RESEARCH REPORT

Paternal Transmission of Complex Phenotypes in Inbred Mice

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Background: Inbred mice are genetically identical but nonetheless demonstrate substantial variability in complex behaviors such as activity levels in a novel environment. This variability has been associated with levels of parental care experienced early in development. Although maternal effects have been reported in biparental and uniparental strains, there have been no investigations of paternal effects in non-biparental strains in which offspring are reared exclusively by mothers.

Methods: In the uniparental inbred Balb/cJ mouse strain, we examined the relationship of paternal open-field activity to the activity of both male and female offspring in the open-field. Potential mediators of paternal transmission of behavior were examined, including maternal care, growth parameters, litter characteristics, and time the father was present with the pregnant mother prenatally.

Results: An association of paternal open-field activity with the open-field activity of female but not male offspring was found. Variation in maternal postnatal care was associated with female but not male offspring activity in the open-field but did not mediate paternal effects on offspring behavior. Paternal effects on offspring growth parameters were present, but these effects also did not mediate paternal effects on behavior.

Conclusions: Paternal transmission of complex traits in genetically identical mice reared only by mothers suggests a nongenetic mechanism of inheritance potentially mediated by epigenetic factors. The exclusion of multiple mediators of paternal effects on offspring suggests the possibility of germline paternal inheritance via sperm of complex phenotypes in inbred mice. Future studies are required to examine these interesting possibilities.

Key Words: Anxiety, Balb, epigenetic, inheritance, mouse, nongenetic, paternal transmission

Despite being genetically homogenous, inbred mice exhibit substantial and stable variability in complex phenotypes such as activity levels during a test of open-field exploration (1). Because they are genetically identical, inbred mice are well-suited for the study of nongenetic factors related to phenotypic variation. There is evidence from a number of experimental paradigms indicating that early life experience affects gene expression and behavior in rodents (1–14). In particular, variability in mother–infant interactions predicts variability in adult offspring on a number of behavioral measures, including the open-field test, and this variability is associated with variability in epigenetic modifications (3,4).

The contribution of maternal factors to offspring behavior is well-studied in both rats and mice (4,7,9–12,15–19). Although studies of paternal factors are few, there have been some reports from animal models of the nongenetic paternal transmission of phenotypes such as coat color and fertility (20,21). Human studies also suggest nongenetic paternal effects on the phenotypes of offspring. For instance, increased paternal age is a risk factor for schizophrenia, autism, and decreased IQ (22–30). In a pilot experiment we found that offspring from fathers separated by extreme differences in open-field behavior had behavioral differences in the open-field test resembling the behavioral differences of their fathers (Figure 1 in Supplement 1). Here we replicated these findings and tested the hypothesis that normal variations in paternal behavior in the open field before mating will predict normal variations in offspring open-field behavior in adulthood. We found this to be true only in female offspring and provide some preliminary evidence that pre- and postnatal effects might override paternal contributions in males. We also provide data to suggest that paternal contributions to offspring phenotype are pleiotropic and sexually dimorphic. The finding of nongenetic paternal contributions to offspring phenotypes has important implications for understanding complex inheritance patterns of psychiatric disorders and might provide a useful model for studying mechanisms underlying the origins of complex disease.

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Methods and Materials

Animals

Balb c/J mice were used for all experiments (Jackson, Bar Harbor, Maine). Before mating and after weaning, mice were group-housed in sex-specific cages (5 mice/cage) and maintained on a 12-hour/12-hour light/dark cycle with food and water available ad libitum. All animal protocols were reviewed and approved as meeting appropriate ethical standards by Columbia University's and New York State Psychiatric Institute's Institutional Animal Care and Use Committee boards.

Mating

Ten-week-old male mice were housed with 2–3 females (10 weeks old) for 2 weeks and then were removed. To score maternal behavior specific to each litter, females were singly housed after 18 days after mating until delivery.

Paternal Behavior

Fathers were tested in a novel open-field test at 9 weeks of age (see following).

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Maternal Behavior

Maternal behavior was scored 2 times/day during the light phase (9:00 AM and 1:00 PM) for a total of 50 measurements 1 min apart during each period (total of 100 measurements/day) for the first week of life. We find, as reported by others, maternal behaviors decrease during the first week of the postnatal period. Behavioral data were log transformed to obtain normal distributions.

