# Mutation in the vasopressin gene eliminates the sex difference in social reinforcement in adolescent rats.

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**Abstract**

The neuropeptide, arginine vasopressin (AVP), is thought to contribute to sex differences in normative and pathological social development by regulating social motivation. Recent studies using Brattleboro rats that have a mutation in the *Avp* gene, however, have suggested that AVP impacts adolescent social behaviors of males and females in a similar manner through actions on behavioral state (i.e., arousal). In the present study, we made use of a recently developed operant conditioning paradigm to test whether the chronic, lifelong AVP deficiency caused by the Brattleboro mutation impacts the reinforcement value of social stimuli during adolescence. Operant responding for access to a familiar conspecific was assessed in male and female adolescent wild type (WT; normal AVP), heterozygous Brattleboro (HET), and homozygous Brattleboro (HOM) rats. Following the social reinforcement test, rats were tested in the same operant paradigm except that the social reinforcer was replaced with a light reinforcer to determine whether effects of the Brattleboro mutation were specific to social stimuli or a general characteristic of operant conditioning. WT males directed a greater proportion of their responding toward the social and light stimuli than WT females; only males exhibited a preference for these reinforcers over unreinforced ports. The sex difference in social reinforcement was absent in HOM rats, whereas the sex difference in light reinforcement was present in all genotypes. These data indicate that adolescent males are more sensitive to the reinforcing properties of social and light stimuli, and that the sex difference in social, but not light, reinforcement depends upon normal levels of AVP. These findings support the hypothesis that AVP plays a critical role in sex differences in social development by acting on factors that influence social motivation.

**Keywords**

Brattleboro rat, vasopressin, sex differences, adolescence, social reinforcement, light reinforcement

## 1. Introduction

Several neurodevelopmental disorders that impact social behavior exhibit striking sex differences in incidence, severity, onset, and/or response to treatment. For example, autism spectrum disorders are more prevalent in boys than girls (4.2 males: 1 female); and schizophrenia, for which social withdrawal is a major symptom, is more prevalent (1.4 males: 1 female), develops earlier, and is more severe in males [1–3]. Sex differences in the neurobiology of social development likely account for why one sex is more vulnerable, and the other more resilient, to the social deficits that accompany neurodevelopmental disorders. Sex differences in the brain and behavior arise from organizational and activational actions of gonadal hormones, direct effects of genes on the sex chromosomes, and environmental factors [reviewed in 4,5]. The downstream neurobiology on which these factors act to regulate sex differences in social development, however, is not understood.

The neuropeptide, arginine vasopressin (AVP), has been implicated in several neurodevelopmental disorders including autism spectrum disorders and schizophrenia [6–9]. AVP regulates several social and anti-social behaviors both in adulthood and during development [reviewed in 10–13]. Notably, AVP's influence on social behaviors often differs depending on sex. For example, AVP infusions into the anterior hypothalamus stimulate aggression in male Syrian hamsters, but inhibit aggression in females [14–16]. During development, ICV and septal infusions of a V1aR antagonist have opposite actions on the social play of male and female juvenile rats [17,18], and septal infusions of AVP enhance social recognition in female but not in male juvenile rats [19]. Hence, AVP is considered a prime candidate substrate for regulating sex differences in social behavior and social development [20].

The Brattleboro rat is a useful model for studying chronic, lifelong disruptions to AVP function. Brattleboro rats have a single base-pair deletion in exon 2 of the *Avp* gene that disrupts the production of AVP [21]. In this model, loss of AVP function at the level of the kidneys leads to the development of diabetes insipidus [22]. Several social behaviors are also impacted, presumably due to the loss of central AVP actions [23–25]. Few studies have tested both sexes in the same experiment, but when comparing across studies adult male Brattleboro rats show deficits in social discrimination, whereas adult female Brattleboro rats do not [26,27]. In juvenile and adolescent rats, however, the Brattleboro mutation affects social interactions of males and females in a similar manner – increases huddling and decreases social play and 50 kHz ultrasonic vocalizations [28,29]. Hence, while acute intracranial pharmacological manipulations of AVP are known to differentially impact male and female social behaviors, the data for chronic AVP disruption are mixed.

AVP is thought to regulate social behavior through actions on social motivation, but few studies directly test this conjecture. Complex behaviors can be influenced by many factors. Indeed, adolescent Brattleboro rats do not exhibit deficits in a simple social approach test, but instead exhibit a hypoaroused phenotype that is correlated with their decreased levels of social play [29]. These findings raise the possibility that AVP influences social behavior through actions on arousal. Recently, operant conditioning paradigms capable of testing social reinforcers have been developed that allow for a direct assessment of social motivation and reinforcement/reward value of social stimuli [30–33]. In the present experiment, we used one such operant paradigm to test whether the Brattleboro mutation affects the social reinforcement of adolescent male and female rats, and if so, whether it impacts the sexes in the same or different manner. A previous study found that the Brattleboro mutation eliminated the sex difference in a non-social learning task (extinction of a conditioned taste aversion) present in adult Long Evans rats [34]. Hence, we also assessed operant responding to a light reinforcer,

which has been shown to function as a non-social reinforcer in operant paradigms [35–37], to assess whether effects seen in the present experiment are specific to social reinforcement or generalize to other types of operant conditioning.

## 2. Materials and Methods

### *2.1. Subjects*

Experimental subjects were 14 wild type (WT; 6 female, 8 male) rats, 30 rats heterozygous for the Brattleboro mutation (HET; 14 female, 16 male), and 20 rats homozygous for the Brattleboro mutation (HOM; 10 female, 10 male) from our breeding colony, which was originally derived from HET rats obtained from the Rat Resource and Research Center (University of Missouri, Columbia, MO). All subjects were generated from HET male x HET female pairings in order to generate offspring of all three genotypes within the same litter. Experimental subjects were derived from 9 litters. All animals within each litter were used in experiments, except when a same-sex, same-genotype cagemate was not available at weaning for pair housing. This resulted in the following mean number of subjects for each sex/genotype per litter: 0.67 female WTs, 0.89 male WTs, 1.56 female HETs, 1.78 male HETs, 1.11 female-HOMs, and 1.11 male-HOMs. All rats were housed in plastic cages (44 cm X 22.5 cm X 20.5 cm) with wood shavings and maintained on a 12 h light/12 h dark cycle throughout the experiment. Food and water were available *ad libitum* and ambient temperature was maintained at 23°C. All experiments were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee at the University at Buffalo, State University of New York.

