate of Dietary Lipid

Because lipids are insoluble in aqueous environments, their digestion, absorption, and transport becomes a challenge to the organism. In the monogastric animal, dietary lipid enters the stomach, where gastric lipase (active at pH 3 to 4) releases fatty acids of short and medium chain length from acylglycerols, which can be directly absorbed by the gastric mucosa. Partial hydrolysis makes fatty acids more susceptible to pancreatic lipase in the small intestine. Upon reaching the proximal part of the duodenum, bile salts are secreted from the gall bladder and act as a detergent to emulsify lipids. Salts of bile acids, such as cholic acid derived from cholesterol, are amphiphilic. This

characteristic allows them to dissolve at an oil-water interface so the hydrophilic surface can interact with the aqueous phase and the hydrophobic surface can interact with the nonpolar phase. The oil droplet at this stage becomes an emulsion of triacylglycerols, cholesterol esters, phospholipids, and vitamin esters. Cholesterol esters are then hydrolyzed and phospholipids converted to their lyso-derivatives. Co-lipase, an enzyme secreted by the pancreas, is adsorbed onto the surface of the oil droplet, acting as an anchor for the attachment of pancreatic lipase at the oil-water interface. The monoacylglycerols, fatty acids, lysophospholipids, cholesterol, and bile salts dissociate from the surface of the oil droplet to form a micellar solution. Micelles orient themselves so that the hydrophobic regions face in and the hydrophilic regions are exposed to the aqueous environment. Pancreatic lipase is unable to hydrolyze the ester bond at the 2-position of the glycerol molecule, so that 2-monoacylglycerol is the major form of acylglycerol that is absorbed from the duodenum. Intestinal digestion of lipids yields free fatty acids, mono- and diacylglycerols; resynthesis of triacylglycerols can also occur during absorption (Brindley, 1985; Matthews and van Holde, 1990).

Absorption occurs mainly in the jejunum when micelles contact the microvillus membranes of the enterocytes. Lipids are transported across the membrane by an energy-independent process, which relies on the maintenance of an inward diffusion gradient. Transportation and metabolism of cholesterol is much slower than triacylglycerols. During absorption, cholesterol becomes

incorporated into the membranes of the enterocytes, diluted with existing cholesterol, and most is esterified to a fatty acid, mainly oleic acid (Brindley, 1985).

Lipids are transported to tissues in the form of lipoproteins, which consist of phospholipids, cholesterol, and apoproteins, of which the polar head groups point towards the aqueous environment. In humans, chylomicrons are the major lipoprotein responsible for transporting triacylglycerol and some cholesterol from the intestine. Size and composition of the lipoproteins varies with the composition of the diet, rate of lipid absorption, apoprotein synthesis, and species of animal. Percentage of cholesterol in triacylglycerol-rich lipoproteins is low, but increases as they are metabolized by lipoprotein lipase and hydrolysis of the triacylglycerol (Brindley, 1985).

Chylomicrons are formed when triacylglycerols are resynthesized in the smooth endoplasmic reticulum of the enterocyte. Triacylglycerols form a lipid droplet that is coated with phospholipids and proteins synthesized in the rough endoplasmic reticulum. Nascent chylomicrons, and to a small extent very low density lipoproteins (**VLDL**), are carried to the lateral surface of the enterocyte and exocytosed. Lipoproteins are secreted into the intercellular spaces that drain into the lymph vessels. Lipoproteins then pass via the thoracic duct into the systemic circulation at the level of the jugular vein (Brindley, 1985).

Because lipoprotein lipase acts on lipoproteins, triacylglycerol is depleted resulting in what is known as remnant particles that are primarily metabolized by the liver (Bisgaier and Glickman, 1982). Remnant particles are more completely hydrolyzed to form low density lipoproteins (**LDL**) rich in cholesterol esters. These

LDL are metabolized by the liver and peripheral tissues (Kovanen, 1987) and function to transport cholesterol to peripheral tissues and regulate endogenous cholesterol synthesis.

High density lipoproteins are another type of lipoprotein high in cholesterol and are secreted by the intestine and liver as nascent disc-shaped particles. Lecithin cholesterol acyl transferase (**LCAT**) transforms these HDL into spherical particles by hydrolyzing linoleic acid from surface lecithin and re-esterifying it to surface cholesterol (Marcel, 1982). A sphere is formed as a portion of the resulting cholesterol ester moves into the core. Larger, denser HDL can be formed by the addition of phospholipid, cholesterol, and protein, that were displaced during metabolism of other triacylglycerol-rich lipoproteins (Nicoll et al., 1980; Tall and Small, 1980).

Upon reaching the target tissue, triacylglycerols can be hydrolyzed to yield free fatty acids and glycerol at the cell surface by lipoprotein lipase found in the capillary beds of extrahepatic tissues, including skeletal muscle and adipose tissue. A lipoprotein lipase lies at the end of a polysaccharide chain and anchors the chylomicron as it interacts with apoprotein C-2 on the chylomicron surface. Free fatty acids carried by serum albumin can cross the plasma membrane, though this mechanism is poorly understood (Schulz, 1985; Matthews and van Holde, 1990).

The metabolic fate of fatty acids depends on the state of the cell into which they are delivered. If uptake exceeds the energy requirement, fatty acids are stored as triacylglycerol in adipocytes (Brindley, 1985). Fatty acids used for energy must be oxidized. Once in the cytosol of the cell, free fatty acid must be

activated to an acyl coenzyme-A (**Co-A**) so that it can be transported via a carnitine shuttle across the mitochondrial membrane to the mitochondrial matrix. The initial oxidation of fatty acyl-CoA occurs at the ß-carbon, with a series of steps releasing two-carbon fragments in the form of acetyl-CoA. Acetyl-CoA can enter the citric acid cycle, where it is oxidized to carbon dioxide generating reduced electron carriers, whose reoxidation in the mitochondria produces ATP through oxidative phosphorylation. For example, one mole of palmitic acid (16C) yields 130 moles of ATP compared to a net of 38 moles of ATP from one mole of glucose (6C carbohydrate). Acetyl-CoA can also be used for synthesis of cholesterol, which will be discussed under **Cholesterol Biosynthesis** (Matthews and van Holde, 1990).

Mobilization of Fatty Acids

The release of fat from adipocytes is hormonally regulated through a cAMP-mediated system to meet the energy requirements of the organism. The hormones involved are glucagon, under fasting conditions, and epinephrine or adrenocorticotropin under stress conditions. Hormonal activation of adenylate cyclase generates cAMP which activates protein kinase A, which in turn phosphorylates triacylglycerol lipase (also known as hormone-sensitive lipase) that hydrolyzes triacylglycerol, causing the release of a fatty acid from the 1- or 3-C position yielding an unesterified fatty acid and diacylglycerol. This is followed by a similar series of reactions for the release of the remaining fatty acids leaving a molecule of glycerol. Non-esterified fatty acids passively diffuse through the cell

membrane and are transported through the bloodstream bound to albumin. The fatty acid is released from this protein and is taken up by tissues, mainly by passive diffusion. Glycerol released into the bloodstream is transported to the liver where it is used as a gluconeogenic precursor to glucose (Matthews and van Holde, 1990).

Acetyl-CoA can accumulate in the liver beyond its capacity to be completely oxidized due to depletion of oxaloacetate, an intermediate in the citric acid cycle. Acetyl-CoA is then metabolized through ketogenesis to ketone bodies; ß-hydroxybutyrate, acetoacetate, and to a small extent acetone. These ketone bodies are transported from the liver to other tissues where they can be reconverted to acetyl-CoA for generation of energy (Matthews and van Holde, 1990).

Ruminants are more susceptible to ketosis than other species, especially cows in early lactation and ewes carrying multiple fetuses, due to high energy demands that exceed energy intake. Ketone bodies are poorly utilized and high levels of acetoacetic acid and acetone can depress the central nervous system. Ruminant animals have limited capacity to export triacylglycerol from the liver (Grummer and Carroll, 1991) and an accumulation can reduce gluconeogenesis (Grummer and Carroll, 1988) and cause development of fatty liver, contributing to ketosis. Symptoms of ketosis include inappetence, incoordination, weight loss, decreased milk production in cows, and even death in ewes (Schultz, 1988).

Biosynthesis of Fatty Acids

Fatty acid biosynthesis utilizes acetyl-CoA derived from a number of different pathways. When carbohydrates are consumed in excess, they are metabolized through glycolysis to acetyl-CoA, which is transported from the mitochondrial matrix to the cytosol through a citrate shuttle where it can be converted to fatty acids. The majority of fatty acid biosynthesis takes place in adipocytes. The first committed step in this synthesis, which is virtually irreversible, is the formation of malonyl-CoA from acetyl-CoA via the enzyme acetyl-CoA carboxylase. The fatty acid chain is built up by successive additions of 2-carbon units; each addition consists of seven reactions associated with fatty acid synthase. The resulting fatty acid is palmitate, which can be elongated in both the mitochondria and the endoplasmic reticulum (Matthews and van Holde, 1990).

Regulation of Fatty Acid Metabolism

Fatty acid biosynthesis is controlled by hormonal mechanisms, as well as concentration of glucose in the blood. After consumption of a meal high in carbohydrates, serum glucose and insulin rise and the latter stimulates fatty acid and glycogen synthesis by activating glucose entry into cells. Glucose in the cytosol undergoes glycolysis and is converted to pyruvate. Pyruvate is then dehydrated to acetyl-CoA via pyruvate dehydrogenase complex; this reaction is stimulated by insulin. At the same time, ß-oxidation of fatty acids and gluconeogenesis is suppressed. Acetyl-CoA is synthesized to fatty acids, which

can then be incorporated into triacylglycerols. These esterified fatty acids are then packaged into VLDL, secreted into the blood and transported to adipose tissue for storage (Brindley, 1985). Triacylglycerols are targeted for adipose tissue because muscle lipase, the enzyme that hydrolyzes fatty acids from glycerol, is stimulated by glucocorticoids rather than insulin (Cryer, 1981). Lipoprotein lipase activity is stimulated by insulin, facilitating the hydrolysis of triacylglycerol for entry into the adipocyte.

When an animal is stressed due to starvation or trauma, plasma insulin concentrations are reduced relative to glucagon, the catecholamines, corticotropin and metabolic glucocorticoids. Lipolysis and release of fat from storage occurs for energy utilization. Glucagon and epinephrine stimulate adenylate cyclase, which leads to phosphorylation and activation of triacylglycerol lipase. Meanwhile, enzymes of triacylglycerol synthesis are inhibited and fatty acid and glycerol are released into the blood (Brindley, 1985).

Digestion and Metabolism of Lipids in Ruminants

Compared to nonruminant animals, which can utilize a diet of 15 to 20% fat, digestive upset can occur in ruminants consuming a diet of more than 8 to 10% fat. The digestion of lipid is vastly different between the two types of species. Ruminants consuming forages typically ingest between 2 to 4% fat (Schingoethe, 1988). High production demands have stimulated researchers and producers to test high energy feeds, such as fats, which are superior over carbohydrate sources

in meeting the increased energy demand of the animal. Feeding lipids to ruminants, however, can be challenging because of the rumen environment.

Lipids reaching the rumen are first degraded by microbes, which possess lipases that influence the digestion of triacylglycerols. Microbial hydrolysis of esterified fatty acids occurs rapidly to release free fatty acids and glycerol, followed by biohydrogenation, adding hydrogen to unsaturated fatty acids. Saturation is usually not complete, resulting in a variety of fatty acids. Feeding high grain diets decreases populations of bacteria responsible for this action, leading to even less complete biohydrogenation, and allowing greater escape of feed and lipids from the rumen resulting in greater availability to the animal. Because of their active contribution to biohydrogenation when populations of protozoa are low, a greater concentration of unsaturated fatty acids reaches the blood, milk, and adipose tissue (Byers and Schelling, 1988).

A diet including lipid at greater than 8 to 10% can decrease overall digestion by inhibiting fiber digestion, reducing microbial cell numbers and growth, and reducing volatile fatty acid production, the major source of gluconeogenic precursors available to the ruminant. Unsaturated fatty acids increase cell surface activity of bacteria, and change the permeability of membranes. Lipid physically coats fiber, preventing bacterial utilization and reducing cation availability through formation of fatty acid soaps (Schingoethe, 1988).

Ruminants have less pancreatic lipase activity than nonruminants, which results in less hydrolysis of triacylglycerol in the small intestine. This does not usually create a problem because rumen microbes can also hydrolyze

triacylglycerols, however, when protected fat (chemically treated fat that can escape rumen degradation) is fed, lipid digestion becomes limited (Schingoethe, 1988).

Lipids reach the duodenum of ruminants as unesterified, highly saturated fatty acids. The pH of the proximal small intestine is low due to rumen ingesta flowing from the abomasum and limited buffering capacity. This results in protonation of fatty acids, hence, fatty acid soaps insoluble in the rumen become solubilized, allowing greater absorption. At this point, digestion of fatty acids becomes similar to that of monogastric animals. Formation of micelles occurs, due to association of bile acids, and absorption occurs by the enterocytes, although at a more proximal point of the small intestine than nonruminants. In the ruminant, fatty acids in the enterocyte are predominantly incorporated into VLDL rather than chylomicrons, due to the saturated nature of the fatty acid, which favors synthesis of VLDL (Byers and Schelling, 1988). There are a few differences between ruminants and nonruminants in post-absorptive synthesis and metabolism of lipids. Fatty acid synthesis is minimal in the liver and more extensive in adipose tissue (Hood, 1982). Acetate, ß-hydroxybutyrate, and lactate act as precursors for fatty acid synthesis, because there is little glucose available for lipogenesis (Byers and Schelling, 1988).

Adverse effects of dietary fat to rumen digestion can be eliminated by the use of ruminally inert fats. Calcium salts added to fatty acids forms insoluble soaps, preventing interaction with rumen microbes (Byers and Schelling, 1988). Protein can also be used to coat the surface of oil droplets and then treated with

formaldehyde. This creates a complex resistant to degradation under rumen conditions, but is dissociated by the acidity of the abomasum. Oil seeds contain naturally encapsulated fat that breaks down slowly providing some unsaturated fatty acids to the small intestine of the ruminant (Van Soest, 1982).

Cholesterol

Biosynthesis

Cholesterol is required for synthesis of new cell membranes, bile acids, and steroidogenesis. *De novo* synthesis occurs in the cytosol and endoplasmic reticulum mainly in the liver and small intestine and is regulated by the rate at which cholesterol enters cells from the bloodstream. Dietary cholesterol efficiently suppresses cholesterol synthesis in the cell.

The first series of reactions begins with the conversion of acetyl-CoA to mevalonic acid. Acetyl-CoA condenses with another molecule of acetyl-CoA catalyzed by a thiolase giving acetoacetyl-CoA. Condensation of yet another molecule of acetyl-CoA by hydroxymethylglutaryl-CoA (**HMG-CoA**) synthase yields HMG-CoA. This molecule is reduced to mevalonic acid by HMG-CoA reductase, which is considered the rate-limiting enzyme of cholesterol synthesis. Tight regulation of HMG-CoA reductase exists in the cellular matrix associated with the level of cellular cholesterol.

The next series of reactions includes the conversion of mevalonic acid to squalene. Mevalonic acid undergoes three successive phosphorylations catalyzed

by separate kinases. Formation of isopentenyl pyrophosphate follows with the release of carbon dioxide, ADP, and inorganic phosphate. Isopentenyl pyrophosphate can isomerize to dimethylallyl pyrophosphate, the latter condensing with another molecule of isopentenyl pyrophosphate to give geranyl pyrophosphate. Another molecule of isopentenyl pyrophosphate condenses with this product to yield a 15-carbon farnesyl pyrophosphate. Two of these molecules join via the enzyme farnesyl transferase, which is bound to the endoplasmic reticulum membrane, yielding presqualene pyrophosphate. Elimination of a pyrophosphate and rearrangement of the molecule produces squalene.

