Effect of Prenatal Testosterone Treatment on Novelty-seeking Behavior in Sheep

by

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Abstract

Prenatal testosterone treatment during a critical period of sexual differentiation of the CNS virilizes external genitalia and the brain of ungulates. Furthermore, the masculinization of genitalia and brain develop under separate mechanisms and can be altered independently. In sheep, we examined the masculinizing effects of prenatal androgen exposure on novelty-seeking behavior. We hypothesized that novelty seeking is sexually differentiated and female sheep treated with testosterone for 30 days during the second half of the critical period of the 147-day gestational period would show more male-typical novelty-seeking behavior than control females. Results partially supported the hypothesis that novelty seeking is sexually differentiated and that prenatal androgen treatment has an effect on novelty-seeking behavior, but which cannot be concluded to be entirely male-typical.

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The prevalence of infants born with ambiguous genitalia is estimated at 1.7% of the population (Blackless et al., 2000). The current medical and social dogma for raising these infants is to do so based on the genital appearance or genetic sex. However, as these infants go through puberty a significant proportion have conflicting gender identity or sexual orientation and experience significant psychosocial distress that can lead to depression, imposing the need for questioning whether the current methods for determining gender in infants can be improved upon (Zucker, 2002). Sexual differentiation of the male brain and external genitalia can occur at different times and, in mammals is mostly controlled by different bi-products of testosterone. For example, sexual differentiation of the genitals in humans occurs within the first two months of pregnancy, much earlier than that of the brain, which occurs in the second half of pregnancy (Bao & Swaab, 2011). Furthermore, the extent of virilization of the external genitalia does not necessarily reflect the extent of masculinization of the brain (Swaab, 2004).

Complete androgen insensitivity syndrome (CAIS), which is caused by genetic mutations that reduce or eliminate the proper function of androgen receptors, necessary for response to the presence of androgens, results in genetic males (XY) that develop female external genitalia and display typical female development and sexual orientation (Wisniewski et al., 2000). When a genetic male fetus has a deficiency in the enzyme 5α-reductase, which prevents the conversion of testosterone into dihydrotestosterone (DHT), the fetus will appear to be a girl with ambiguous genitalia and an enlarged clitoris (Bao & Swaab, 2011). As the child grows, however, the excess testosterone during puberty allows further penile development and descent of the testicles, and the child's phenotype is masculinized. Despite initially being raised as a girl, 60% of these

children will choose to live as heterosexual males (Swaab, 2004; Swaab & Garcia-Falgueras, 2009).

Genetic girls (XX) affected by congenital adrenal hyperplasia (CAH), which is caused by mutations in the genes regulating the release of cortisol by the adrenal gland, are exposed to high levels of androgens as a fetus, which results in the development of ambiguous genitalia. Feminizing surgery is overwhelmingly performed at an early age as a standard treatment, so that the appearance of external genitalia is congruous with the XX genotype of the child (Rangecroft, 2003). As these children grow, they exhibit play behavior, artistic expression, and aggression typical to male children. For example, children with CAH are more likely to play with toy cars than dolls and engage in rough-and-tumble play, both of which are male-typical (Barnes et al., 1994; Nordenstrom, 2002). One problem with this treatment is that there is little consideration for the potential for a masculinized brain, which may lead to a conflicting sense of gender identity, gender role, and sexual orientation. Thus, the goal of the present study was to investigate early behavior of sheep following a short duration of prenatal androgen exposure that leaves genitalia largely female but results in adults that demonstrate masculinized reproductive behavior (Roberts et al., 2008).

Sheep are a good animal model for several reasons. Their 5-month gestation and 7-month period of development from birth to puberty are short enough to make their study practical and cost-effective, while long enough to provide a significant breadth of observation that can be linked to varying stages of development. As a gregarious species, sheep engage in extensive social behavior, which is important when studying novelty-seeking behavior. Finally, studies have uncovered many sexually dimorphic qualities and behaviors in sheep as well as reaction to prenatal androgen exposure that bear similarities to humans (Clarke & Scaramuzzi, 1978).

The Role of Androgens in Sexual Differentiation

In mammals, sexual differentiation is first driven genetically to determine the gonadal sex that will initiate the production or absence of androgens, and the formation of secondary sex characteristics to complement the genetic sex under normal circumstances. Extensive research in various animal models uncovered that exposure to prenatal testosterone results in masculinized sex behavior and virilized genitalia, which can be independent of the genetic sex of the mammal. The aromatization of testosterone has a particular role in masculinizing and defeminizing the CNS and driving male-typical behavior (Clarke et al., 1976). By a separate mechanism, virilized external genitalia are a result of testosterone being converted to dihydrotestosterone (DHT), the 5α-reduced product of testosterone (Steckler et al., 2007). To illustrate this point, genetic (XY) males that have a disorder with the production of the enzyme, 5α-reductase, may not be able to form DHT from testosterone, resulting in an animal with a masculinized brain and female phenotype, similar to CAH females (Bao & Swaab, 2011). However, excess exposure to androgens, not necessarily DHT, is sufficient to masculinize external genitalia (Grino et al., 1990). It follows then that the lack of prenatal androgen exposure, either due to lack of an androgen source or functioning androgen receptors, will result in a feminine brain and feminine secondary sex characteristics (Breedlove & Hampson, 2002).

