Abstract

Wolfe, Jason R. THE EFFECTS OF PREGNANCY OR PROGESTERONE ON ETHANOL CONSUMPTION IN HIGH ETHANOL PREFERRING RATS. (Under the direction of Dr. Brian A. McMillen). Department of Biology, East Carolina University. July, 1999.

A significant fraction of women continue to drink heavily during pregnancy, which is associated with fetal alcohol syndrome, alcohol-related birth defects, alcohol-related neurodevelopmental disorder, and spontaneous abortion. Previous reports indicated that outbred rats, induced to drink ethanol, would decrease their volitional intake during pregnancy. This study examined the selectively bred genetic drinking HEP rat. Rats from the F7 generation were screened by a ten day 3-30% v/v ethanol concentration 'step up' procedure in order to determine the concentration which resulted in maximal drinking with an ethanol solution to total fluid ratio closest to 0.5. After baseline drinking of the fixed concentrations was established, female HEP rats were randomly selected for mating and their ethanol removed. Upon finding a 'sperm plug', male rats were removed and the ethanol was returned. A second group received injections of progesterone in sesame oil beginning with a 1.0 mg/kg/day dose which was increased to 3.0 mg/kg/day on days 5-20. Vaginal smears confirmed that the progesterone made the rats anestrous. One half of the control rats received no injections and one half received injections of sesame oil. Neither pregnancy nor progesterone changed either the amount or proportion of ethanol consumed compared to the baseline period. The rats drank an average of 8.4 g/kg daily throughout pregnancy. A sharp drop in food intake was noted the day after mating. However, only one of the pregnant rats successfully delivered a litter. These rats were taken off the ethanol and mated again:

seven of the eight rats delivered litters. Beginning on day 13, it was observed that the pregnant rats showed a marked increase in the variance for body weight. A similar sustained increase in variance for proportion, but not amount of ethanol consumed, was also noted for this group. These two findings led to the conclusion that the pregnant females must have begun to lose their litters on or after day 13. Pregnancy does not affect the consumption of ethanol in the HEP rat. The HEP rat could therefore serve as a model for the female type 2 alcoholic. In addition, due to the fact that drinking by HEP rats during pregnancy leads to such a high rate of resorption of the fetus, this strain may also constitute a useful model for the study of alcohol-induced spontaneous abortion.

THE EFFECTS OF PREGNANCY OR PROGESTERONE ON ETHANOL CONSUMPTION BY HIGH ETHANOL PREFERRING RATS

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements of the Degree

Masters of Science in Biology

by

Jason R. Wolfe

July 1999

THE EFFECTS OF PREGNANCY OR PROGESTERONE ON ETHANOL CONSUMPTION IN HIGH ETHANOL PREFERRING RATS

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Introduction

The Myers HEP Rat and the Genetics of Alcoholism

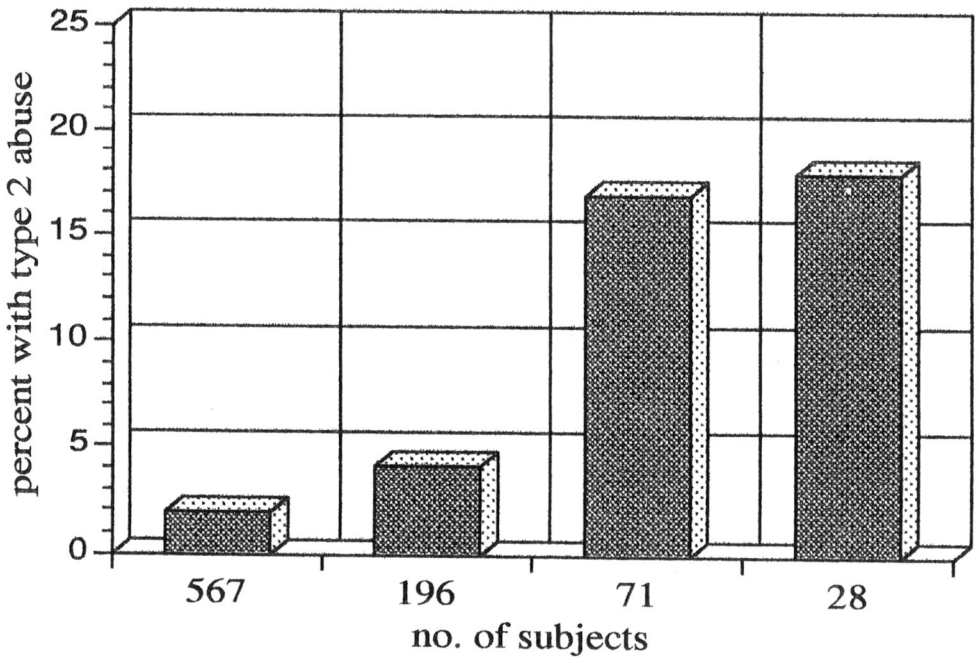
The primary objective of this research was to investigate the effects of pregnancy or progesterone on the consumption of ethanol by the Myers High Ethanol Preferring (HEP) rat in order to find a valid model for the severe female alcoholic. This new strain of rat was derived initially by crossing female Harlan Sprague Dawley rats with male ethanol preferring (P line) rats selectively bred from Wistar rats at Indiana University (Myers et al., 1998). Myers and co-workers (1998) discovered accidentally that a variant strain of Harlan Sprague Dawley rats displayed an unusual preference for ethanol. Therefore, a decision was reached to breed these Sprague Dawley female rats with the high ethanol preferring (P) male rats. The resulting hybrid provides a genetic model of the human type 2 alcoholic. Although alcoholism is a disease that can take on multiple forms, there are two main classes of alcoholics: type 1 and type 2 (Cloninger et al., 1981). The type 2 alcoholic has a genetic predisposition to drink and begins drinking at an early age. The type 1 alcoholic, on the other hand, has no genetic element associated with his or her drinking and usually begins drinking at a later age (Cloninger et al., 1981; Cloninger, 1987).

It has been clear for many years that genetics plays a role in alcoholism. It was perhaps best illustrated by Cloninger and coworkers' studies on the effects of genetic and environmental history on male Swedish adoptees (Cloninger et al., 1987). They demonstrated that genetics, much more so than the environment, was the contributing factor behind male Swedish adoptees developing type 2 abuse (Figure 1).

Figure 1

Effects of genetic and environmental history on adult male Swedish adoptees (Cloninger et al., 1981). The predominant role that genetics plays over the environment is illustrated. Adult adoptees were interviewed in order to determine whether they met criterion for Type 2 alcoholism. Genetics indicates whether one or both biological parents were alcoholic. Environment indicates whether one or both adoptive parents were alcoholic.

Effect of Nature vs. Nurture on Alcoholism: Swedish Adoption Study



genetic	no	no	yes	yes
environ.	no	yes	no	yes

The HEP rat meets the two criteria which must be met in order for an animal to be considered a valid model of the human alcoholic (McMillen et al., 1998). First, it imbibes high concentrations and copious amounts of ethanol in water for a pharmacological effect (which is indicated by high blood ethanol levels). Second, it continues to do this when a nutritious, highly palatable alternative, such as a chocolate solution, is offered in addition to an ethanol solution and water (Lankford et al., 1991; Myers et al., 1998). The sixth generation of female HEP rats consumed more ethanol (at an average of 10.3 g/kg and at an average concentration of 15.7%) than any other rat previously genetically bred to drink alcohol, including the HEP males. Another noteworthy characteristic is that ethanol consumption does not vary during the estrus cycle (Myers et al., 1998) which has been apparent in outbred strains of rats (Forger and Morin, 1982). In addition, the HEP rat drinks immediately at first exposure, even at thirty days of age (Myers et al., 1998).

Consumption of Ethanol During Pregnancy

Data collected on humans, suggest that most nonalcoholic women decrease their alcohol consumption during the first trimester of pregnancy, citing the reason for the reduction being a distaste for alcohol. In one study of pregnant women, 53% reported a distaste for alcohol that arose during pregnancy, and 65% of the women in the same study reported that consuming alcohol resulted in illness (stomach irritation, nausea, and headache) (Little et al., 1976). Another study conducted on 530 pregnant women, 90% of whom drank before and during their pregnancies, demonstrated that the proportion of drinking women decreased with advancing gestational age. Fifty percent of the women retrospectively reported drinking after 32 weeks and only 20% reported drinking during the last week of

gestation (Halmesmaki et al., 1987).

There are two main problems with data collection on ethanol consumption in pregnant humans. One is that collecting data often involves retrospective self-report which is invariably influenced by self image consequences. The other is that concern for fetal welfare often elicits a decrease in alcohol consumption during pregnancy. To truly determine if a biological feto-protective mechanism exists for the fetus, such confounding, cognitive factors should be eliminated from a study. The women tested would have to be unaware of the harmful effects that ethanol has on the fetus. Finding such a group today would be difficult because most women are educated on this matter by their doctors.