The final series of reactions occurs in the endoplasmic reticulum, converting squalene to cholesterol. Cyclization of squalene to lanosterol occurs in two steps through a mixed-function oxidase, squalene monooxyenase, and a cyclase enzyme. Nineteen more reactions follow, including reduction of double bonds and three demethylations (Fielding and Fielding, 1985; Matthews and van Holde, 1990).

Lipoprotein Cholesterol Uptake by Steroidogenic Cells

Low density lipoprotein serves to transport cholesterol to the cell for uptake. In most tissues, HDL functions in removing excess cholesterol from the cell (Oram, 1986); however, in steroidogenic tissues, HDL serves to provide cholesterol to the cell, much like LDL (Andersen and Dietschy, 1981; Strauss et al., 1982). Studies have shown that serum lipoproteins are required for maximal luteal progesterone synthesis (Pate and Condon, 1982; O'Shaughnessy and Wathes, 1985). Both LDL

and HDL cholesterol can be utilized for steroidogenesis, however, the mechanism of uptake appears to differ between the two lipoproteins.

Low density lipoprotein cholesterol is taken up by fibroblasts through a receptor-mediated endocytosis (Brown and Goldstein, 1986). Endocytosis is the process by which cells take in large molecules from the extracellular environment. Low density lipoproteins possess a protein on the hydrophilic portion of the molecule, apoprotein B-100, that acts as a ligand for the LDL receptor. In fibroblast cells, these lipoprotein receptors are clustered in a structure called a coated pit, an invagination of the plasma membrane surrounded by the protein, clathrin (Paavola et al., 1985). Following the binding of ligand and receptor, the complex is internalized. The plasma membrane fuses around the LDL molecule becoming an endocytotic vesicle. Several of these vesicles fuse to become an endosome, which then fuse with a lysosome. Lysosomes contain enzymes that function in hydrolysis of the protein component of LDL to amino acids and cholesterol esters to free cholesterol and fatty acids. The receptor is recycled and translocated to the plasma membrane to pick up more LDL. Approximately 10 minutes is required to complete one cycle.

Much of the free cholesterol is transferred to the rough endoplasmic reticulum, where it is used for membrane synthesis. Cholesterol has three regulatory effects on the cell: (1) suppression of HMG-CoA reductase, the rate-limiting enzyme in *de novo* cholesterol synthesis, and suppression of transcription of the gene encoding this enzyme, (2) activation of acyl-CoA: cholesterol acyltransferase (**ACAT**), which re-esterifies cholesterol to a long chain acyl-CoA for

cellular storage, and (3) down-regulation of LDL receptor synthesis by lowering the mRNA content for the receptor, thus inhibiting further cholesterol uptake (Brown and Goldstein, 1986).

The mechanism by which HDL are taken up by the cell is less clear. There are a number of different mechanisms proposed. First, some cell types appear to take up cholesterol from HDL by its association with the cell and transfer of cholesterol by passive diffusion (Karlin et al., 1987; Oram et al., 1987; Johnson et al., 1988). Second, HDL has been hypothesized to transport cholesterol to cells and endocytosis occurs, the same as for LDL-mediated endocytosis. This occurs only in those HDL possessing apoprotein E, which is recognized by the LDL receptor (Mahley et al., 1984; Driscoll et al., 1985). Apoprotein E is found on cholesterol ester-rich HDL obtained from cholesterol-fed swine, rats, dogs, monkeys, rabbits, and humans (Mahley, 1983), but not cattle (Cordle et al., 1985; Brantmeier et al., 1988).

The third mechanism proposed is that of internalization of HDL by a non-lysosomal endocytotic pathway in ovarian luteal cells (Paavolo and Strauss, 1983; Rajan and Menon, 1987; Rahim et al., 1991) that requires microtubules (Rajan and Menon, 1985) and releases apoproteins without degrading them (Schreiber et al., 1982; Rajan and Menon, 1987). A HDL-binding protein has been identified, which acts as a receptor for the uptake of HDL cholesterol. Studies have demonstrated the presence of a HDL-binding protein associated with the plasma membrane of bovine luteal tissue (Ferreri and Menon, 1992) and rat ovary (Ferreri and Menon, 1990). The HDL receptor binds to apoproteins A-1, A-2, and possible other

homologous apoproteins (Hwang and Menon, 1985; Fong et al., 1987), but not apoprotein B (Rajandran et al., 1983) or apoprotein E (Rifici and Eder, 1984). Maximal uptake of HDL cholesterol esters requires apoprotein A-1 (Rajan and Menon, 1988). Over-expression of HDL-binding protein in mammalian cells is associated with enhanced HDL-binding activity, supporting its role as a HDL receptor (Graham and Oram, 1987). Further evidence for the HDL-binding protein acting as a receptor has been presented for rat luteal cells; cells treated with a proteolytic enzyme inhibiting HDL binding displayed decreased progesterone synthesis (Aitken et al., 1988).

The Role of Lipoproteins in Progesterone Production

The major source of cholesterol for *in vivo* production of steroids in rat adrenal, ovary, and testis is serum lipoproteins (Andersen and Dietschy, 1978). *In vitro* progesterone production by granulosa cells (bovine: Savion et al., 1982; porcine: Veldhuis et al., 1984) and luteal cells (ovine: Wiltbank et al., 1990; bovine: Pate and Condon, 1982; O'Shaughnessy and Wathes, 1985; Carroll et al., 1992; rat: Schuler et al., 1981; human: Carr et al., 1981) in culture is greatly enhanced by the presence of serum lipoproteins, although LDL uptake by luteal cells declines with advancing age of the corpus luteum (porcine: Brannian et al., 1994). Similarly, *in vivo* experiments in the cow suggest a role of lipoproteins in progesterone synthesis (Talavera et al., 1985). These latter researchers demonstrated that a diet-induced increase in serum concentrations of total cholesterol (which includes

free cholesterol and that associated with lipoproteins) results in increased progesterone production during the mid- to late luteal phase.

Whether HDL or LDL is used as the predominant lipoprotein for progesterone synthesis is dependent on species. Vascularized steroidogenic tissue can use cholesterol from either HDL or LDL (Grummer and Carroll, 1988), however, HDL is the only lipoprotein found in follicular fluid of some species (bovine: Savion et al., 1982; Brantmeier et al., 1987; porcine: Chang et al., 1976; human: Simpson et al., 1985; Enk et al., 1986). This suggests that progesterone synthesis in granulosa cells of these species uses HDL rather than LDL cholesterol. It was determined that ovine luteal cells also prefer HDL over LDL for progesterone production (Wiltbank et al., 1990). Rat luteal and adrenal cells prefer LDL to HDL cholesterol as a substrate (Andersen and Dietschy, 1978; Schuler et al., 1981). On the other hand, human luteal cells prefer LDL to HDL for progesterone synthesis (Carr et al., 1981).

Carroll et al. (1992) determined that bovine luteal cells can utilize cholesterol that was not associated with lipoproteins for progesterone synthesis. Phosphatidylcholine liposomes or bovine serum albumin (BSA) containing non-esterified cholesterol stimulated progesterone production by 50 and 108% of that obtained with HDL or LDL. Cholesterol esterified to linoleic acid did not increase progesterone production by the cells. This suggests that free cholesterol, but not cholesterol esters, are imported to the cell by a receptor-independent process. Similarly, in rat luteal cells treated with reconstituted liposomes containing free cholesterol with or without apoprotein A-1, progesterone synthesis was enhanced

(Rajan and Menon, 1988). Liposomes containing cholesterol esterified to linoleic acid did not increase cell production of progesterone except in the presence of apoprotein A-1, suggesting that cholesterol esters must be taken up by the cell by a receptor-dependent process.

Lipoprotein utilization for progesterone synthesis may be regulated by prostaglandins. Pate and Nephew (1988) revealed that *in vitro* lipoprotein-stimulated synthesis of progesterone by bovine luteal cells was inhibited by $PGF_{2\alpha}$. Subsequent studies suggested that $PGF_{2\alpha}$ is inhibitory by allowing entry of lipoproteins into the cell, but preventing their utilization for steroidogenesis (Pate and Condon, 1989). They also discovered that $PGF_{2\alpha}$ can suppress *de novo* cholesterol synthesis and decrease the conversion of cholesterol to progesterone. Wiltbank et al. (1990) determined that $PGF_{2\alpha}$ acts to suppress lipoprotein-stimulated progesterone synthesis in ovine large luteal cells through the protein kinase C second messenger pathway. Luteal cells were made protein kinase C-deficient by treatment with a phorbol ester. Progesterone production was reduced in normal large cells but not those treated with phorbol ester. Progesterone production was increased by the presence of lipoproteins in both normal and protein kinase C-deficient cells, but when $PGF_{2\alpha}$ was added to medium, progesterone production was inhibited in normal, but not protein kinase C-deficient cells.

Eicosanoids

Eicosanoids are a class of lipids consisting of 20-carbon fatty acids derived from arachidonic acid (5-cis-,8-cis-,11-cis-,14-cis-eicosatetraenoic acid) that function as local hormones. Prostaglandins, thromboxanes, and leukotrienes are synthesized in nearly every known cell type and perform a wide variety of functions, including regulation of water balance, lipolysis, and body temperature, induction of platelet aggregation, chemotactic and myotropic activities, and luteolytic and luteotropic effects on the corpus luteum. Eicosanoids are not stored and are rapidly catabolized to inactive forms, mainly by the lung, which makes it difficult to study the actions of the free acid forms of these biochemicals (Smith and Borgeat, 1985).

Biosynthetic Pathway

Linoleic acid, which has 18C and two double bonds (C18:2), and linolenic acid (C18:3) are two essential fatty acids required in the mammalian diet. These molecules are the only fatty acids that can be converted to arachidonic acid and their availability is the major determinant of the amount of prostaglandins, thromboxanes or leukotrienes synthesized. To form arachidonic acid, linoleic acid condenses with Co-ASH and undergoes two desaturations and an elongation to form arachidonyl-CoA. Most of this is used for the synthesis of phospholipids, whereas arachidonic acid used for eicosanoid biosynthesis is derived from hydrolysis of phospholipids (Matthews and van Holde). Arachidonic acid is found

to a large extent esterified to membrane phosphoglycerides, which when acted upon by a phospholipase hydrolyzes the acid to its free form. Physiological stimuli of arachidonic acid release include hormones such as angiotensin II, bradykinin, and epinephrine; proteases such as thrombin; and certain antibody-antigen complexes. Each cell capable of eicosanoid synthesis is acted on by a factor specific to that cell. Arachidonic acid can follow two pathways of oxygenation to form cyclooxygenase or lipoxygenase products (Smith and Borgeat, 1985).

The formation of cyclooxygenase products, which includes prostaglandins and thromboxanes, begins with the conversion of arachidonic acid to PGH_2 by the enzyme PGH synthase. Actually, this membrane-bound hemoprotein is a complex consisting of cyclooxygenase, which oxygenates arachidonic acid with two molecules of oxygen to form PGG_2, and hydroperoxidase, which involves a two-electron reduction of PGG_2 to PGH_2. Cyclooxygenase requires a hydroperoxide to catalyze the oxygen insertion reaction and is capable of undergoing a self-catalyzed destruction both *in vivo* and *in vitro*. This limits the production of prostaglandins over time. Finally, PGH_2 is converted to the biologically active products PGE_2 or PGD_2 by isomerases, $PGF_{2\alpha}$ by a reductase, PGI_2 (also called prostacyclin) or thromboxane by synthases, each reaction dependant on the cell type. Prostaglandin E_2 can also be reduced to $PGF_{2\alpha}$ by PGE_2-9-ketoreductase in ovine ovarian and uterine tissue (Beaver and Murdoch, 1992). The enzymes catalyzing prostaglandin formation are associated with the endoplasmic reticulum. The prostaglandins formed differ from each other in the number of double bonds and the locations of oxygen groups.

Prostaglandin formation is suppressed by glucocorticoids, as well as nonsteroidal anti-inflammatory drugs, such as aspirin, indomethacin, and ibuprofen, which inhibit cyclooxygenase activity. These drugs compete with arachidonic acid for binding to the cyclooxygenase active site, preventing the initial oxygen insertion reaction. Aspirin causes a covalent modification of PGH synthase by acetylating a serine residue, presumably at or near the active site, an irreversible reaction. Cells must then synthesize new enzyme to restore prostaglandin biosynthetic activity. Conversely, aspirin and indomethacin have no effect on hydroperoxidase activity (Smith and Borgeat, 1985). Oldick et al. (1994) have demonstrated inhibition of $PGF_{2\alpha}$ synthesis in bovine uterine tissue incubated with high concentrations of linoleic acid (25 and 200 μM vs. 10 μM) over time and no increase in synthesis with the addition of arachidonic acid. Linoleic, as well as oleic and linolenic acid inhibits prostaglandin synthesis likely by competing with arachidonic acid for binding of cyclooxygenase (Pace-Asciak and Wolfe, 1968). Other inhibitors of cyclooxygenase activity include some, but not all, epoxyeicosatrienoic acids (Fitzpatrick et al., 1986) and docosahexaenoic acid, a major polyunsaturated acid component of fish oil (Corey et al., 1983).

Arachidonic acid can also be acted upon by lipoxygenase to yield hydroperoxy-eicosatetraenoic acids (**HPETEs**), which can then be converted to either hydroxy-eicosatetraenoic acids (**HETEs**), dihydroxy-eicosatetraenoic acids (**diHETEs**), or an epoxy fatty acid. This is a simple dioxygenase reaction, unlike PGH synthase which catalyzes a net oxidation-reduction reaction. Epoxy fatty acids can be transformed to a number of epimeric diHETEs or to leukotrienes.

The synthesis of HPETEs and HETEs is generally associated with cells of the immune system: neutrophils, eosinophils, monocytes, mast cells, macrophages, and platelets. Synthesis of leukotrienes requires the release of arachidonic acid and activation of lipoxygenase mainly by immunological and inflammatory stimuli (Smith and Borgeat, 1985). Less is known about how these compounds function in reproduction, though it is likely they play a role.

The Role of Prostaglandins in Reproduction

Prostaglandin E_2 and $PGF_{2\alpha}$ play an essential role in many reproductive functions. Cyclooxygenase inhibitors have aided in determining the *in vivo* requirement of prostaglandins in specific tissues followed by local administration of exogenous prostaglandins. For example, studies have revealed enhanced fertility in dairy cattle routinely injected with $PGF_{2\alpha}$ (Young et al., 1984; Benmrad and Stevenson, 1986) and premature luteolysis is associated with $PGF_{2\alpha}$ administered to ewes early in the cycle (Inskeep et al., 1975). Luteotropic effects have been exhibited in small luteal cells incubated with $PGF_{2\alpha}$, PGE_2, and PGI_2, and in large luteal cells cultured with PGE_2 and PGI_2 (Alila et al., 1988). Also cows given exogenous PGI_2 (Milvae and Hansel, 1980) or PGE_2 (Thibodeaux et al., 1992) displayed increased progesterone production.

Villeneuve et al. (1987) demonstrated an increase in follicular recruitment in beef cattle given exogenous $PGF_{2\alpha}$. Lucy et al. (1990) reported elevated levels of $PGF_{2\alpha}$ in dairy heifers infused with long chain fatty acids, which was associated with larger follicle number and diameter. A direct mechanism of effect can be

explained by $PGF_{2\alpha}$'s apparent mitogenic action which occurred in rat intestine (Johnson and Guthrie, 1976) and may also occur in granulosa cells of the follicle. An indirect effect of $PGF_{2\alpha}$ may be to enhance the release of pituitary gonadotropin, which has been demonstrated in cows (Hafs et al., 1974). Alternatively, $PGF_{2\alpha}$ may act to enhance estradiol production (Carlson et al., 1973), which is associated with increased follicular recruitment (Lucy et al., 1990).