### *2.2. Experimental Timeline*

Rats were genotyped on postnatal day (P)15 and subsequently weaned on P21 into same-sex, same-genotype pairs; day of birth = P0. Between P21-P23, rats were transferred from the

North Campus animal facility to the animal facility at the Clinical and Research Institute on Addictions (University at Buffalo, SUNY) for behavioral testing, at which point the time of lights off was shifted from 6PM to 7AM EST to facilitate testing during the dark phase. All rats were given at least 6 days to acclimate to the new building and altered light cycle. Rats were trained and tested on a social reinforcement task (training P28-P34; testing P35-P41) then a light reinforcement task (training P42-48; testing P49-P55; see methods below for details of the operant apparatus and procedure). Hence, training and testing occurred from early to late adolescence as defined by Vetter-O'Hagen and Spear [38]; early/mid adolescence = P28-42, late adolescence = P42-55. After testing was complete, rats were transferred back to the North Campus animal facility where they were individually housed and tested for 24-hour water intake measures between 10-13 weeks of age.

*2.3. Genotyping Procedure*

Rats were genotyped prior to weaning using the method developed by Paul et al. [28]. Between P13-P15, ear tissue was collected from rat pups using a sterile ear punch. Ear tissue was digested and DNA extracted using the REDExtract-N-Amp Tissue PCR Kit (SigmaAldrich). The DNA surrounding the single base pair Brattleboro deletion was amplified by PCR using the forward primer, GACGAGCTGGGCTGCTTC, and reverse primer, CCTCAGTCCCCCACTTAGCC. The amplified DNA was then incubated at 37°C for 24 h with the restriction endonuclease, Bcg1 (New England BioLabs). Following incubation with the restriction endonuclease, samples were run on a 2% agarose gel using gel electrophoresis and genotype designations determined by assessing the number and weight of DNA bands: WT = a single 222 bp band; HOM = a single ˜95 bp band; HET = two bands, one at 222 bp and one at ˜95 bp.

### *2.4. Operant Testing Apparatus*

Social reinforcement was measured in a set of locally constructed operant chambers (Figure 1), as previously published [31, for video, see supplemental material or http://ratgenes.org/social-reinforcement-monitor/]. The three-chamber apparatus was housed inside of a cooler (Model # 3000000187, Coleman, Wichita, KS), blocking external stimuli. The center of the test chamber was made of a clear acrylic tube (diameter: 22.75 cm, height: 25.5 cm) and set on a grid floor (1/8 inch stainless steel rods, 0.7 cm apart). Social stimulus chambers (diameter: 21.5 cm, height: 16.5 cm) were located on the right and left sides of the test chamber. Lateral observation ports made of 2-inch PVC pipe provided access between the test chamber and the social stimulus chambers, allowing test and social stimulus rats to contact snouts and vibrissae, as well as the passage of odor cues. The test chamber also had a center observation port located on the far side of the chamber from the front face, capable of providing access to liquid reinforcers; liquid reinforcers were not used in the present study, but the center port was available as an unreinforced port. Left and right stimulus lights were located in the roof of the test chamber above the social stimulus chambers. A ventilation fan was located on the back wall of the enclosure. Air vents were placed in the bottom of the social stimulus chambers and led to air holes in the bottom of the sound and light attenuating enclosures. A lid was placed on the tops of the social stimulus chambers, ensuring that the ventilation fan would draw air through the bottom of the social stimulus chambers into the observation port connected to the central test chamber, thereby presenting the test rat with olfactory cues from stimuli placed into the social stimulus chambers. Two obstruction bars (8-32 × 2.5 bolts placed 17.5 mm apart) were placed in the PVC pipe between the test chamber and the social stimulus chambers to prevent the test and social stimulus rats from escaping into the alternative chamber. Figure 1D illustrates the sliding door that controls access of the test rat to the social stimulus chambers. Infrared photo sensors bisected the observation port 17.5 mm from the test chamber wall and detected snout pokes into the observation port. The swinging door was used to open or close

the passageway between the test and social stimulus chambers. The swinging door was normally closed and was opened by operating a 24-volt rotating solenoid (Ledex H-1141-033, Johnson Electric, Hong Kong). Operation of the solenoid opened the door and then held it open against the stop, which allowed physical contact between the test and stimulus rats and the free passage of odor cues. The photo detector circuit input and the solenoid output were connected to a computer interface (Med Associates, St. Albans, VT) allowing the computer to control access contingent upon snout poke responses.

*2.5. Operant Procedure*

A social stimulus (the cage-mate) was first placed into either the left or right stimulus chamber (counterbalanced). Stimulus chambers were removable and could be placed on either side of the apparatus. Separate chambers were designated for stimulus animals versus empty chambers; stimulus animal chambers were never used as empty chambers and vice versa to prevent accumulation of odors in the empty chambers. For each test rat, the same rat served as the stimulus rat for all the test sessions. The test rat was placed into the center test chamber, and the number of snout poke responses into the three observation ports during an 18-min test session was recorded. The opening of the sliding doors to all observation ports was response-contingent according to a variable-interval (VI) 1 min schedule of reinforcement, separate for each port. Each rat was given 1 week to train on this task then tested during the second week. Training/testing sessions occurred three days per week (every other day) for a total of six training/testing sessions. Data from the three test sessions for each rat were combined and used for analysis. The order in which animals were tested and the side of reinforcement were randomized. House light stimuli used in the light reinforcement task (see below) were never turned on during the social reinforcement portion of the experiment.

Following social reinforcement training and testing, rats completed an additional two weeks of training and testing using the same schedule except that the social stimulus was replaced with a house light. The light reinforcement port and active house light were on the opposite side to the prior social reinforcement port and social stimulus. The same VI 1 min schedule of reinforcement was used. Once activated, the light remained on for 5 seconds. As with social reinforcement, the last three test sessions were combined and used for analysis.