Open-Field Behavior

Offspring were tested at 9 weeks of age. Activity in an open field is quantified in four Plexiglas open-field boxes 43 $cm^2 \times 43$ cm^2 with two sets of 16 pulse-modulated infrared photobeams (MED Associates, Georgia, Vermont). Data were analyzed on the basis of 2 zones: center (25% total area) and surround (75% total area). Behavioral data were log transformed to obtain normal distributions. In the case of offspring, mice were tested both under light (200 lux, first test) and on the following day in dark conditions (second test). Testing in the dark increases the spread of data by increasing the number of animals and amount of time that animals will spend in center regions. Light and dark measures are highly correlated, suggesting that both measures are testing similar constructs (1). In the present study, behavioral measures from the dark are used for analyses. Similar but weaker paternal effects were seen for measures of offspring behavior in the light.

Weight Measurements

Offspring were weaned at 4 weeks and weighed at this time. Adult body weight was measured at the time of death. Body weight was measured on a scale with sensitivity to .001 g. Brain weights were measured on a scale with sensitivity to .0001 g. Mice were killed by cervical dislocation; brains were removed and weighed. The brain was then hemisected, and the right and left hippocampus were removed and weighed individually. Total hippocampal weight was calculated by adding left and right measurements.

Statistical Analyses

Statistical analyses were performed with StatView software (College Station, Texas). To control for multiple testing, factor analysis and multiple regression analysis was used.

Factor Analysis

Open-field measures were highly correlated within individuals (Figure 1 in Supplement 1). Factor analysis was used to reduce five open-field behavioral measures (distance traveled in the center area [cen], percent of total distance traveled in the center area [% *p*], time in the center [time], entries into the center [ent], and total distance traveled [tp]) to a single latent variable. Open-field factor scores were used as a measure of open-field behavior for subsequent analyses.

Multiple Regression

Multiple regression was used to control for potential confounders and mediators of the associations we found. In each case we first created a model with a single dependent and independent variable. Next we added potential confounders to the model. Finally, we added potential mediators to the model containing potential confounders. Variables were median normalized and center on the mean before regression analysis.

Results

Paternal Contributions to Offspring Phenotype

There was a significant positive association of paternal behavior in the open-field test before mating with female but not male offspring behavior in the open field (Table 1). A number of potential confounders and mediators of this effect were entered into correlation matrices for female (Table 1) and male offspring (Table 1). The relationship of litter-specific variables to each other was also assessed (Table 1). In females but not males, there was a negative association of paternal open-field behavior with female hippocampus weight and a trend ($p = .1$) for a positive association of maternal arched-back nursing with open-field behavior. In males but not females, there were significant positive associations of paternal open-field behavior with body weight at weaning and brain weight in adulthood and a trend ($p < .1$) for an association with adult body weight. There was a trend ($p < .1$) for a positive association of maternal arched-back nursing with adult body weight. Litter size was positively associated with male but not female weight at weaning, adult body weight, and hippocampus weight. At the litter level there was a trend ($p < .1$) for an association of paternal open-field behavior with the percent females in a litter.

In sum the relationship of fathers' open-field behavior to offspring phenotype differed between male and female offspring with an increase in open-field behavior of fathers before mating predicting an increase in adult levels of open-field behavior in female but not male offspring and a decrease in the weight of the female offspring's hippocampus. Whereas in males, fathers' open-field behavior predicted differences in general growth parameters including increased weight at weaning, adult body weight, and total brain weight but no change in the weight of the hippocampus (Table 1). Additionally, male but not female offspring appeared sensitive to litter size with negative effects on open-field behavior and positive effects on weaning weight, adult weight, and hippocampus weight (Table 1).

Multiple regression analysis was performed in a stepwise fashion on the association of the open-field behavior of fathers and female offspring. First, maternal care including arched-back nursing and licking and grooming were entered into the model as possible confounders. The association remained significant (Table 2). Next, potential mediators were entered into the model. These included: maternal arched-back nursing, maternal licking and grooming, litter size, percent females in litters, weight at weaning, adult body weight, brain weight, and hippocampus weight. When all variables were entered into the model, the association of paternal open-field behavior with female offspring open-field behavior continued to be significant (Table 2). A similar analysis was performed on male offspring that indicated the association of litter size with male offspring behavior remained highly significant in the complete model (Table 2).

