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ORIGINAL RESEARCH

Genetic Analysis of the Stereotypic Phenotype in *Peromyscus maniculatus* (deer mice)

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Abstract

Peromyscus maniculatus, including the laboratory stock BW, have been used as a model organism for autism spectrum disorder and obsessive–compulsive disorder because of the high occurrence of stereotypy. Several studies have identified neurological and environmental components of the phenotype; however, the heritability of the phenotype has not been examined. This study characterizes the incidence and heritability of vertical jumping stereotypy (VS) and backflipping (BF) behavior in the BW stock of the *Peromyscus* Genetic Stock Center, which are indicative of autism spectrum disorders. In addition, interspecies crosses between *P. maniculatus* and *P. polionotus* were also performed to further dissect genetically stereotypic behavior. The inheritance pattern of VS suggests that multiple genes result in a quantitative trait with low VS being dominant over high VS. The inheritance pattern of BF suggests that fewer genes are involved, with one allele causing BF in a dominant fashion. An association analysis in BW could reveal the underlying genetic loci associated with stereotypy in *P. maniculatus*, especially for the BF behavior.

Keywords *Peromyscus* · Stereotypy · Genetic analysis · Autism spectrum disorders · Obsessive–compulsive disorder

Introduction

The genus *Peromyscus*, commonly known as the deer mouse, is the most abundant mammal in North America and these small rodents caught in the wild can be reared using standard *Mus* housing and husbandry conditions (Crossland et al. 2014). *Peromyscus* are widely used in ecology and evolution studies as well as behavioral, physiology and developmental areas. *Peromyscus* research in the field and more observational research has merged with more genetic and genomic approaches now available to be able to determine the genetic basis of variant phenotypes (Weber et al. 2013). In addition, transcriptomic methods can identify changes in gene expression under varying environmental, behavioral, and physiological conditions (Storz and Cheviron 2016), (Munshi-South and Richardson 2017). A compelling case can be made for the use of *Peromyscus* as a useful model for research as the animals are the result of natural selection and represent genotypes occurring in the natural environment (Bedford and Hoekstra 2015).

The *Peromyscus* stocks at the *Peromyscus* Genetic Stock Center (PGSC) are derived from wild populations from natural environments and are maintained as outbred stocks. The genetic diversity of the BW stock is illustrated by whole genome sequence analysis of BW individuals, which identified a single nucleotide polymorphism (SNP) approximately every 200 bp in the BW population (Lucius et al. 2021). The total number of SNPs identified in six BW individuals, 17.52 million, is greater than the total number of private SNPs, 1.24 million, identified across 25 common inbred laboratory strains of *Mus musculus* when compared to the C57BL/6 J reference genome, and is equivalent to the total number of private SNPs from four inbred strains derived from wild *Mus* stocks (PWK/PhJ, CAST/EiJ, WSB/EiJ, and MOLF/EiJ 12.54 million) or the total number of SNPs in the inbred strain derived from wild stocks of *Mus spretus* (SPRET/EiJ, 23.46 million). The value of *Peromyscus* as a model for the

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human condition and biomedical research has been emphasized in a thorough review (Bedford and Hoekstra 2015).

P. maniculatus (BW stock) animals maintained at the PGSC display stereotypic behaviors and have been used as a model for both autism spectrum disorder (ASD) and obsessive–compulsive disorder (OCD). BW stock animals cannot model the entire autism spectrum found in humans. However, part of the autism spectrum in humans is repetitive movement often in a rhythmical way and in the same spatial direction (Powell et al. 1999). These movements are similar to the vertical stereotypy and backflipping behaviors observed in BW animals, as the vertical stereotypy and backflipping are repetitive and occur in the same location. While vertical stereotypy and backflipping models the rhythmicity of and spatial direction of the human stereotypy it does not model the insistence for sameness demonstrated by humans.

Previous analysis of stereotypy in *P. maniculatus* used automated beam-break systems to measure stereotypy in a large group of randomly selected BW animals. Mice were analyzed weekly after weaning and a developmental trajectory determined such that mice were grouped into high, low, and intermediate levels of stereotypy (Tanimura et al. 2010b, a), and similar groupings were identified in a similar analysis (Korff et al. 2008). Stereotypic behavior reached maximal levels and plateaued approximately 6 weeks postweaning.

Animals reared in enhanced environments (toys, ladders, etc.) have reduced stereotypy levels (Powell et al. 2000) as well as improvements in procedural and reversal learning scores compared to those reared in standard cages (Tanimura et al. 2008). Neuronal activity is elevated in non-stereotypic mice and in mice reared in enhanced conditions (Turner et al. 2002). Of particular interest is that there appears to be a transgenerational effect of the enriched environment on stereotypy (Bechard et al. 2016).

Associations of various neuronal chemical levels with stereotypy levels have been observed, including cAMP and the enzyme PDE4 (Korff et al. 2008) and enkephalin (Presti and Lewis 2005). Levels of Dopamine, DOPAC, Homovannillic Acid, and Serotonin levels were not statistically different in the striatum in stereotypic and non-stereotypic animals. A separate study that examined various neurochemicals in different brain regions of low stereotypic and high stereotypic animals did not find a significant difference in the concentrations of the neurochemicals examined between the two phenotypes. However, they did observe an increased redox state in the frontal cortex of high stereotypic animals (Guldenpfennig et al. 2011).

A number of drugs, including receptor agonists and antagonists, have been used to examine the mechanisms controlling stereotypy in *P. maniculatus*, using the laboratory stock. Briefly, intrastriatal injections of NMDA and dopamine D_1 receptor agonists reduced stereotypy in the high stereotypy animals (Presti et al. 2003), while dopaminergic agonists failed to induce high level stereotypy (Presti et al. 2004). Drugs targeting Dopamine D_2, Adenosine A_{2A}, and Glutamate $mGlu_5$ receptors as a triple-dose cocktail was shown to reduce repetitive behaviors in BW animals and supports the role of the indirect basal ganglia pathway, which expresses the targeted receptors (Lewis et al. 2019) and (Tanimura et al. 2010b, a).