*2.6. Water Intake Measures*

We have previously validated our genotyping procedure using sequencing [28]. In the present experiment, we further confirmed HOM genotype designations by assessing each animal's 24-h water intake. Rats were individually housed and the weights of their water bottles were recorded. The bottles were weighed again 24 and 48 hours later and the average 24-h decrease in weight over this period was used as a measure of water intake, reported in milliliters (1g water = 1ml water).

*2.7. Statistical Analyses*

Social and light reinforcement measures were calculated as the proportion of responses directed at the reinforcer (reinforcer nose pokes/total nose pokes). Group differences in reinforcement and total responses during the testing phase as well as during the first session of the training phase were assessed using a 2 x 3 ANOVA, with Sex and Genotype as the independent variables. Reinforcement across the 3 training sessions for each genotype was analyzed using repeated-measures ANOVA, with Sex and Session as independent variables. If main effects or interactions of the overall ANOVA were significant, *post hoc* tests were conducted using Fisher's PLSD. Preference for and aversion to the social or light reinforcer were determined by comparing the confidence intervals of reinforcement measures for each experimental group with the proportion of responses expected by chance. Given that there

were 3 possible ports, a random distribution of port responses would result in 33% of responses directed toward each port, i.e. a proportion of 0.33 for each port. Hence, if the lower confidence interval for the reinforced port was greater than 0.33, a preference was assumed and if the higher confidence interval for the reinforced port was less than 0.33 than an aversion was assumed. Outliers were identified using the Box and Whiskers plot on SPSS, which defines outliers as measures greater than 1.5 times the interquartile range and were removed prior to conducting the overall ANOVA or assessing preference; see Supplemental Table for number of outliers within each group for each measure. Final sample sizes are indicated within the bars of each figure or in the figure legend. All analyses were conducted using SPSS v23.0 (IBM). Significance was assumed when $P<0.05$.

## 3. Results

### *3.1. Lifelong AVP-deficiency eliminates the sex difference in social reinforcement*

During the testing phase, there was a significant main effect of Sex on social reinforcement ($P<0.05$, ANOVA). This was due to WT males directing a higher proportion of responses toward the social reinforcer than WT females (Fig. 2A; $P<0.05$, Fisher's PLSD). This sex difference, however, was not significant in HET rats ($P=0.19$, Fisher's PLSD) and was absent in HOM rats ($P=0.90$, Fisher's PLSD). WT, HET, and HOM males as well as HET and HOM females responded greater than chance for the social reinforcer indicating a preference for the social port over the unreinforced ports, a preference not present in WT females (Table 1). While the WT females did not prefer the social port, they also did not find it aversive (Table 1). Total responding during the social reinforcement test was not impacted by the sex or genotype of the animals (Fig. 2B; $P>0.21$, main effect of Sex, main effect of Genotype, and the interaction between Sex and Genotype, ANOVA).

Analysis of the training phase revealed that the sex difference in social reinforcement was not present upon first exposure to the operant procedure and there were no differences among the genotypes (Fig. 3A; $P>0.39$, main effect of Sex, main effect of Genotype, and the interaction between Sex and Genotype on session 1). However, a sex difference in social reinforcement emerged in WT and HET animals over subsequent sessions (Fig. 3B,C; $P<0.05$, main effect of Sex in WT animals, interaction between Sex and Session in HET animals, repeated-measures ANOVA). In HET animals, the sex difference was significant on sessions 2 and 3 ($P<0.05$, Fisher's PLSD), whereas in WT animals the sex difference was significant on session 3 only ($P<0.05$, Fisher's PLSD). As seen during the testing phase, there was no evidence of a sex difference during the training phase in the HOM rats (Fig. 3D; $P>0.31$, main effect of Sex, main effect of Session, and the interaction between Sex and Session, repeated measures ANOVA).

*3.2. Males, but not females, exhibit a preference for light reinforcement*

During the testing phase of light reinforcement, there was also a main effect of Sex, with males directing a higher proportion of responses to the light reinforcer than females (Fig. 4A; $P<0.05$, ANOVA). In contrast to social reinforcement, however, this sex difference was present in all genotypes ($P<0.05$, WT, HET, and HOM males versus WT, HET, and HOM females, respectively, Fisher's PLSD). Furthermore, males, but not females, of all genotypes exhibited a preference for the light port over the unreinforced ports (Table 1). Total responding during the light reinforcement test was also impacted by sex, but in this measure females exhibited greater responding than males (Fig. 4B; $P<0.05$, main effect of Sex, ANOVA). Although the mean total responses of females was higher than that of males for each genotype, these differences fell short of significance in the post hoc tests ($P=0.11$, 0.08, and 0.10 for WT, HET, and HOM comparisons, respectively, Fisher's PLSD).

In contrast to that seen on the first day of social reinforcement training, there were group differences on the first day of light reinforcement training. There was a significant interaction between Sex and Genotype on the first session of the training phase (Fig. 5A; $P<0.05$, ANOVA). This was due to male-specific differences across genotypes: lower light responding in HOM males compared to HET and WT males ($P<0.05$ for both comparisons, Fisher's PLSD). Although there was a trend toward Sex differences in WTs (males > females) and HOMs (females > males) on the first day of training, these comparisons did not reach significance ($P=0.09$ for WT males vs. WT females; $P=0.07$ for HOM males vs. HOM females; Fisher's PLSD). Note that the trend toward a sex difference on Session 1 for HOM rats reached significance in the repeated-measures analysis due to the removal of 1 male that was an outlier on session 3 (Fig. 5D; $P<0.05$, Fisher's PLSD). Analysis across the three training sessions indicated that light responding for all groups was at their highest level on the first session (Figs. 5B-D). WT male and female rats maintained the same level of responding throughout the training phase ($P>0.54$, main effect of Session and interaction between Sex and Session, repeated-measures ANOVA). For HET and HOM rats, there was a female-specific decline in light responding on session 2 that was maintained on session 3 ($P<0.05$ for HET and HOM comparisons of session 1 vs. session 2 and 3, Fisher's PLSD). The sex difference in light responding seen in the testing phase (males > females) tended to be present in WT rats throughout the training phase ($P=0.055$, main effect of Sex, Repeated-measures ANOVA), but this was only significant on session 3 ($P<0.05$, Fisher's PLSD). For HET rats, this sex difference emerged on session 2 ($P<0.05$, Fisher's PLSD), but was not significant on session 3 ($P=0.16$, Fisher's PLSD). In HOM rats, females initially exhibited higher light responding on session 1 ($P<0.05$, Fisher's PLSD), but this sex difference was absent on sessions 2 and 3 ($P>0.25$, session 1 vs. session 2 and 3, Fisher's PLSD).