Perhaps the best place to look for such a group of test subjects would be in a developing country where pregnancy education is often minimal at best. To date, only one such study, which was conducted in Papua, New Guinea, has been carried out. Surprisingly, even in this developing country, many women were aware of alcohol's potential harmful effects on the fetus. Due to their knowledge, the factor or factors causing the reported decrease in alcohol consumption found in the study (Marshall, 1985) cannot be ascertained.

A study conducted on one hundred pregnant women in Ireland, 89% of whom drank alcohol prior to pregnancy, attempted to determine the relationship between alcohol consumption and the level of knowledge to its potential adverse effects. Eleven of the women completely stopped drinking when they became pregnant and 66% of the women decreased their alcohol consumption considerably, but this left a considerable percentage who continued to drink throughout pregnancy. Forty-two percent of the women were unaware of the harmful effects of alcohol during pregnancy (Daly et al., 1992). Although it is unclear how

many of the unaware women decreased their alcohol consumption, it can be concluded that something other than cognitive factors were playing a role in the decrease of alcohol consumption during pregnancy.

The trend of decreasing alcohol consumption during pregnancy occurs in other animals that drink alcohol including mice (Emerson et al., 1952), rats (Means and Goy, 1982; Sandberg et al., 1982), hamsters (Carver et al., 1953), and monkeys (Elton and Wilson, 1977). The fact that this occurs in a number of different species in conjunction with the observation that a substantial amount of women acquire a taste aversion for alcohol during pregnancy (Little et al., 1976) suggests that there is a protective mechanism for the fetus, mediated by reproductive hormones, that has evolved to cause the gravid female to reject potentially toxic substances.

To date, a limited number of studies have utilized genetic female drinking rats, and no research has been done on these animals during pregnancy. Two independent studies have shown that outbred strains of rats decreased their proportion and consumption of ethanol during pregnancy (Means and Goy, 1982; Sandberg et al., 1982), but these rats were probably drinking for either caloric value or taste. The high ethanol preferring rat (HEP), on the other hand, consumes ethanol for a pharmacological effect, and will develop significant blood alcohol concentrations (Myers et al., 1998).

Hormones and Consumption of Ethanol

Investigations on the influence of ovarian hormones upon ethanol consumption in rats have provided mixed results. This ambiguity is probably due to the choice of and dose of hormones, the concentration of ethanol solutions (Forger and Morin, 1982) and the different

strains of rats used.

However, estradiol enhances ethanol consumption in intact (ovaries are present) rats, but decreases ethanol consumption in ovariectomized rats (Forger and Morin, 1982). The exact mechanism by which the enhancement in the intact females occurs remains unclear, but seems to be regulated by an interaction between the ovarian hormone estradiol and unidentified adrenal hormones (Forger and Morin, 1982). Ovariectomy produces a hormonal state that to some extent mimics pregnancy. Similar to the pregnant rat, the ovariectomized rat is characterized by diminished ethanol consumption, high levels of progesterone, and low levels of estrogen (Forger and Morin, 1982). The effect of ovariectomy on the HEP rat is unknown since this procedure has never been tested with this rat. Morin and Forger (1982) suggested that high levels of progesterone during pregnancy may directly inhibit ethanol consumption or indirectly do so by blocking estradiol activity. It is possible that the decrease in consumption brought about by the administration of estradiol to the ovariectomized female was due to the fact that estradiol acted to increase the concentration of progesterone binding sites at a time when the level of progesterone was high. Progesterone has a wide range of effects on the endocrine systems and behaviors of many species, and is quite an effective blocker of estradiol (Morin, 1977). In further support of the hypothesis that progesterone, not estrogen, is the inhibitor of ethanol consumption, estradiol was found to have no effect on alcohol consumption in normal women (Roehrs and Samson, 1982). Yet, as previously suggested, many women significantly decrease their ethanol consumption during pregnancy.

The pregnant human female maintains high levels of both estrogen and progesterone during pregnancy, which later decrease during lactation (Tortora and Grabowski, 1996).

Conversely, the states of pregnancy and lactation in the rat are characterized by low levels of estrogen and high levels of progesterone. Due to this fact, it remains unclear why rats in one study showed a decrease in ethanol consumption during pregnancy, but an increase in ethanol consumption during lactation (Means and Goy, 1982). Perhaps this was due to the fact that the demand for calories and fluids which increases dramatically during lactation prevailed over the inhibitory effect of progesterone.

Alcohol Related Birth Complications

It has long been known that ethanol freely crosses the placenta (Tortora and Grabowski, 1996), and that high alcohol consumption during pregnancy can cause a number of negative impacts including spontaneous abortion as well as the birth defects collectively labeled fetal alcohol syndrome (FAS). In humans, the effects of this teratogen can be devastating to the fetus. Fetal alcohol syndrome is a condition that is characterized by growth deficiency, a specific pattern of facial anomalies, and central nervous system damage. Drinking associated with FAS has its primary effect on the brain (Streissguth, 1997) and is now considered to be the most common nongenetic cause of mental retardation (Davidson and Alden, 1981). The incidence of FAS has been estimated to vary from 0.6 to 3 per 1000 live births in most communities, although some show much higher rates (Stratton et al., 1996). From this estimation it can be projected that anywhere from 2,000 to 12,000 of the 4 million infants born each year in the United States will have FAS (Streissguth, 1997).

But FAS is just "the tip of the iceberg" in terms of alcohol-related birth disorders. Alcohol is associated with spontaneous abortion (Harlap and Shiono, 1980; Kline et al., 1980; Windham et al., 1997). Alcohol-induced spontaneous abortion usually terminates the fetus

and can imperil the life of the mother. In addition, two other alcohol-related birth disorders have also been defined: Alcohol-related birth defects (ARBD) and alcohol-related neurodevelopmental disorder (ARND). Alcohol related birth defects refers to alcohol-induced physical anomalies; and ARND refers to alcohol-induced cognitive and behavioral problems without the hallmark facial and growth abnormalities. These two disorders in conjunction with FAS significantly increase the magnitude of the overall problem (Stratton et al., 1996). Fetal alcohol syndrome, ARBD, and ARND may occur on average in as many as 6 per 1000 live births (May et al., 1983). However, this estimate may be very low due to the fact that the diagnosis of FAS, ARBD, and ARND is often very difficult (Stratton et al., 1996). Therefore, many cases are unreported. Fetal alcohol syndrome and related disorders are often overlooked at birth and treated later by community professionals--many times unknowingly (Streissguth, 1997). The highest prevalence rate ever recorded anywhere occurred in a Native Canadian community which was noted for its high rates of alcohol consumption. In this small population, it was estimated that the prevalence of FAS, ARBD, or ARND was 190 per 1000 live births (Robinson et al., 1987).

Fortunately, FAS, ARBD, ARND, and alcohol-induced spontaneous abortion are completely preventable complications. Preventative efforts have primarily focused on two viable approaches: encouraging women to abstain from alcohol use during all phases of pregnancy; or alternatively, advising women who drink alcohol not to consider trying to conceive unless they can control their drinking (Stratton et al., 1996).

Unfortunately, heavy drinkers or alcoholics, who are most likely to give birth to children with FAS, are least likely to lower alcohol consumption on their own during

pregnancy (Stratton et al., 1996). Typically though, alcohol-focused prevention research does not target the actual treatment of alcohol abuse and alcoholism. Such treatment could prove to be very effective at preventing alcohol-related birth disorders. In support of this idea, the Institute of Medicine stated, "Treatment of alcohol abuse and dependency in a pregnant woman is also prevention of FAS in her fetus" (Stratton et al., 1996).

The current study demonstrates that the HEP rat, much like the severe human female type 2 alcoholic, continues to drink large quantities of alcohol during pregnancy. This strain could therefore, be very useful in testing new treatments for alcohol abuse and alcoholism. Finding a model to help develop treatments for the female alcoholic could significantly decrease the incidence of FAS and other alcohol related birth disorders.

Materials and Methods

Twenty-five female rats from the F7 generation of the high ethanol preferring colony started at East Carolina University were either impregnated by F7 male HEP rats, given injections of progesterone to mimic pregnancy, or served as controls while their drinking was monitored. The twenty-five rats were divided into two groups: a group of eleven and a group of fourteen. The difference between the two groups was the environment in which they were tested. The first group of eleven rats were housed on the stainless steel battery. Due to the fact that three of four presumed pregnant rats in this group failed to deliver pups, it was thought that the stainless steel cages may have been a factor in the pregnancy failures. Therefore, the second group of fourteen were housed in standard plastic cages with corncob bedding which is the environment normally used by the Department of Comparative Medicine for breeding. This change necessitated two separate protocols: one for the first group of eleven rats and another for the second group of fourteen.