It is hypothesized that the ratio of uterine secretion of PGE_2 to $PGF_{2\alpha}$ influences the maintenance of the CL (Beaver and Murdoch, 1992). It is possible that regulators exist that control PGH_2 conversion to PGE_2 or $PGF_{2\alpha}$, dependent on stage of the estrous cycle. It is known that PGE_2 can be reduced to $PGF_{2\alpha}$ by the enzyme PGE_2-9-ketoreductase, which has been isolated from ovaries (Watson et al., 1979; Murdoch and Farris, 1988) and uteri (Kruger and Schlegel, 1986) in several species. Beaver and Murdoch (1992) demonstrated decreased activity of this enzyme on days 13 and 16 of pregnancy in ewes, a time when maintenance of the CL is critical for embryo survival. Perhaps in the nonpregnant animal there are regulatory factors controlling $PGE_2/PGF_{2\alpha}$ ratio, which may influence cycle length. Lucy et al. (1990) observed a lipid-induced increase in $PGF_{2\alpha}$ in heifers, which resulted in shortened estrous cycles; levels of PGE_2 were not measured. Conversely, treatment with PGE_2 has been associated with extended cycle length (ewes: Pratt et al., 1977; cows: Gimenez and Henricks, 1983). Fitz et al. (1993) demonstrated a biphasic effect of PGE_2 on ovine luteal cells: low concentrations stimulated progesterone secretion, whereas high concentrations reversed this luteotropic effect. Similar results were observed when luteal cells were incubated

with $PGF_{2\alpha}$ alone or in combination with PGE_2, suggesting the luteolytic role of $PGF_{2\alpha}$ in preventing PGE_2-induced stimulation of luteal cell steroid synthesis. On the other hand, it has been demonstrated that PGE_2 attenuates $PGF_{2\alpha}$-induced luteolysis in several species (Reynolds et al, 1981; Zelinski-Wooten and Stouffer, 1990) by causing inhibition of intracellular calcium release induced by $PGF_{2\alpha}$ (Wiepz et al., 1993). It is difficult to ascertain this delicate balance between the luteotropic and luteolytic prostaglandins *in vivo* and what mechanisms cause a shift of dominating action on luteal function from one to the other. Under *in vivo* conditions, endogenous biosynthesis of eicosanoids, as well as other factors such as change in metabolism, could influence luteal cell function.

Lipid Emulsions

Intravenous glucose and saline infusions were developed in the late 1800's. Lipid emulsion infusions were developed in the 1940's to supply adequate quantities of energy for post-operative procedures in humans. As outlined earlier, it is difficult to study the effects of specific fatty acids in the ruminant animal because they are hydrolyzed and hydrogenated in the rumen to saturated fatty acids. Also, diets containing greater than 10% lipid can cause digestive disturbances. Lipid emulsions can be used as a tool in ruminants, as well as other species, to define the role of fatty acids.

Schuberth and Wretlind (1961) generated a lipid emulsion consisting of soybean oil and egg yolk phospholipids that did not cause toxic reactions as did earlier lipid emulsions. Other lipid emulsions that were less successful consisted

of cottonseed oil as the lipid and soybean phospholipids as the emulsifier. When infused into dogs, there were adverse effects such as severe anemia, blood urine and feces, diarrhea, vomiting, jaundice, and death (Hakansson, 1968). Similarly, soybean oil emulsion with soybean phospholipids produced toxic reactions. When 2.4% egg yolk phospholipids were administered in a soybean oil emulsion there were no adverse reactions, however, blood lipids became extremely high (triacylglycerol rose from 22 to 606 mg/100 ml; cholesterol rose from 155 to 2280 mg/100 ml; phospholipids from 291 to 6100 mg/100 ml); on the other hand, a soybean oil emulsion containing 1.2% egg yolk phospholipids did not significantly alter blood lipids (Hakansson, 1968). Therefore, the composition of the lipid emulsion may be extremely important from a metabolic standpoint. Either glycerol, xylitol or sorbitol must also be included to maintain isotonicity with the blood. The only commercially available lipid emulsion in the United States today is Intralipid® (Kabi Pharmacia, Clayton, NC) which consists of either 10% or 20% soybean oil and 1.2% egg yolk phospholipids and 2.25% glycerol.

The fat particles in Intralipid® are metabolized similarly to that of chylomicrons. When the emulsion is infused into the jugular vein, it enters circulation at approximately the same point as chylomicrons. Chylomicrons are 1 μm or less in diameter, compared to 0.5 μm diameter of the emulsified fat particles in Intralipid®. Carlson and Hallberg (1963) reported that the elimination rate of chylomicrons and Intralipid® was the same in fasting dogs. Wretlind (1975) reported that the disappearance rate of the lipid emulsion was dependent on energy reserves. There is no fat loss observed in urine or feces (Shenkin and

Wretlind, 1978). When 10% Intralipid® was infused for 4 hours into humans, maximum serum triacylglycerol levels were found at the fourth hour and returned to preinfusion levels within 2 hours (Wretlind, 1975). Serum free fatty acids were maximal at 4 hours and declined to preinfusion levels within 8 hours (Wretlind, 1975).

There is a change observed in pulmonary function due to infusion of 10% Intralipid® in humans (Wretlind, 1975). Steady state pulmonary diffusion capacity and membrane-diffusing capacity decreased for at least 4 hours following infusion in 6 of 10 volunteers. These changes returned to control levels within 24 hours. McKeen et al. (1978) reported that infusion of 10% Intralipid® into sheep caused a dose-dependent increase in pulmonary artery pressure and lung microvascular filtration and a decrease in arterial oxygen tension with no effect on vascular permeability. These changes returned to control levels within 2 to 4 hours after infusion ended. Administration of indomethacin blocks these effects, suggesting prostaglandins are involved in this regulation.

Fatty Acids as Precursors in Reproduction

Supplemental lipid has been shown to influence reproductive phenomenon in ruminant animals. For example, blood concentration of progesterone increases in cows (Talavera et al., 1985; Carroll et al., 1990), plasma $PGF_{2\alpha}$ increases in heifers, resulting in shortened estrous cycles (Lucy et al., 1990), number of ovarian follicles increases (Lucy et al., 1990; 1991a; Hightshoe et al., 1991; Wehrman et al., 1991), and ovulation rate increases in dairy cows (Lucy et al., 1991a). Dietary

lipid may provide additional energy to exert a direct effect on the ovary or may provide precursors for synthesis of biochemicals. An increase in dietary lipid is associated with an increase in cholesterol-rich lipoproteins (Talavera et al., 1985), which may provide a substrate for progesterone synthesis. *In vitro* culture of luteal and granulosa cells in the presence of lipoproteins results in increased production of progesterone (Pate and Condon, 1982; Savion et al., 1982; O'Shaughnessy and Wathes, 1985). Elevated levels of essential fatty acids, such as linoleic acid, can be converted to arachidonic acid, which may enter the pathway for prostaglandin synthesis, having either luteolytic ($PGF_{2\alpha}$) or luteotropic (PGE_2) actions. Other cyclooxygenase and lipoxygenase products may also rise to cause changes in the uterus or ovary that have yet to be discovered.

Changes in Concentration of Total Cholesterol and Steroidogenesis

Several researchers have reported an increase in total cholesterol in blood when feeding supplemental lipid (cows: Bohman et al., 1962; Bitman et al., 1973; Talavera et al., 1985; sheep: Nestel et al., 1978), as well as in bovine ovarian follicular fluid (Wehrman et al., 1991). Both HDL and LDL cholesterol concentrations have been shown to increase in the blood (Storry et al., 1980; Talavera et al., 1985) and mainly HDL in follicular fluid (Wehrman et al., 1991). This occurs through an increase in *de novo* synthesis of cholesterol in the small intestine of sheep, and to a small extent, decreased fecal excretion of bile acids (Nestel et al., 1978). It is possible that once cholesterol is absorbed into enterocytes, there is a failure of feedback inhibition on cholesterol synthesis in the

small intestine causing hypercholesterolemia (Nestel and Poyser, 1976; Whyte et al., 1977). *In vitro* synthesis of cholesterol in the ovine liver is suppressed due to supplemental lipid, which suggests an increased requirement for cholesterol synthesis by the small intestine for transport of triglyceride in chylomicrons (Nestel et al., 1978). Talavera et al. (1985) caution that the stage of the estrous cycle should be considered when examining the effects of dietary lipid on levels of total cholesterol. Serum total cholesterol declines during the luteal phase of unsupplemented cows, possibly due to increased uptake and utilization by luteal tissue, or factors causing suppression of lipoprotein or lipoprotein receptor synthesis.

Concurrent with the rise in total cholesterol, levels of progesterone during the estrous cycle and pregnancy also increase when cows are fed supplemental lipid (Williams, 1989; Carroll et al., 1990; Sklan et al., 1991), though this may be dependent on the stage of the estrous cycle (Talavera et al., 1985). The latter study reports an increase in serum progesterone during the mid- to late luteal phase. It is difficult to determine whether an increase in progesterone is due to increased luteal synthesis or decreased clearance rate in the blood.

It is not surprising that levels of estradiol-17ß do not increase due to increased dietary lipid, because cholesterol is not an immediate precursor (Talavera et al, 1985). It is believed that the major precursor for estrogen synthesis by granulosa cells is derived from thecal cells (Fortune, 1986).

Changes in Follicular and Luteal Development

It is believed that fat feeding leads to enhanced resumption of normal ovulatory cycles after parturition in cattle (Wehrman et al., 1991). Williams (1989) reported an increase in plasma progesterone in hyperlipidemic postpartum beef cows and associates this to an extension of luteal life span. There was a higher incidence of normal *versus* short luteal phase in cows supplemented with whole cottonseed following GnRH treatment compared with controls. Normally functioning CL in these animals was attributed to an early capacity to develop transitional luteal structures. Oss et al. (1993) describe an increase in the length of the second, but not the first, cycle in beef cows fed calcium soaps of fatty acids. They suggest this occurred through an extension of CL life span in normal estrous cycles, but does not affect luteal function during short cycles.

Supplemental dietary lipid has been associated with stimulated development of follicles in a number of studies. Wehrman et al. (1991) report a greater number of medium class follicles (3.1-9.9 mm in diameter) and fewer smaller class follicles ($\leq$ 3 mm; nonsignificant) and no change in large class follicles ($\geq$ 10 mm) in postpartum beef cows fed whole cottonseed compared with controls. Similarly, Hightshoe et al. (1991) revealed an increase in larger class follicles (> 10 mm) in postpartum beef cows fed calcium soaps of fatty acids. It is hypothesized that populations of smaller class follicles move into a larger class, resulting in earlier ovulation and increased ovulation rate (Lucy et al., 1991b). Indeed, in this latter experiment, postpartum dairy cows fed calcium soaps of fatty acids experienced earlier day to first ovulation and higher ovulation rates.

Lucy et al. (1991b) suggested that this change in follicular dynamics may occur by manipulating the energy balance of postpartum cows from negative to positive. These researchers observed fewer smaller class follicles (3-9 mm) and more larger class follicles (10-15 mm) with increasing positive energy balance. When supplemental lipid acts to improve energy balance there may be a change in the relationship between the hypothalamus/pituitary and the ovary resulting in initiation of the estrous cycle (Nett, 1987). Cows in extreme negative energy balance exhibit inhibited LH secretion, maintaining the animal in the anestrous state (Lucy et al., 1990). When a more positive energy balance is attained through feeding calcium soaps of fatty acids, LH pulse amplitude and diameter of dominant follicle increases (Lucy et al., 1991a).

Lucy et al. (1992) attribute enhanced follicular development to effect of fatty acids and not to a direct effect of additional energy, because cows fed calcium soaps of fatty acids possessed larger preovulatory follicles than controls, both diets being equivalent in energy (Mcal/kg of dry matter). Linoleic acid is a common fatty acid found in calcium soaps of fatty acids, as well as other fat sources that have thus far been mentioned. Supplementation of protected linoleic acid to the ruminant animal may overcome an essential fatty acid deficiency created by rumen biohydrogenation, hence increasing prostaglandin synthesis.

Villeneuve et al. (1987) detected an increase in recruitment of ovarian follicles in early postpartum beef heifers given exogenous $PGF_{2\alpha}$. Lucy et al. (1990) revealed an increase in plasma $PGF_{2\alpha}$ metabolite during diestrus in dairy heifers given a jugular infusion of Intralipid® containing 20% soybean oil (50% linoleic acid),

or abomasal infusion of safflower oil (70% linoleic acid) or olive oil (8% linoleic acid). Heifers infused with soybean oil emulsion had a greater number of follicles per ovary and a greater cumulative follicular diameter per ovary. It was suggested that $PGF_{2\alpha}$ directly and/or indirectly stimulates follicular growth to an extent that unsupplemented animals could not attain.

Lucy et al. (1990) also found a nonsignificant increase in plasma estradiol, either due to a nonspecific effect of fatty acid or increased $PGF_{2\alpha}$, which suggests occurrence of enhanced follicular recruitment. Other studies have demonstrated increased plasma estradiol as a result of injecting arachidonic acid directly into the bovine CL *in vivo* (Shemesh and Hansel, 1975) or administering exogenous $PGF_{2\alpha}$ into ewes (Carlson et al., 1973).

EXPERIMENTS 1 AND 2: INTRAVASCULAR INFUSION OF LIPID INTO EWES STIMULATES PRODUCTION OF PROGESTERONE AND PROSTAGLANDIN

INTRODUCTION

Dietary lipid has been shown to affect ovarian function in ruminant species. Supplementing diets with lipids might represent a practical way to influence follicular development, hence leading to an increase in ovulation rate. As examples, cows fed or infused with long chain fatty acids (either abomasally or via jugular vein) prior to mating had an increased number of follicles (Lucy et al., 1990; 1991a; Ryan et al., 1992) and development of smaller into larger class follicles was enhanced (Hightshoe et al., 1991). Effects of feeding fat to ewes prior to breeding have not been examined; however, grain feeding, which elevated dietary energy, increased follicular development at estrus (Bellows et al., 1963). Feed-induced follicular development may involve an increase in energy level or specific fatty acids that may provide precursors to alter reproductive function. Alternatively, providing additional energy may increase production or release of gonadotropins (Bellows et al., 1963). Ewes fed less dietary energy had reduced secretion of GnRH and concomitantly lower serum concentrations of LH and FSH compared to those fed a maintenance diet (Kile et al., 1991). When these animals were given exogenous GnRH, synthesis and secretion of gonadotropins were restored.

Experiments have shown that increasing dietary lipid in cows resulted in a rise in progesterone (P_4) production (Talavera et al., 1985), possibly by acting

directly on the CL or decreasing metabolic clearance rate. Alternatively, P_4 synthesis may be enhanced due to an elevation in serum cholesterol. In most species, the cholesterol used in steroidogenesis by luteal cells is derived from serum low density lipoprotein (LDL) and/or high density lipoprotein (HDL) (Savion et al., 1982; O'Shaughnessy and Wathes, 1985; Wiltbank et al., 1990). Supplemental lipid increased systemic plasma LDL and/or HDL cholesterol concentration in cattle (Talavera et al., 1985) and sheep (Nestel et al., 1978; Lough et al., 1991), which reflects a higher concentration of total cholesterol (TC).

Administration of lipids containing essential fatty acids to ruminants may affect uterine synthesis and secretion of $PGF_{2\alpha}$, which has been shown to cause luteal regression in ewes (Inskeep et al., 1975) and cows (Louis et al., 1973). Infusion of lipid into cows during the mid-luteal phase of the cycle resulted in a shortened cycle, presumably due to a premature increase in plasma $PGF_{2\alpha}$ (Lucy et al., 1990). Production of PGE_2 might also change due to increased availability of fatty acids. Exogenous PGE_2 has been shown to be luteotropic and causes an increase in P_4 secretion in ewes (Inskeep et al., 1975).

The present study was conducted to examine the effects of i.v. infusion of lipid emulsions containing high and low concentrations of the essential fatty acid, linoleic acid, on serum concentrations of total cholesterol, prostaglandins and luteal P_4 production in cycling ewes. Whether infusion of lipid would increase subsequent ovulation rate was also examined.