### *3.3. Confirmation of HOM genotype designation by drinking phenotype*

As expected, all rats designated as HOM by our genotyping procedure drank markedly more water than WT and HET rats (Fig. 6), confirming HOM genotype assignments.

## 4. Discussion

The present findings demonstrate that chronic disruption of AVP disrupts sex differences in adolescent social behavior, specifically the sex difference in adolescent social reinforcement. Male adolescent WT rats directed a greater proportion of responses toward the social stimulus than female adolescent WT rats. Indeed, WT males showed a significant preference for the social stimulus over the unreinforced ports, whereas WT females did not. This sex difference depended on a functional *Avp* gene: it was inconsistent in HET rats (i.e., present at the end of training, but not during testing) and was completely absent in HOM rats. HET rats exhibit a partial reduction in AVP mRNA expression and pituitary protein content [39]. Hence, a partial reduction in AVP appears sufficient to diminish the sex difference in adolescent social reinforcement. These findings support the hypothesis that AVP plays an important role in sex differences in adolescent social development.

To our knowledge, the present study is the first to demonstrate increased operant responding for social stimuli in male compared to female adolescent rats. Adolescent male rats often (but not always) exhibit higher levels of social interactions, particularly social play behavior, than their female counterparts [40–42][but see 43,44]. The sex difference in social reinforcement of WT rats seen in the present study adds to the small but growing literature indicating that the greater levels of social interactions in adolescent male rats is due, in part, to a greater sensitivity of males to the reinforcing/rewarding properties of social stimuli compared to females. Adolescent males exhibit a greater conditioned place preference (CPP) than females to a compartment previously paired with social interactions [45]. The sex difference in CPP to social

interaction depends on housing conditions, being present in single-housed, but not pair-housed rats [45–47]. In contrast, we detected the sex difference in social reinforcement preference even though rats were pair-housed throughout the experiment. This suggests that the greater sensitivity of males to social reinforcement/reward is present in group-housed rats and that operant paradigms may be more sensitive in detecting such preferences than CPP.

Although often assumed, few studies directly test whether AVP influences social behavior by regulating social motivation or reinforcement/reward value of social stimuli. Increased social motivation is typically inferred by shortened latencies to approach another individual or increased time spent in social contact [48]. The operant conditioning paradigm used in the present experiment allowed us to more directly assess the role of AVP in social reinforcement. The present findings support the hypothesis that AVP influences social behavior by modulating the reinforcement/reward value of social stimuli.

We have previously found that male and female adolescent Brattleboro rats exhibit 1) an atypical social behavior profile characterized by decreased active social behaviors (e.g., social play, 50 kHz ultrasonic vocalizations) and increased passive social behaviors (e.g., huddling) and 2) a hypoaroused phenotype characterized by decreased locomotor activity in an open field and decreased marble burying [28,29]. Notably, decreased behavioral arousal is correlated with decreased social play suggesting that the more passive social behavior phenotype of Brattleboro rats is due, in part, to AVP's actions on arousal [29]. Perhaps AVP's actions on arousal impact males and females to influence active versus passive social behaviors similarly in both sexes, whereas AVP's actions on social reinforcement differentially impact the sexes to modulate sex differences in social behaviors. AVP also regulates other factors that likely impact complex behaviors – circadian timing and social recognition [19,49–51]. Hence, it is becoming

clear that AVP acts through multiple mechanisms to influence social behavior (e.g., social motivation, behavioral state, behavioral timing, and social memory).

The results of the social reinforcement test are unlikely to be the result of sex-specific effects of the Brattleboro mutation on learning. This possibility is raised by studies demonstrating 1) sex differences in the performance on several learning and memory paradigms [52,53] and 2) AVP influences on both social and non-social learning tasks, including operant conditioning [54,55]. Similar to the present findings, a previous study found that the Brattleboro mutation eliminates the sex difference in extinction of a conditioned taste aversion to a sucrose-lithium chloride pairing [34]. Nevertheless, in the present study rats learned the social reinforcement task within the first session, at which point their social responding did not differ between groups. Instead, the sex difference emerged in WT and HET animals as the social responding of females declined in later training sessions. These data suggest that the loss of the sex difference in the social reinforcement in HOM rats seen in the present study is not due to effects of the Brattleboro mutation on learning.

Loss of the sex difference in operant responding of Brattleboro rats was specific to the social stimulus, with males of all genotypes exhibiting a greater proportion of responses for the light stimulus than their female counterparts during the testing phase. This too argues against a general effect of the Brattleboro mutation on operant responding in the current behavioral paradigm. Caution is warranted here, however, because all rats had undergone social reinforcement testing before being subjected to light reinforcement. Hence, it is possible that the different results seen for social and light reinforcers in HOM rats are due to interactions between AVP-deficiency and the order of testing, with animals more adapted to the apparatus and testing procedures during light reinforcement. In addition, the differing ages or pubertal stages of animals during social versus light reinforcement could have also influence the results.

Adolescence is a time of remarkable neural, behavioral, and reproductive development, with marked changes in social behavior and cognitive performance in both humans and rodents [56]. Pubertal factors, which differ markedly between early/mid-adolescence (when social reinforcement was tested) to late adolescence (when light reinforcement was tested) [38], could impact learning and memory although this topic is understudied [57]. The most compelling data indicate a role for pubertal status and pubertal hormones in PFC-dependent cognitive flexibility [58,59]. Given that the number of AVP cells and fibers increases during adolescence [60,61], one would predict that AVP-deficiency would have a greater effect in late as opposed to early adolescence, which is opposite to that seen in the present study. Nevertheless, future studies are needed to determine whether AVP's role in reinforcement conditioning changes across adolescence or is impacted by testing experience. Assessment of the training phase for light reinforcement was also less clear. As seen with the social reinforcement task, rats learned the light reinforcement task within the first session. Unlike in the social reinforcement task, however, sex differences in light reinforcement were significant (in HOM rats) or approached significance (in WT rats) on this session, raising the possibility of learning differences in the light reinforcement task among groups. Here too, the order of testing could have impacted the data as animals were extinguishing the social reinforcement task while acquiring the light reinforcement task.