Protocol # 1

Initially, each of the eleven female HEP rats went through two ten day step-up procedures adolescence and adulthood, respectively. Ethanol was presented (in a 24 hour unlimited beginning at approximately 40 and 80 days of age, which represent the beginning of access paradigm) ethanol in tap water in one calibrated drinking tube, tap water alone in a second drinking tube, and a third drinking tube left empty. Each day the concentration of ethanol was increased according to the following sequence: 3%, 4%, 5%, 7%, 9%, 11%, 13%, 15%, 20%, and 30%, and the tubes were rotated in a semi-random fashion to avoid the

development of a position habit (Lankford et al., 1991). The concentration of ethanol solution that resulted in maximal g ethanol/ kg drinking with a proportion of ethanol solution to total fluid fluids consumed nearest 0.5 was chosen as the preferred concentration that was used for the rest of the experiment for each rat. In this way, each rat selected the concentration at which it drank (Myers et al., 1991; McMillen and Williams, 1995). The proportion of 0.5 was chosen to allow for any large increases or decreases in proportion throughout the test period.

After a ten day stabilization period of drinking ethanol at each rat's preferred concentration, the eleven rats were divided into three groups (pregnant, progesterone injection, or control). This division was based on each rat's daily g/kg consumption of ethanol so that each of the three groups had approximately the same level of intake. This division resulted in four rats that were impregnated, three rats that received progesterone injections, and a group of four controls.

To impregnate the females, the alcohol tube was removed each night and a high consuming male HEP rat was placed in the cage for cohabitation. The male rat was removed in the morning and the alcohol bottle returned. This was repeated until a sperm plug was found in the litter tray under each cage of the four rats. The day the sperm plug was observed was designated day 1 of pregnancy or gravid day 1 (GD-1). The rats were then, once again, allowed 24 hour access to the alcohol solution. Each day, the volume of fluids, body weight, and amount of food (Pro Lab Chow) consumed was recorded. At GD-17, a paper towel was placed in each of the cages on the battery that contained a pregnant female so she could begin nest-building activities. In addition, a piece of screen was placed on the floor of each cage

so that the newborn pups would not fall through the grid floor. The screen was replaced every couple of days because the females regularly bit large holes in the screen. Three of the females in this group did not give birth and were reimpregnated in the absence of alcohol to show that they were capable of delivering a litter.

The progesterone group of three rats received injections of progesterone in sesame oil to mimic the pregnant state. The sesame oil was used as a depot to slow and prolong the absorption of the progesterone. On days 1 and 2, the rats received a 1.0 mg/kg dose of progesterone in sesame oil. On days 3 and 4, the rats received a 2.0 mg/kg dose of progesterone in sesame oil. And on days 5 through 20, the rats received a 3.0 mg/kg dose of progesterone in sesame oil. The 3.0 mg/kg dose of progesterone is reported to inhibit cycling by females and mimic the pregnant state (Zarrrow et al., 1964). As with the impregnated dams, the body weight, food intake, and fluid consumption was recorded. Vaginal smears were also performed on this group in order to confirm that the progesterone inhibited cycling.

The control group of four rats received no treatment. After data were collected for this group, the four females were impregnated to show that they were capable of reproduction. Therefore, any differences in consumption between the controls and the experimental groups could not be attributed to differences in reproductive potential (Means and Goy, 1982). Vaginal smears were conducted on the three that did not deliver after they had been taken off of alcohol in order to confirmed that these three were still cycling.

Protocol #2

As with the first group, the second group of rats went through a ten day step-up

procedure with 3-30% ethanol at 40 and 80 days of age while they were on the battery. From this, a preferred concentration was assigned to each rat. Then the rats went through the standard ten day stabilization period on the battery. It was at this point that the decision was made to alter the living environment to increase the likelihood of the females giving birth. The entire group of fourteen female rats was taken off the battery and placed in individual plastic cages. Therefore, the stabilization period was repeated with the rats in their new environments.

After this second ten day stabilization period was repeated, the fourteen rats were divided into three groups (pregnant, progesterone injection, or injection control) as before. This division was based on each rat's g/kg ethanol consumption during the stabilization period so that each group had approximately the same level of ethanol consumption. The division resulted in a group of four impregnated dams, five rats that received injections of progesterone, and a group of five controls which received injections of sesame oil. Combined with the rats from protocol # 1, this resulted in a total of eight pregnant females, eight females that received injections of progesterone, and nine controls for the entire experiment.

To impregnate the four females, they were taken out of their standard plastic cages each night and placed on the battery with two water bottles and a high consuming male HEP rat. The female was placed back in her standard cage with alcohol each morning and back on the battery at night. The day the sperm plug was found was designated GD-1 and the female was returned to 24 hour access to the alcohol solution. Each day, the volume of fluids, body weight, and amount of food consumed was recorded. At GD-17, bedding materials were placed in each of the four cages so that the pregnant mothers could begin nest-

building activities. None of the four females delivered, so they were reimpregnated in the absence of alcohol to show that they were capable of delivering a litter. One of these four failed to deliver. Therefore, a vaginal smear was conducted in order to confirm it was cycling.

The injection group of five rats was administered subcutaneous injections of progesterone in sesame oil to mimic the pregnant state in the same manner as in protocol #1. The only difference was that these females were in standard cages as opposed to the cages on the battery.

The control group received injections of sesame oil only, to test for the possibility that the injections themselves or the vehicle had an effect on ethanol consumption. After data were collected for this group of controls, each of the five females were impregnated for the same reason stated in protocol # 1. Vaginal smears were conducted on the four that did not deliver in order to confirm that these four were still cycling.

Statistical Methods

Intra-group g/kg consumption, proportion of ethanol to total fluid consumed, changes in body weight, and food consumed were analyzed for significance by repeated measures one-way analysis of variance utilizing GB-STAT 4.4 (Dynamic Microsystems Inc. Silver Spring, MD). Individual time points were compared to baseline, an average of the four days prior to day 1, using Dunnett's (treatments vs. control) test. Inter-group comparisons of the same dependent variables were also analyzed for significance by one-way analysis of variance. Most data are expressed as mean ±standard error with significance ($p<0.05$) displayed with a symbol (either an asterisk, a plus sign, or a letter) on the figures. Some data are expressed as standard error of the mean (s.e.m.) in order to illustrate changes in variance.

Results

Pregnancy Outcomes

In the first group of eleven females, one of four pregnant females successfully delivered. The one HEP rat which did deliver did so rather unexpectedly three to four days later than normal. After no pups could be palpated in the rat's abdomen on day 23, it was taken off the battery and put in a standard cage with just a water bottle. On day 25, it gave birth to a small litter of eight pups (six viable, two dead), with the six weighing a total of 36g. It was this unexpected result that led to the change in environment in which the second group of fourteen rats was housed. This was done in an effort to determine if the environment offered by the small stainless steel cages of the battery was leading to the high rate of fetus resorption. Although the high rate of resorption could have been due to the fact that the pregnant mothers were imbibing copious amounts of ethanol, it is also possible that the confined environment in which they live elicited the resorptions. Pregnant rats have been shown to eat their offspring when they are in an environment that is not conducive to raising healthy young.

None of the females in the second group of fourteen delivered. All of the females that failed to deliver (seven of eight in the pregnant group) apparently resorbed their fetuses because there was no evidence of parturition in any of the three cages.

Vaginal Smears

Vaginal smears confirmed that the 3.0 mg/kg daily dosage of progesterone made the rats anestrous. Anestrous is the nonreceptive period of the estrous cycle, and is characterized

by small, round, immature epithelial cells with large nuclei. A smeared slide typically has a few cells: two to five per low power field (Figure 2a).

Confirmation of cycling by the control rats which were re-mated and subsequently failed to deliver, and the one rat from the pregnant group which also failed to deliver after being taken off of the alcohol, was also provided by vaginal smears. After having been taken off of ethanol, estrous in these rats was demonstrated by the large numbers of angular, anuclear, cornified epithelial cells found in clumps on smeared slides (Weil, 1996) (Figure 2b).

Consumption of Ethanol

Consumption of ethanol for each of the three groups of HEP rats (pregnant, progesterone, and control) primarily ranged from 6-10 g/kg. Overall, daily consumption during the baseline period averaged 7.10 ±0.65 g/kg for the controls, 7.69 ±0.94 g/kg for the pregnant rats, and 7.02 ±1.21 g/kg for the progesterone treated rats. The preferred concentration of ethanol for each group was 14.4%, 17.9%, and 16.0%, respectively. Drinking was remarkably steady over the twenty-five day testing period with two exceptions: on day 16 the control group's consumption, 9.71 ±1.42 g/kg, varied from baseline ($p<0.01$), and on day 17 the pregnant group's consumption, 12.17 ±3.4 g/kg, varied from their baseline ($p<0.05$). The pregnant group exhibited a decrease in drinking following cohabitation at day 1, and an increase at day 3, neither of which were statistically significant (Figure 3). Pregnancy had the greatest affect on the variance of ethanol consumption. Increases in variance were observed on days 3 and 17, with standard error of the mean values reaching 2.9 and 3.4, respectively. The progesterone group showed an increase in variance of ethanol

Figure 2

a. A vaginal smear performed on a progesterone treated female HEP rat. The smear demonstrates that the 3.0 mg/kg/day dose inhibited cycling and made the rat anestrous. Shown are a scant number of immature epithelial cells which are commonly found during anestrous. 100X.

b. A vaginal smear taken from one of the females in the control group. The smear demonstrates that the female was still cycling which is evidenced by the numerous cornified epithelial cells typically found during estrous. 100X.