MATERIALS AND METHODS

Experiment 1

Exp. 1 was conducted to examine the effects of long chain fatty acids on serum concentrations of TC and P_4 in the ewe and to determine the response of ewes to the infusion of lipid over a 5 day period. In January 1993, six mature Hampshire ewes with body condition scores of approximately 3.5 (thin = 1, good = 3, fat = 5) were assigned randomly in equal numbers to receive either a jugular infusion of saline (S) or a 20% soybean oil emulsion (SB; 20% Intralipid®; Kabivitrum, Clayton, NC; 2 Mcal/L; fatty acid content listed in Table 2). To avoid hydrogenation of fatty acids to non-essential fatty acids in the rumen (Tove and Mochrie, 1963), lipid was infused directly into the jugular vein rather than simply including fat as a dietary component.

Prior to and throughout the infusions, ewes were fed a diet of moderate quality grass hay and whole corn to meet maintenance requirements (NRC, 1985) and had access to water at all times. Ewes were housed in metabolism crates throughout the study. Prior to initiation of treatment, a catheter (Angiocath, 1.7 mm x 8.3 cm, Becton-Dickinson Deseret Medical, Sandy, UT) was placed into the jugular vein and maintained throughout the 5 days of infusion. Ewes were infused for a period of 5 h each day beginning at 0700 with 250 ml of S or SB from Day 9 through 13 of the estrous cycle (first day of detected estrus = Day 0) using a 4-channel peristaltic pump (Gilson Medical Electronics, Middleton, WI). Ewes were

Table 2. Composition of fatty acids in lipid emulsions.

Lipid Emulsions	Fatty Acid Components, %				
	Palmitic C16:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Linolenic C18:3
Soybean Oil	10	4	26	50	9
Olive Oil	11	2	72	8	1

checked for behavioral estrus using vasectomized rams twice daily (morning and evening) prior to and after the end of treatment period.

Blood (10 ml) was collected via jugular vein catheter during treatment at 0 (0700), 5 and 9 h of sampling period. On the first day of infusion, additional samples were taken at 6, 7, and 8 h of the sampling period. Blood was allowed to clot overnight at 4°C, centrifuged (2540 x g) for 15 min and the resulting sera were stored at -20°C.

Experiment 2

Exp. 2 was performed to assess the effects of long chain fatty acids on ovulation rate and to determine whether the anticipated changes in reproductive function were due to fatty acids in general or to a specific effect of essential fatty acids. Fifteen four-year-old Polypay ewes were assigned randomly in equal numbers to receive either a jugular infusion of saline (S), soybean oil emulsion (SB; 20% Intralipid®; 2 Mcal/l), or olive oil emulsion (**OO**) (20% olive oil; 2 Mcal/l; fatty acid contents of the emulsions are listed in Table 2). One ewe infused with saline did not cycle after treatment and was not included in the analysis.

The infusion period occurred in August during the second seasonal estrous cycle. Ovulation rates are optimal in ewes in good body condition. In order to determine the effectiveness of lipid infusion as a source of supplemental energy, the ewes were subjected to a reducing diet (limited access to pasture of high quality to 1 to 2 h per day) for 6 wk to attain a moderate body condition score of 2.23 ± 0.1.

Prior to and throughout the infusions, ewes were fed a diet of pelleted rye grass seed screenings (12% crude protein and 30% acid detergent fiber, dry matter basis) to meet maintenance requirements (NRC, 1985). Ewes were contained in metabolism crates during infusion then placed in a common pen with wood shavings as bedding. The day before infusion began, ewes were fitted with a catheter in each jugular vein; one for infusing and one for collecting blood. Ewes were infused for a period of 5 h each day with 200 ml of S, SB, or OO from Days 9 through 15 of the estrous cycle (estrus = Day 0). On each day, all ewes were infused with 2 ml oxytetracycline HCl (Bio-Mycin C®, BioCeutic Division, Boehringer Ingelheim Animal Health, Inc., St. Joseph, MO) to prevent any infection that might occur as a result of infusing OO possibly contaminated during its preparation in the laboratory (see below). Ewes were checked for behavioral estrus using vasectomized rams twice daily (morning and evening) prior to and after the end of treatment period. After the final day of infusion, ewes were fed free choice pasture, which by that time was of moderate quality.

Blood (10 ml) was collected via jugular vein catheter on Day 9 (first day of infusion) at 0 h (immediately before infusion) and each half hour up to 8 h of the sampling period. On Days 10 through 15 of the cycle, 10 ml blood was collected at 0, 2.5, 5, 6 and 8 h. Blood was processed and the sera stored as described for Exp. 1.

On Day 14 of the succeeding estrous cycle laparotomy was performed to determine number of CL (as an indication of ovulation rate) and number and diameter of follicles > 4 mm. Briefly, animals were initially anesthetized with

sodium thiamylal (10 ml 2% Bio-Tal®, Bio-Ceutic Division) then maintained on halothane-oxygen inhalation. All surgical procedures were performed aseptically.

Preparation of Olive Oil Emulsion

The olive oil emulsion was prepared in the laboratory similar to the process used to create Intralipid®. Because a small hand-held homogenizer was used only 300 ml was generated at a time. Six grams of L-α-phosphatidylcholine (2% w/v; catalogue # P-5394, Sigma Chemical), a 60% egg yolk phospholipid used as an emulsifier, was mixed at high speed in a Sorvall Omnimixer with 233.26 ml sterile distilled water. This mixture, along with 60 ml autoclaved Pomace olive oil (20% v/v) and 6.74 ml glycerol (2.25% v/v; catalogue # G-2025, Sigma Chemical), which was added to maintain osmolality when infused in the blood, was again mixed at high speed for 5 min. The pH of the emulsion was then adjusted to 8.0 with sodium hydroxide. This emulsion was then homogenized twice, placed into a sterile bottle, and stored at 4°C. The shelf life is approximately 30 d. Aseptic technique was used throughout the procedure because this emulsion cannot be autoclaved or filter sterilized. Therefore, it is possible some preparations were contaminated with airborne microorganisms.

Cholesterol Colorimetric Assay

Total cholesterol concentration in serum samples was measured by the procedure of Wybenga et al. (1970). This assay is a simple, direct and specific

method, requiring no serum extraction and a single stable reagent. Volumes used for this assay for serum and reagent were half the volume recommended by the original procedure. The cholesterol reagent [520 mg ferric perchlorate (catalogue # 40-G, Smith Chemical Co., Columbus, OH) dissolved in 600 ml ethyl acetate added to 400 ml cold sulfuric acid] was added to samples and then the mixture was placed in a heating block to reach 65°C. Then, tubes were heated for 105 sec, immersed in ice for 3 min and the liquid transferred to cuvettes. Absorbance was measured using a spectrophotometer at 560 nm wavelength and compared to that of standards. Standards ranged from 100 to 300 mg/dl. The intra- and interassay CV were 1.6 and 4.7%, respectively in Exp. 1 and 4.7 and 7.1%, respectively in Exp. 2. There was no cross-reactivity between cholesterol reagent and SB.

Progesterone RIA

Serum was analyzed in duplicate for P_4 concentration according to Koligian and Stormshak (1976) after hexane:benzene (2:1) extraction. For unknown samples, serum (100 μl) was combined with 2 ml benzene:distilled hexane in a 13 x 100 test tube and vigorously vortexed for 30 sec. Tubes were incubated at -20°C overnight to freeze the aqueous phase or snap frozen using an ice bath of dry ice and ethanol. To correct for procedural loss due to extraction, 100 μl of tracer, which consisted of [1,2,6,7-^{3}H]progesterone (^{3}**H-P$_4$**; 12 x 10^3 dpm; Dupont New England Nuclear, Boston, MA) in distilled ethanol diluted to 6,500 dpm/100 μl, was added to a third tube containing an aliquot of the sample, as well as to two

scintillation vials to attain total counts. The organic phase was poured into scintillation vials with 6 ml Ecolume counting cocktail (ICN Biomedicals, Inc., Costa Meca, CA) and counted. Extraction efficiency was calculated for each assay and this value was used as a correction factor in determining the final concentration of P_4. In Exp. 1 the mean extraction efficiency was 92% and in Exp. 2 the extraction efficiency was 99% throughout the assay so that the correction factor used to determine the final concentration was 1.

The organic phase of serum samples was decanted into a clean 12 x 75 mm test tube, saved and dried under air for approximately 10 min. Standards (100μl) in a range of 0.6 to 20 ng/ml were pipetted into 12 x 75 mm test tubes, 100 μl gel-phosphate buffered saline (GPBS) was added to total count and non-specific binding (NSB) tubes, and 100 μl 1:3500 antibody:GPBS (#337 anti-progesterone-11-BSA; Gordon Niswender, Colorado State University) was added to standards and unknowns. All tubes were vortexed for 5 sec and incubated at room temperature for 30 min. Next, 100 μl of an aqueous tracer (^{3}H-P_4 diluted with GPBS to 3.5 x 10^4 dpm/100 μl) was added to all tubes. Tubes were incubated overnight at 4°C. Bound P_4 was separated from free by adding 1 ml ice cold dextran coated charcoal to all tubes except the total count tubes; to these 1 ml GPBS was added. Tubes were vortexed for 5 sec, incubated in ice for 15 min, and centrifuged at 2540 x g (Beckman) for 10 min at 4°C. Supernatant was decanted into scintillation vials containing 6 ml Ecolume, capped, inverted, and counted. The sensitivity of the assay was 0.1 ng/ml. The intra- and interassay CV were 9.1 and

18.0%, respectively for Exp. 1 and 10.6 and 12.0% for Exp. 2. There was no cross-reactivity between P_4 antisera and SB.

Prostaglandin $F_{2\alpha}$ Metabolite RIA

Prostaglandin $F_{2\alpha}$ metabolite was analyzed and the assay validated for sheep using the RIA procedure of Guilbault et al. (1984) modified with acquisition of antiserum, rabbit J57 anti-13,14-dihydro-15-keto $PGF_{2\alpha}$ (W.W. Thatcher, University of Florida, personal communication). Standard for PGFM was obtained from Cayman Chemical Company (Ann Arbor, MI) and 13,14-dihydro-15-keto [5,6,8,9,11,12,14(n)-^{3}H] $PGF_{2\alpha}$ (185 Ci/mmol specific activity) from Amersham (Arlington Heights, IL). Prostaglandin-free serum (PFS), used in the standard curve, was prepared by administering a cyclooxygenase inhibitor, flunixin meglumine (Banamine, Schering-Plough Animal Health Corp., Kenilworth, NJ) at 50 mg/ml to a ewe (Day 15 of the estrous cycle) twice at a 16 h interval. The ewe was bled 4 h after the second injection.

The standards were constructed by pipetting 200 µl PFS into all 12 x 75 tubes and 100 µl of standards ranging from 25 to 10,000 pg/ml into appropriate tubes. An aliquot of 200 µl 0.05 M Tris-HCl (7.88 g/l Tris-HCl, pH 7.5) was added to NSB tubes. A volume of 200 µl of sample and 100 µl Tris buffer were pipetted into the tubes. Next, 100 µl 0.5% bovine gamma globulin (catalogue # G5009, Sigma Chemical) were added to all tubes and the contents incubated at room temperature for 15 min. A volume of 100 µl of 1:8000 antiserum:Tris buffer was added to all tubes except NSB tubes and all were incubated at room temperature

for 30 min. A volume of 100 µl ^{3}H-PGFM (18,000 dpm) was added as a tracer to all tubes and to two scintillation vials. Tubes were incubated at room temperature for 1 h and then overnight at 4°C. A volume of 750 µl 40% polyethylene glycol (PEG), to separate bound PGFM from free, was added to all tubes and they were vortexed for 1 min. Tubes were centrifuged for 30 min at 1620 x g (Beckman) at 4°C, then placed in foam racks and inverted to drain supernatant. The pellet was redissolved in 750 µl Tris buffer and tubes were vortexed for 1 min. Next, 750 µl PEG were added to all tubes, which were then vortexed for 1 min, and centrifuged for 30 min at 1620 x g at 4°C. Tubes were inverted to drain supernatant, the pellet redissolved in 1 ml Tris buffer, vortexed for 3 min and immediately poured into scintillation vials containing 6 ml Ecolume, and counted. Sensitivity of the assay was 50 pg/ml.Intra- and interassay CV were 17.6 and 3.3%, respectively.

Bicyclo Prostaglandin E_2 ELISA

Serum concentration of PGE_2 was determined by analyzing bicyclo PGE_2 using a competitive enzyme-linked immunoassay (EIA) kit (Cayman Chemical). Prostaglandin E_2 is rapidly converted to its metabolite 13,14-dihydro-15-keto PGE_2 in vivo, however, it is not chemically stable. When all of the PGE_2 metabolites are converted to bicyclo PGE_2 in vitro, a stable derivative can then be quantified by EIA. The assay is based on the competition between free bicyclo PGE_2 and a bicyclo PGE_2 tracer for a limited number of specific rabbit antiserum binding sites. Sensitivity of the assay is 8 pg/ml. The intra- and interassay CV were 18.8 and 6.0%, respectively.

Statistical Analysis

Analysis of variance was used to analyze changes in serum P_4, TC, and prostaglandins using the general linear models procedure of SAS (1993) with repeated measurements over hour of sampling and day of infusion. Samples collected during the first day of infusion in Exp. 1 and Exp. 2 were analyzed independently from those taken on Days 9 through 13, 0, 5, and 9 h for Exp. 1 and Days 9 through 15, 0, 2.5, 5, 6, and 8 h for Exp. 2. For the initial day of infusion, analysis of variance with repeated measurements over hour of sampling was used. For data on serum levels of PGFM, only Days 13 through 15 were analyzed and for PGE_2 Day 9 and Days 13 through 15 were examined (hours 0, 2.5, 5, and 8). Data for duration of the estrous cycle of ewes in Exp. 1 were analyzed by a one-tailed t-test while data for Exp. 2 were subjected to analysis of variance (SAS, 1993). The relationship between concentrations of TC and P_4 over all ewes was evaluated both for Day 9 alone and Days 9 through 13 or Days 9 through 14 using Pearson's coefficient *rho* through SAS (1993). Analysis of variance was used to determine differences in follicle size (> 4 mm), largest follicle size, number of follicles and CL among treatments. Differences among least squares (LS) means due to treatment, hour, and day were evaluated for significance by use of least significant differences test. Level of statistical significance was assigned as $p < 0.05$ for main effects.

In Exp. 2, analysis of Day 15 was excluded for P_4 and TC because some ewes had declining P_4 concentrations, presumably due to regression of CL, thus invalidating any statistical comparison. One animal was not included in the

analysis of P_4 in the OO treatment group because she had negligible levels of the steroid throughout.

Four outliers in the range of 781 to 1000 pg/ml representing ewes from each treatment were removed for PGFM analysis because the high concentrations recorded were probably due to an endogenous surge of PGFM concentrations (Fairclough et al., 1983). Values falling below the detectable level of the assay (50 pg/ml) were arbitrarily assigned a value of 25 pg/ml.

RESULTS

In Exp. 1, changes in serum TC and P_4 of ewes in response to saline and SB infusions on Day 9 of the estrous cycle are shown in Figures 2 and 3. Serum TC concentrations were not affected by infusion of lipid ($p = 0.14$; Fig. 2); however, mean concentrations of TC increased by hour 5 ($p = 0.01$) concomitant with the increase in P_4 concentrations (Fig. 3) and then declined by hour 9. Compared to S-infused ewes, serum P_4 in SB-infused ewes increased 3.3-fold by the end of infusion (hour 5), and by hour 9 (4 h post-infusion) returned to a level comparable to that of S-infused ewes (treatment x time interaction, $p = 0.008$). The correlation coefficient between TC and P_4 concentrations was significant (*rho* $= 0.40$; $p = 0.02$). Concentrations of P_4 may be lower than values in the literature because the experimental period occurred at the end of the breeding season.