Germline Versus Prenatal Effects of Fathers on Offspring Phenotype

A possible explanation for paternal effects on offspring phenotypes was that fathers who behaved differently in the open field had different effects on the prenatal environment (Table 3). In our experimental design, fathers were housed with pregnant females for between 7 and 14 days after conception. We reasoned that an effect of fathers on the prenatal environment would be greater when the father was present for a longer period. To examine this we calculated the time that a father spent

Table 1. Correlation Matrices

Female Offspring											
$n = 40$ mice											
Litters = 17											
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Father OF factor	(1)	1.00	.37[a]	.21	.02	.25	.07	.18	−.31[a]	.01	.44
Offspring OF factor	(2)		1.00	.30[b]	−.20	.01	.09	.03	−.18	.16	−.03
Ln (Mother AN % time)	(3)			1.00	.11	−.20	−.20	−.21	−.13	.25	.16
Ln (Mother GP % time)	(4)				1.00	.17	.11	.06	.07	−.30	.09
Weight at weaning	(5)					1.00	.74[a]	.88[a]	.17	.17	.07
Adult body weight	(6)						1.00	.73[a]	.22	.09	−.04
Brain weight	(7)							1.00	.44[a]	.21	−.01
Hippocampus weight	(8)								1.00	.20	−.28[b]
Litter size	(9)									1.00	.07
Percent female	(10)										1.00
Male Offspring											
$n = 58$ mice											
Litters = 19											
		(2)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Father OF factor	(1)	1.00	.05[a]	.37	.07	.39[a]	.24[b]	.27[a]	.15	.38	.22
Offspring OF factor	(2)		1.00	.04	.05	.06	.02	−.02	−.07	−.38[a]	.14
Ln (Mother AN % time)	(3)			1.00	−.05	.11	.22[b]	−.05	−.02	.41	−.34
Ln (Mother GP % time)	(4)				1.00	.06	.08	.18	−.03	−.24	.09
Weight at weaning	(5)					1.00	.66[a]	.70[a]	.34[a]	.39[a]	.03
Adult body weight	(6)						1.00	.64[a]	.15	.25[b]	−.16
Brain weight	(7)							1.00	.34[a]	.19	.10
Hippocampus weight	(8)								1.00	.31[a]	.05
Litter size	(9)									1.00	.07
Percent female	(10)										1.00
Litters											
$n = 19$											
Fathers = 17											
Mothers = 19											
		(3)	(2)	(3)	(4)	(5)					
Father OF factor	(1)	1.00	.24	.02	.24	.42[b]					
Ln (Mother AN % time)	(2)		1.00	−.02	.28	−.13					
Ln (Mother GP % time)	(3)			1.00	−.26	.01					
Litter size	(4)				1.00	.06					
Percent female	(5)					1.00					

To assess variables for possible contributions to offspring phenotypes, linear correlations are evaluated in female and male offspring. For litter-specific variables, linear correlations are also evaluated separately. Table contains Pearson *r* correlations. Significant correlations and trends are noted.

OF, open field; Ln, natural log; AN, arched-back nursing; GP, grooming pups.

[a] $p < .05$.

[b] $p < .1$.

with a pregnant female and entered this into the regression model. The calculation was based on a fixed 2-week period that males were housed with females, an estimated 21 day gestation period, and the date of birth (e.g., pups that were born 21 days after males were housed with females had fathers present for 14 days of the prenatal period). Entering the time that fathers spent with pregnant mothers into the regression model had no effect on the association of paternal and female offspring behavior, consistent with a germline mechanism of transmission. Suggesting that prenatal effects of fathers might be important in males, there was a significant association of paternal time with the pregnant mother and male offspring open-field behavior factor scores. Of note, there was a significant negative interaction of paternal open-field behavior with the amount of time spent with the mother (i.e., the less time that fathers were present post conception the more of an effect there was of a father's open-field behavior on male offspring open-field behavior). This result suggests the interesting possibility that paternal prenatal effects might override paternal germline effects on male offspring open-field behavior. Future experiments using in vitro fertilization would help to validate the presence of germline transmission.

Paternal Contributions to Growth Parameters of Offspring

Significant correlations from Table 1 indicated that fathers might influence other offspring phenotypes. We used multiple regression models to further assess the associations of paternal open-field behavior with female offspring hippocampus weight and male offspring weight at weaning. The association of paternal behavior with female hippocampus weight was unchanged and remained significant in the complete model (Table 4). There was a significant positive association of brain weight with hippocampus weight and a significant negative association of weight at weaning with the adult weight of the hippocampus in female offspring (Table 4). The association of the father's open-field behavior with the weight of male offspring at weaning became stronger in the complete model (Table 4). Male offspring weight at weaning was significantly associated with multiple variables.