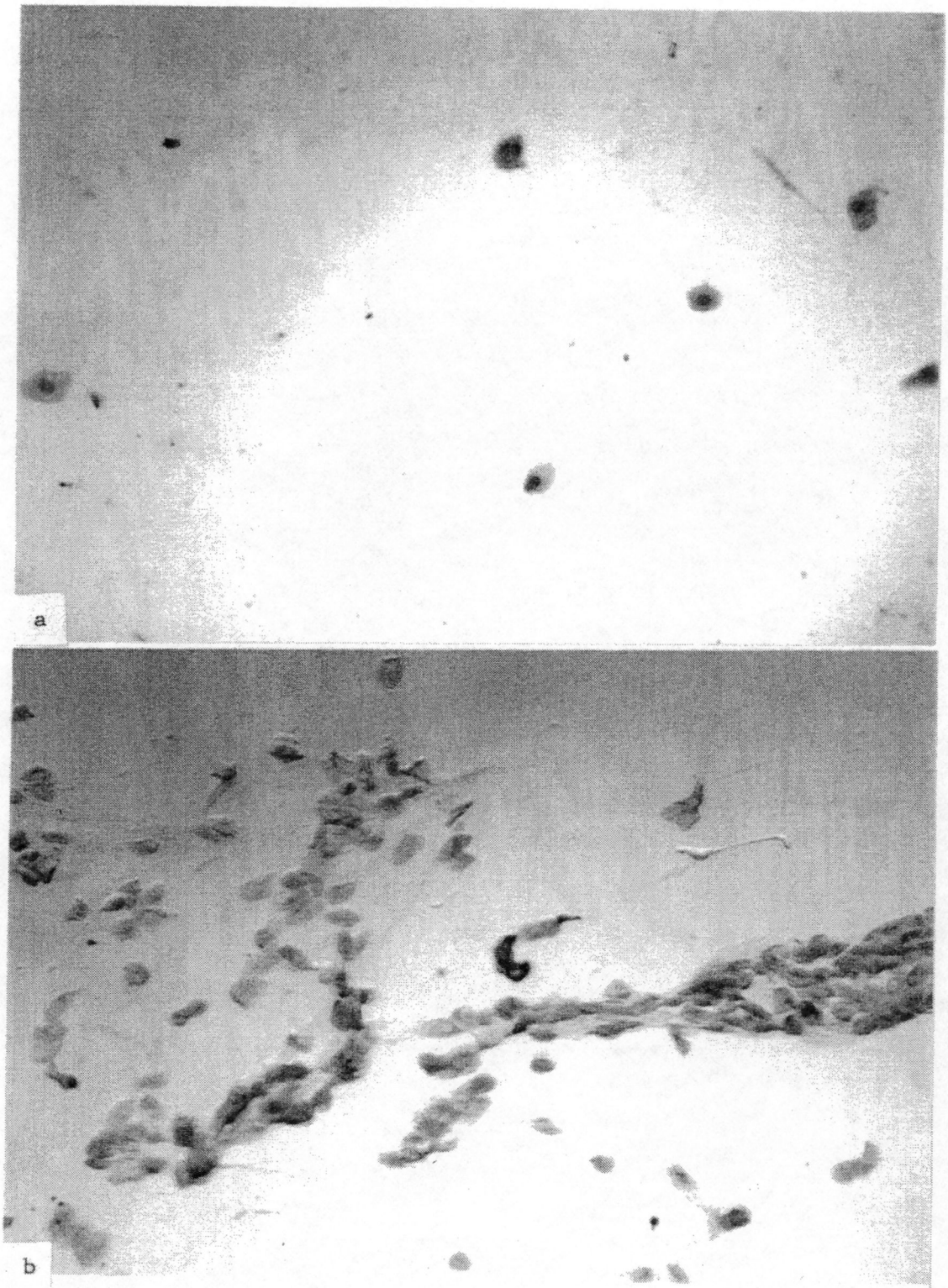
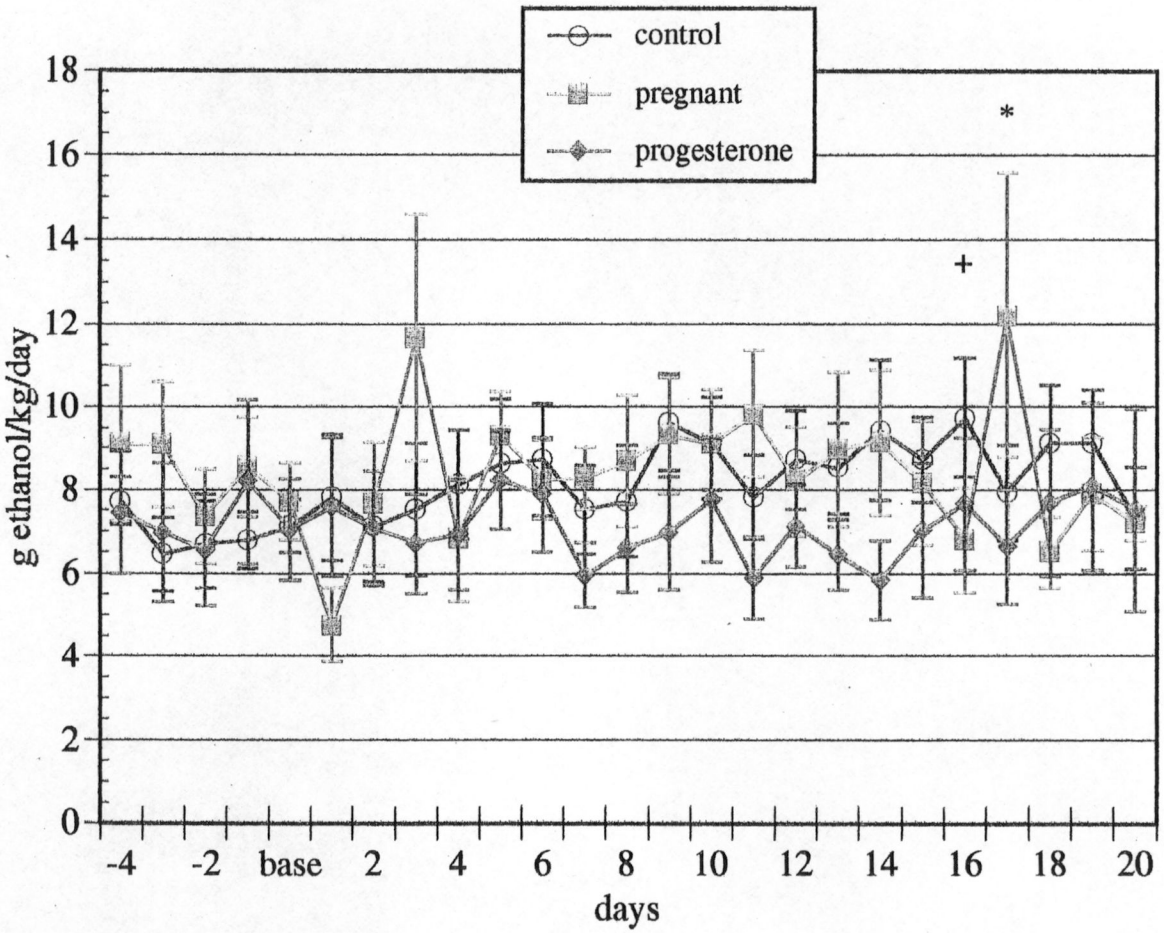

a
b

Figure 3

Amount of ethanol consumed by HEP rats during pregnancy or injections of progesterone. Twenty-four hour access to water or alcohol was allowed, and daily consumption of each was recorded.
Control group's consumption was significantly different from the baseline period (four days prior to mating or injections of progesterone), + $p<0.01$.
Pregnant group's consumption varied significantly from baseline, * $p<0.05$.


control
pregnant
progesterone
*
+
g ethanol/kg/day
0
2
4
6
8
10
12
14
16
18
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

consumption which began on day 18 and lasted until the end of the treatment period (Figure 4).

Proportion

No significant differences were noted for all three groups with regards to the proportion of ethanol to the total volume of fluids consumed. Average proportion was approximately 0.5, with a range of 0.4-0.6 (Figure 5). The concentration of alcohol was selected for each rat to be near a proportion of 0.5.

An increase in variance was demonstrated by the pregnant group which began on day 14 and continued until day 19. The increase in variance was not due to a consistent change in one or two females, but rather all of the females exhibited an increased variance. By comparing the range of values for proportion for each rat on days 8-13, the five days prior to the significant increase in variance, to the range of values on days 14-20 it was discovered that every rat had at least one day with a lower value for proportion, and five of the eight had at least one day higher. For example, one rat had values for proportion that ranged from 0.50 to 0.625 during days 8-13. This same rat had values for proportion that ranged from 0.327 to 0.785 during days 14-20. These highs and lows occurred on different days for different rats. No significant increases in variance of proportion were noted in the control or progesterone treated groups (Figure 6).