Changes in mean serum concentrations of TC and P_4 in response to infusion of lipid on Days 9 through 13 in Exp. 1 are depicted in Figures 4 and 5. On each of Days 9 to 13, infusion of lipid into SB ewes caused a significant

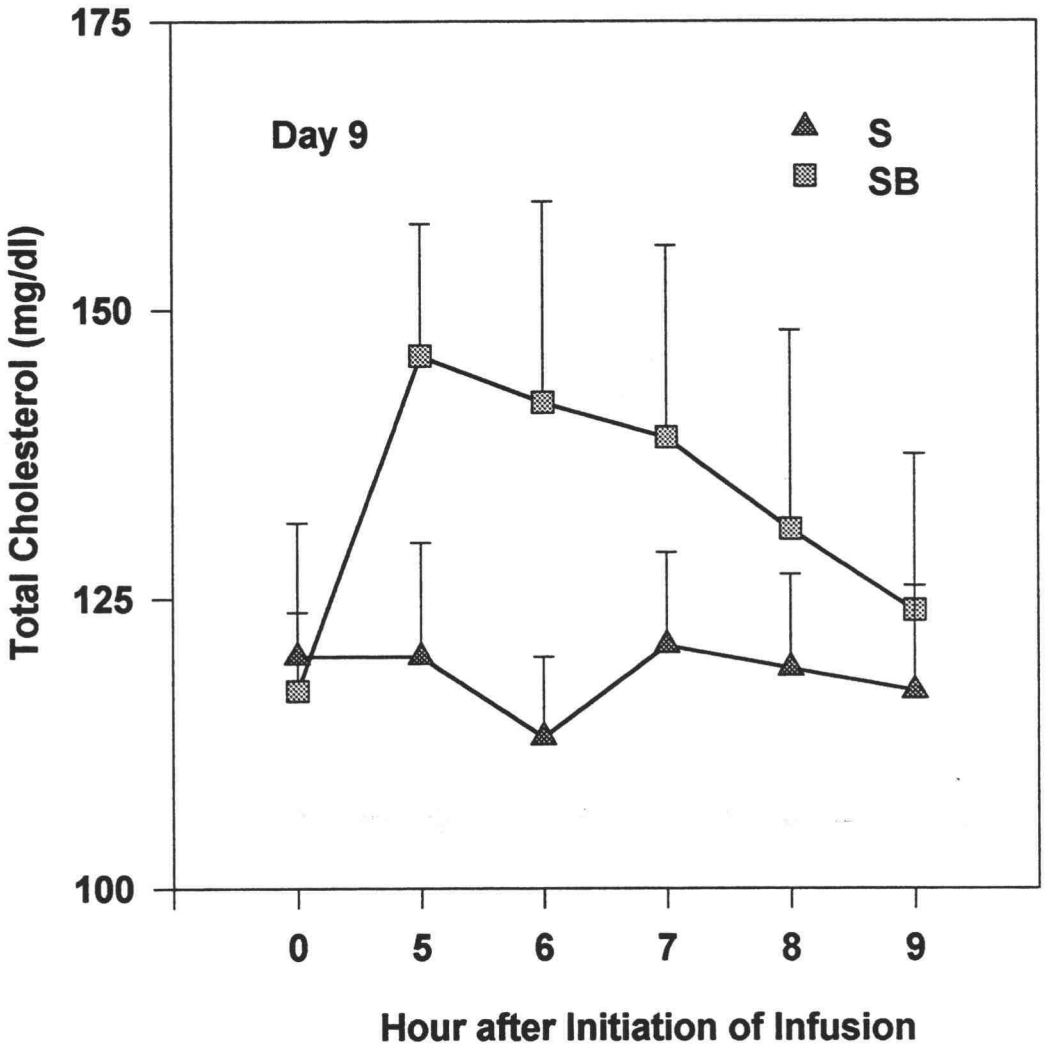


Figure 2. Mean (± SE) serum concentrations of TC in ewes (n = 3/treatment) infused with saline (S) or soybean oil emulsion (SB) for 5 h. Means represent serum levels at 0, 5, 6, 7, 8, or 9 h of infusion on Day 9 of the estrous cycle.

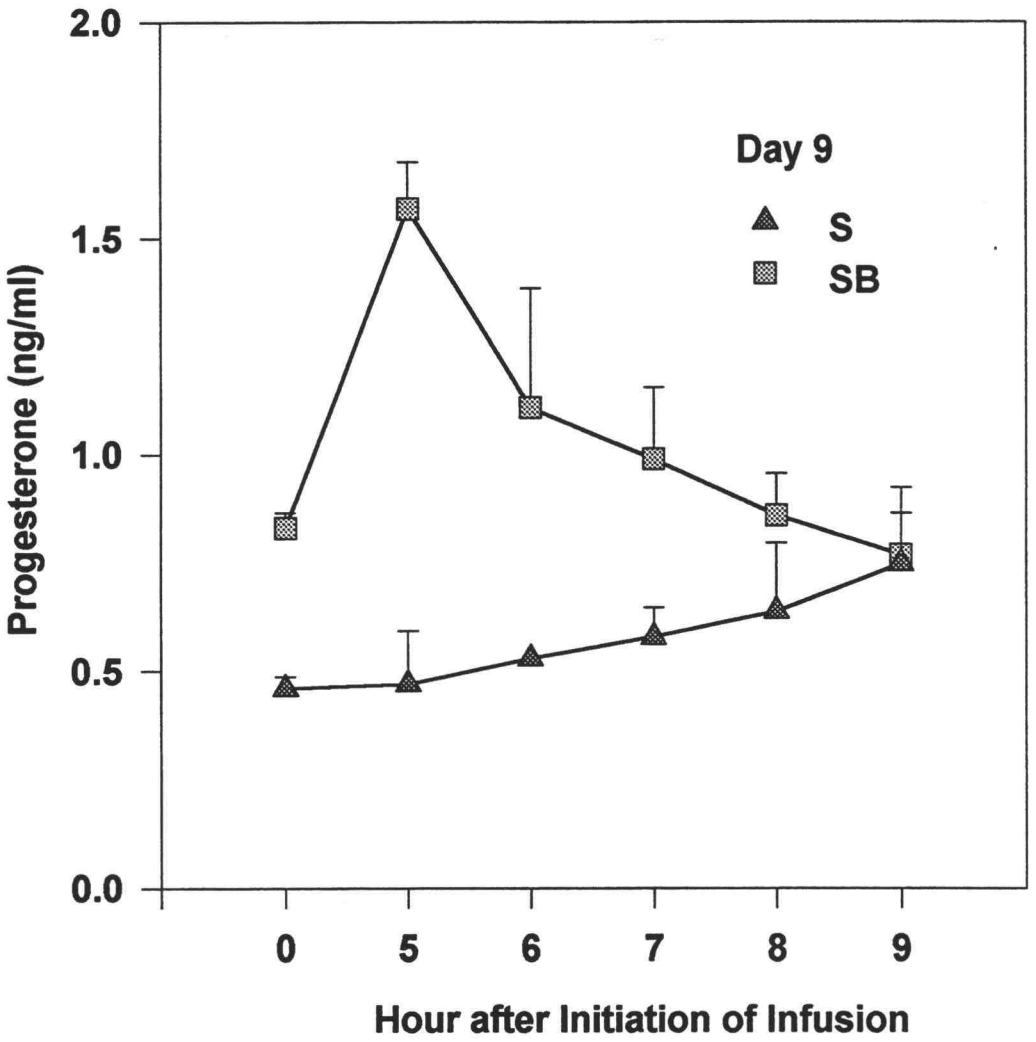


Figure 3. Mean (± SE) serum concentrations of P_4 in ewes (n = 3/treatment) infused with saline (S) or soybean oil emulsion (SB) for 5 h. Blood samples were collected at 0, 5, 6, 7, 8, or 9 h of infusion on Day 9 of the estrous cycle. A treatment x hour interaction was detected (p = 0.008).

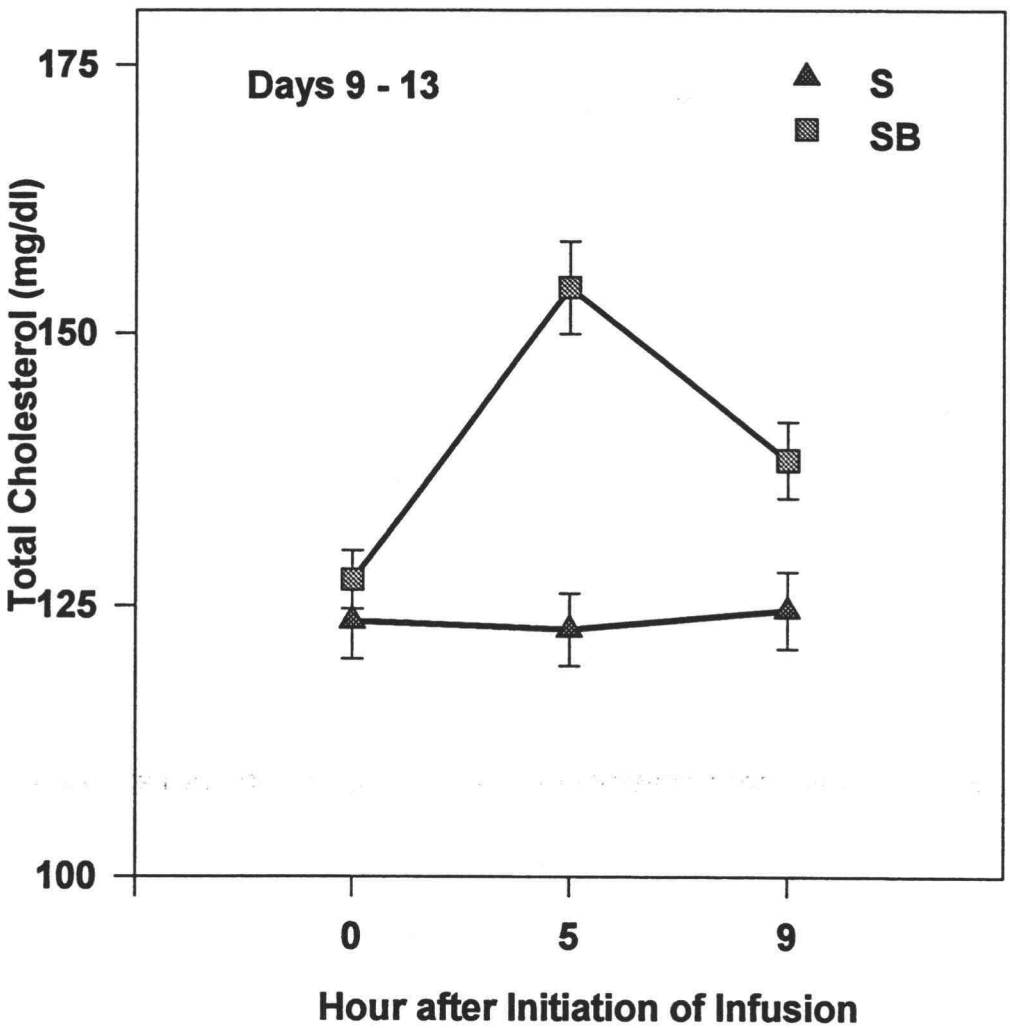


Figure 4. Mean (± SE) serum concentrations of TC in ewes (n = 3/treatment) infused with saline (S) or soybean oil emulsion (SB) for 5 h daily. Means represent samples taken at 0, 5, and 9 h and on each of Days 9, 10, 11, 12, and 13 of infusion. A treatment x hour interaction over days was detected (p = 0.0002).

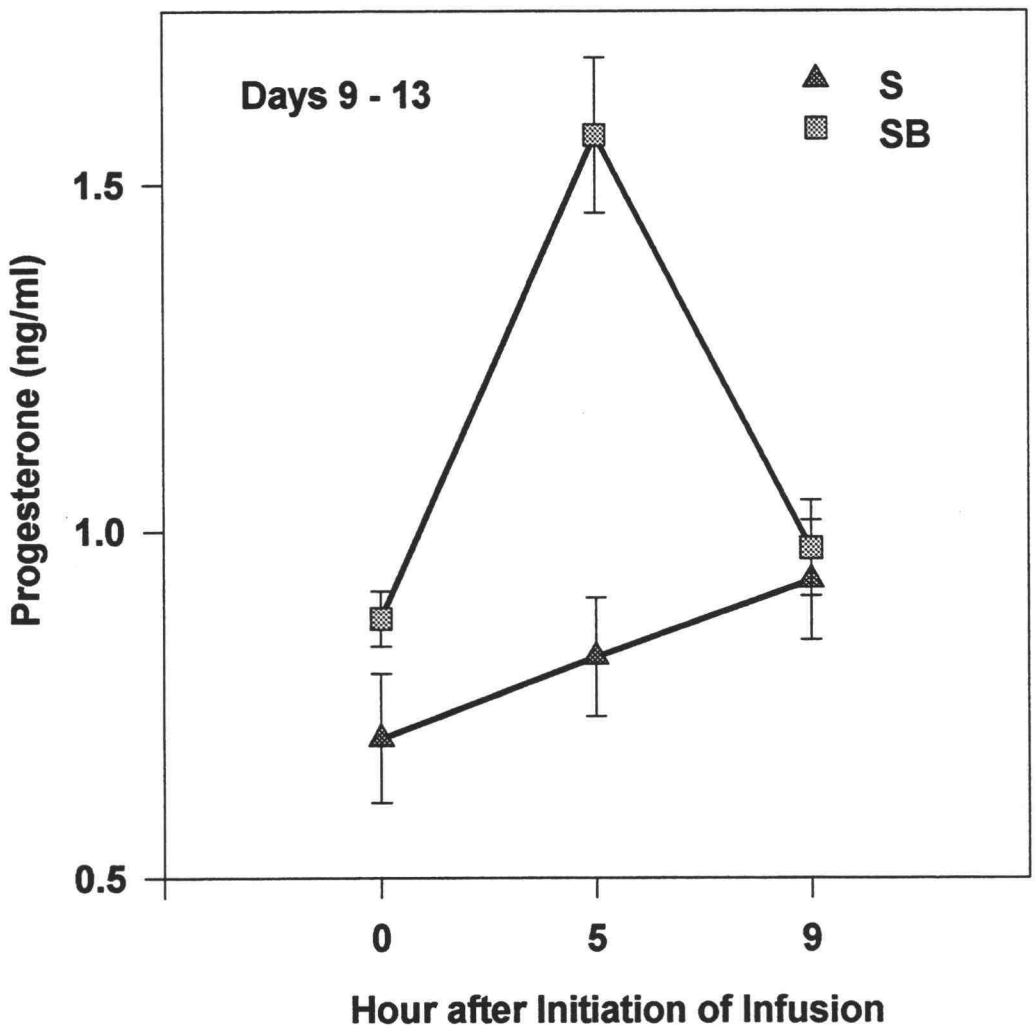


Figure 5. Mean (± SE) serum concentrations of P_4 in ewes (n = 3/treatment) infused with saline (S) or soybean oil emulsion (SB) for 5 h daily. Means represent samples taken at 0, 5, and 9 h and on each of Days 9, 10, 11, 12, and 13 of infusion. A treatment x hour interaction over days was detected (p = 0.003).

increase in TC ($p = 0.0002$) and P_4 ($p = 0.003$) concentrations by hour 5. Comparable to the responses of lipid-infused ewes observed on Day 9, the concentrations of these steroids were reduced to control levels by 4 h after end of infusion. The correlation coefficient between TC and P_4 concentrations was significant (*rho* = 0.53; $p < 0.0001$).

Although animals were assigned randomly to treatments in Exp. 2, the mean concentration of TC at 0 h on Day 9 was different ($p = 0.002$) between lipid-infused and control ewes (Fig. 6). Maximum concentrations of TC were attained by 4.5 h of infusion in SB-infused ewes and 4 h in OO-infused ewes (treatment x time interaction, $p = 0.08$) and values remained relatively constant in control ewes (Fig. 6). All concentrations at hour 0 were in the normal range for sheep (mean of 90.0 mg/dl, standard deviation of 18.2, and range of 50.0 to 140 mg/dl; Mitruka et al., 1977). Means differed among all treatments ($p < 0.0001$). On the initial day of infusion, there was a significant difference in serum concentrations of P_4 among treatments ($p = 0.01$; Fig. 7) and the means differed ($p < 0.0001$) among all groups. There was a significant correlation on Day 9 between serum concentrations of TC and P_4 (*rho* = 0.29; $p < 0.0001$).