The critical period for sexual differentiation in humans begins with the genitalia in the first two months of pregnancy and differentiates the brain in the second half of pregnancy (Bao & Swaab, 2011; Merke et al., 2005). Critical periods have also been characterized for other animal models, including, guinea pig (Phoenix et al., 1959), rat (Wolf et al., 2002), and rhesus macaques (Goy et al., 1988), all of which display timing separation of brain and genital sexual differentiation. The critical period for sexual differentiation of the hypothalamic-pituitary-

gonadal (HPG) axis of sheep, which spans from Days 30-90 of their 147-day gestational period, is the window in which prenatal testosterone treatment has an effect on sexual differentiation of sheep (Robinson et al., 2002; Wood et al., 1998). Female sheep that received androgen treatment for the entire critical period, that is for 60 days total between Gestational Days (GD) 30 - 90, developed completely virilized external genitalia with a functioning pseudopenis and empty scrotal sac instead of a vaginal opening (Wood et al., 1998). These (T60) females also sustained neuroendocrine alterations like LH hypersecretion and disrupted estradiol negative and positive feedback (Padmanabhan et al., 2006; Robinson et al., 2002). Furthermore, they exhibited altered estrus cycles and more male-typical reproductive behavior than controls. For example, T60 females followed, called, nudge/sniffed, flehmened, pawed, did head overs, and mounted at significantly higher rates than control females. In addition, they did not exhibit any female-typical reproductive behavior in contrast to control females (Roberts et al., 2008).

To obtain a more robust understanding of the role of prenatal testosterone in sexual differentiation, it was necessary to alter the androgen treatment. Reducing the duration of treatment from 60 days of exposure to the last 30 days of gestation dampened the extent of neuroendocrine and phenotypic alterations in sheep (Wood et al., 1998). T50 females, exposed from GD 50 - 100, exhibited a masculinized urinal stance and restricted vaginal openings, but lacked viriziled genitalia like a pseudopenis or scrotal sac (Clarke et al., 1976). When females were tested with a reduced, 21-day exposure at GD 65 – 86, they exhibited female-typical external genitalia. When the same exposure was given earlier in gestation, at GD 30 – 51, the genitalia was virilized, suggesting that the earlier in the critical period the treatment is started, the more masculinized the genitalia will be. Despite these latter two groups exhibiting different effects on genitalia, it was determined that both produced partially male-typical neuroendocrine

patterns of tonic LH secretion (Wood et al., 1998). While the timing of exposure in gestation affected development of external genitalia, the neuroendocrine organization was not different, suggesting that the critical timing of sexual development in the brain is different from that of the external genitalia (Roberts et al., 2008).

Females that received androgen treatment for the second half of the critical period, 30 days total at GD 60 – 90, developed normal external female genitalia (Steckler et al., 2007). However, these (T30) females still exhibited more male-typical behavior, similar to T60 females, which included a masculinized urinary posture and masculinized reproductive behavior. They differed statistically from T60 females by also engaging in some female-typical reproductive behavior like proceptive behavior, where the female initiates reproductive behavior with the male. The rate of proceptive behavior in T30 animals was intermediate between T60 females, which did not engage in any, and control females. Interestingly, within the T30 female group, there was a greater variation in sex behavior than among T60 females, with some exhibiting more male-typical behavior than others (Roberts et al., 2008). In summary, T60, virilized females, exhibited solely male-typical reproductive behavior, while T30, non-virilized females, exhibited mostly male-typical behavior but retained some female-typical behavior, and control females exhibited nearly exclusive female-typical sex behavior (Roberts et al., 2008).

Sexually Dimorphic Reproductive and Social Behavior in Sheep

When speaking of male-typical or female-typical behavior, it is important to get a sense of what types of behaviors are typical of each sex. In terms of reproductive behavior of sheep, some male-typical behavior includes following a female, low-rumbling calls towards females, nudging the female's flank with its muzzle, sniffing the female's genitalia and/or urine in a gesture known as a flehmen, pawing by lifting the leg up and down in the direction of the female,

placing its head and neck over the female's back, and mounting. Male-typical behavior also involves aggressive behavior towards other males for access to food or mates, often in the form of head butts or body slams. Some female-typical behavior includes receptive behavior, whereby the sheep stands still and positions itself in front of a male, and proceptive behavior, including approaching the male, passing by the male, standing in front of and looking back at the male, wiggling the ears back and forth, and tail wagging. While this list is not exhaustive, it consists of the major reproductive behaviors that are commonly seen.

Sexually differentiated behaviors are not limited only to reproductive behavior, but are also relevant in social behavior and social ranking as prenatal androgen treatment has been shown to have an impact on social dynamics and ranking in adult sheep (Bouissou & Gaudioso, 1982). The differences in reproductive behavior between T30, T60, and control females have spurred an interest in studying these others forms of social behavior that may predict later adult sex behavior, like novelty seeking. A novel object, placed in a familiar environment of a sheep pen, has been shown to induce anxiety or fear, as measured by latency of approach to the object, eating habits, immobilization, glancing, vocalization, and social behavior (Romeyer & Bouissou, 1992). In a follow-up study comparing sex differences in fear reactions, control female sheep exhibited more fear than control males in various tests that have been linked to fear, including reactions to novel objects in a familiar environment, particularly by spending less time near the object (Vandenheede & Bouissou, 1993a). The conclusion that the male is typically less fearful than the female is in agreement with other studies of mammals including dogs (Goddard & Beilharz, 1983), primates, and humans (Buirski et al., 1978; Crepeau & Newman, 1991; Gray, 1987). In order to characterize the sexual differentiation of fear behavior, female sheep treated prenatally with testosterone were introduced as a test group and exhibited significantly less fear