Stereotypy in the BW stock has been considered as a model of obsessive–compulsive disorder (OCD) (Wolmarans et al. 2018). Using high and low stereotypic animals as a model, response to various drugs mimics the response to those drugs used in humans with OCD. Attenuation of stereotypy in the BW stock occurs with treatment with $5HT_{2A/C}$ and dopamine D_2 receptor agonists, which is similar to the human response (Korff et al. 2008). A brief review has discussed the value of *P. maniculatus* as an OCD model (Wolmarans et al. 2018).

A number of studies have identified neurochemical differences between high and low stereotypic animals as well as different effects of drugs and receptor antagonists and agonists. This might suggest that multiple genes could be involved in a quantitative way to control this phenotype. In addition, the enrichment studies have found that mice reared in enriched cages compared to normal cages have reduced vertical stereotypy, but have increased backflipping behavior (Powell et al. 1999). Also, animals reared in enhanced environmental conditions that had reduced stereotypy levels compared to those raised under standard conditions performed better in learning and cognitive flexibility tests (Tanimura et al. 2008).

Initial experiments to understand the genetic basis of stereotypy were to examine phenotypic variation among *Peromyscus species* and mutants (Shorter et al. 2014). *P. maniculatus* (BW stock) and *P. polionotus* (PO stock) differ in a number of characteristics yet BW females mated to PO males can produce fertile but small offspring. The F_1 progeny are also fertile. However, the reciprocal cross produces fetus/offspring with developmental defects and rarely survive (Vrana et al. 2000). Using simple open field behavioral testing, it was found that BW mice have higher percent (approximately tenfold) repetitive behavior (jumps, circle running) during a 5-min test than PO animals. All testing was done with 12 males and 12 females. In addition, F_1 (BWxPO) mice exhibit low percent repetitive behavior essentially the same as the PO parent. This strongly suggests that low stereotypy is dominant over high stereotypy. Interestingly, a coat color mutant, A^{nb}, called wide-band agouti, maintained on a BW background, had a low stereotypy score but higher than PO animals. These preliminary studies suggested that a larger study using controlled matings might determine the inheritance pattern of vertical jumping or backflipping.

Identification of the genetic underpinnings of behavior has been a long-standing goal of geneticists that has made significant progress through the application of genome wide association studies (GWAS), quantitative trait locus (QTL) mapping, and the application of next generation sequencing techniques. A range of animals, including insects, birds, and voles, have been used to identify specific genetic variations associated with behavior (Niepoth and Bendesky 2020). Studies in *Peromyscus* have contributed to these successes. Results from the genetic analysis of burrowing behavior in *Peromyscus* provides support that *Peromyscus* is a viable model for discovery of the genetic basis of complex behavior. W. D. Dawson, founder of the PGSC, began genetic analysis of the behavior differences in burrowing between *P. maniculatus* and *P. polionotus.* Behavior in nature was recapitulated in captivity and F_1 progeny had the more complex burrowing phenotype of *P. polionotus,* which included an escape tunnel and long entrance tunnel. Additional phenotyping of backcross progeny suggested that two or more loci controlled the length of the entry tunnel (Dawson et al. 1988). Further analysis of this difference in burrowing habit between *P. maniculatus* and *P. polionotus* also found that the presence of an escape tunnel in F_1 animals suggested dominance of this trait. Tunnel length was a quantitative trait and was found using backcross animals to be controlled by at least 3 genomic regions (Weber et al. 2013).

P. maniculatus and *P. polionotus* display phenotypic differences in paternal parenting behaviors, including nest building, with *P. polionotus* males building higher quality nests (Bendesky et al. 2017). This paternal parenting behavior is heritable, and a QTL mapping analysis identified a region of chromosome 4 associated with the nest building behavior. While there were few coding sequence variants in the QTL between *P. maniculatus* and *P. polionotus* in likely candidate genes, there was a difference in the expression level of vasopressin, with higher levels of mRNA produced in the hypothalamus of *P. maniculatus*. Administration of vasopressin to *P. polionotus* males inhibited nest building behavior. These results suggest that *Peromyscus* is a useful model system for identification of genomic loci and specific genes that underpin complex behaviors and that identifying genes controlling stereotypy (VS and BF) in this species is likely tractable. The study presented here is the first study, to our knowledge, to examine the inheritance pattern of offspring from matings designed to gain insight into the genetic control of stereotypic behavior in *Peromyscus*.

Materials and Methods

Animal crosses

All animals were obtained from the breeding colony at the *Peromyscus* Genetic Stock Center at the University of South Carolina. All crosses were established from parents that were phenotyped in the PGSC. Initially, animals of breeding age were randomly chosen from the large colony of *P. maniculatus* (BW stock) animals within the colony. The goal was to establish matings that should be informative. A mating involved one female and one male. The mating cages were maintained in the PGSC, and weaning was supervised by the Colony Manager. Offspring were weaned at about 21 days and sorted into male and female cages. All offspring were identified by an ear notch system. Each animal has a 5-digit number assigned, which is simply a continuation of all breeding that occurs in the PGSC.

Phenotypic scoring

When animals reached 60–70 days of age, an animal was captured by the tail and scruff of the neck and gently placed into a large rat cage 17.5 long × 9.5 wide × 8 inches deep. The cage was covered with a clear plastic lid with holes for ventilation 1 inch apart and ¼ inch in diameter around the perimeter of the lid.

The cage was in a back corner of the home cage room. The cage floor was lightly covered with sani chip bedding. The same amount was used each time by measuring the bedding in a 1-L beaker. The animal was filmed for 5 min with a Sony HD digital camera directly over the middle of the cage using a tripod. Three sample videos showing the range of phenotypic behavior are included as supplemental material. All attempts were made to maintain quiet in the room and the room almost always had only a single person present, who remained out-of-sight of the rat cage during filming. After filming 5 min the animal was removed and placed in a new, clean cage. Five large cages were available for filming. After filming, bedding was removed to disposal, the cage was washed well with very hot water, drained, and then sprayed with 70% ethanol and wiped dry and allowed to sit. The lid was also washed and cleaned with 70% ethanol.