HOM and WT rats differ in their performance on a visual attention test – lateralized reaction time task to a house light stimulus [62]. Notably, genotype differences are dependent on the duration of the house light, with WT rats outperforming HOM rats at short durations (˜0.2s) and HOM rats outperforming WT rats at longer durations (˜2s). The authors attributed these findings to genotype differences in attention rather than light perception because varying the brightness of the house light had no effect on performance in either genotype. Importantly, WT and HOM rats performed similarly when the house light was presented for 4s, which is similar to the duration of

the light stimulus in the present study (5s). Hence, it is unlikely that genotype differences in the perception of the house light influenced the light reinforcement in the present study. Furthermore, genotype differences in light perception would not account for the presence or absence of sex differences within a given genotype.

Activity can also impact performance on operant responding [reviewed in 52], and previous studies have demonstrated sex differences in locomotor activity (females > males) as well as decreased locomotor activity in HOM Brattleboro rats [29,63–66]. However, in contrast to the effect on social reinforcement, the Brattleboro mutation impacts locomotor activity of adolescent males and females in a similar manner [29]. Furthermore, in the present study there were no sex or genotype differences in the total number of responses during the social reinforcement task and data were analyzed as proportion of responses directed toward the social stimulus, thereby controlling for any potential differences in activity. Hence, the present findings are more consistent with differences in goal-directed responses than in overall activity.

Sex differences in brain and behavior arise from organizational actions of prenatal, early postnatal, and pubertal gonadal hormones; activational actions of gonadal hormones; direct effects of genes on the sex chromosomes; and environmental factors [reviewed in 4,5]. The sex difference in adolescent social reinforcement is likely the result of several of these factors acting on AVP circuits, the primary candidate being the sexually dimorphic AVP pathway that originates from cells in the bed nucleus of the stria terminalis (BNST) and medial amygdala (MeA). Males of most species that have been assessed have greater numbers of AVP cells and more dense projections than females [reviewed in 67,68] due to interactions between organizational hormone actions, activational hormone actions, and direct effects of genes on the sex chromosomes [69–73]. Given that the BNST and MeA of rodents receive extensive chemosensory input, sex differences in this pathway could contribute to sex differences in social

behavior through differential modulation of chemosensory input [74]. Nevertheless, selective ablation of AVP cells in the BNST decreased same-sex social investigation by males, without altering their detection of social odors or habituation and discrimination of non-social odors [75]. These findings suggest that BNST AVP can act through non-chemosensory mechanisms to regulate sex differences in social behavior. The BNST/MeA pathway is thought to link brain areas that regulate social behavior (the Social Behavior Network) with the mesocorticolimbic dopamine system [12,76–78]. In juveniles/early adolescents, AVP manipulations in the septum, a projection area of BNST/MeA cells, modulate local dopamine release [79] and social play [17,18] in sex-specific ways. Furthermore, septal injections of the dopamine agonist, apomorphine, counteract the depressive effects of a vasopressin receptor 1a antagonist on play behavior [79]. These data suggest that vasopressin interacts with dopamine in the lateral septum to regulate sex differences in reward-associated social behaviors of juveniles/early adolescents. Future studies using social reinforcement paradigms are needed to determine whether this AVP/dopamine mechanism in the septum (or elsewhere) regulates sex differences adolescent social behaviors through sex-specific modulation of social motivation.

## 5. Conclusions

The present findings demonstrate that AVP plays a critical role in sex differences in adolescent social reinforcement. This adds to the growing list of social behaviors that are influenced by AVP during adolescence (e.g., social play, social recognition, 50 kHz USVs, huddling) [17,19,28,80]. Furthermore, it supports the hypothesis that AVP influences adolescent social behaviors, in part, by modulating the reinforcement/reward value of social stimuli. AVP has also been implicated in several neurodevelopmental disorders that impact social behavior [8,9,81–87]. Hence, understanding the neurobiology through which AVP influences social development is critical for our understanding of normative and pathological development, both of which exhibit striking sex differences in humans [1–3,56,88,89].

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## Declarations of Interest

None

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## Figure Legends

Figure 1. Pictures and illustration of the operant testing apparatus from the (A,B) side and (C) top view. (D) Observation port door that opens to the social stimulus or empty chamber.

Figure 2. The Brattleboro mutation eliminates the sex difference in social reinforcement. (A) Mean (±s.e.) proportion of responses directed toward the social reinforcement port in wild type rats (WT), heterozygous Brattleboro rats (HET), and homozygous Brattleboro rats (HOM). (B) Mean (±s.e.) total responses directed toward the social reinforcement and unreinforced ports. *Indicates significant sex difference within genotype ($P<0.05$, Fisher's PLSD).

Figure 3. Sex differences emerge in WT and HET rats, but not HOM rats, during the latter stages of the social reinforcement training phase. Mean (±s.e.) proportion of responses directed toward the social reinforcement port in WT, HET, and HOM rats on the (A) first training session and (B-D) across all training sessions. Sample sizes for panels B-D: WT females = 6, WT males = 8, HET females = 10, HET males = 16, HOM females = 9, HOM males = 10. *Indicates significant sex difference within genotype ($P<0.05$, Fisher's PLSD). Abbreviations defined in Figure 1.

Figure 4. Males show a greater preference than females for the light reinforcer, irrespective of genotype. (A) Mean (±s.e.) proportion of responses directed toward the light reinforcement port. (B) Mean (±s.e.) total responses directed toward the light reinforcement and unreinforced ports. *Indicates significant sex difference within genotype ($P<0.05$, Fisher's PLSD). Inset of panel B illustrates the main effect of Sex on total number of responses ($P<0.05$, ANOVA). Abbreviations defined in Figure 1.

Figure 5. Acquisition data for light reinforcement training. Mean (±s.e.) proportion of responses directed toward the light reinforcement port in WT, HET, and HOM rats on the (A) first training session and (B-D) across all training sessions. Sample sizes for panels B-D: WT females = 6, WT males = 8, HET females = 13, HET males = 16, HOM females = 10, HOM males = 9. *Indicates significant sex difference within genotype ($P<0.05$, Fisher's PLSD). #Indicates significant difference between HOM male rats and males of other genotypes ($P<0.05$, Fisher's PLSD). Abbreviations defined in Figure 1.