behavior than control females during a test with a novel object. The treated group received 56 days of testosterone exposure, presumably within the critical period of sexual differentiation of the HPG axis, although this is not disclosed (Vandenheede & Bouissou, 1993b). Along with fear, sexual differentiation has also been established in risk-taking and exploratory behavior, a characteristic that is also relevant to interacting with a novel object. In most species, males are more aggressive and competitive in nature, react less fearfully to stressful or novel stimulants, and engage in more risk-taking behavior than females, which tend to be more fearful and cautious (Maccoby & Jacklin, 1974). In ungulates, males show patterns of being less fearful than females in response to novel or fear-inducing stimuli and are therefore more willing to explore novel environments or novel stimuli in familiar environments (Vandenheede, 1993a).

This provides a precedent for studying sex differences in novelty seeking in sheep as a vehicle for expanding the research on CNS sexual differentiation through behavior. Previous study of T60 females also suggests a behavioral pattern in novelty-seeking behavior that is more male-typical than female-typical (Carroll & Lee, unpublished raw data). To expand on the research, this present study tested the novelty-seeking behavior of T30 females, compared to control males and females. T30 females provide a good test group based on a previous study that showed that despite their non-virilized genitalia and limited alteration to the HPG axis, T30 females mated at rates much lower than control females and failed to successfully conceive when bred (Steckler et al., 2007). Since T30 females exhibited defeminized sex behavior, it is valuable to ask if reducing and delaying the prenatal testosterone treatment will have an effect on novelty-seeking behavior, despite the fact that the animal did not experience any phenotypic masculinization. Supporting this hypothesis is a study that characterized the varying masculinizing and defeminizing effects of the timing and duration of prenatal testosterone on

female sheep, which hypothesized that the behavioral effects of T30 females may be impacted more heavily than their phenotype (Roberts et al., 2008). This led us to propose that the T30 cohort of sheep, not only the T60, might show significant alterations in novelty seeking. Therefore, we hypothesize that novelty-seeking behavior is sexually differentiated and that T30 female sheep will show more male-typical, increased novelty-seeking behavior than control females when encountering a novel object in a familiar environment.

Method

Subjects

All animal procedures used on the subjects in this research were approved by the University Committee on the Use and Care of Animals (UCUCA). The Suffolk ewes were purchased from local breeders and housed in the Sheep Research Facility at the University of Michigan, a farm inspected and approved by both the U.S. Department of Agriculture and the University of Michigan Department of Laboratory Animal Medicine. Breeding occurred between mid-October and mid-November with timed impregnation. In order to determine the date of impregnation, the chest of the rams was marked with an oil-based dye, which leaves a mark on the rump of the ewes when mounted. The ewes were subsequently monitored daily for marks left by the rams and the date of breeding was recorded. All subjects had numbers painted across their sides for identification. When ewes were discovered to be marked again following a previous record of mating, it was considered a rebreed and the date of mating was changed accordingly, because the ewe would not have been receptive to the male had she been impregnated successfully the first time. Six weeks before expected lambing, the pregnant ewes were fed an enriched diet of 0.5 kg shelled corn, 2 kg alfalfa hay, and 250 mg chlortetracycline (aureomycin) per day, a practice that helps prevent infection-related abortions. Lambs were weaned at

approximately eight weeks of age and then kept with their birth group so that both the control group and treatment group could interact freely. Lambs had access to an unlimited supply of commercial sheep food pellets and alfalfa hay until reaching the weight of 40 kg, upon which they received a diet consisting of 15% crude protein until 6 months of age. Water was always available to all lambs and ewes.

Prenatal Androgen Treatment

For generation of the T30 female cohort, 2-mL intramuscular injections of 100 mg testosterone propionate (Sigma-Aldrich Corp., St. Louis, MO, USA) was administered to pregnant ewes twice-weekly in cottonseed oil for the 30 days between GD 60 – 90 of the 147-day gestational period. A number of the pregnant ewes received a vehicle injection of 2-mL of cottonseed oil to make up the control group. After birth, the lambs were dam-reared and experienced frequent contact with humans, facilitating adequate habituation to our presence. To provide an adequate number of controls, additional control lambs were purchased from the same breeders that provided the ewes for the experimental group and brought to the facility at ten weeks of age following weaning, after which they were exposed to the same environment as the lambs reared at the facility.

Experimental Set-up

A novel stimulus was placed along the path that the sheep take to cross between the shaded barn and the feed troughs. The novel stimulus consisted of a blue tarp providing a base on top of which a large, colorful Pilates ball was taped. This object was chosen for its previously established qualities for piquing the interest of sheep (Carroll & Lee, unpublished raw data). The object was distanced so that the subjects would easily notice the object but would be far enough

away so that they could choose whether to approach or not. Two fixed cameras were set up to record approach to object and any relevant behavior.