Table 2. Paternal OF Behavior Predicts Female Offspring Behavior

Variable	Model 1 Coeff	SEM	Model 2 Coeff	SEM	Model 3 Coeff	SEM
Female Offspring OF Factor						
Father OF factor	.416[a]	.170	.361[a]	.167	.443[a]	.214
Ln (mother AN % time)			.520	.307	.561	.369
Ln (mother GP % time)			2.639	1.647	2.151	2.021
Weaning weight					−2.526	3.763
Adult body weight					4.565	
Brain weight					3.286	9.293
Hippocampus weight					−2.288	3.330
Litter size					.058	.867
% Females in litter					−.609	.458
Male Offspring OF Factor						
Father OF factor	.052	.133	.037		.142	.156
Ln (mother AN % time)			.046	.213	.291	.244
Ln (mother GP % time)			.459	1.374	−.957	1.329
Weaning weight					2.543	1.447
Adult body weight					−.968	2.489
Brain weight					−2.642	4.698
Hippocampus weight					.335	1.658
Litter size					−2.097[b]	.532
% Females in litter					−.110	.282

Multiple regression models for offspring open-field (OF) behavior. Regression models are created in a step-wise manner. The dependent variable is offspring OF behavior. Model 1 assesses the relationship to the father's OF behavior. Model 2 adds the possible confounders of maternal care. Model 3 includes all hypothesized confounders and mediators. Male and female offspring are evaluated separately. Significant effects are noted.

Coeff, coefficient; Ln, natural log; AN, arched-back nursing; GP, grooming pups.

[a] $p < .05$.

[b] $p < .0005$.

There was a positive association with litter size, prenatal time with father, and maternal licking and grooming. There was a negative association with maternal arched-back nursing. Interestingly, as was the case with male offspring open-field behavior, there was a negative interaction of a father's open-field behavior with the time spent with the pregnant mother, supporting the possibility that fathers influence male offspring through both germline and prenatal mechanisms.

Discussion

We examined the relationship of paternal behavior in the open field to offspring phenotypes in Balb/cJ mice. Balb/cJ mice are highly anxious-like and have been found to be sensitive to early developmental interventions (10,31,32). They are also noted to have a high degree of variability in brain development (33–35). Large within-strain variability and sensitivity to environmental factors make Balb/cJ mice ideal for the study of nongenetic factors contributing to phenotypic variation.

Table 3. Paternal Prenatal Time with Pregnant Mother

Variable	Model 1 Coeff	SEM	Model 2 Coeff	SEM
Female Offspring OF Factor				
Father OF factor	.416[a]	.170	.418[a]	.192
Prenatal time with father			.027	.139
Time × father OF factor			−.008	.131
Male Offspring OF Factor				
Father OF factor	.052	.027	.233	.144
Prenatal time with father			.281	.084[b]
Time × father OF factor			−.204	.080[a]

Assessing for prenatal effects of fathers on offspring behavior in the open-field (OF) test. The amount of time that a father was present after conception and an interaction term of this amount of time × father's OF behavior are entered into the regression model for the association of the father's and offspring's OF behavior. Male and female offspring are evaluated separately. Significant effects are noted.

Coeff, coefficient.

[a] $p < .05$.

[b] $p < .005$.

We found that the behavior of female but not male offspring in the open field was associated with the behavior of fathers in the open-field test before mating. The association of paternal behavior with the behavior of female offspring in genetically identical mice indicates that fathers can influence complex phenotypes of their offspring through nongenetic mechanisms even when they are not present during rearing. Importantly, the association of father's premating open-field behavior was not limited to offspring behavior but also influenced the size of the female offspring hippocampus and the weight of male offspring at weaning, thus indicating that whatever is being transmitted has pleiotropic and sexually dimorphic effects. These findings might offer some insight into the sexual dimorphism within human mental disorders and their association with other medical problems such as diabetes and heart disease (36,37).

The mechanism for the transmission of paternal effects on offspring phenotypes is currently unknown. Epigenetic variability has been associated with variability in rodents for many behaviors, including activity in the open field, and has also been associated with the effects of maternal care on offspring behavior (3–5,7). Thus, epigenetic factors are excellent candidates for mediating paternal effects. Prenatal stress has also been found to affect the behavior of offspring (11). Arguing against prenatal effects in females, the time fathers spent with pregnant females did not influence paternal effects on female offspring. However, in male offspring, there was evidence for a prenatal effect of fathers where the presence of a father for longer periods was positively associated with male offspring open-field factor

Table 4. Paternal OF Behavior Predicts Female Hippocampal Weight and Male Weight at Weaning