Films were scored manually to record the number of vertical jumps or backflips in 5 min. The scoring is rather precise because it is only numerical for how many vertical leaps occurred or how many backflips occurred. It is possible to stop the video and scroll back if there is any concern about "lost count" or a disruption. With this visual scoring, "cage running" was not assessed.

The protocol for these studies has been approved by the IACUC at the University of South Carolina. The University is accredited by the AAALAC.

Results

Stereotypy within the *P. Maniculatus* (BW) Stock

In order to ascertain the level of stereotypy within the BW colony, random animals were analyzed for the phenotype. Stereotypy consisted of vertical jump or backflips. A total of 113 females and 174 males obtained from 24 different matings were scored. Not only was this to assess the distribution of stereotypy within the colony but selected animals were used for establishing matings for subsequent genetic analysis. The average and standard deviation of vertical stereotypy (VS) level in males and females screened is shown in Table 1. The number of animals that did backflips (BF) are also shown. Among the 287 animals scored, no animal displayed both vertical stereotypy and backflipping. Vertical stereotypy was observed, backflipping was observed, or the animal did neither. For VS, 5 vertical jumps in 5 min was set as the minimum number of jumps in order to assign the VS phenotype. For females, 68.1% displayed some form of VS, while 64.4% of males displayed VS. A z-test to compare the proportion of females and males with VS is insignificant ($z = 0.66$, $p = 0.51$), and a t-test to compare the mean VS between females and males is also insignificant ($t = 0.077$, $p = 0.94$). For BF, if one backflip was observed in 5 min, the animal was scored with BF behavior. For females, 13.3% displayed BF, while 7.5% of males demonstrated the behavior. A z-test to compare the proportion of females and males with BF is insignificant ($z = 1.62$, $p = 0.11$), and the mean number of BF is also insignificantly different between females and males ($t = 0.0095$, $p = 0.99$). These data suggest that neither VS nor BF is influenced by sex. Further analysis of the distribution of vertical stereotypy level in males and females by total number of jumps also suggests the phenotype is equally distributed in males and females (Table 2).

Table 1 Vertical stereotypy and backflipping in a random set of BW animals scored as total observations in 5 min*

Sex	Female	Male
Number Scored	113	174
Number with > 5 VS	77	112
Avg Vertical Stereotypy	36.33	35.55
Standard Deviation	50.23	47.47
Backflipping animals	15	13
Avg Backflipping	24.4	46.2
Standard Deviation	21.7	34.4

*Statistical analysis of vertical stereotypy was based on all the animals scored and includes animals with no vertical stereotypy. The smaller number of backflipping animals were used to determine average and standard deviation within that group and does not include non-backflip animals

Matings Designed to Determine the Inheritance Pattern of VS

To determine if the stereotypic behavior is heritable, ten matings employing males and females that had been scored for stereotypy within the PGSC colony were established. Animals used in these matings received a designation of high (H), low (L), or no (N) stereotypy. Established crosses included HxH, LxL, NxN, LxN, and NxH matings, where the first symbol represents the female and the second symbol the male in the cross (Table 3). The offspring from these ten matings were scored for stereotypy. Five additional matings employed offspring from these initial crosses. The offspring received a two-letter designation that represents the stereotypy score of the parents. For instance, LHxLH represents a female offspring whose mother had low stereotypy and the father had high stereotypy crossed with a male offspring that was also produced from a LxH cross. The phenotypes and numbers of offspring produced by the different matings are presented in Figs. 1, 2, 3, 5, and 7. Supplemental Table 1 gives numerical values of averages and numbers of animals and phenotypes.

For Figs. 1, 2, 3, 5, and 7, the legend at the top of each graph gives the mating number of the cross and the phenotypes of the parents. All stereotypy scores are numbers of vertical leaps (VS) or backflips (BF) in the five-minute test. L = low vertical stereotypy (below 20 VS.). N = 0 VS. H = high VS usually above 50 and usually much higher. L/N would mean that parent is an offspring of a cross with the female parent L and the male parent N phenotypes.

The orange points in the graphs are parents and the round point is female, and the square point is male. Female parents are always listed first. The VS and BF points are described in the legend. In some instances, a number of zero stereotypy animals are not given because of extensive overlap and density of points. These are used in determining the average and SEM, however (see Table 3).

"High" X "High" Matings Produce more VS Than "Low" x "Low" Matings

The phenotypes and numbers of animals obtained from LxN and NxN matings are presented in Fig. 1A and Fig. 1B.

Table 2 Distribution of vertical stereotypy level in male and female BW animals

Vertical Stereotypy	0–4	5–20	21–50	51–100	= or > 100
Females	36	26	24	14	13
Percent	31.6	23	21.2	12.4	11.5
Males	62	29	37	26	20
Percent	35.6	16.7	21.3	14.9	11.5

Table 3 Pairwise comparison of mean vertical stereotypy in offspring from matings LxL 2048, LxL 2050, HxH 2074, HxH 2075, LxL 2103, LxL 2104, HxH 2076, and HxH 2096, using Tukey HSD following one-way ANOVA