Novelty-seeking Test and Analysis

For this study, 13 T30 females, 20 control females, and 5 control males were tested, at approximately 20 weeks of age. Males were post-pubertal and females were pre-pubertal. The test was started in the morning shortly after the sheep had been fed, to maximize the subjects' awareness of the novel stimulus when traffic between the food troughs and the shaded barn is high and the sheep are not hungry. The test was ended after 5 hours. The main behavior considered was the latency to approach, which is presumed to be inversely correlated with the subjects' fear from a novel stimulus. Whether they approached alone or in proximity to other sheep was also recorded because the highly gregarious species prefer to explore novel stimuli together rather than alone. Other data recorded included behavior in response to first noticing the novel object and behavior involved in interacting with the novel object. For a complete list of behaviors tested, an ethogram is provided in the appendix. The behaviors recorded in the video were transcribed into an Excel spreadsheet. In order to avoid bias, observers were blind to treatment groups; the sheep numbers were only linked to a treatment group after all behaviors were transcribed. Statistics analysis of variance between treatment groups for each of the transcribed behaviors was run using Systat Version 13 Copyright SPSS Inc., 2008. A p-value of $p < .05$ was considered significant and a p-value of $p < .08$ was considered approaching significance.

Results

Fourteen tests of behavior were recorded by videotape and transcribed. The means of all behaviors tested are summarized in Table 1. Multivariate Analysis of Variance was used to

determine overall significant differences of all behaviors tested between treatment groups. Wilks's Lambda multivariate test revealed a statistically significant overall difference between treatment groups and behaviors tested ($F(18,52) = 2.720$, $p = 0.003$). This suggests that there is some sexually differentiated behavior associated with novelty seeking. Some specific behaviors out of those tested showed significant results that warranted individual comparisons within treatment groups. Back-up behavior was tested because male sheep tend to display this behavior when preparing to ram another male, so we proposed that they might display this behavior when confronted with the novel stimulus, which they may perceive as a threat. The MANOVA for this behavior among all the others was found to be approaching statistical significance ($F(2,34) = 2.665$, $p = 0.084$). Another behavior we found interesting in context with novelty seeking is ear pointing. In ungulates, ears pointed forward, that is, towards the stimulus, and backward, that is, in the opposite direction to the stimulus suggest curiosity, fear or anxiety (Christensen et al., 2006; Lansade et al., 2008; Wolff et al., 1997). We found the MANOVA for ears backward behavior to be statistically significant ($F(2,34) = 10.125$, $p = 0.000$). The touch object behavior was tested to investigate the behavior toward the novel object, including touching, sniffing, or nibbling the object. The MANOVA for this behavior was found to be approaching significance ($F(2,34) = 2.803$, $p = 0.075$).

Once we discovered statistically significant variance among multiple behaviors, we sought to test behaviors by treatment to identify sexual differentiation in specific behaviors. We found that there were statistically significant differences between control males and females that suggest sexual differentiation. Wilks's Lambda multivariate test revealed a statistically significant overall difference between control males and females among all behaviors tested ($F(9,15) = 2.635$, $p = 0.047$). ANOVA was used to determine differences between treatment

groups for each individual behavior. The difference between mean count of approaches towards the novel object was found to be approaching significance ($F(1,23) = 3.925$, $p = 0.060$), indicating that control males were more likely to approach the object than control females (Figure 1). Control males also exhibited more back-up behavior than control females ($F(1,23) = 7.331$, $p = 0.013$; Figure 2) and more touch object behavior than control females ($F(1,23) = 7.636$, $p = 0.011$; Figure 3). Chi-square analysis was also utilized in assessing whether out of the sheep that interacted with the novel object, there was a statistically significant difference in how they approached, that is if they approached alone, following other sheep, or in a group. Control males showed a difference from control females approaching significance ($X^2 = 6.515$, $p = 0.08$). These data, therefore, provide evidence that novelty-seeking behavior is sexually differentiated.

Since we wanted to investigate the effect of prenatal testosterone treatment on behavior, it was necessary to examine differences between control females and T30 females. Wilks's Lambda multivariate test revealed a statistically significant overall difference between control females and T30 females among all behaviors tested ($F(9,22) = 4.522$, $p = 0.002$). ANOVA was used to determine differences between treatment groups for each individual behavior. Control females exhibited a greater mean count of ears forward than T30 females that approached significance ($F(1,30) = 4.065$, $p = 0.053$; Figure 4) and a smaller mean count of ears backward than T30 females, which was statistically significant ($F(1,30) = 16.760$, $p = 0.000$; Figure 5). These data provide evidence that prenatal androgen treatment had an effect on novelty-seeking behavior.

To strengthen our understanding of the effects of prenatal androgen treatment, it was also important to look for differences between control males and T30 females. Wilks's Lambda multivariate test revealed no significant overall difference between control males and T30

females among all behaviors tested ($F(7,9) = 1.809$, $p = 0.223$). ANOVA was used to determine differences between treatment groups for each individual behavior. Only one behavior, mean count of ear backwards, showed a statistically significant difference between control males and T30 females ($F(1,15) = 5.226$, $p = 0.037$; Figure 5), where control males exhibited less ear backward behavior than T30 females.