Figure 6. Boxplot of mean daily water intake of rats genotyped as WT, HET, and HOM rats (see methods for genotyping details). The lower and upper ends of the boxes represent the first and third quartile range, respectively. Lines within the boxes represent the median. Whiskers represent the lowest and highest values within the group, excepting outliers. The single outlier is indicated by a shaded circle. Abbreviations defined in Figure 1.

Table 1. Lower and upper confidence intervals for proportion responding to social and light reinforcers

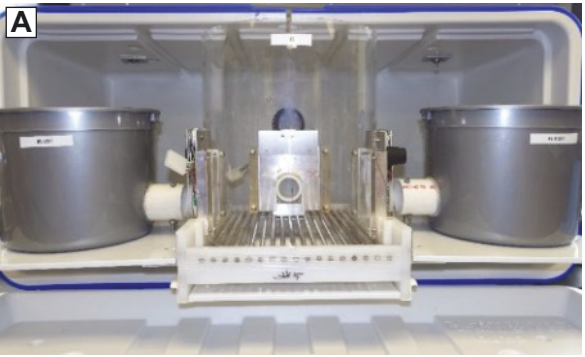
| | | Social Responding | | Light Responding | |
|---|---|---|---|---|---|
| Genotype | Sex | Lower CI | Upper CI | Lower CI | Upper CI |
| WT | Females | 0.28 | 0.45 | 0.27 | 0.47 |
| | Males | 0.40* | 0.61 | 0.42* | 0.62 |
| HET | Females | 0.35* | 0.44 | 0.31 | 0.49 |
| | Males | 0.38* | 0.53 | 0.45* | 0.56 |
| HOM | Females | 0.34* | 0.57 | 0.32 | 0.41 |
| | Males | 0.38* | 0.51 | 0.41* | 0.54 |

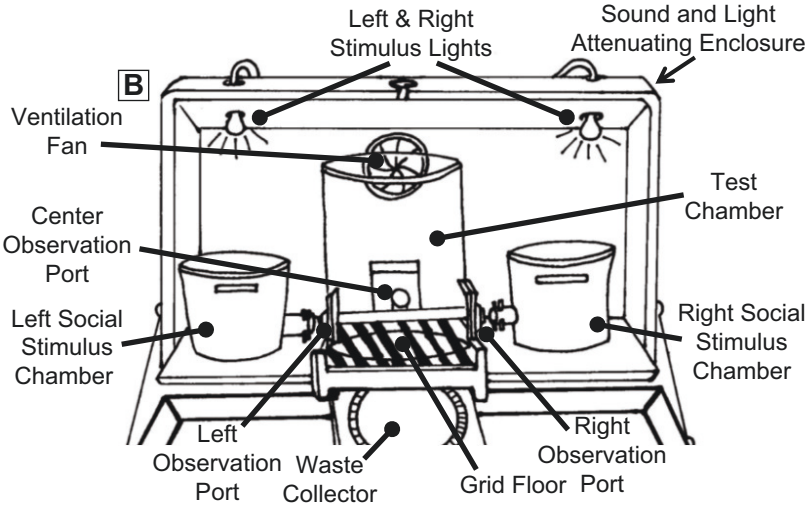
*Indicates significant preference for social or light reinforcer.

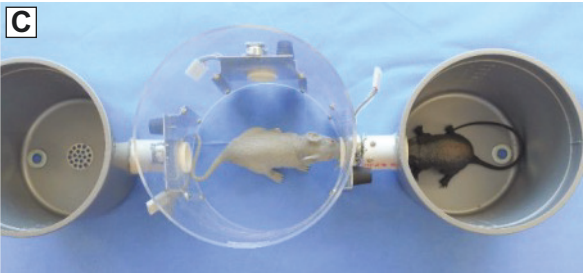
Supplemental Table. Outliers removed in each analysis.

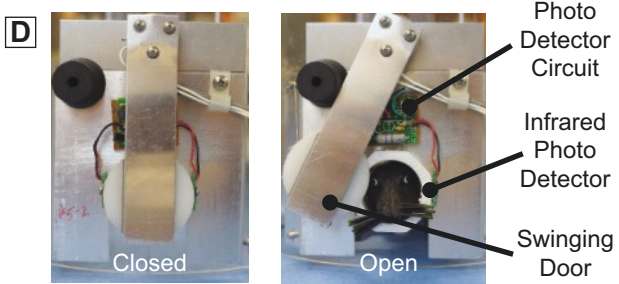
| | | Social Reinforcement | | | | Light Reinforcement | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Social Responses | | | Total Responses | Light Responses | | | Total Responses |
| Genotype | Sex | Training Session 1 | All Training Sessions | Testing Phase | Testing Phase | Training Session 1 | All Training Sessions | Testing Phase | Testing Phase |
| WT | Females | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Males | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| HET | Females | 0 | 4 | 0 | 1 | 1 | 1 | 0 | 1 |
| | Males | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| HOM | Females | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 |
| | Males | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 |

# Figure 1

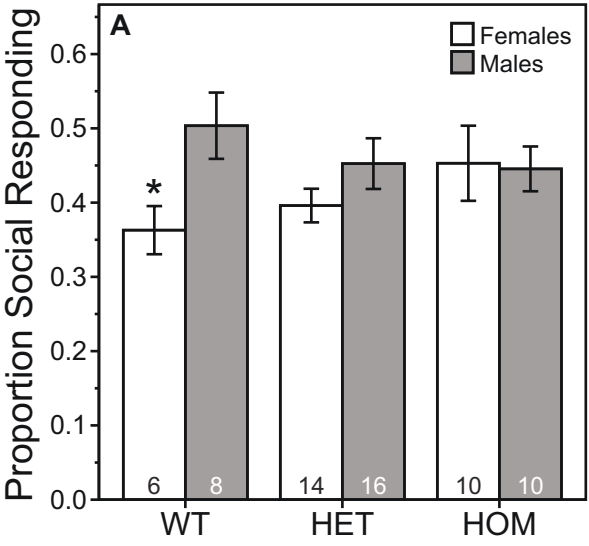

A


B
Left & Right
Stimulus Lights
Sound and Light
Attenuating Enclosure
Ventilation
Fan
Test
Chamber
Center
Observation
Port
Left Social
Stimulus
Chamber
Right Social
Stimulus
Chamber
Left
Observation
Port
Waste
Collector
Grid Floor
Right
Observation
Port