Changes in Body Weight

Overall, the weights of each group increased during the twenty-five day test period. The pregnant group, by far, exhibited the most weight gain, 28g, which was an expected result. Weight gain for this group reached a significant level at day 5, with a mean weight of

Figure 4

Effect of pregnancy or progesterone on the variance of consumption of ethanol. The s.e.m. from the data in figure 3 were plotted.

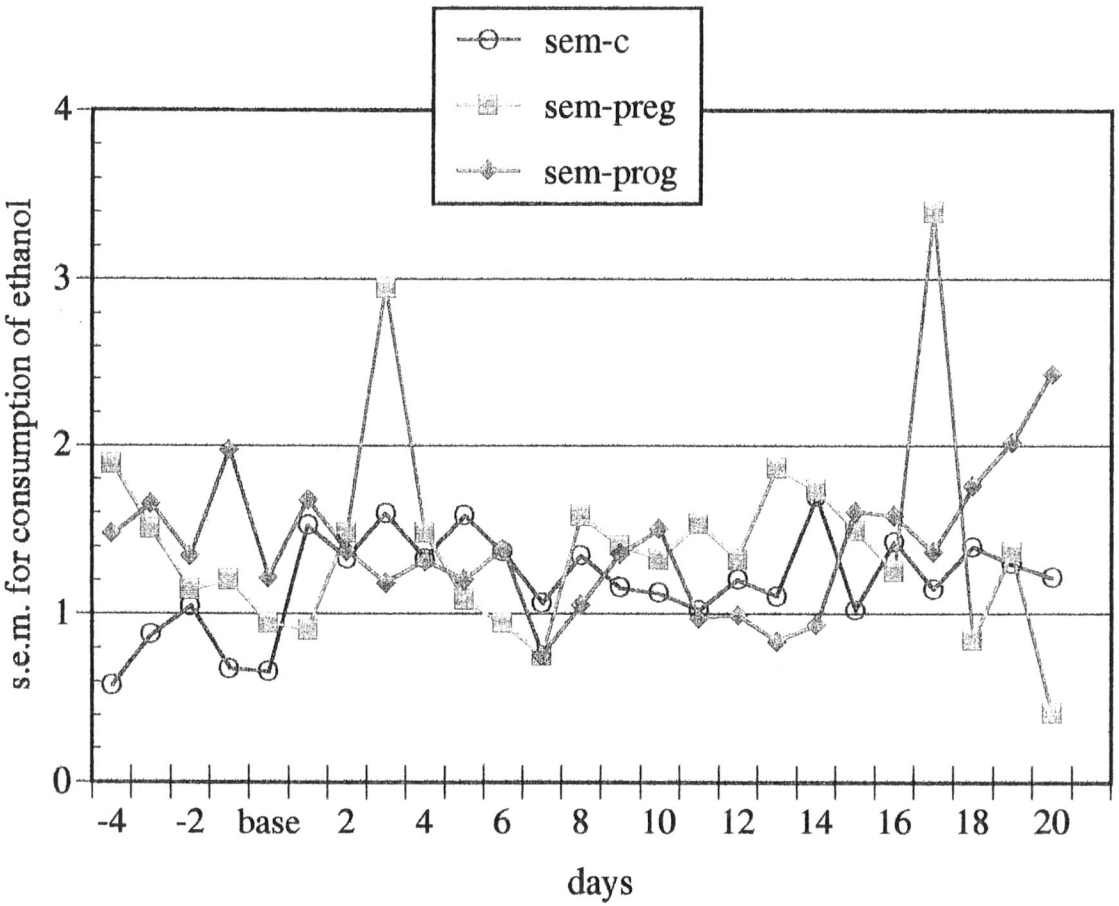

sem-c
sem-preg
sem-prog
s.e.m. for consumption of ethanol
0
1
2
3
4
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

Figure 5

Proportion of ethanol to total fluid volume consumed by HEP rats.
Proportion = ml ethanol / (ml water + ml ethanol).

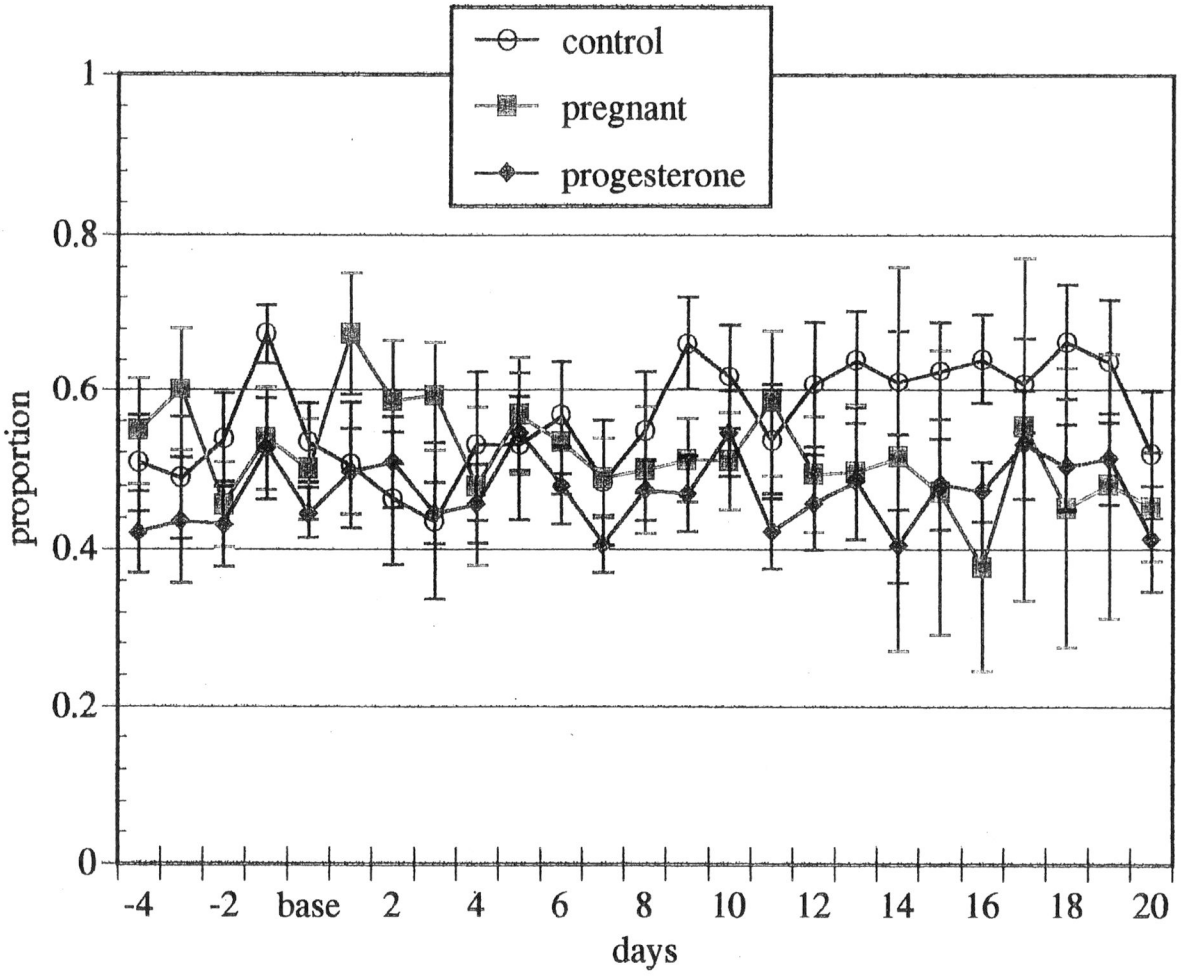
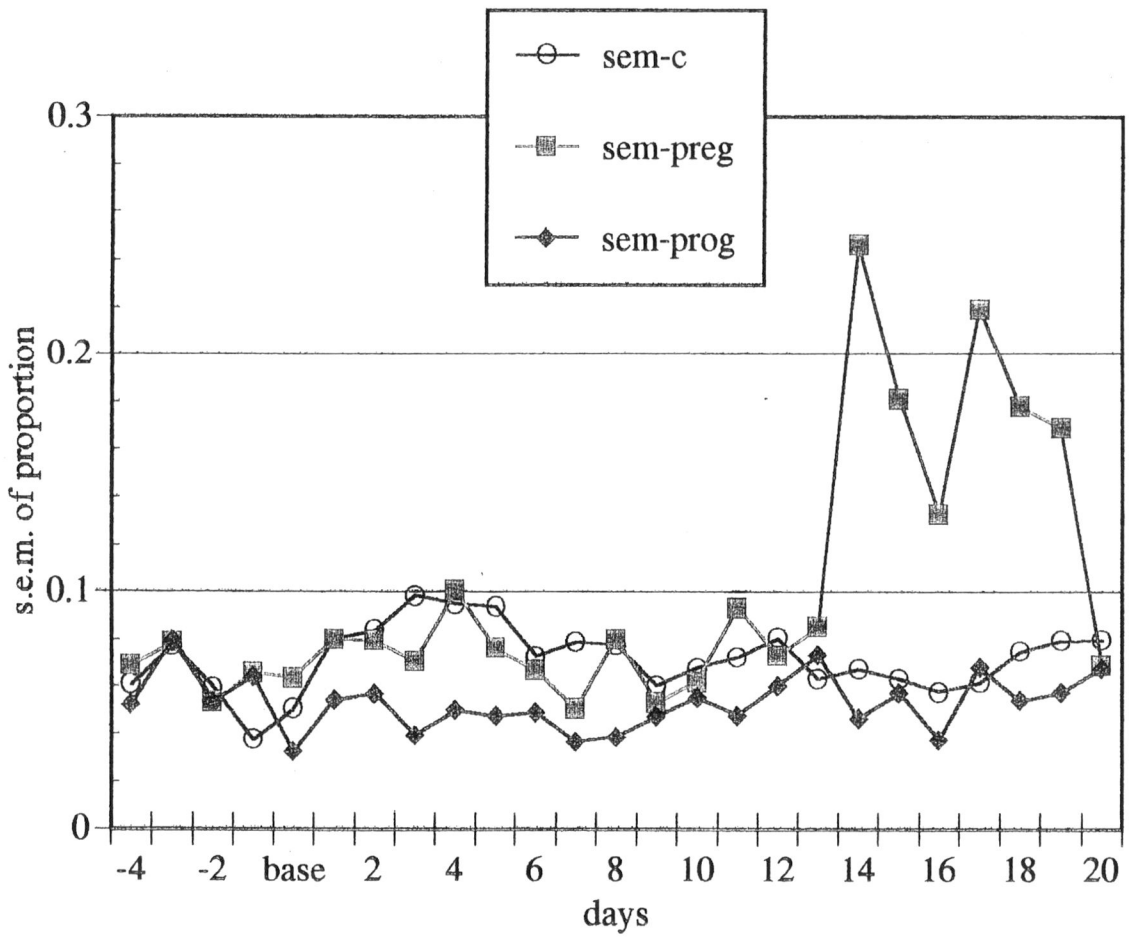