Discussion

Our hypothesis was that novelty-seeking behavior was sexually differentiated and T30 females would exhibit masculinized behavior that was more male-typical and less female-typical. The data provide evidence that some novelty-seeking behavior is sexually differentiated. Furthermore, T30 females exhibited behavior that was different from control females and similar to control males, strengthening our hypothesis. However, the overall trend of novelty-seeking behavior of T30 females did not correlate with the sexually differentiated reproductive behavior of the same cohorts, where we see behavior of T30 females that is in the middle of the range of male-typical to female-typical behavior (Lee, unpublished raw data). It is clear, therefore, that while novelty-seeking behavior may not correlate with sex behavior, there is evidence to support our hypothesis that novelty seeking is sexually differentiated and is affected by prenatal androgen treatment. However, none of the behaviors tested showed sexual differentiation combined with differences between T30 females and control females, which would provide clear cut evidence that prenatal androgen treatment masculinizes novelty-seeking behavior so that it is more male-typical than female-typical. Therefore, our data supported our hypothesis only partially, indicating that novelty-seeking behavior is sexually differentiated and affected by prenatal androgen treatment, but not necessarily that prenatal androgens produce male-typical novelty-seeking behavior in females. While previous study of T60 females suggested a trend that

we expected in T30 females, that study did not provide extensive statistical analysis to arrive at a conclusion (Carroll & Lee, unpublished raw data). More research of T60 and T30 females is needed, therefore, to determine if prenatal androgen treatment masculinizes novelty-seeking behavior.

The behaviors were split into three main groups: (1) if and how the sheep approached, (2) general behavior associated with noticing the novel object, and (3) behavior involved in interacting with the novel object. To summarize the data involved in approach, three out of 38 animals did not approach the novel object. Of those, all the control males approached, while one control female did not approach and two T30 females did not approach. However, Chi-square analysis revealed no significance between the groups in terms of sheep that approached and sheep that did not. A majority (71%) of the animals approached more than once. 80% of control males, 70% of control females, and 69% of T30 females approached more than once. Again, Chi-square analysis did not reveal any significance between groups regarding sheep that approached once against sheep that approached more than once. The result that all males approached while not all control females did, seems to suggest an expected trend that males are more likely to approach because they are less fearful (Vandenheede & Bouissou, 1993a). However, having only five males precluded any statistical significance. Surprisingly, a lower percentage of T30 females approached at least once, compared to control females. Also, fewer T30 females approached multiple times, suggesting an opposite trend than what we might expect, namely, that females treated with androgens are less likely to approach.

The latency to first approach was timed and included in the testing. The three sheep that did not approach were prescribed latencies slightly longer than the span of the experiment to prevent skewing of the data as outliers. ANOVA tests with Scheffe post-hoc did not reveal any

statistically significant differences in approach latency between groups. However, latency to approach data suggest a trend supporting the hypothesis where on average, males were the quickest to approach the object, T30 females in the middle, and control females the slowest. A possible explanation for this is that a masculinized brain encourages behavior that is less inhibited, resulting in sheep that are quicker to approach a novel stimulus, but eventually all the sheep will approach regardless of masculinization. In spite of this explanation, the mean count of approaches of control males was indeed greater than that of control females, approaching statistical significance, suggesting that even after prior investigation, males are still more likely to approach multiple times. However, since T30 females exhibited no significant difference from either control males or females, we cannot determine if the possible sexual differentiation is due to prenatal testosterone or some other independent factor correlated with sex.

Another aspect of approach we investigated was whether sheep approached alone or in company. As a gregarious species, studying the social dynamics in the context of novelty-seeking and sexually differentiated behavior is important for understanding social behavior and the effects of androgens. We would expect males to be more inclined to approach alone than females, based on general social proximity behavior and attachment to kin (Dwyer & Lawrence, 1999). We did not find any statistically significant differences between groups in mean count of approaching alone, following other sheep, or in a group. Interestingly, all three groups of sheep preferred approaching the novel object alone, on average, but due to a lack of statistical significance, we cannot conclude whether this says something profound about social behavior. To further explore this point, we decided to add a new test to redefine approach more conservatively as occurring only when the sheep interacts with the object. With this new definition, we again sought a statistical difference between groups on how they approached, and

indeed Chi-square analysis revealed a difference between control males and females that approached significance, suggesting that females were more likely to approach alone than males, which we did not expect. Interestingly, of the sheep that interacted with the object, 20% of control males approached alone, while more than 50% of control females approached alone, a trend that does not support our proposal that males would be more likely to approach without the social support of other sheep, when compared to control females. It should be noted, however, that all the control males interacted with the object, while 75% of control females interacted with the object. Table 2 summarizes this data.

The next group of behaviors tested was general behavior associated with noticing the novel object. Sheep often glanced at the object for a length of time before either approaching or walking away. We thought this would be a good behavior to test because it may reflect a level of inhibition in the animal deciding whether it is safe to approach or not. We proposed that males would be more inclined to approach because previous research has found them to be less fearful, which would result in a shorter glance duration mean and a decreased mean of counts of glances compared to females (Vandenheede & Bouissou, 1993a). A lack of statistical significance, however, suggests that there is no sexual differentiation of glance behavior.

Another behavior involved in noticing the object that we investigated was ear pointing. Some sheep pointed their ears forward or backward when glancing at or approaching the object and we wanted to consider sexual differentiation of this behavior, which may reflect curiosity, fear or anxiety (Christensen et al., 2006; Lansade et al., 2008; Wolff et al., 1997). According to our results, ear pointing behavior does not seem to be sexually differentiated because control males and females did not exhibit statistically significant differences. Interestingly, T30 females exhibited less ear forward behavior than control females, which approached statistical

significance, and exhibited more ear backward behavior than both control females and males, which was statistically significant. Since control males and females were not statistically different from each other in ear pointing, we cannot conclude that the behavior is sexually differentiated, but this result does support our hypothesis that prenatal testosterone treatment has an effect on behavioral interaction with novel objects.