C


D
Closed
Open
Photo
Detector
Circuit
Infrared
Photo
Detector
Swinging
Door

# Figure 2


A
Females
Males
Proportion Social Responding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*
6
8
14
16
10
10
WT
HET
HOM

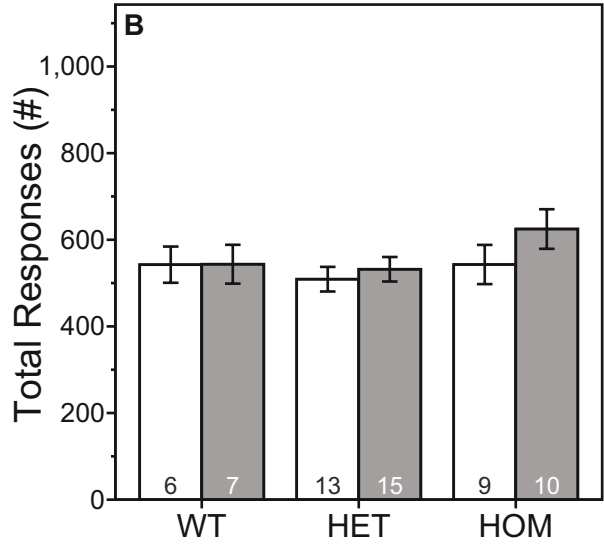
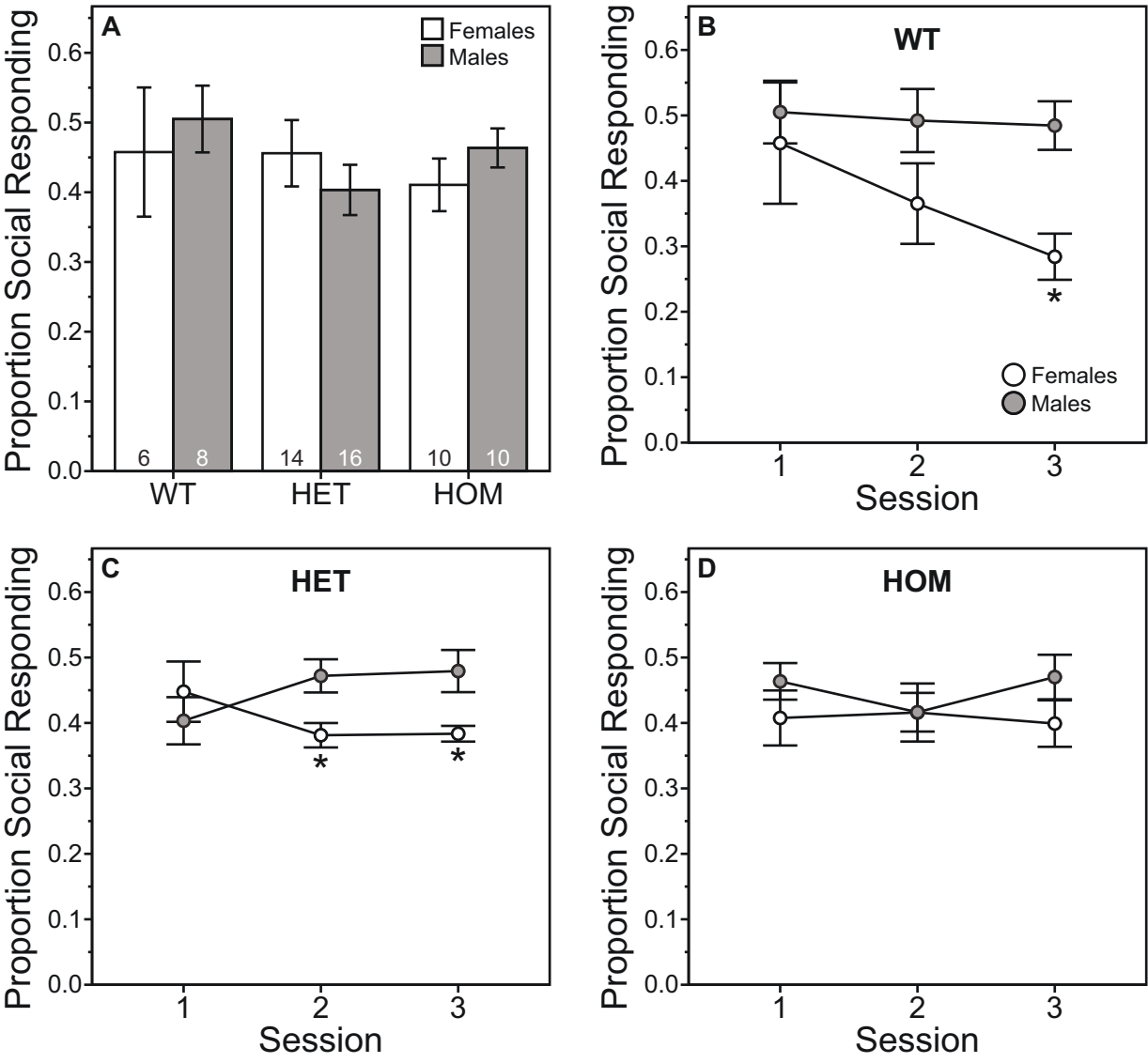

B
Total Responses (#)
0
200
400
600
800
1,000
6
7
13
15
9
10
WT
HET
HOM

Figure 3


A
Females
Males
Proportion Social Responding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
6
8
14
16
10
10
WT
HET
HOM
B
WT
Females
Males
*
1
2
3
Session
C
HET
*
*
D
HOM