Comparison	Tukey HSD Q statistic	Tukey HSD p-value	Inference
LxL 2048 vs LxL 2050	0.67	0.90	insignificant
LxL 2048 vs HxH 2074	1.34	0.90	insignificant
LxL 2048 vs HxH 2075	0.84	0.90	insignificant
LxL 2048 vs LxL 2103	0.48	0.90	insignificant
LxL 2048 vs LxL 2104	0.50	0.90	insignificant
LxL 2048 vs HxH 2076	5.98	0.00	** $p<0.01$
LxL 2048 vs HxH 2096	3.48	0.22	insignificant
LxL 2050 vs HxH 2074	2.11	0.78	insignificant
LxL 2050 vs HxH 2075	1.53	0.90	insignificant
LxL 2050 vs LxL 2103	1.08	0.90	insignificant
LxL 2050 vs LxL 2104	0.11	0.90	insignificant
LxL 2050 vs HxH 2076	7.05	0.00	** $p<0.01$
LxL 2050 vs HxH 2096	4.25	0.06	insignificant
HxH 2074 vs HxH 2075	0.43	0.90	insignificant
HxH 2074 vs LxL 2103	0.65	0.90	insignificant
HxH 2074 vs LxL 2104	1.75	0.90	insignificant
HxH 2074 vs HxH 2076	5.10	0.01	* $p<0.05$
HxH 2074 vs HxH 2096	2.43	0.65	insignificant
HxH 2075 vs LxL 2103	0.26	0.90	insignificant
HxH 2075 vs LxL 2104	1.27	0.90	insignificant
HxH 2075 vs HxH 2076	4.92	0.02	* $p<0.05$
HxH 2075 vs HxH 2096	2.62	0.57	insignificant
LxL 2103 vs LxL 2104	0.89	0.90	insignificant
LxL 2103 vs HxH 2076	4.39	0.05	* $p<0.05$
LxL 2103 vs HxH 2096	2.57	0.59	insignificant
LxL 2104 vs HxH 2076	5.85	0.00	** $p<0.01$
LxL 2104 vs HxH 2096	3.69	0.16	insignificant
HxH 2076 vs HxH 2096	1.73	0.90	insignificant

Most of the ♀ and ♂ offspring from both matings have a LVS phenotype with only one animal (Fig. 1A) expressing a reasonably high VS phenotype (50VS). Most of the other progeny essentially have a VS value between the two parents (Fig. 1A). A single animal expressing the BF phenotype was found among the progeny. We describe these backflip animals as "unexpected" as neither parent displays backflip behavior. These unexpected BF animals appear occasionally among the crosses designed to test vertical stereotypy. All the animals produced from the A2050 (NxN) mating have very low stereotypy values showing that the low stereotypy phenotype is heritable.

The two HxH matings (Fig. 1C, 1D) produced a number of offspring with much higher VS values than found among progeny of the A2048 (LxN) and A2050 (NxN) matings. The offspring from the two HxH crosses have a larger distribution of stereotypic phenotypes with many animals expressing higher VS values than offspring from the LxN and NxN matings. A possible explanation is that multiple loci with additive effect control the VS phenotype and those loci are heterozygous, producing a large distribution of phenotypes. The results of a second set of similar crosses are shown in Fig. 2. These results suggest that VS is a quantitative phenotype; therefore, we graph the data for each individual parent and offspring as they are likely to represent unique genotypes. The two LxL crosses (Fig. 2A, B) produce offspring with low VS scores with an average score of 16.9 and 5.5, respectively, while the two HxH progeny have VS scores of 65.5 and 73.4 (Table 1).

Combined together four HxH matings produced 83 total offspring with a mean VS of 57.5 and standard deviation of ± 58.4, while four LxL matings produced 38 total offspring with a mean VS of 7.5 and standard deviation of ± 13.6. The means of these two populations are significantly different from each other (two-tailed t-test, $t=7.34$, $p=5.9\times10^{-11}$, Hedge's $g=1.42$), demonstrating that HxH matings produce more offspring with higher VS scores. The BW population is outbred, and each parent likely represents a unique combination of alleles that produces offspring with a quantitative phenotype. The offspring data was also analyzed by ANOVA with a post hoc Tukey test to compare the mean VS for offspring among the eight individual matings (4 LxL and 4 HxH). There is a significant difference among the offspring ($F_{7,113}=7.33$, $p=3.02\times10^{-7}$, $\eta^2_p=0.31$), with offspring from HxH mating 2076 being significantly different from all other mating offspring, except HxH mating 2096. This suggests that the parents in mating 2076 likely had more alleles that promote stereotypic behavior (Table 3).

"Low" VS is Partially Dominant to "High" VS

The results of crosses to examine the phenotypes of offspring from "low" x "high" type matings are shown in Fig. 3. Crosses shown in Fig. 3A, B are essentially the same except the female in Fig. 3A came from a LxN mating whereas the female in Fig. 3B was from a NxN mating. Both females exhibited zero VS. The phenotypes of offspring from both matings were heavily skewed towards low VS suggesting a partial dominance at least of the "low" VS phenotype. Two females from the A2055 cross had the BF phenotype.

The results shown in Fig. 3C, D are both from LHxH crosses testing if more offspring have a higher VS phenotype since both parents have "H" contributions. Again, the offspring skewed towards the low VS phenotype in both male and female offspring. Nearly all offspring had lower VS scores than either parent. Again, one aberrant BF animal appeared among the progeny of A2085 (LHxH).

The offspring phenotypes shown in Fig. 3E–G are all from LHxLH crosses. For these matings, both parents were low VS, but many offspring had much higher VS scores than