control
pregnant
progesterone
1
0.8
0.6
0.4
0.2
0
proportion
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

Figure 6

Effect of pregnancy or progesterone on the variance of proportion of ethanol consumed. The s.e.m. from the data in figure 5 were plotted.


sem-c
sem-preg
sem-prog
0.3
0.2
0.1
0
s.e.m. of proportion
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

281 ±6.7g ($p<0.01$), and remained significant for the remainder of the experiment. The control group and the progesterone treated group also showed significant, but smaller gains in weight. For the control group, weight gain reached a significant level at day 10, with an average weight of 277 ±6.7g ($p<0.01$). Total weight gain for this group over the test period was 11g. Weight gain reached a significant level at day 12 for the progesterone group, with a mean weight of 283 ±7.1g ($p<0.01$). The composite weight gain for this group was 15g (Figure 7). Beginning at day 13, s.e.m. for the body weight of the pregnant group markedly increased, reaching a peak of 0.011 on day 20. Variance for the other two groups remained fairly constant at lower levels (Figure 8).

Food Consumption

No significant increases in food consumption were found; however, significant decreases were noted for all three groups. Of the three, the pregnant group exhibited the greatest decrease in food consumption which occurred on day 1, with a mean consumption of 7.73 ±1.26g ($p<0.01$). Significant decreases were noted on days 7, 15, and 17 for the progesterone group with mean consumptions of 10.83 ±1.68g, 11.95 ±0.75g, and 11.83 ±1.26g, respectively. The control group consumed significantly less food on day –1 with a mean consumption of 11.06 ±2.62 g (Figure 9). Two of the animals in this group did not eat on that day.

Variance of food consumption was highly erratic for each of the three groups throughout the experiment. The control group exhibited a sharp increase in variance on day -1 with the standard error of the mean reaching a value of 2.6. The pregnant group showed

Figure 7

Effect of pregnancy or progesterone on body weight of HEP rats. Daily weights were recorded.
P Pregnant group's weight reached a significant level which remained significant, $p<0.01$
c Control group's weight reached a significant level which remained significant, $p<0.01$..
p Progesterone group's weight reached a significant level which remained significant, $p<0.01$.

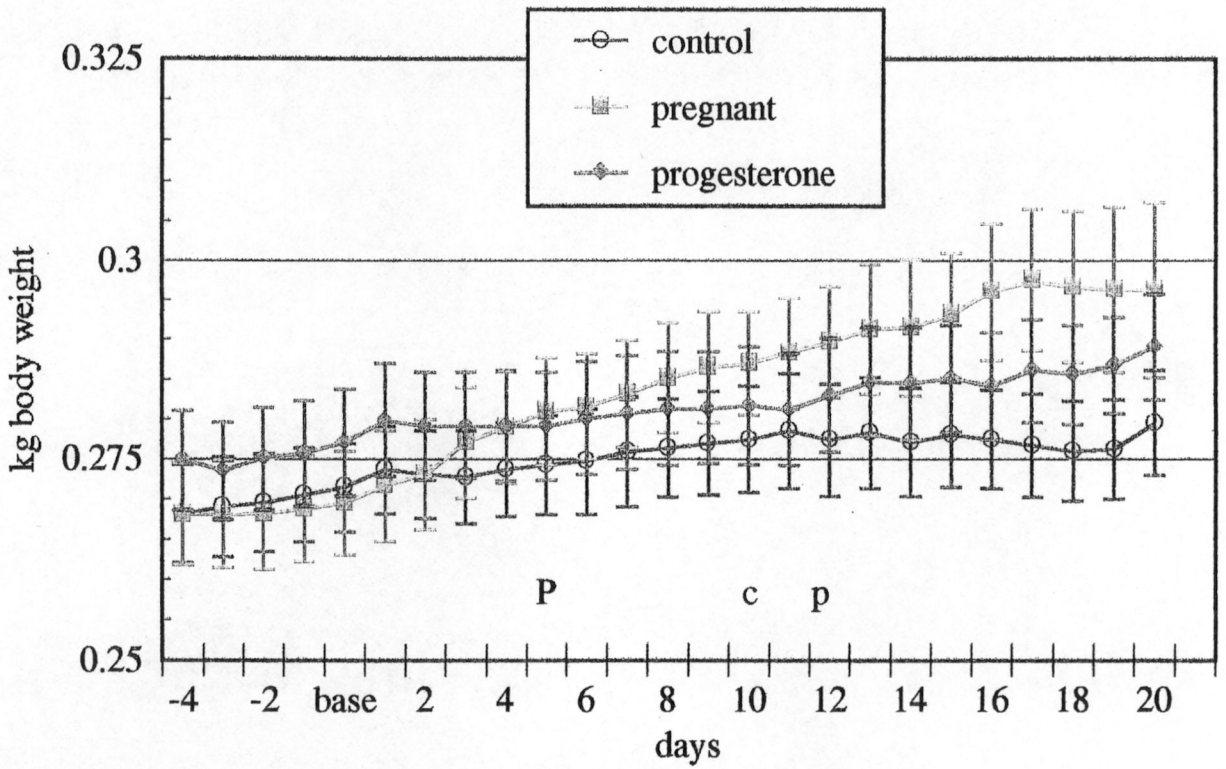

control
pregnant
progesterone
0.325
0.3
0.275
0.25
kg body weight
P
c
p
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

Figure 8

Effect of pregnancy or progesterone on the variance of body weight. The s.e.m. from the data in figure 7 were plotted.