# Figure 4

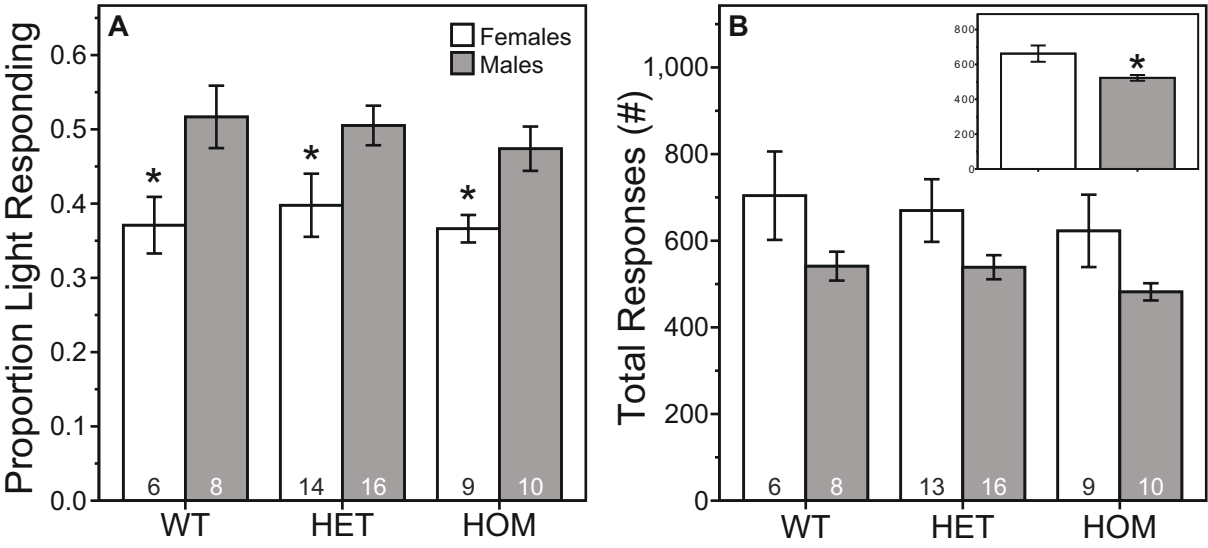
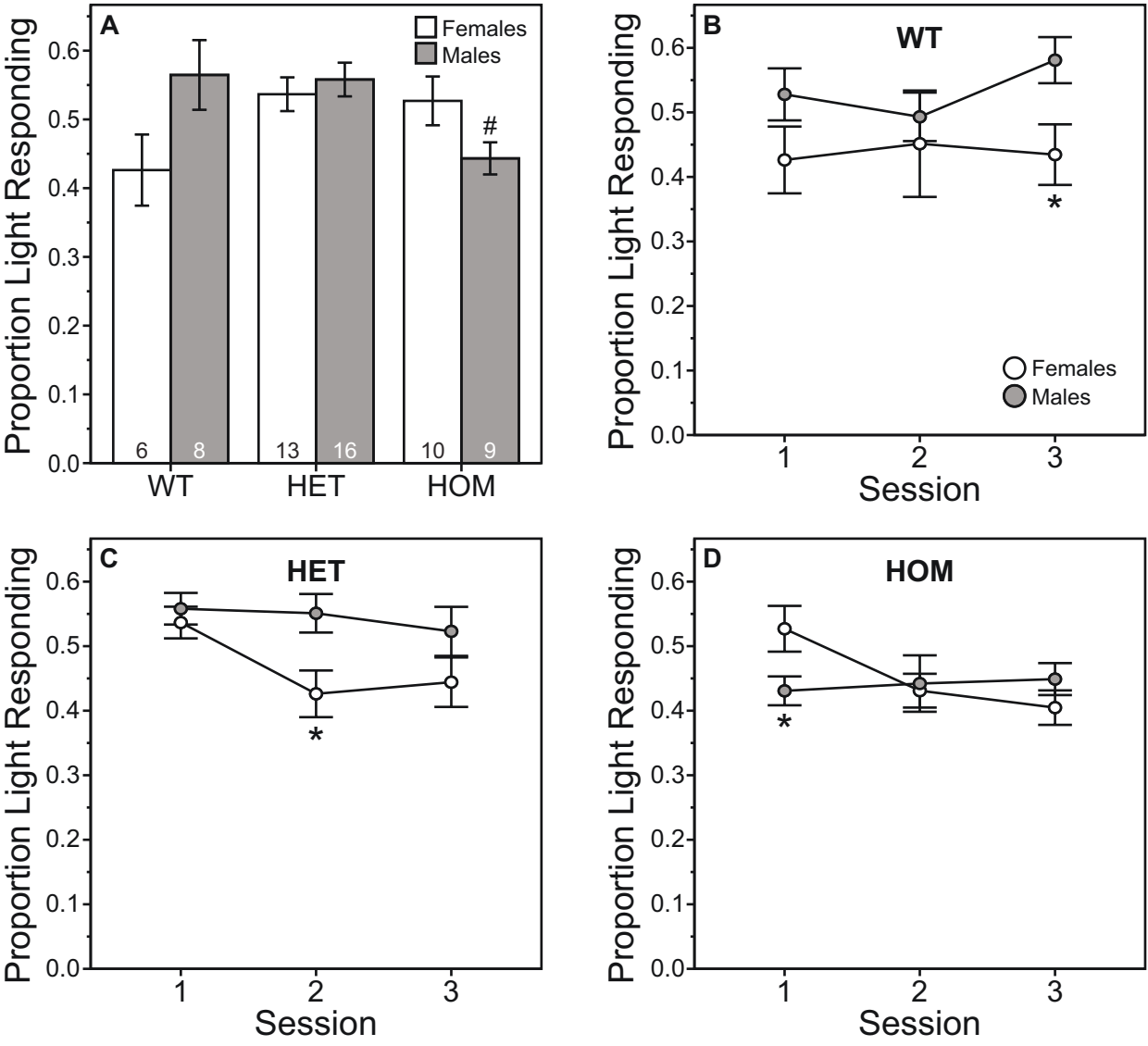

A
Females
Males
Proportion Light Responding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
*
*
*
6
8
14
16
9
10
WT
HET
HOM
B
Total Responses (#)
0
200
400
600
800
1,000
800
600
400
200
0
*
6
8
13
16
9
10
WT
HET
HOM

Figure 5


A
Females
Males
Proportion Light Responding
0.0
0.1
0.2
0.3
0.4
0.5
0.6
6
8
13
16
10
9
#
WT
HET
HOM
B
WT
Females
Males
*
Session
1
2
3
C
HET
*
D
HOM
*

# Figure 6