Infusion of both OO and SB, increased serum concentrations of TC on Days 9 through 14 so that maximum levels were reached by hour 5 and declined thereafter (treatment x hour interaction, $p = 0.009$, Fig. 8). Corresponding serum concentrations of P_4 were markedly increased before hour 5, then declined to control levels ($p = 0.005$, Fig. 9) and the means for treatment were different among all groups for both TC and P_4 ($p < 0.0001$, $p < 0.0001$, respectively).

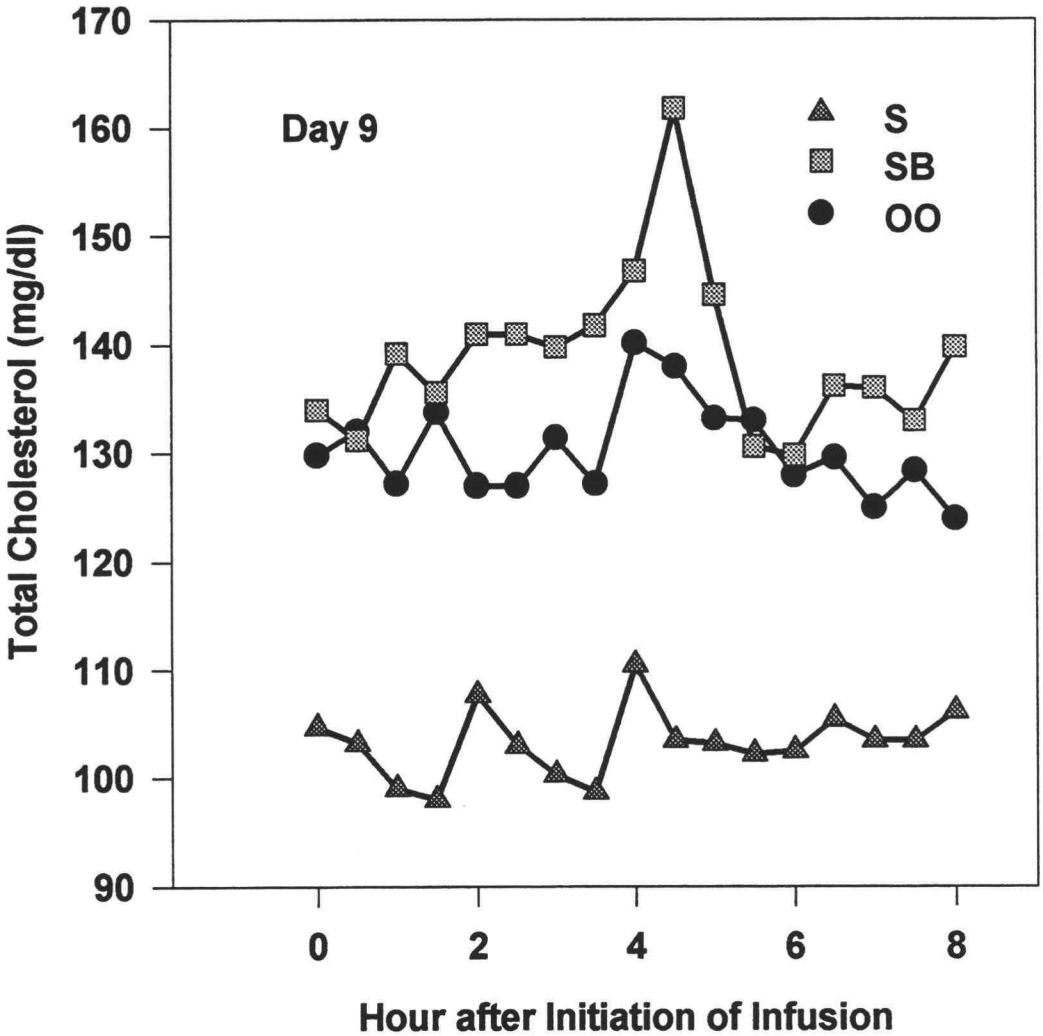


Figure 6. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of TC. Blood samples were collected every 30 min from 0 to 8 h after initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean of treatments with pooled LS SE for S = 0.07, SB = 0.06, OO = 0.06.

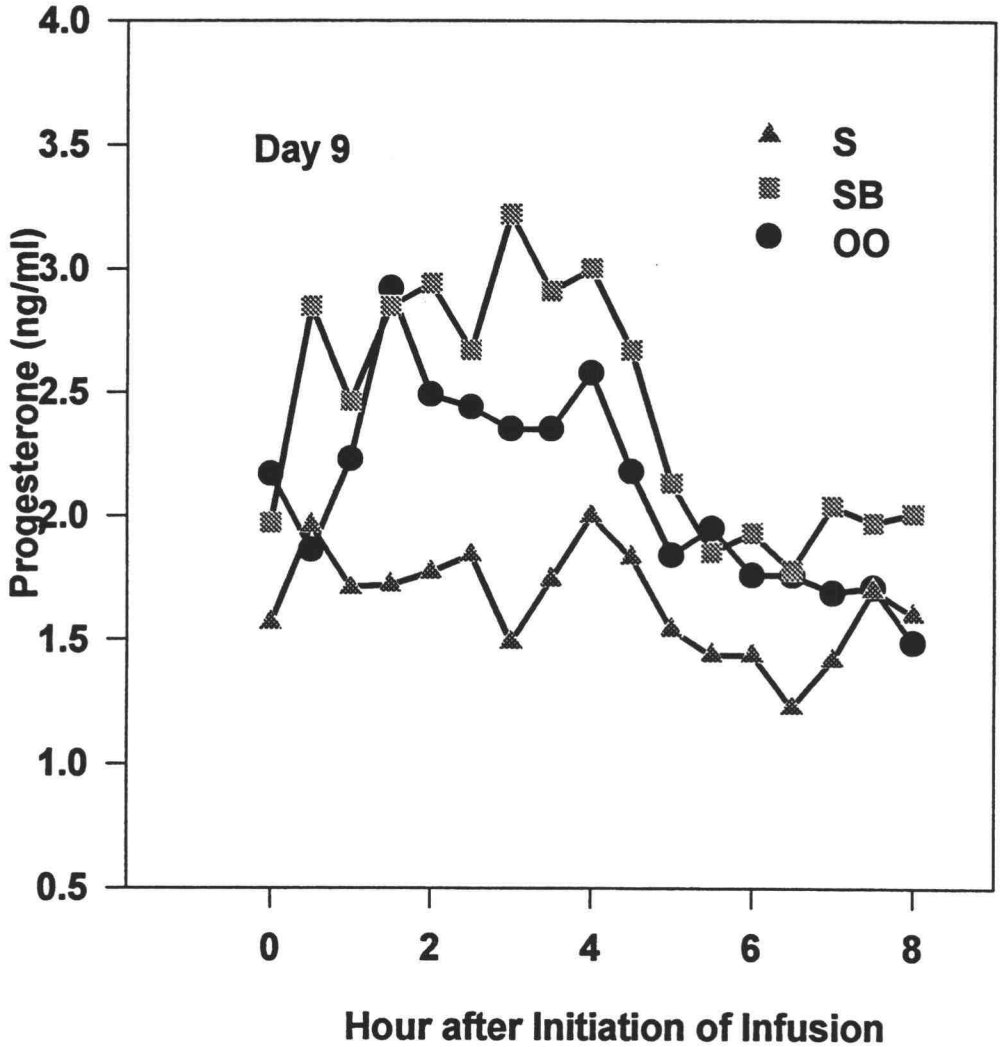


Figure 7. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 4) on serum concentrations of P_4. Blood samples were collected every 30 min from 0 to 8 h after initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean of treatments with pooled LS SE for S = 1.0, SB = 0.8, OO = 0.8.

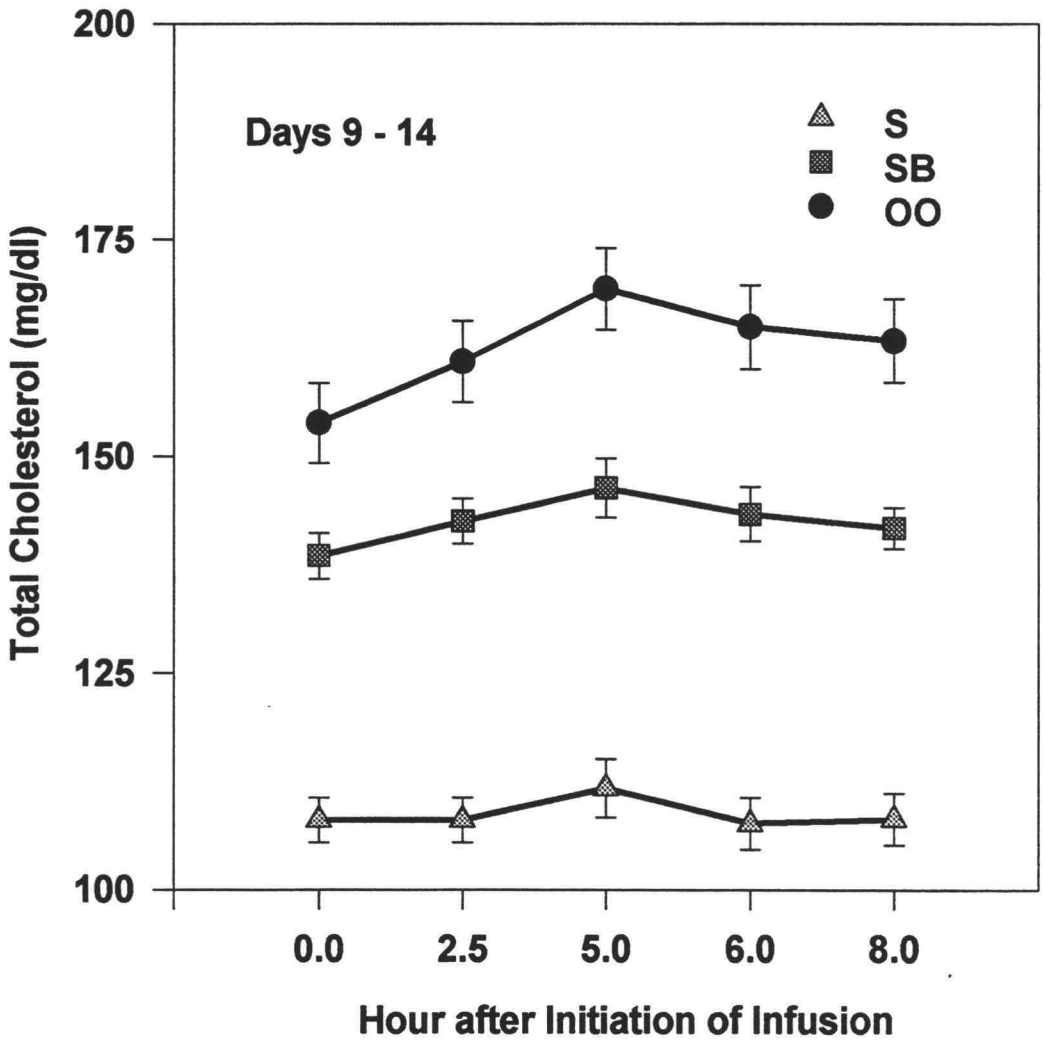


Figure 8. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of TC on Days 9 through 14 of the estrous cycle. Blood samples were collected at 0, 2.5, 5, 6, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) over day for each treatment.

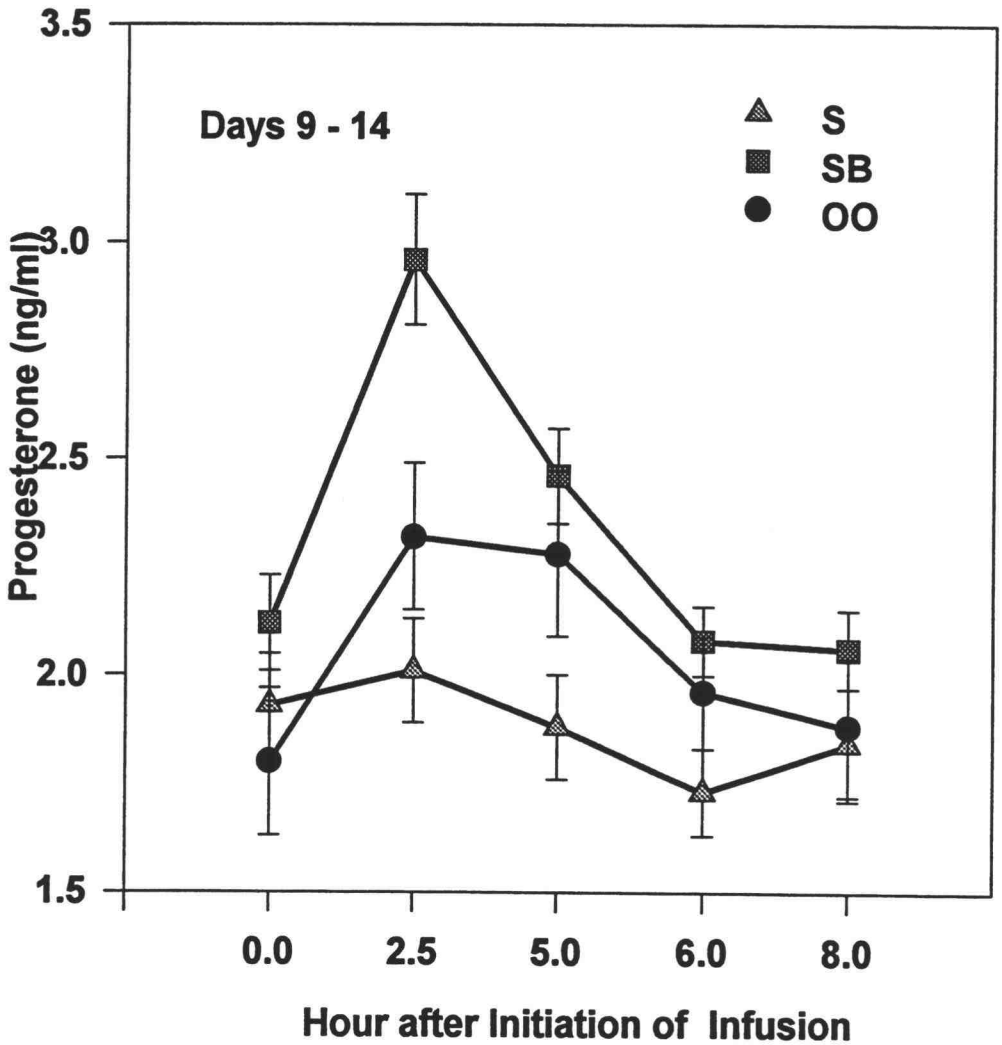


Figure 9. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 4) on serum concentrations of P_4 on Days 9 through 14 of the estrous cycle. Blood samples were collected at 0, 2.5, 5, 6, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) over day for each treatment.

Analysis of means with day as a variable, revealed that maximal serum concentrations of both TC and P_4 were attained on Day 13 ($p = 0.009$, $p < 0.0001$, respectively) and declined thereafter on Day 14 (data not shown).

On Days 13 through 15 of the estrous cycle, the pattern of $PGF_{2\alpha}$ concentration as determined by measurement of PGFM, differed over time among treatments (treatment x hour x day interaction, $p = 0.03$). On Day 13 and 14 infusion of SB or OO provoked an increase in PGFM compared with S-infused ewes ($p = 0.02$, $p = 0.05$, respectively; Fig. 10, Fig. 11, respectively), while on Day 15, infusion of OO was linked to an increase in PGFM compared with SB and S-infused ewes ($p = 0.0005$, Fig. 12).