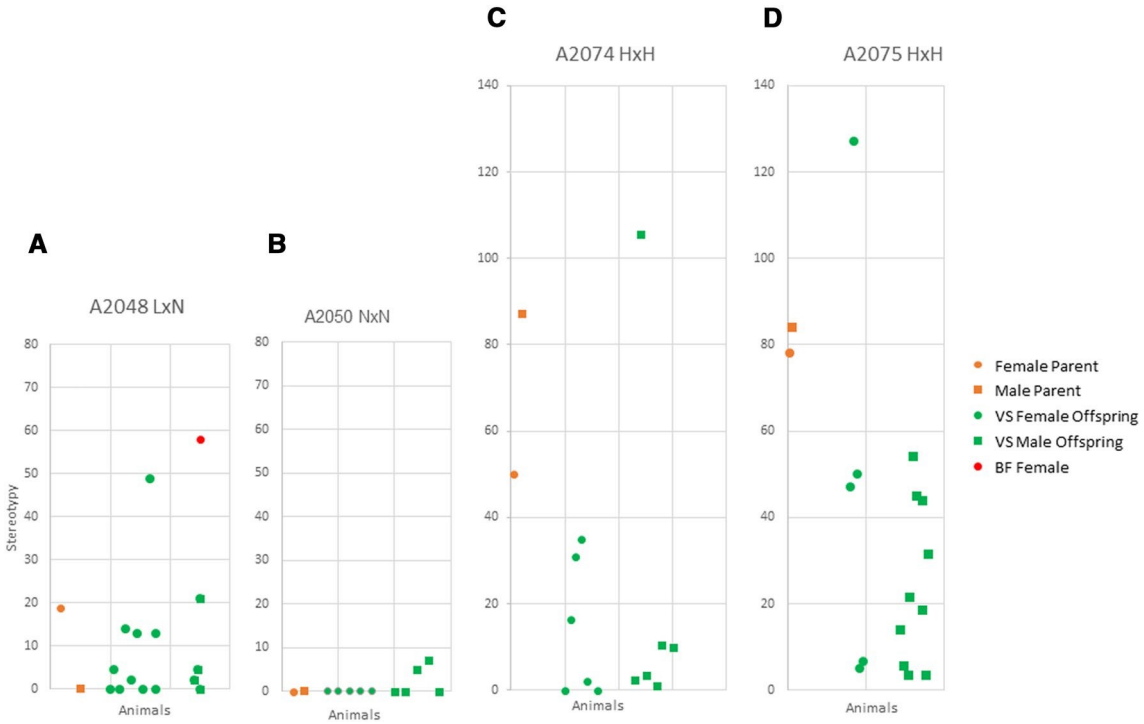

Fig. 1 **A** L x L VS cross A2048. **B** N x N VS cross A2050. **C** H x H VS cross 2074. **D** H x H VS cross 2075. Note the different scales on the y-axis between the low and high VS crosses

either parent. Two animals (one female and one male) had the BF phenotype. A regression on offspring versus mid-parent VS was performed across all VS matings ($R^2 = 0.596$, $F(1, 19) = 26.56$, $p = 6.66 \times 10–5$) to determine the heritability of the VS phenotype and was found to be $h^2 = 0.37$, demonstrating a moderate heritability (Fig. 4).

Inheritance Pattern of the BF Phenotype

The BF phenotype was found among BW animals tested in the animal facility and others occurred sporadically among offspring of matings between animals with no BF behavior (5 BF offspring in 15 matings) (Fig. 1–3). Three BFxL matings and 2 LxBF matings were established. Figure 4A shows the offspring obtained from a BFxLN mating. Among the progeny were 14 BFs out of 40 total progeny. Figure 4C shows the phenotypes of progeny from a BFxL cross and there were 5BF among 9 total progeny. Among the reciprocal LxBF matings (Fig. 5D–F) there were 2BF among 11 total progeny and 1BF among 2 total progeny, respectively. Summing across all BF matings, there are 22 BF offspring among 62 total progeny. A mid-parent–offspring regression across all matings for BF number ($R^2 = 0.204$, $F(1, 19) = 4.6$, $p = 0.046$) shows an $h^2 = 0.21$, demonstrating a low level of heritability (Fig. 6). There are 33 animals that displayed BF behavior among 301 total animals analyzed and two matings, from 20 total matings, produced 19 of the 33 animals with BF behavior. Therefore, heritability in our analysis, while significant, is likely influenced by a small sample size.

If BF behavior is controlled by a single gene, and the BF allele is dominant, then half of the offspring from a mating with one BF parent should display BF behavior. If the BF allele is recessive, then none of the offspring should display BF behavior. While less than half of the offspring display BF behavior, the number generated is not significantly different from number expected ($\chi^2 = 2.67$, $p = 0.102$). These results are consistent with the BF phenotype being controlled by a dominant gene and the BF parents in these matings being heterozygous. The appearance of BF animals from parents with no BF behavior argues against a single dominant allele for backflipping. There are multiple possibilities for the spontaneous appearance of BF behavior, including that there is a BF allele that is recessive. If this were true, then when a BF animal is crossed with a non-BF animal, then the offspring should not have the BF behavior, unless the non-BF animal is heterozygous for the BF allele. This possibility is not likely because when the BF behavior is observed from parents with no BF behavior, and that should be heterozygous, much less than 25% of the littermates have BF behavior. Another possibility is