Variable	Model 1 Coeff	SEM	Model 2 Coeff	SEM	Model 3 Coeff	SEM
Female Offspring Hippocampus Weight						
Father OF factor	−.023[a]	.012	−.032[b]	.011	−.024[a]	.012
Ln (mother AN % time)			.007	.020	.001	.033
Ln (mother GP % time)			.050	.102	.139	.119
Adult body weight			−.325	.242	−.094	.239
Brain weight			1.006[b]	.284	1.840[c]	.450
Weaning weight					−.564[a]	.209
Litter size					.008	.073
% Females in litter					−.016	.026
Prenatal time with father					−.007	.014
Time × father OF factor					.006	.010
Male Offspring Weight at Weaning						
Father OF factor	.055[b]	.017	.057[b]	.019	.060[b]	.019
Ln (mother AN % time)			−.009	.028	−.135[b]	.042
Ln (mother GP % time)			.037	.179	.441[a]	.179
Litter size					.459[c]	.102
% Females in litter					.016	.039
Prenatal time with father					.070[b]	.020
Time × father OF factor					−.034[a]	.017

Multiple regression models for female offspring hippocampus weight and male offspring weight at weaning. Regression models are created in a step-wise manner. Model 1 assesses the relationship to the father's open-field (OF) behavior to the dependent variable. Model 2 adds the possible confounders to the Model. Model 3 includes all hypothesized confounders and mediators. In female offspring potential contributors to variance in hippocampus weight are evaluated. In male offspring potential contributors to variance in weight at weaning are assessed. Significant effects are noted.

Coeff, coefficient; Ln, natural log; AN, arched-back nursing; GP, grooming pups.

[a] $p < .05$.

[b] $p < .005$.

[c] $p < .0005$.

scores. There was a negative interaction of the father's open-field behavior and prenatal time, suggesting the possibility that a father's prenatal effects override germline effects and that an effect of fathers on male offspring behavior might be seen if germline effects could be isolated with in vitro fertilization.

Germline paternal transmission across multiple generations in rats has been documented for the effects of endocrine disrupters on male fertility (38,39). In mice, fathers heterozygous for a mutation in the gene, *Smarca5*, that encodes the chromatin remodeler Snf2h transmitted effects on coat color to offspring who did not inherit the mutation (21). The effects of a mutation in the mouse Kit gene that leads to a heritable white tail phenotype were transmitted to nonmutant offspring through transfer of Kit-specific microRNAs (20). In humans, paternal age was associated with increased rates of schizophrenia and autism and decreased IQ in offspring (24,25,28). Starvation during the prepubertal slow growth phase in grandfathers was found to have effects on the health and longevity of grandsons but not granddaughters (40–42). Germline transmission of behavioral traits has potentially important therapeutic implications. Nongenetic factors such as epigenetic modifications and levels of microRNAs can be influenced by experience (7,15,43,44). Therefore, the presence of nongenetic germline inheritance introduces the possibility of influencing heritable factors through treatment before conception. Definitive evidence for germline transmission of a paternal effect could be obtained with studies using in vitro fertilization to isolate paternal germline contributions from possible effects on prenatal or postnatal environments. The study of paternal contributions to normal variation in Balb/c mice, therefore, represents a potentially useful model for studying heritable nongenetic mechanisms contributing to the development of disorders with complex patterns of inheritance such as those present in most psychiatric disorders.

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Supplementary material cited in this article is available online.