Analysis of serum samples collected from all ewes at 0 h on Day 9 for PGE_2 resulted in a baseline value of 18.83 (± 4.14) pg/ml, which did not differ significantly among treatments. On Day 9 (Fig. 13) and Days 13 through 15 (Fig. 14) of the estrous cycle, infusion of OO increased production of PGE_2 with the highest concentration measured occurring at hour 5 and declining thereafter (treatment x hour interaction, Day 9, $p = 0.01$; Days 13 through 15, $p < 0.0001$). This pattern of secretion was not apparent in S- or SB-infused ewes and serum concentrations of PGE_2 in these ewes did not differ. On Day 9, means were different between OO-infused ewes and S- or SB-infused ewes ($p = 0.0001$), but not between the latter groups ($p = 0.10$). On Days 13 through 15 means were not different among days, but were different among all treatments ($p < 0.0001$).

Effects of lipid infusions on ovarian characteristics are presented in Table

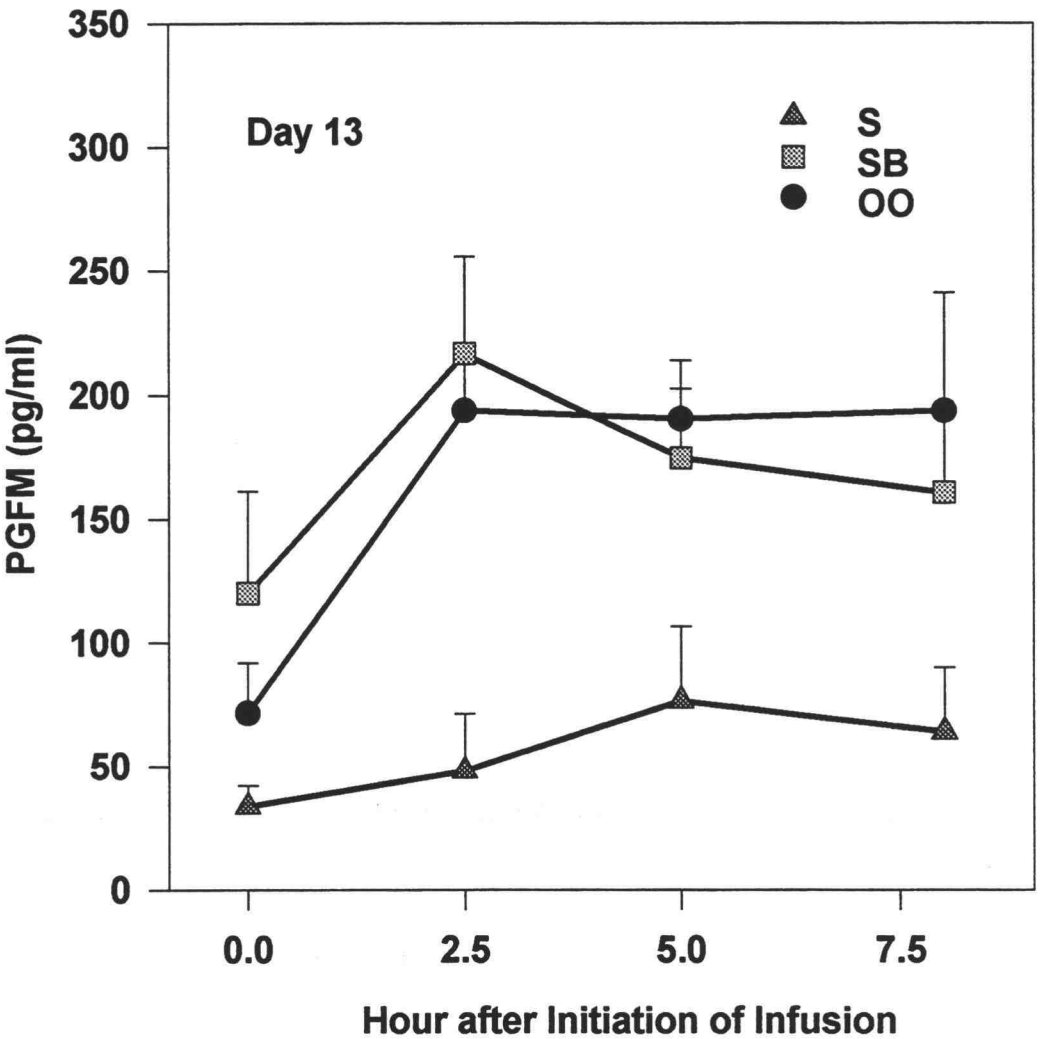


Figure 10. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of PGFM on Day 13 of the estrous cycle. Blood samples were analyzed for 0, 2.5, 5, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) for each treatment.

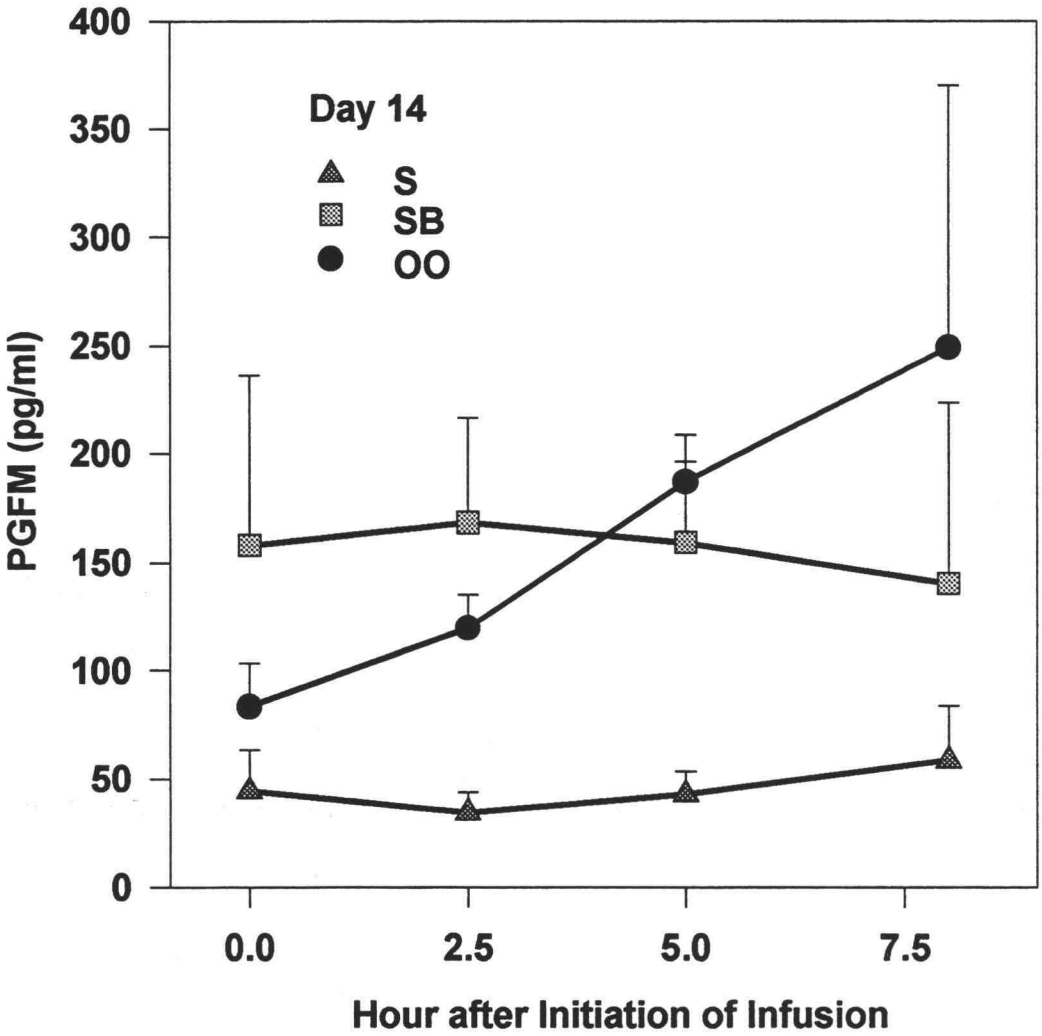


Figure 11. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of PGFM on Day 14 of the estrous cycle. Blood samples were analyzed for 0, 2.5, 5, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) for each treatment.

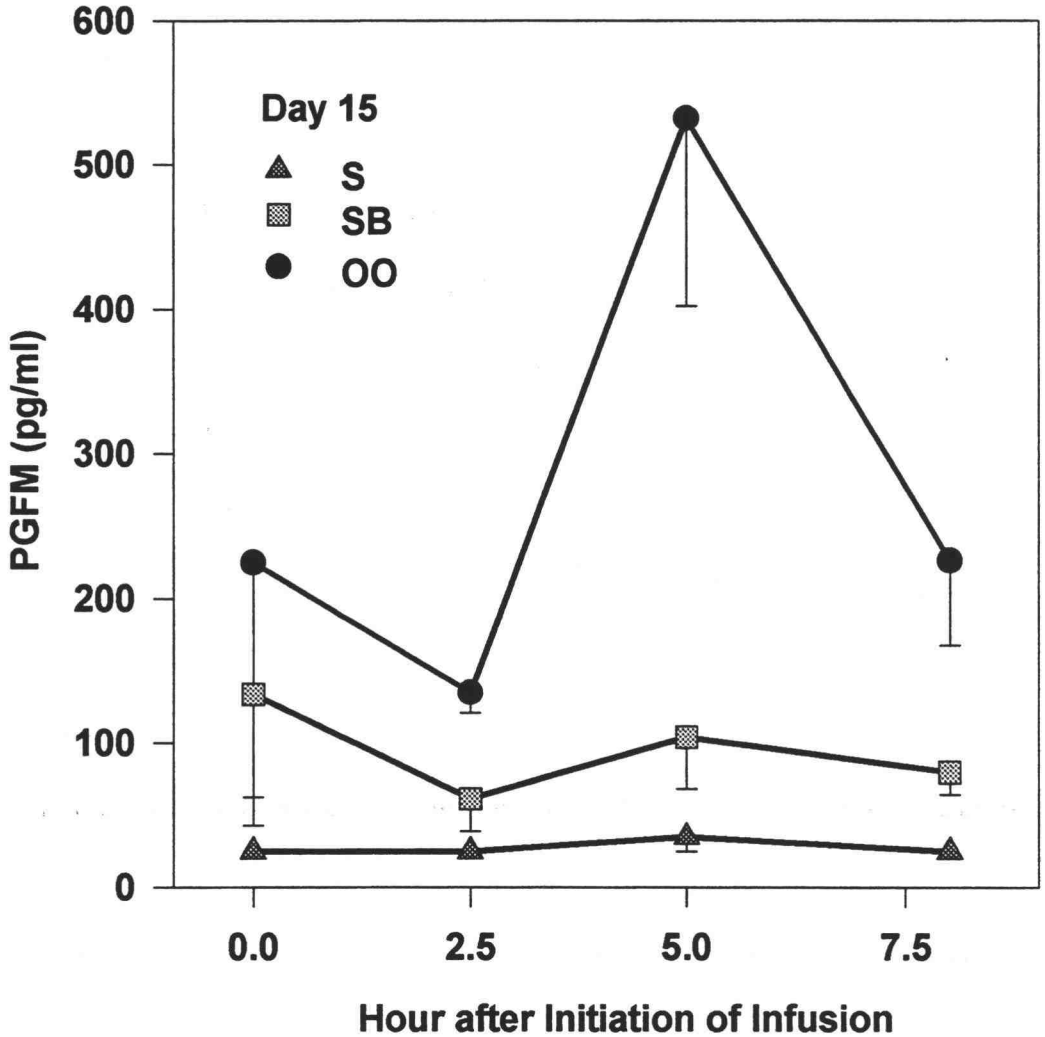


Figure 12. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of PGFM on Day 15 of the estrous cycle. Blood samples were analyzed for 0, 2.5, 5, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) for each treatment.

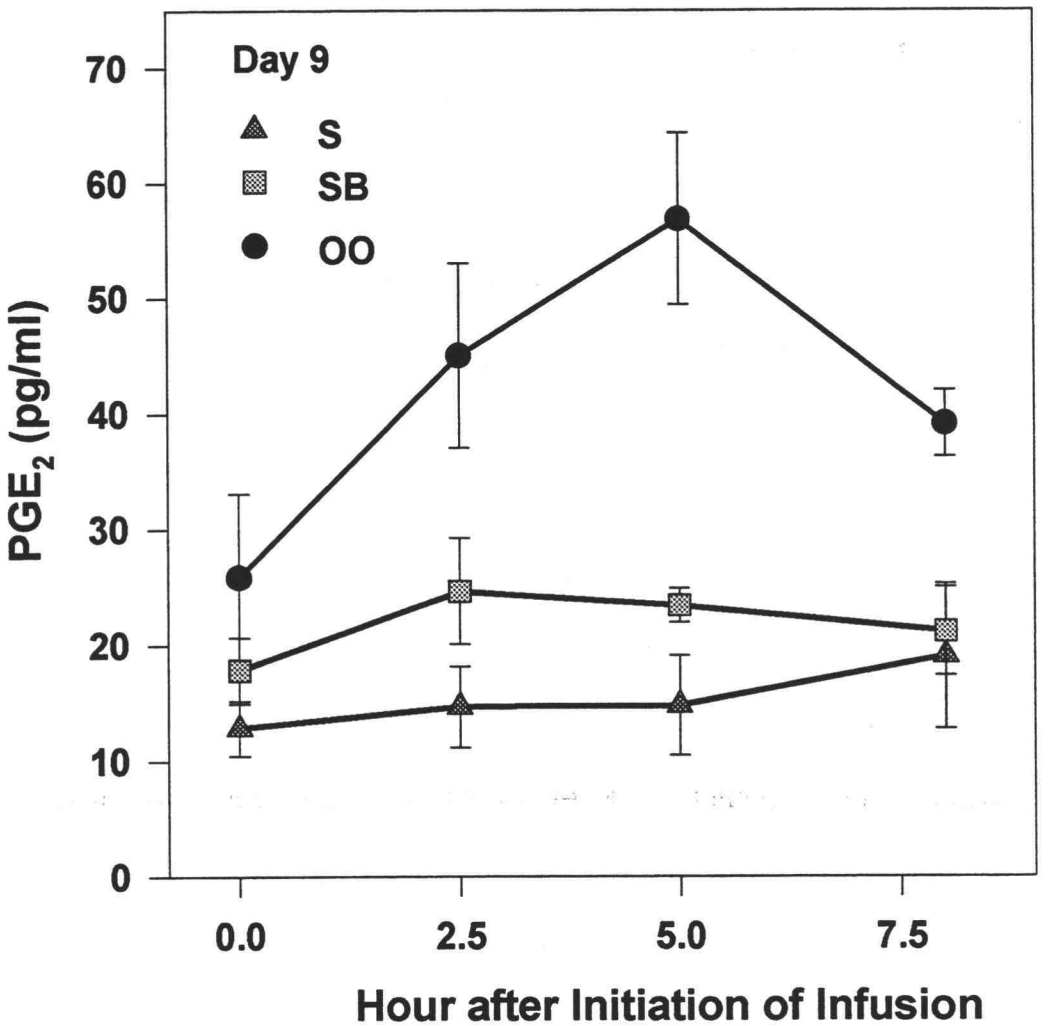


Figure 13. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of PGE_2 on Day 9 of the estrous cycle. Blood samples were analyzed for 0, 2.5, 5, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) for each treatment.