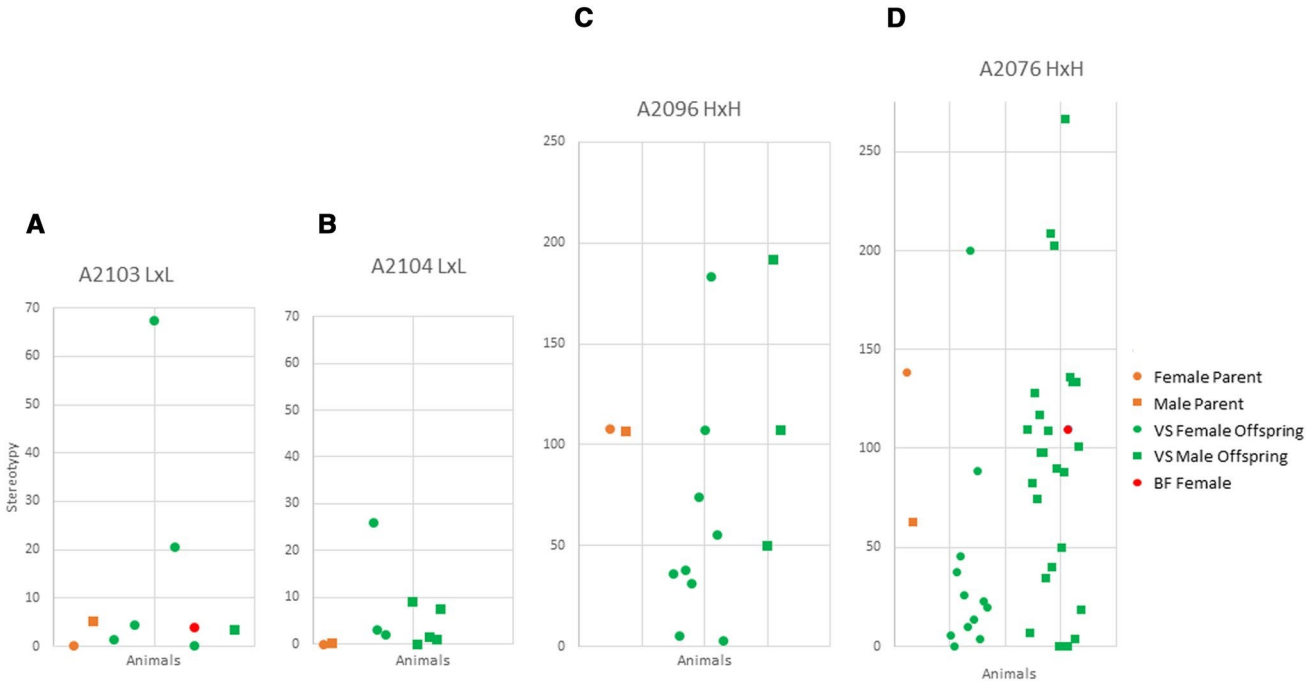

Fig. 2 **A** L x L VS cross A2103. **B** L x L VS cross A2104. **C** H x H VS cross 2096. **D** H x H VS cross 2076. Note the different scales on the y-axis between the low and high VS crosses

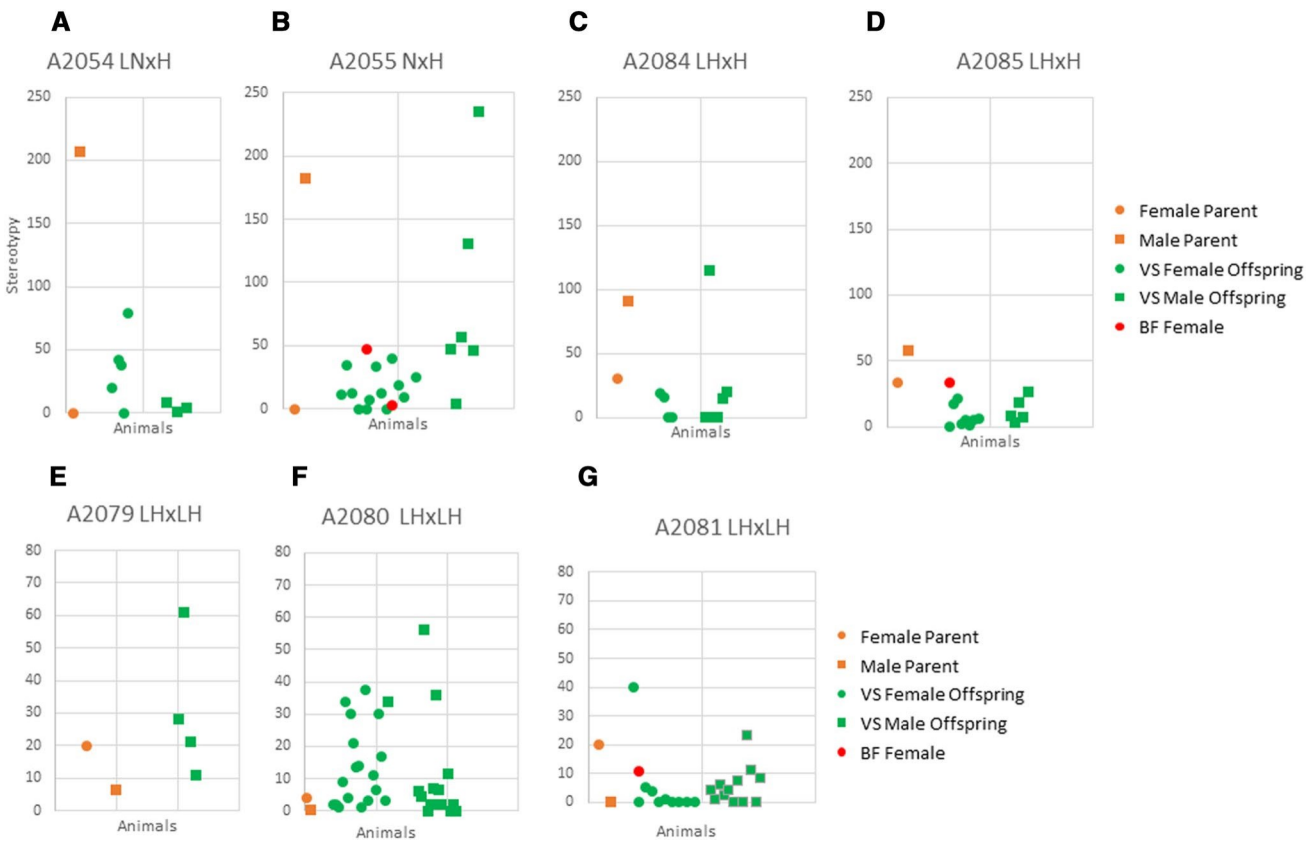

Fig. 3 **A** LN x H VS cross A2054. **B** L x H VS cross A2055. **C** LH x H VS cross 2084. **D** H x H VS cross 2076. **E** LH x LH VS cross A2079. **F** LH x LH VS cross A2080. **G** LH x LH VS cross A2081. Note the different scales on the y-axis between the low and high VS crosses