Back-up behavior was exhibited by sheep in response to noticing the object, an aggressive behavior that male sheep tend to display when preparing to ram another male. Control males engaged in back-up behavior at a higher rate than control females, which was statistically significant, suggesting that males may have engaged in more male-typical aggressive behavior than females when perceiving the novel stimulus as a possible threat. One explanation for the result that T30 females did not exhibit statistically different back-up behavior from either control groups suggests that the 30 days of prenatal androgen treatment was not robust enough to affect back-up behavior to the extent that would make the behavior male-typical. It is also possible that testosterone is not the cause for back-up behavior and further research should investigate other differences in sex that could contribute to these results.

Behavior concerned with interaction with the novel object is important to novelty-seeking research. Sheep behavior involved in touching the tarp or ball revealed a statistically significant sexual differentiation, where control males interacted with the object more than control females. Again, T30 female differences were insignificant, suggesting that a longer testosterone treatment may be needed to elicit male-typical behavior. This result provides further evidence for supporting our hypothesis that novelty-seeking behavior is sexually differentiated.

Future Directions

This study has elucidated several avenues to improve and build on this research and arrive at more definitive conclusions. One important lesson learned is to use larger populations of treatment groups. Five control males used in this study produced too large of a standard error that most likely precluded statistically significant results in some behaviors. Therefore, it would be useful to do another experiment with similar conditions but with a larger sample size. Another aspect of the particular population used in this study was a questionable degree of controls. Some of the control ewes were purchased rather than raised at the sheep research facility, so that while all the lambs were raised similarly, it is hard to control for any epigenetic differences in stress development that could have formed between the purchased and familiar lambs. This is another possible explanation for the limited evidence for significance between control females and T30 females in behaviors that showed sexual differentiation. Furthermore, these control females were subjected to consistent handling and testing for other experiments, which could have raised their stress and cortisol levels and defeminized some behavior. This would serve to blend the behavior of female-typical and male-typical behavior and make it more difficult to discern. Repeated exposure to different people and tests may also have habituated the sheep to novel stimuli, homogenizing the different treatment groups to similar reactions to the novel object, drowning effects from sexual differentiation or testosterone exposure. With all this in mind therefore, it would be useful to conduct an experiment with more ideal conditions, like testing lambs raised from the same population of ewes, that experience limited exposure to people or novel stimuli, and that undergo limited testing to reduce the chance for inducing masculinizing effects from stress.

An important variable that should be investigated within the context of novelty-seeking behavior is age. Younger lambs are more curious by nature and are more interested in exploring

unfamiliar stimuli than older sheep. A study could be designed so that novelty-seeking behavior of the treatment groups would be tested at different times in the age of the sheep, to obtain a more robust understanding of effects not only by sex or testosterone treatment, but also by age.

Finally, a test could be devised to better identify the effect of prenatal testosterone treatment on novelty-seeking behavior. While certain novelty-seeking behaviors may not be dependent on prenatal androgen treatment, the administration of testosterone over 30 days during the critical period of sexual differentiation may not provide enough androgen exposure to elicit a noticeable response in some behaviors. If this is the case, including both T30 females and T60 females in the same study may provide more convincing evidence that the masculinization of novelty-seeking behavior is dependent on the degree of exposure to androgens. Furthermore, providing an additional test group consisting of gonadectomized male sheep, which are not exposed to androgens, may help support our hypothesis if the males exhibit less male-typical novelty-seeking behavior, and further characterize whether the effects are a result of the genetic sex or the HPG axis.

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Table 1

Summary of Mean Data for Novelty-seeking Behavior

Behavior	Control Male Mean	Control Female Mean	T30 Female Mean
Count of Approach	3.8 ± 0.8	2.2 ± 0.4	2.9 ± 0.5
Latency of Approach (mins)	72.1 ± 29.6	94.0 ± 17.8	74.9 ± 22.0
Count of Alone	1.4 ± 0.5	1.1 ± 0.2	1.5 ± 0.4
Count of Follow	1.0 ± 0.4	0.6 ± 0.2	0.6 ± 0.2
Count of Group	1.4 ± 0.5	0.6 ± 0.2	0.5 ± 0.2
Count of Ear Forward	1.4 ± 0.7	1.5 ± 0.3	0.5 ± 0.3
Count of Ear Backward	0.4 ± 0.2	0.5 ± 0.2	2.3 ± 0.5
Count of Behavior	9.0 ± 2.4	6.4 ± 1.2	6.5 ± 1.3
Count of Glance	5.4 ± 1.2	4.2 ± 0.9	3.5 ± 0.8
Duration of Glance (sec)	27.2 ± 7.7	24.0 ± 5.3	26.8 ± 6.9
Count of Back Up	2.0 ± 1.0	0.5 ± 0.2	0.7 ± 0.5
Count of Touch Object	4.4 ± 1.9	1.4 ± 0.3	2.8 ± 1.0
Duration of Touch Object (sec)	43.8 ± 8.9	25.0 ± 7.3	30.7 ± 15.8

Table 2

Summary of Chi-square Data for Type of Approach

Treatment Group	Proportion Approaching Alone	Proportion Approaching By Following	Proportion Approaching With Group	Proportion That Never Approached
Control Males	1/5	1/5	3/5	0/5
Control Females	8/20	2/20	5/20	5/20
T30 Females	7/13	2/13	1/13	3/13