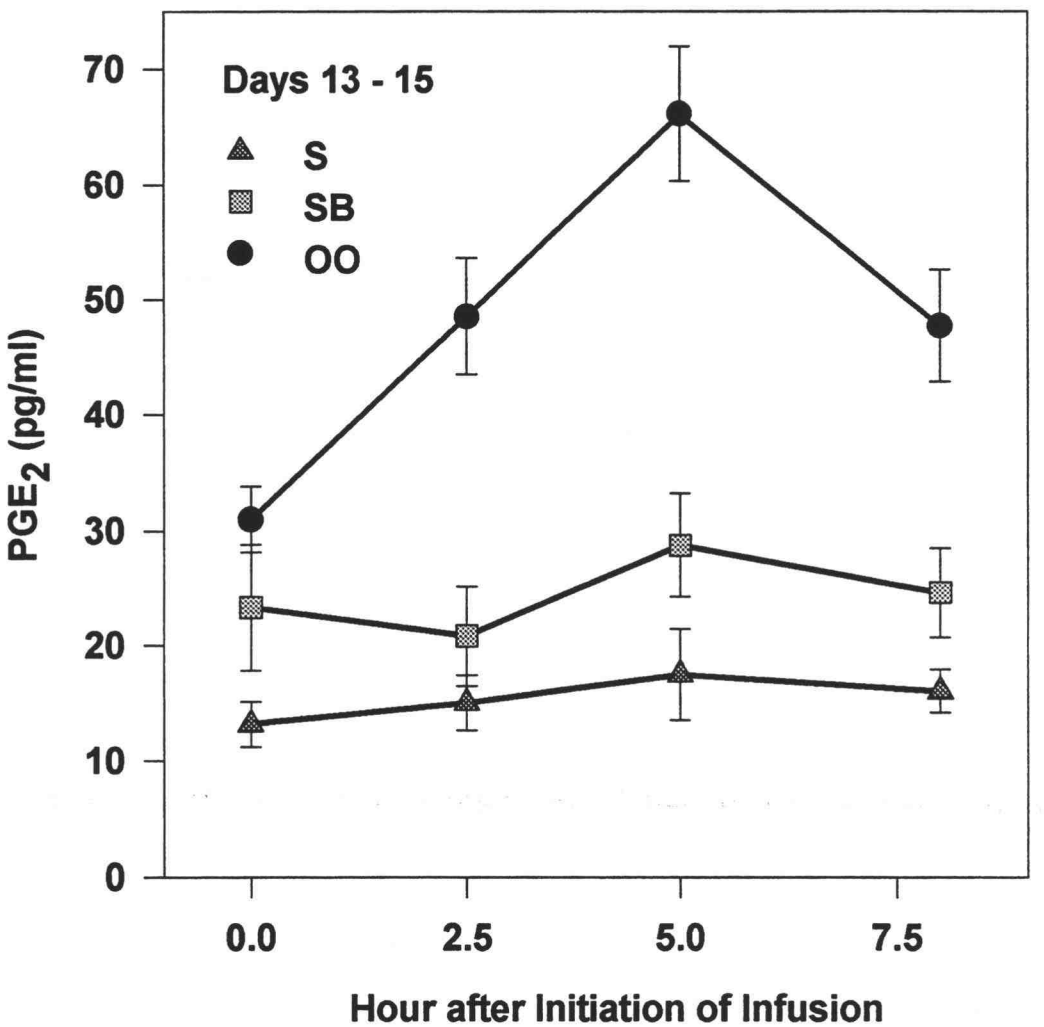


Figure 14. Effect of infusion of saline (S; n = 4), soybean oil emulsion (SB; n = 5), or olive oil emulsion (OO; n = 5) on serum concentrations of PGE_2 on each of Days 13 through 15 of the estrous cycle. Blood samples were analyzed for 0, 2.5, 5, and 8 h from initiation of infusion. Ewes were infused from 0 through 5 h. Data are expressed as mean (± SE) for each treatment and day.

3. Infusion of OO significantly shortened the duration of the estrous cycle compared with SB and S-infused ewes ($p = 0.002$). There was no difference in ovulation rate as measured by number of CL, or in number of follicles and diameter of the largest follicle among treatments.

DISCUSSION

Results of the present study indicate that luteal and uterine function are altered by infusion of both SB and OO in mid- to late diestrus. In Exp. 1 and 2 the increase in serum concentration of TC in treated ewes likely stimulated luteal steroidogenesis leading to an increase in serum concentrations of P_4. This is consistent with data of Carroll et al. (1990), who reported that cows fed 5% long chain fatty acids (average energy density of 1.78 Mcal NE_l/kg vs. 1.65 Mcal NE_l/kg for controls) from Day 5 to 100 postpartum had a 22% increase in TC compared with control animals. In addition, due to the additional dietary fat, serum concentrations of P_4 increased in the mid- to late luteal phase of the second and third estrous cycles. Feeding heifers a diet containing whole sunflower seeds, which are high in linoleic acid, increased TC concentrations by 70% relative to levels present after feeding a control diet (Talavera et al., 1985).

In Exp. 2, concentrations of TC on Day 9 were greatest in SB-infused ewes, however, on subsequent days of infusion, OO-infused ewes had significantly higher concentrations. As noted earlier, the OO was prepared in our laboratory similar to that of the commercially-available SB. Emulsified fat particle size was not

Table 3. Effects of lipid infusion on length of estrous cycle and ovarian characteristics on Day 14 of subsequent cycle.

Treatment	Length of Cycle (d)	No. of CL	No. of Follicles (> 4mm)	Diam. Lg. Foll. (mm)
Saline	18.0 ± 0.0	2.5 ± 0.3	3.5 ± 0.5	5.6 ± 0.6
Soybean Oil	17.2 ± 0.2	2.4 ± 0.2	4.2 ± 0.4	6.1 ± 0.4
Olive Oil	16.2 ± 0.4*	2.4 ± 0.2	4.6 ± 0.2	6.5 ± 0.2

*Mean (± SE) is different from other column means , p = 0.002.

determined in the OO, whereas SB particle size is 0.5 μm. Rate of metabolism of the two emulsions may be different if size of emulsified fat particles differs. It was noted that on the initial day of infusion OO-infused ewes were depressed and appetite poor, however, this never occurred on subsequent days of infusion. Perhaps these ewes adapted metabolically to utilize the OO as efficiently as SB-infused ewes.

The increase in serum P_4 in ewes during infusion of lipid is in contrast to the decrease in plasma concentrations of P_4 reported in heifers when they were infused with Intralipid® from Days 9 to 13 of the estrous cycle (Lucy et al., 1990). However, it has been shown that effects of supplemental fat on P_4 secretion in cattle is dependent upon the stage of the estrous cycle (Talavera et al., 1985). Similarly, Carroll et al. (1990) reported a decrease in P_4 concentrations in fat-fed cows in metestrus or early luteal phase of the first cycle. There have been no reports on effects of supplemental lipid on P_4 production in sheep. Further research is needed to determine whether, as in cows, serum P_4 in ewes decreases due to supplemental fat provided during metestrus to the early luteal phase.

It is unlikely that concentrations of P_4 increased as a result of changes in pituitary gonadotropins. Estienne et al. (1990) infused Intralipid® into ovariectomized ewe lambs and found that mean serum concentrations of LH and LH pulse frequency did not differ from those of S-infused lambs. It is possible that the increase in TC provided a substrate for steroidogenesis for maximal production of progesterone, which has been documented *in vitro* (Savion et al., 1982). It is also possible that the clearance rate of P_4 may have decreased due to an increase

in catabolic enzymes in the liver, resulting in greater serum concentrations of the steroid.

Serum concentrations of P_4 were lower in Exp. 1 than in Exp. 2 probably due to the experiment being conducted late in the breeding season. Ewes stopped exhibiting behavioral estrus shortly after the experimental period ended. In addition, Hampshire ewes are known to be less prolific than Polypay ewes, which may result in less production of P_4 from fewer CL present. The change in serum P_4 from before infusion began to hour 5 in lipid-infused ewes was greater in Exp. 1 than Exp. 2 possibly due to the difference in body condition between the two experiments. The Hampshire ewes in good body condition probably did not require as much energy for metabolic processes other than reproduction, because this requirement was already satisfied. Therefore, more precursors were available for reproductive functions than for those of Polypay ewes in Exp. 2. It is apparent that in both experiments for SB-infused ewes, the cholesterol precursors were similarly elevated by the end of infusion, supporting the premise of providing a percentage of additional nutrients for metabolic functions other than reproduction in ewes of Exp. 2.

Dietary linoleic acid is a precursor of arachidonic acid, which can be converted to prostaglandins. The enzyme complex responsible for this conversion is PGH synthase which consists of cyclooxygenase and hydroperoxidase. Pace-Asciak and Wolfe (1968) reported that linoleic acid is a competitive inhibitor of arachidonic acid for PGH synthase in sheep vesicular glands. Results of *in vitro* experiments indicated that when bovine caruncular tissue explants were cultured

with increasing concentrations of linoleic or arachidonic acid, synthesis of $PGF_{2\alpha}$ declined at the higher concentrations of linoleic (25 and 200 μM vs. 10 μM) but not of arachidonic acid [Oldick et al., 1994; W.W. Thatcher, personal communication]. The results of PGFM and PGE_2 analyses in the present study indicate that a component of the OO, likely linoleic acid, stimulated prostaglandin synthesis and/or release on Days 13 through 15 of the estrous cycle. There was less serum PGFM and PGE_2 apparent in SB-infused ewes compared with those infused with OO, but more than in S-infused ewes. It is possible that the higher concentration of linoleic acid in the SB caused decreased synthesis of prostaglandins compared with that of the OO. Alternatively, increased availability of fatty acids to tissues caused an increase in linoleic acid in phospholipids of cell membranes resulting in a change in cell membrane permeability, which might affect rate of secretion of prostaglandins. In support of this concept, Thatcher et al. (1994) reported greater plasma concentrations of PGFM in Holstein cows abomasally infused with tallow (2% linoleic acid: 0.45 kg/d) compared with those infused with yellow grease (20% linoleic acid; 0.45 kg/d) on Day 15 of the estrous cycle when challenged with oxytocin. They suggested that infusion of yellow grease inhibited the ability of the uterus to secrete $PGF_{2\alpha}$. Perhaps the lower concentration of linoleic acid in the tallow infusion and the OO in the present study provided a more optimal quantity compared to that of the yellow grease or the SB and caused little if any inhibition of prostaglandin synthesis.

In humans, conversion of linoleic acid to $PGF_{2\alpha}$ was slow, and 4 to 5 days of ingesting the essential fatty acid was required before an increase was detected

(Adam et al., 1982). In ruminant animals the response in prostaglandin synthesis from exposure to essential fatty acids appears to occur more rapidly. Infusion of OO caused an immediate increase in serum PGE_2 on Day 9 and each of Days 13 through 15 and of PGFM on Days 13 and 15, whereas levels of PGE_2 declined after infusion was terminated. It may be that the rapid increase in serum prostaglandins detected in ewes occurred due to production of these eicosanoids by a number of tissues besides the uterus. Similarly, Lucy et al. (1990) found an increase in plasma concentrations of $PGF_{2\alpha}$ in heifers infused abomasally with either safflower (70% linoleic) or olive oil (8% linoleic) after 4 h.

Infusion of lipid into cows during the mid-luteal phase of the cycle resulted in a shortened cycle (Lucy et al., 1990), which was attributed to a premature increase in plasma $PGF_{2\alpha}$, measured as PGFM. Hence, it was anticipated that the duration of the estrous cycle in ewes receiving lipid would be shortened. In Exp. 1, estrous cycles tended to be shorter in SB-infused ewes relative to those of control ewes (16.7 ± 0.3 vs. 18 ± 0.6 days, $p < 0.10$); however, in Exp. 2, duration of the estrous cycle was shortened in OO-infused ewes, but not SB-infused ewes. Concentrations of PGFM increased on Day 14 by hour 8 of the sampling period in OO-infused ewes, and were greatest on Day 15 (Fig. 11, Fig. 12, respectively), suggesting that luteolysis was initiated on Day 14 or 15. Inskeep et al. (1975) reported decreased plasma concentrations of P_4 in ewes within 1 h of follicular exposure to exogenous $PGF_{2\alpha}$ compared with control ewes. Upon examining serum concentrations of P_4 in individual ewes, levels began declining on Day 14 in three OO-infused ewes, whereas this trend was not apparent in SB- or

S-infused ewes. On Day 15, levels had declined markedly in all OO-infused ewes and in three SB-infused ewes compared with only one S-infused ewe (data not shown). The increase in PGE_2, which occurred in OO-infused ewes, did not likely cause a luteotropic effect because it could not counteract the effects of increased levels of $PGF_{2\alpha}$ present in the ewes at this time of the reproductive cycle.

Mean number of follicles > 4 mm was greatest in the OO then in SB-infused ewes, although this was not a significant difference. This follows a similar trend that occurred in cattle (Lucy et al., 1990; Ryan et al., 1992) in which administration of long chain fatty acids caused a significant increase in the number of large follicles. An increase in follicle number may result in a subsequent increase in ovulation rate; however, this did not occur in the present experiment. Perhaps supplying a concentrated source of energy for a longer period of time may have influenced ovarian characteristics significantly in ewes. In fact, when ewes are fed an elevated level of energy prior to breeding, ovulation rate increases (Allen and Lamming, 1961; Coop, 1966a,b; Rhind et al., 1989), but maximal response is achieved after 30 to 60 days (Allen and Lamming, 1961; Bellows et al., 1963).

CONCLUSIONS

Summary

In conclusion, ewes infused with lipid during mid- to late diestrus in both Exp. 1 and Exp. 2 had increased serum concentrations of total cholesterol, which may have provided a substrate for increased progesterone synthesis. Alternatively,

lipid infusion may have caused a decreased clearance rate of progesterone, hence increasing serum concentrations. In Exp. 2, OO stimulated production of $PGF_{2\alpha}$ and PGE_2 compared with ewes infused with S or SB. It is likely that the low concentration of linoleic acid in OO provided a precursor for prostaglandin synthesis by the uterus or other tissues and the high concentration of linoleic acid in SB competed with arachidonic acid for binding to cyclooxygenase, thus yielding less prostaglandin than that of OO-infused ewes, yet greater than that of control animals. To support this, the duration of the estrous cycle of OO-infused ewes was shortened compared with S- or SB-infused ewes, probably due to an increase in $PGF_{2\alpha}$ causing the demise of the CL. Ovulation rate and follicular dynamics were not different between treatments, however, numerical values demonstrate that OO-infused ewes experienced increased follicular development compared with that of control and SB-infused ewes; the latter also had greater follicular development compared with control animals.

Recommendations for Future Research

Future research must focus on the effects of lipid administration to ewes immediately after breeding. It is possible that an increase in progesterone due to treatment may improve embryo survival; however, it is not known whether the increase in progesterone as detected in the present study was dependent on stage of the cycle. It is an exciting possibility that concentrations of prostaglandins can be manipulated through nutrition to alter reproductive function. It must be determined whether the increase in prostaglandin production in OO-infused ewes

was due to a general effect of fatty acids or to a specific effect of linoleic acid. In addition, future research should examine how supplemental fatty acids might alter antiluteolytic mechanisms in early pregnancy in the ewe.

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APPENDIX

List of Abbreviations

ACAT	acyl-CoA:cholesterol acyltransferase
cAMP	cyclic adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
bTP-I	bovine trophoblast protein-I
C	carbon
CoA	coenzyme A
CL	corpus luteum, corpora lutea
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
FSH	follicle-stimulating hormone
GnRH	gonodotropin-releasing hormone
HDL	high density lipoprotein
HETE	hydroxy-eicosatetraenoic acid
HMG-CoA	hydroxymethylglutaryl-CoA
HPETE	hydroperoxy-eicosatetraenoic acid
3ß-HSD	3ß-hydroxysteroid dehydrogenase
IFN-gamma	interferon-gamma
IL-1	interleukin-1
LCAT	lecithin cholesterol acyl transferase
LDL	low density lipoprotein
LH	luteinizing hormone
LS	least squares
MFO	mixed function oxidase
MHC	major histocompatibility complex
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NE	net energy
NSB	nonspecific binding
OO	olive oil emulsion
OT	oxytocin
oTP-I	ovine trophoblast protein-I
OVX	ovariectomized
P_4	progesterone
PFS	prostaglandin-free serum
PG	prostaglandin
PGFM	13,14-dihydro-15-keto $PGF_{2\alpha}$
PMSG	pregnant mare serum gonadotropin
RIA	radioimmunoassay
RNA, mRNA	ribonucleic acid, messenger RNA
S	saline
SB	soybean oil emulsion

List of Abbreviations (Continued)

SCC	side chain cleavage
TC	total cholesterol
TNF-α	tumor necrosis factor alpha
TRIS	tris(hydroxymethyl)aminomethane
VLDL	very low density lipoprotein