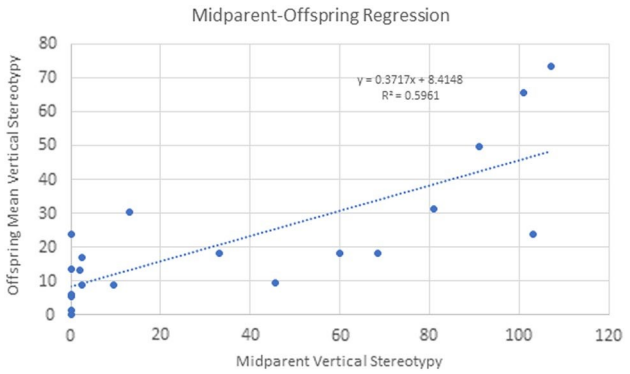

Fig. 4 Offspring versus mid-parent regression analysis for vertical stereotypy. The mean VS for all offspring in a mating was plotted versus the average VS of the two parents. A linear regression generates a slope, which is equal to the heritability ($h^2 = 0.37$)

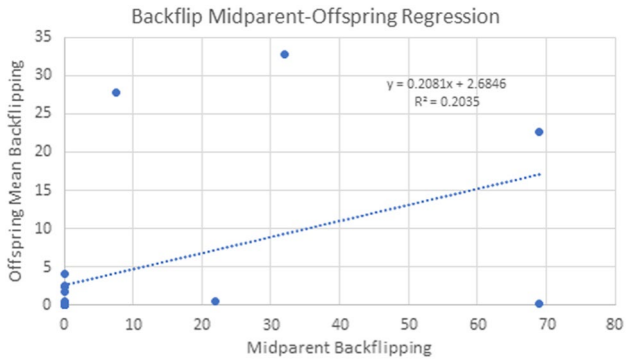

Fig. 6 Offspring versus mid-parent regression analysis for vertical stereotypy. The mean BF for all offspring in a mating was plotted versus the average BF of the two parents. A linear regression generates a slope, which is equal to the heritability ($h^2 = 0.20$)

that backflipping behavior is regulated by more than one gene. The data could be explained by a dominant BF allele that can be masked by a dominant allele at a second modifier gene. In this scenario, backflipping animals that are heterozygous for BF would be homozygous for the recessive (permissive) allele of the modifier gene. When crossed with a non-BF animal that is heterozygous for the dominant (masking) allele of the modifier gene, one quarter of the offspring should display BF behavior. The number of BF produced across all BF matings is not significantly different from the number of expected BF animals produced in this scenario ($\chi^2 = 3.22$, $p = 0.73$). Discriminating between these possibilities will require additional matings and offspring. Three BFxBF matings were also established, but no offspring were produced.

BW female x PO male matings produce viable and fertile offspring. PO is the *P. polionotus* stock maintained in the PGSC. This is a valuable asset for genetic analysis since these two species have many phenotypic differences in many traits. PO animals exhibit little stereotypy compared to BW animals (Yadon et al. 2019). Several matings were established between BW♀ and PO♂ animals. Two matings were BW H VS x PO (L). Surprisingly, the phenotypes of the offspring had mostly low VS but a number of BF animals were obtained (Fig. 7A). In fact, 10 of 23 animals exhibited the BF phenotype. In another identical mating, 2/6 animals were of the BF phenotype (Fig. 7B).

Two matings of BW BF phenotypes with PO animals were established. One mating produced 3/26 animals with the BF phenotype (Fig. 7C) and the other produced 13/29 animals that were BF phenotype.

Summary and Discussion

In this study animals similar or differing in stereotypy values were crossed with the goal of understanding more about the genetic basis of stereotypy within the *P. maniculatus* stocks housed and maintained by the PGSC. Both vertical stereotypy and backflipping stereotypy are heritable but display differing patterns of inheritance. Vertical stereotypy appears to be a quantitative trait with multiple loci that contribute to the phenotype, in combination with the environmental

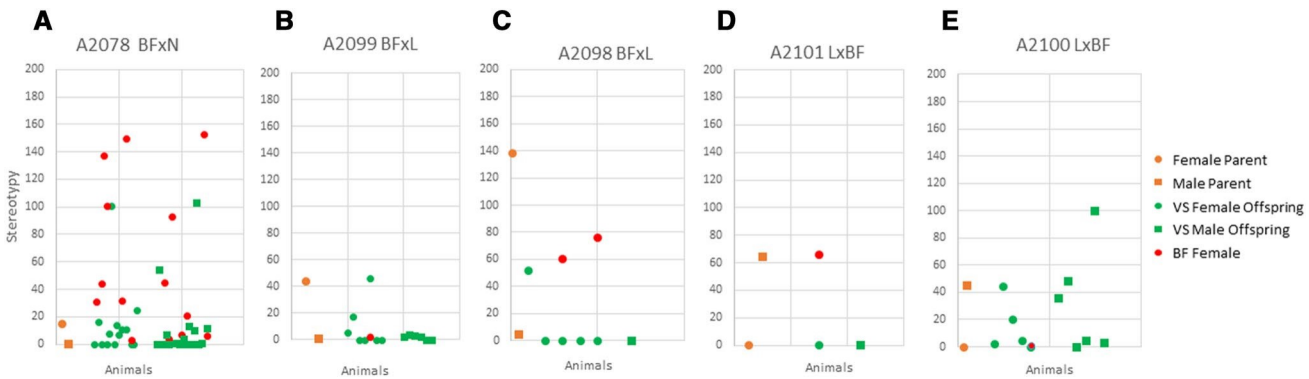

Fig. 5 **A** BF x N BF cross A2078. **B** BF x L BF cross A2099. **C** BF x L BF cross 2098. **D** L x BF BF cross 2101. **E** L x BF BF cross A2100