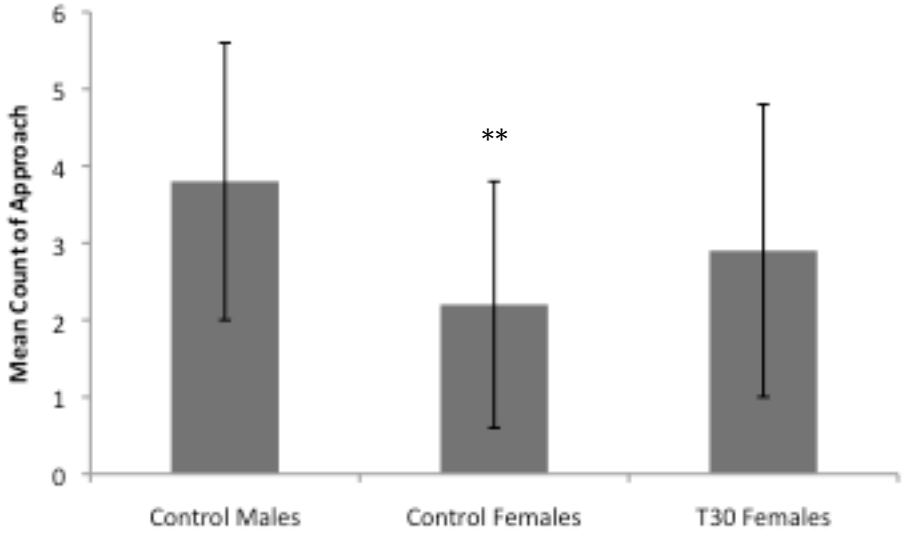

Figure 1. Mean count of approaches to the novel object. Analysis of treatment groups shows control males (n=5) approached more than control females (n=20), approaching significance, $F(1,23) = 3.925$, $p = 0.060$. ** Notes approaching significant difference, $p < 0.08$.

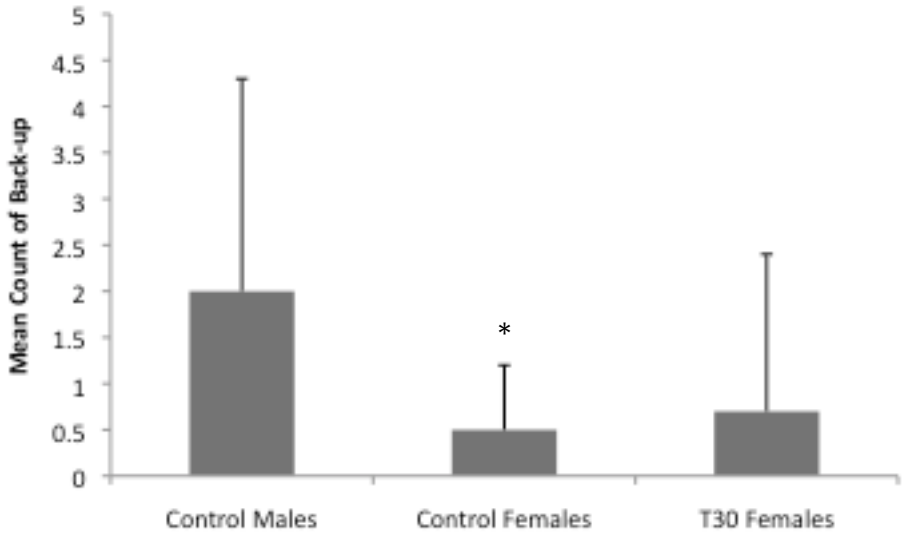

Figure 2. Mean count of back-up behavior directed at the novel object. Analysis of treatment groups shows control males (n=5) exhibited back-up behavior significantly, $F(1,23) = 7.331$, $p = 0.013$, more than control females (n=20). * Notes significant difference, $p < 0.05$.

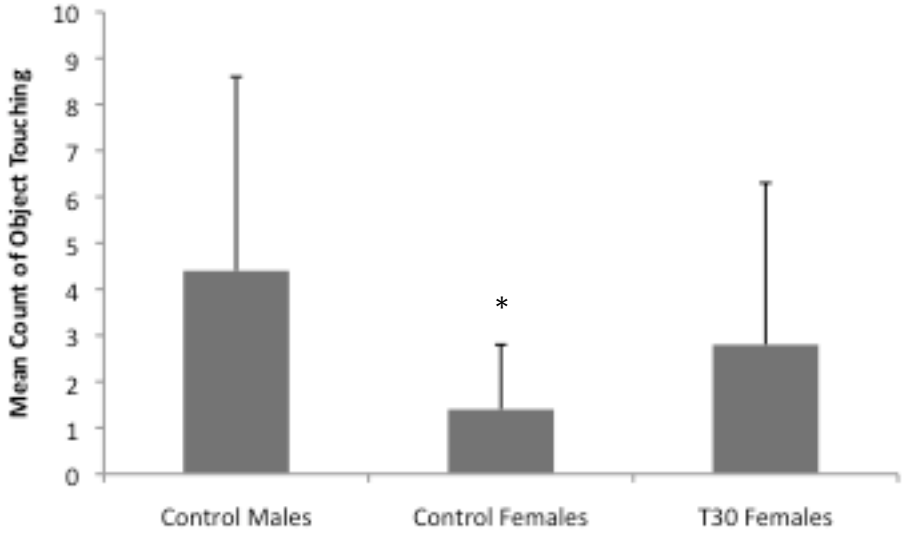

Figure 3. Mean count of touching of novel object. Analysis of treatment groups shows control males (n=5) touched the object significantly, $F(1,23) = 7.636$, $p = 0.011$, more than control females (n=20). * Notes significant difference, $p < 0.05$.