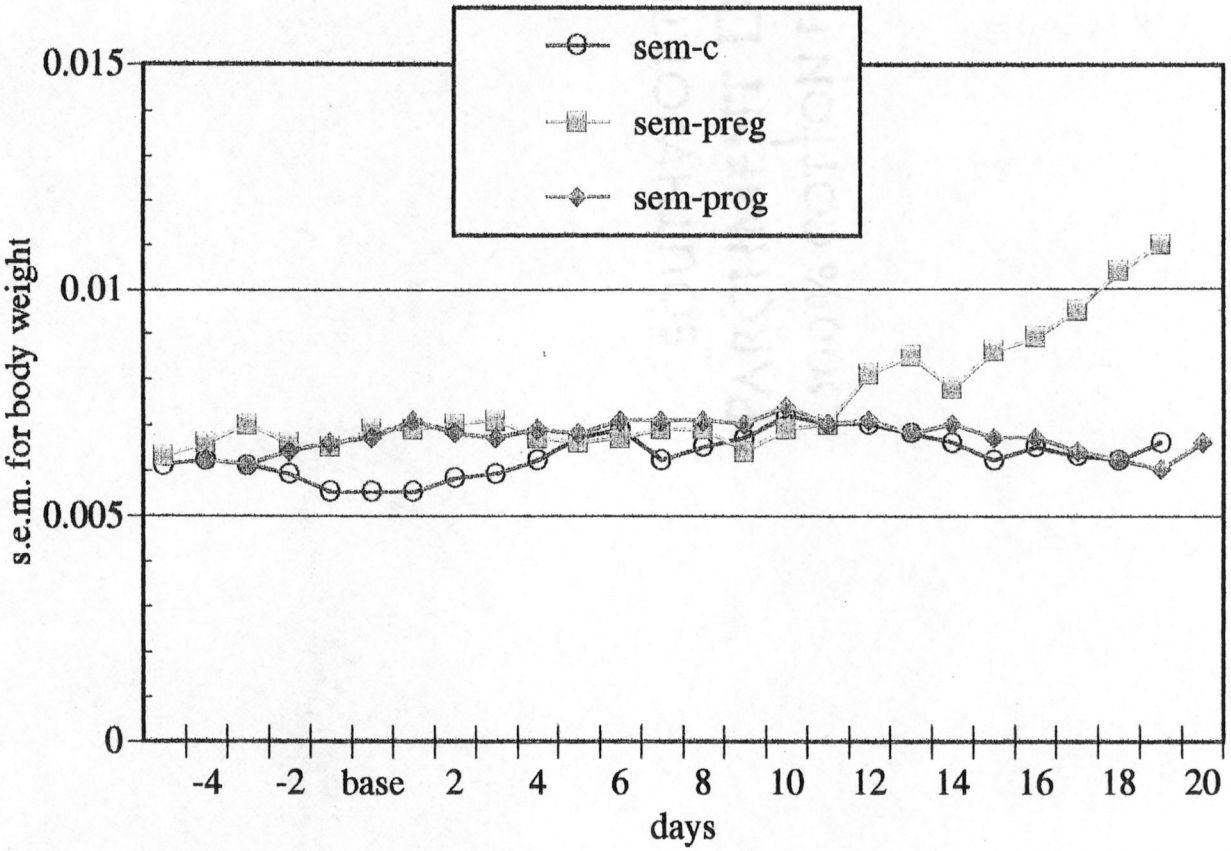
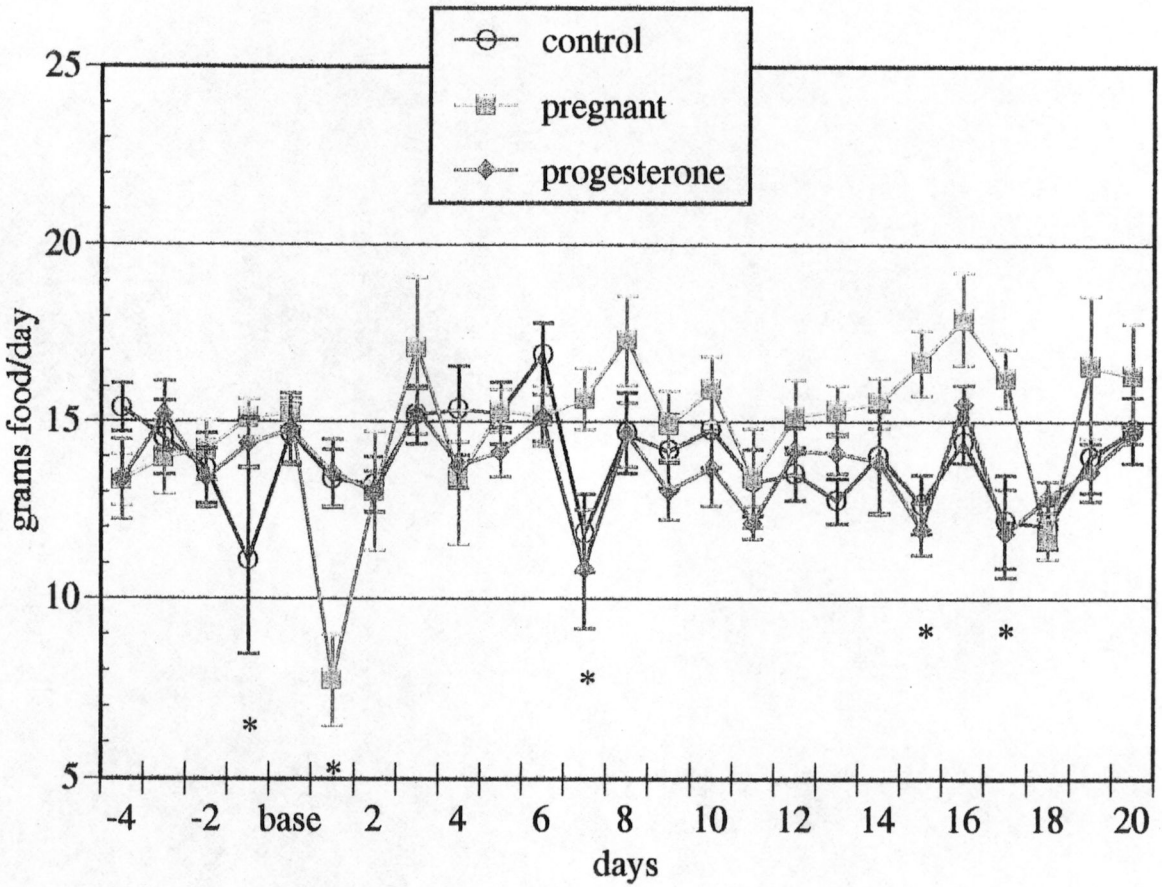

sem-c
sem-preg
sem-prog
0.015
0.01
0.005
0
s.e.m. for body weight
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

Figure 9

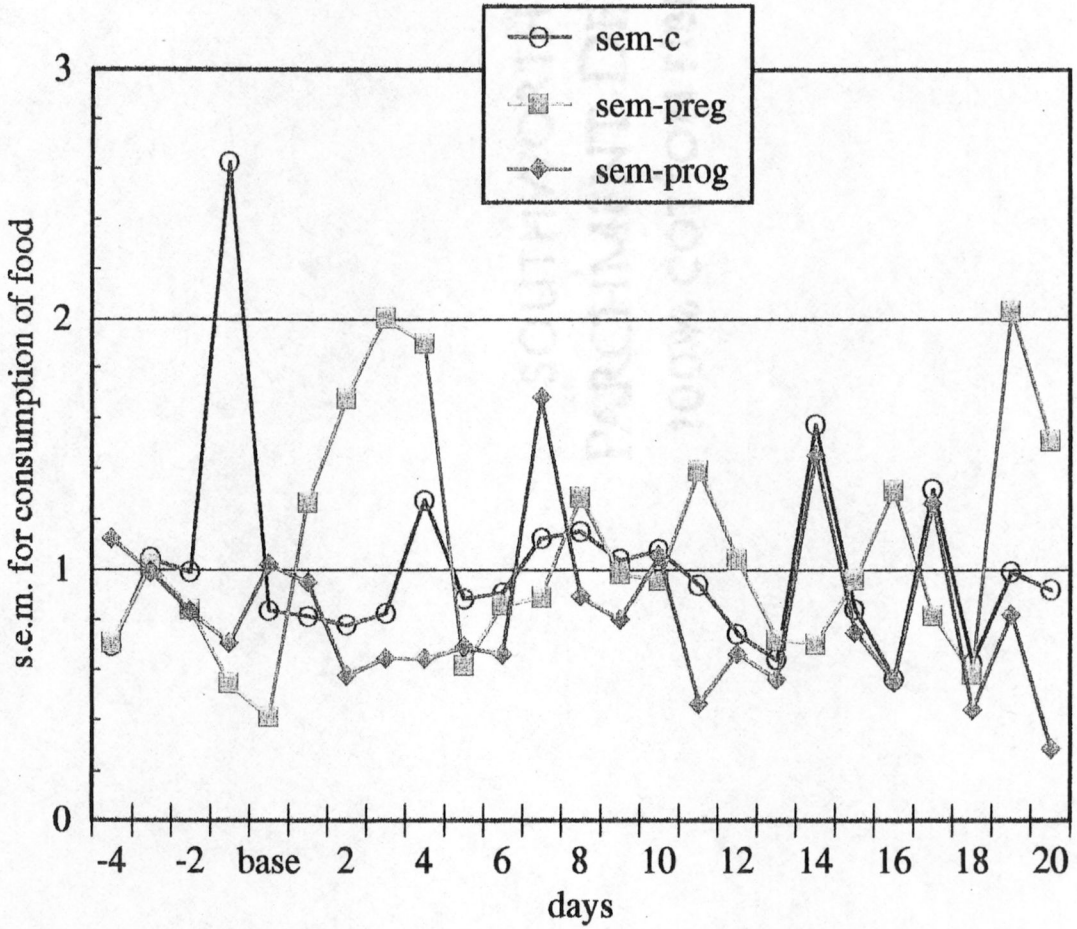
Amount of food consumed by the HEP rats during pregnancy or injections of progesterone.
* Food consumption differed significantly from the baseline: Control group (day –1), pregnant group (day 1), and progesterone group (days 7,15, and 17), $p<0.01$.


control
pregnant
progesterone
25
20
15
10
5
grams food/day
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days
*
*
*
*
*

an increase in variance early, which began on day 1 and continued through day 4 with the standard error of the mean reaching a value of 2.0 on day 3. The same value was reached by this group on day 19, after most of the rats had apparently lost their pregnancies (Figure 10).