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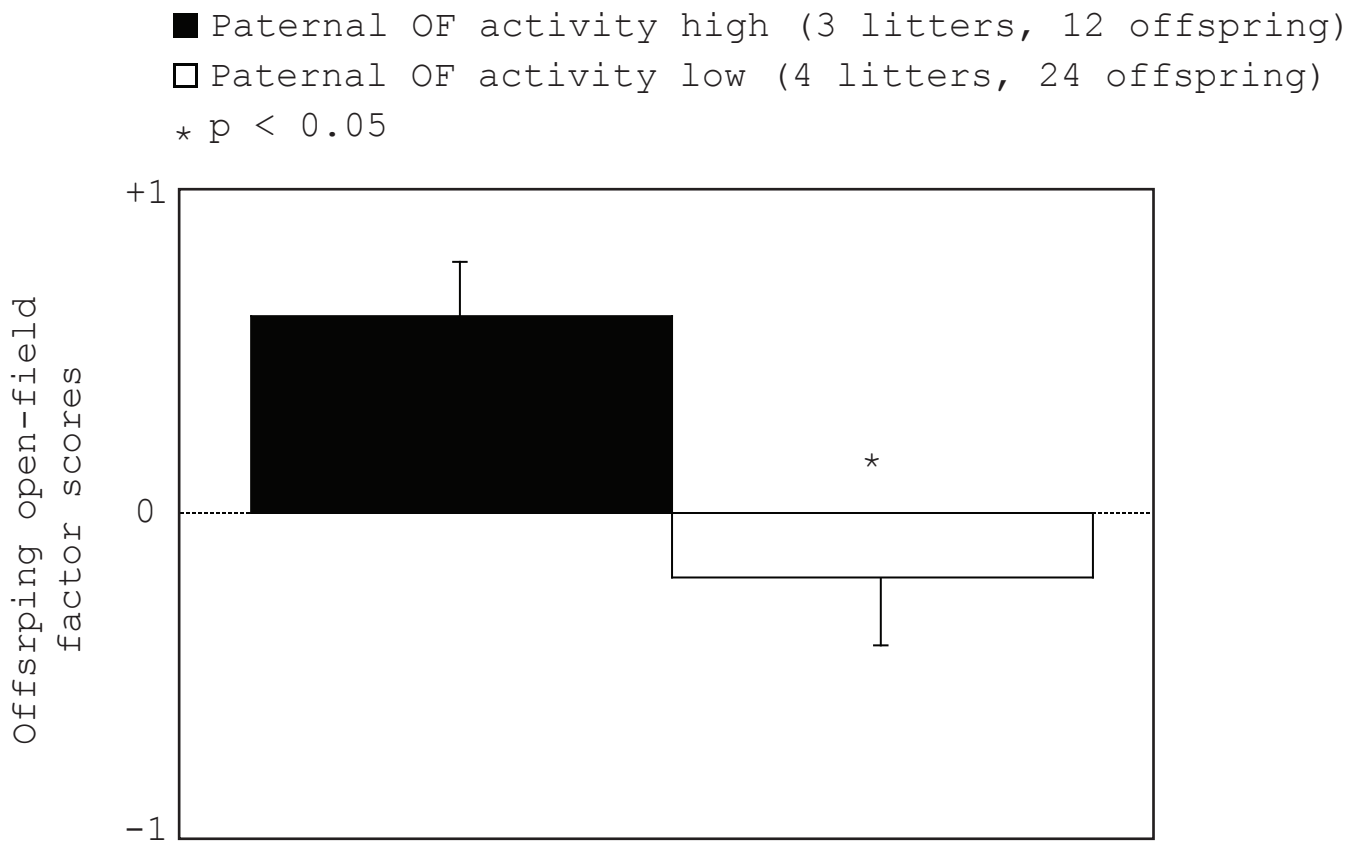
	Father OF factor	Father ln(cen)	Father ln(%p)	Father ln(time)	Father ln(ent)	Father ln(tp)	Offspring OF factor	Offspring ln(cen)	Offspring ln(%p)	Offspring ln(time)	Offspring ln(ent)	Offspring ln(tp)
Father OF factor	1.000	.959	.810	.968	.981	.793	.164	.151	.129	.162	.205	.125
Father ln(cen)		1.000	.891	.871	.914	.656	.150	.138	.114	.155	.192	.108
Father ln(%p)			1.000	.698	.749	.324	.133	.133	.085	.152	.174	.083
Father ln(time)				1.000	.979	.813	.196	.182	.163	.187	.232	.162
Father ln(ent)					1.000	.780	.184	.168	.149	.180	.221	.152
Father ln(tp)						1.000	.070	.056	.068	.053	.103	.053
Offspring OF factor							1.000	.964	.933	.968	.964	.908
Offspring ln(cen)								1.000	.962	.889	.875	.841
Offspring ln(%p)									1.000	.853	.851	.755
Offspring ln(time)										1.000	.982	.859
Offspring ln(ent)											1.000	.856
Offspring ln(tp)												1.000

Supplemental Table 1. Correlation matrix of paternal and offspring open-field measures. Demonstrates a high level of correlation between separate open-field behavioral measures. High between measure correlations allowed 5 measures to be reduced to a single latent variable in fathers and offspring for use in subsequent analyses.



Supplemental figure 1. Pilot experiment:Paternal open-field behavior predicts offspring open-field behavior. Open-field factor scores of offspring from fathers separated by extremes of open-field behavior show that offspring of fathers with high levels of open-field activity have significantly higher levels of open-field activity than offspring of low activity fathers.