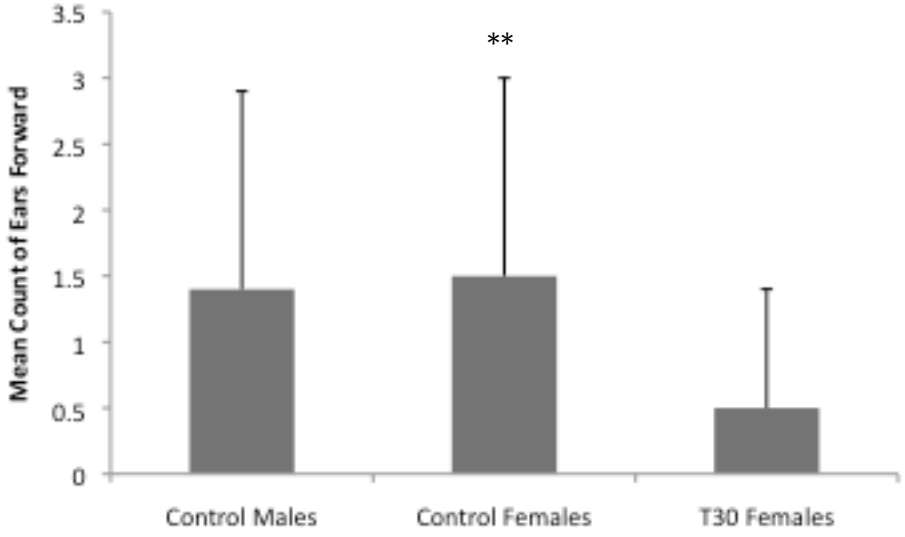

Figure 4. Mean count of ears forward behavior in reaction to the novel object. Analysis of treatment groups shows control females (n=20) exhibited more ears forward behavior than T30 females (n=13), approaching significance, $F(1,30) = 4.065$, $p = 0.053$. ** Notes approaching significant difference, $p < 0.08$.

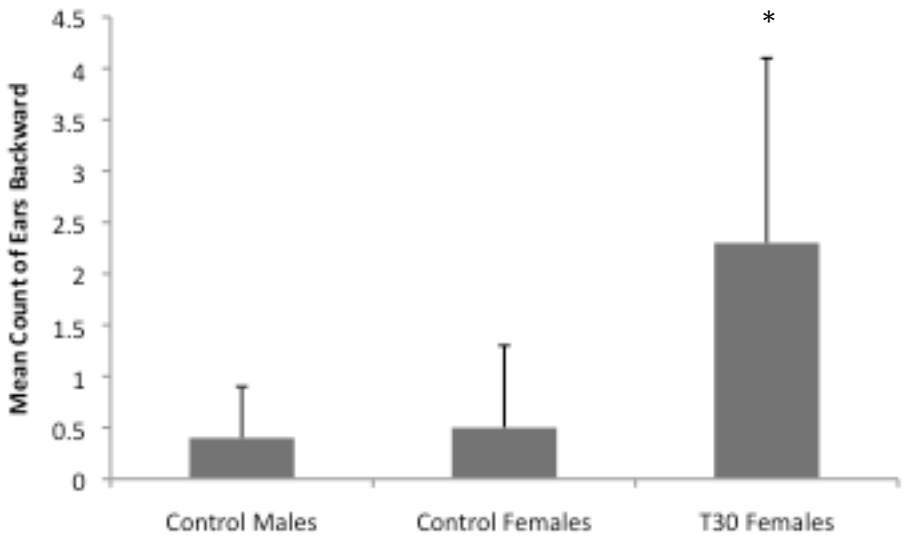

Figure 5. Mean count of ears backward behavior in reaction to the novel object. Analysis of treatment groups shows T30 females (n=13) exhibited significantly, $F(1,30) = 16.760$, $p = 0.000$, more ears backward behavior than control females (n=20). T30 females also exhibited significantly, $F(1,15) = 5.226$, $p = 0.037$, more ears backward behavior than control males (n=5). *Notes significant difference, $p < 0.05$.

Appendix

Ethogram for Novelty-seeking Behaviors Tested

Behavior	Description
Approach	Directed, investigative movement towards the novel object.
Latency to Approach	Measured time from start of experiment until first directed, investigative movement towards the novel object.
Alone Approach	Movement towards the novel object alone and not in the proximity of other sheep.
Follow Approach	Movement towards the object in response to another sheep approaching the object.
Group Approach	Movement towards the object with a group of sheep.
Ears Forward	Movement of ears in the forward direction, which can be associated with curiosity.
Ears Backward	Movement of the ears in the backward direction, which can be associate with anxiety or fear.
Back-up	Rearing backwards in preparation for aggressive actions towards the novel object.
Glance	A look towards the novel object for a length of time.
Touch object	Touching, sniffing or nibbling the object