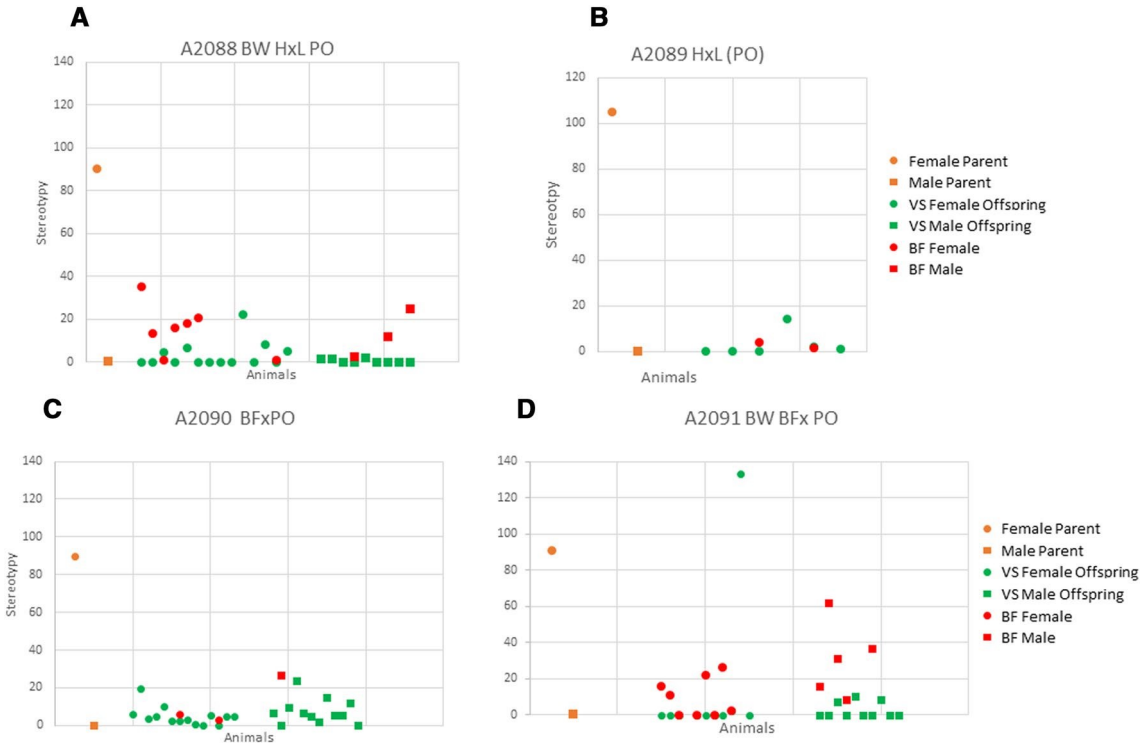

Fig. 7 **A** BW H x PO L cross A2088. **B** BW H x PO L cross A2089. **C** BW BF x PO L cross A2088. **D** BW BF x PO L cross A2091

impacts that have been identified by other studies. Backflipping may have fewer loci contributing to the stereotypy phenotype, potential with one dominant allele and a modifier locus.

For vertical stereotypy, four matings of low stereotypic parents (LVS) indicated the phenotype is heritable with the offspring exhibiting vertical stereotypy scores in the low range (20 vertical leaps in 5 min). Additional matings between high vertical stereotypy animals (HVS) showed some variable outcomes among the progeny. All matings produced offspring with vertical stereotypy scores higher than the offspring from LVS x LVS matings. Two of the HxH matings produced offspring with very high VS scores. Both A2096 and A2076 produced both higher and lower VS animals than the parents. This would suggest the phenotype is controlled by several quantitative loci with variable levels of heterozygosity between HVS animals. HVS animals may therefore produce offspring with high vertical stereotypy phenotype but with a number of different genotypes. Testing this hypothesis will require a quantitative trait loci analysis to identify the loci associated with the phenotype.

A number of matings were constructed with parents differing in VS phenotype, and some of these matings were between animals with vastly different VS scores. In almost all cases except one (Fig. 3) the offspring had a much lower VS score than the HVS parent. Only one mating produced offspring on average much higher than the LVS parent. This would appear that the LVS phenotype is somewhat dominant to the HVS phenotype. With the variability within this wild-derived and outbred animal model, we expect that more than a single locus controls this phenotype, producing a quantitative trait.

The backflip (BF) phenotype is of particular interest as it appears to be controlled by a single dominant locus and a potential modifier gene. Six spontaneous BF phenotypes occurred among the offspring of vertical stereotypy matings, and crosses with a single BF animals and non-BF animals suggests BF is dominant and behaves as a heterozygote (Fig. 5). BF offspring are produced among interspecific crosses where *P. maniculatus* (BW) BF animals are crossed to *P. polionotus* (Fig. 6). This result differs from crosses between BW with vertical stereotypy and PO, where the low stereotypy PO trait dominates. Because of the likelihood of the BF phenotype being under single gene control, it would be a good candidate for gene discovery.

The demonstration that VS and BF stereotypy in *P. maniculatus* is heritable provides a tractable animal model system to identify the loci associated with stereotypic behaviors. Identifying these loci through a quantitative trait loci analysis or genome wide association study will identify the genetic determinants of these behaviors, providing loci that may be conserved in humans and that can be screened for variations in people with ASD and OCD. Identifying the genetic determinants will also augment the extensive body

of knowledge developed on the environmental components of stereotypy in *P. maniculatus* providing a fuller understanding of these complex and multifactorial phenotypes.

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Data Availability All data generated or analysed during this study are included in this published article [and its supplementary information files]. Videos are available upon reasonable request to the authors. *P. maniculatus* and *P. polionotus* are available for purchase from the *Peromyscus* Genetic Stock Center at the University of South Carolina, https://sc.edu/study/colleges_schools/pharmacy/centers/peromyscus_genetic_stock_center/

Code Availability Not applicable.

Declarations

Conflict of interest Davis, Shannon W., Kiaris, Hippokratis, Kaza, Vimala, Felder, Michael R., that they have no conflict of interest.

Ethical Approval All animal experiments were approved by the University of South Carolina Institutional Animal Care and Use Committee under animal use protocol number 2356–101506-042720.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

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