Figure 10

Effect of pregnancy or progesterone on the variance of food consumption. The s.e.m. from the data in figure 9 were plotted.


sem-c
sem-preg
sem-prog
s.e.m. for consumption of food
3
2
1
0
-4
-2
base
2
4
6
8
10
12
14
16
18
20
days

Discussion

Consumatory Behavior of the HEP Rat

Neither pregnancy nor injections of progesterone given to the HEP female rats modified their consumption of ethanol. The proportion of ethanol to total fluid consumed also was unaffected. Both of these findings conflict with findings from other studies conducted on outbred strains of rats. Outbred strains of rats, Wistar and Long-Evans hooded rats, reduced their intake and proportion of ethanol to total fluid during pregnancy (Means and Goy, 1982; Sandberg et al., 1982). The HEP rats continued to consume copious quantities of ethanol at a proportion near 0.5. Thus inbred genetic drinkers did not follow the pattern set previously by outbred strains.

Another interesting aspect of HEP rats' drinking was that they drank in just seconds after the ethanol bottle was presented, often immediately almost attacking the bottle. It is known that the smell of alcohol will increase the activity of the alcohol preferring (P) rat (Myers et al., 1996). Therefore, it can be concluded that they must respond to the odor of the ethanol.

Weight gain by the pregnant group was considerably less than what is normally expected by pregnant rats. Pregnant Sprague-Dawley rats housed under the same conditions without alcohol exhibited a 60% increase in body weight (an approximate gain of 150g) during pregnancy and had an average litter size of fourteen pups (Henderson, 1990).

Despite only having eight pups, the one pregnant female that did deliver still managed to gain slightly over 100g. Most of this weight gain, 70g, came during the final week of

pregnancy, which precisely coincides with the time that it appears the other seven pregnant females began losing their pregnancies. Small litter size and delayed delivery are just two of many deleterious effects caused by alcohol on pregnant rats and their offspring (Streissguth, 1997). The weight gain exhibited by both the control group and the progesterone group was considered normal for a twenty day time period.

A general trend of increased food consumption was exhibited by the pregnant group. Although this increased consumption was not significant on any given day, it was expected due to the fact that the demand for nutrients and calories increases during pregnancy. It is unknown why the pregnant animals significantly decreased their consumption of food immediately following cohabitation at day 1. Perhaps, the stress of mating mediated the reduction.

A marked increase in the variance for proportion, but not amount of ethanol consumed, was shown by the pregnant group beginning at day 14. Drinking during that last week of pregnancy greatly fluctuated. Perhaps this was mediated by the females apparently undergoing resorptions. Due to the fact that the amount of ethanol consumed did not change, with the exception of day 17 when an increase was noted, the HEP rats must have been drinking more or less water during that time period (depending on the day in question). In fact, each rat had an increased range of values for proportion during the last week of the experiment.

A very similar increase in the variance for body weight was also observed in the pregnant animals beginning at day 13. This finding, in conjunction with the increase in variance for proportion, which both began around the same time, led to the conclusion that

the pregnant females must have begun to lose their litters at various times thereafter.

The copious quantities of ethanol that the pregnant females imbibed led to a high rate of fetus resorptions. Seven of the eight females in the pregnant group apparently had resorbed their fetuses. But, when these seven were removed from the ethanol and mated again, six successfully delivered litters. Clearly, ethanol was adversely affecting pregnancy outcomes. Padmanabhan and Hameed (1988) by maternally administering an acute dose of ethanol (0.03 ml/g body weight of 25% v/v absolute ethanol) found that alcohol exposure on gestation days 1-6 markedly increased prenatal mortality (resorptions) in mice. This also suggests that ethanol is lethal to the developing embryo.

It is unknown why many of the controls and one of the females in the pregnant group were unable to deliver after being taken off ethanol. Vaginal smears confirmed each rat was still cycling or was again cycling. One possibility is that the rats were simply too old. The attempted matings were performed after the experimental phase of the research when all of the rats were older than seven months of age, which is a month beyond what is generally considered the prime reproductive period for these animals.

In the same manner that fecundity decreases with age, drinking during early development has also been shown to decrease fecundity. In fact, the HEP rats were drinking large quantities of ethanol as early as 40 days of age during the first screen. Cebral and co-workers (1997) observed significantly decreased in vitro fertilization rates when oocytes from prepubertal and pubertal ethanol treated female mice were inseminated with spermatozoa from adult control males. The aim of that particular study was to investigate the effects of low chronic alcohol intake on fertility. Ethanol in tap water at a concentration of 5% (a low

dose) was administered to hybrid F_1 mice (C57/Bl x CBA) for four weeks. Low chronic ingestion of ethanol sufficiently reduced the percentage of activated oocytes, and increased the number of fragmented oocytes taken from immature females, such that in vitro fertilization rates decreased.

Other deleterious effects of ethanol on immature female rats include: delayed vaginal opening, decreased uterine and ovarian weights, and depressed ovarian function (Gavler et al., 1980; Bo et al., 1982). In addition, ovarian failure has been found to occur in rats that are fed high doses of ethanol (Van Thiel et al., 1978). Ethanol is undoubtedly a reproductive toxin.

Spontaneous Abortion in Women

Contemporary studies have shown a correlation between alcohol consumption and an increased risk of spontaneous abortion in women. In the United States, women who had been clinically diagnosed as alcohol abusers were twice as likely as controls to have suffered three or more spontaneous abortions (Sokol et al., 1980). A survey of clinical literature regarding FAS found that out of ninety women who had given birth to children with FAS, 52% had had at least one spontaneous abortion, and the average rate of spontaneous abortion per mother was 2.2 (Abel, 1990).

In spite of the plethora of studies which have been conducted on the matter of spontaneous abortion, no threshold level or critical time period of consumption leading to the occurrence of spontaneous abortion has been established. In a retrospective study, Wilsnack and co-workers (1984) estimated the threshold for spontaneous abortion at six or more drinks per day consumed at least three times per week. This high level of consumption would be

typical of an alcoholic.

Lower thresholds, which suggest alcohol may harm the fetus not only when alcohol is abused, but also when taken in moderation, have been proposed. In a study of second trimester losses, which in the rat is equivalent to gestation days 14-20 (the time period in which the females presumably lost their litters), one or two drinks daily doubled the risk of spontaneous abortion, and more than three drinks daily more than tripled the risk (Harlap and Shiono, 1980). Windham and co-workers (1998) found a two-fold increase of the risk of spontaneous abortion with an average consumption of seven or more drinks per week during the first trimester. A doubled risk was also found by Kline and co-workers (1980) for a weekly consumption of two to six drinks. In addition, risk increased an average of 25% for each additional ounce of alcohol consumed by pregnant women in a study performed by Russell and Skinner (1988).

Significance

Some 6 to 20% of women have been reported to drink heavily during pregnancy (Halmesmaki et al., 1987). Reductions in alcohol consumption in human females tend to be inversely proportional to prior consumption. Therefore, women who drink heavily prior to pregnancy (alcoholics) are most often those who continue to drink heavily throughout their pregnancies (Little et al., 1976). Like these women, female HEP rats do not curtail their drinking during pregnancy, and could therefore be considered valid models of the severe type 2 alcoholic.

The HEP rat's high level of alcohol consumption during pregnancy led to a high rate of fetus resorptions. Due to this fact, the HEP rat could also potentially offer a second model

for alcohol research--a model of alcohol-induced spontaneous abortion.

Such models could make additional research possible which in turn could substantially contribute to our understanding of unclear matters such as: the relationship of dosage, developmental timing, gender differences, genetic susceptibility, and differences in tolerance elicited by hormonal changes during pregnancy. In France, the HEP rat has already been utilized in genetic studies attempting to identify genes that lead to alcoholism (Jones and Mormede, personal communication, 1999). These rats could also be used for testing drug and behavioral treatments. Such research could point towards additional therapeutic approaches that could be used on alcohol abusing women during their pregnancies. New treatments could significantly improve the quality of life of females afflicted with the disease of alcoholism. This in turn would help decrease the prevalence of completely preventable disabilities such as FAS, ARBD, and ARND as well as decrease the incidence of alcohol-induced spontaneous abortions.

There are many possibilities for future studies utilizing the HEP rat. Ovariectomies could be performed to determine what effect, if any could be imposed by such a state on alcohol consumption. It may also be worthwhile to determine whether or not a two hour limited access paradigm would decrease overall consumption, and lead to a lower rate of fetus resorptions. Myers and co-workers (1998) demonstrated that nonpregnant female HEP rats from the F6 generation drank an average of 3.0 g/kg in such a paradigm, which is less than half of their mean daily consumption. If a higher percentage of pregnant females could successfully deliver as a result of switching to such a paradigm, studies involving alcohol consumption during lactation and investigations of physical and neurodevelopmental

abnormalities in their offspring could be performed. Also, studies of drug or behavioral modification of drinking behavior could be performed.

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