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Social Olfaction in Maternally Separated CD-1 Mouse Pups

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Abstract

Olfaction is important in infants' attachment to their mother. Previous studies have shown that maternal odors attract infant rabbits, humans, nonhuman primates and mouse pups. This study investigated whether removing infant mice from their mother for three hours daily during the first two weeks of life weakens their preference for familiar odors. Two hundred and forty CD-1 mouse pups from thirty litters were used. There were four rearing conditions: Handled (H), Maternally Separated Clean (MSc), Maternally Separated Nest (MSn), and Animal Facility Reared (AFR). All litters were weighed daily on Postnatal days 1-14 except for the AFR mice. Handled litters were then returned to the home nest with their mothers. Maternally Separated Clean litters were separated from their mother for three hours daily and placed in a Plexiglas cages with hardwood shavings. Maternally Separated Nest mouse pups were separated from their mother for three hours daily and were placed in Plexiglas cages with some of their nest shavings. The AFR mouse pups were the control and they were always with their mother except for testing days. Testing took place on postnatal day 10 and 14. The results showed significance differences for AFR mouse pups at PN 14 for different scores compared to H, MSc, and MSn mouse pups when tested in familiar odors. The results suggest that maternal odors play a very crucial role in development of mouse pups.

Table of Contents

Introduction

The purpose of this study was to determine whether removing infant mice from their mother for three hours daily during the first two weeks of life will weaken preference for nest odors. This study also saw whether allowing pups to remain in the nest during separation maintained nest odor preferences at normal levels. This procedure has been used to model the effects of rearing human infants in environments that prevent normal bonding with a caretaker. Some examples of this are orphanages or successive foster homes. In previous studies this procedure has also been used by removing the pups from their nest odors and then placing them in "clean" bedding during separation. This process does not allow the possible effects of maternal odor cues to be distinguished from the pups being away from their mother's tactile stimulation. The present research addressed this issue by adding a condition that had never been used by keeping the pups in their home nest odor during the period of separation.

Olfaction is very important in infant's attachment to their mothers. Maternal odor cues are important in attracting infants. Studies have shown that maternal odor cues attract infant rabbits (Bilko A, Altbaacker V, Hudson, 1994), mice (Coppola, 1997), and humans (Macfalane, 1998). Studies have shown that pre and postnatal olfactory exposure influence which odors elicit newborn rats' nipple attachment. This shows that maternal odor cues before and after birth are important (Pedersen, 1982).

Maternal odor cues attract infant rabbits (Bilko , Altbaacker , and Hudson, 1994). In this study rabbit pups were being raised by their mothers. However, the rabbit pups were fed different diets during pregnancy. Rabbit pups showed a clear preference for the diet of their mother at weaning. This study investigated the importance of prenatal experience in utero, fecal pellets deposited by the mother in the nest, and the contact with the mother during nursing in determining pups later food preference. The three means of transmission that were effective are 1) pups raised from normal fed mothers raised in fecal pellets from juniper fed mothers 2) pups raised from juniper fed mothers cross fostered to normal fed does right after birth 3) pups from normally fed mothers nursed by juniper fed does all showed as strong a preference for juniper as pups raised by juniper fed mothers. This study has strong significance because it shows maternal odor cues are important in attracting infants in rabbits (Bilko A, Altbaacker V, and Hudson, 1994).

Coppola (1997) article showed that maternal odor cues are important in attracting mice. In this study on the day before birth, mouse fetuses were tested for their behavioral responses to iso-amyl acetate and iso-valeric acid. This was delivered in the nasal cavity (liquid). The most important part of the study is the second experiment. When the two odorants were administered; the fetus was able to differentiate between iso-amyl acetate and iso-valeric acid. The results showed that the fetuses had different responses to the two odorants. According to (Coppola, 1997), "given the immaturity of the mouse's accessory olfactory system before birth and the observed responses to

concentrations of odorants below the threshold of the trigeminal system, the results suggest that the mouse fetus has a functionally competent main olfactory system." Olfactory cues are important in mouse pups because the mouse is not able to see their mothers when they are a couple of days old. In (Coppola, 1997), study the only way the mice were able to discriminate between iso-amyl acetate and iso-valeric acid is through their olfactory cues.

Pedersen (1982) consisted of two experiments. In experiment one; rats were exposed to a lemon scent prenatally and after birth. The rats that were exposed to the lemon scent preferred the nipple washed with lemon scent over it. However, the rats did not suck the unwashed nipple of the dams. In experiment two; rats were exposed to citral either in utero, immediately after birth, both pre and postnatally, or not at all. Only the rats exposed to citral both pre and postnatally did not suck the normal unwashed nipples that elicited sucking in the control rats. These findings show that pre and postnatal exposure influenced the newborns rats' first nipple attachment. The findings from this study show that maternal attachment on the basis of odors occurs both before and after birth.

Studies have shown that early olfactory experience have long term effects. A previous study by Shah et al. (2002) demonstrated these effects in mice. They hypothesized that female rats receiving exposure to an unusual odor in the nest as pups would be more responsive as adults to pups scented with the (same) exposed odor than to unscented pups. The results showed that the females had a strong preference for pups scented with the familiar odors.

The Shah et al (2002) study correlates with this current study because it shows that early olfactory exposure has a tendency for long term effects. This present study shows that mice tend to spend more time in familiar odors than novel odors.

All of these studies have great significance to the present research. Maternal odor cues are important in attracting infants from various species. The present research investigated whether maternal separation alters odor preferences in infant mice. The research also examined whether exposure to maternal nest odors during separation maintained normal odor preference development.

The hypothesis of this study is that the Handled mouse pups should be more attached to their mother compared to the Maternally Separated Nest and Maternally Separated Clean mouse pups. However, the Maternally Separated Clean will be more likely to explore novel odors because they do not have a close attachment to their mother since they are separated from her daily. The Maternally Separated Nest pups should be more like the Handled mouse pups. This is because the Maternally Separated Nest mouse pups are separated from their mother daily but they are placed in nest shavings during separation. The AFR mouse pups are the control group and should be attached to their mother since they are never separated from her.

Method

Two hundred and forty CD-1 mouse pups from thirty litters were used. All litters were reared in hardwood shavings. On postnatal day 1 (PN1 the day

after birth) each litter was culled to five males and five females. The experimental males and females were assigned to either maternally separated/clean bedding (MS/Clean), maternally separated/nest bedding (MS/Nest), or handled (H) rearing conditions. The control, animal facility reared (AFR) condition animals were left in their Plexiglas cages with the dam. Experimental litters were weighed daily until PN-14. Handled litters were returned to their mother in the home nest after being weighted. MS litters were caged away from their mothers for three hours daily in either clean bedding or their home nest bedding. Then the MS pups were housed in another room with a temperature maintained at 29+/-2 degrees Celsius.

On Postnatal day 10 two females and two males from each litter were given olfactory preference test. On Postnatal day 14 another two males and two females from the same litters were also tested. The apparatus for odor preference testing is a rectangular box with Plexiglas walls and a screen floor. Underneath the rectangular box were two compartments that held wood shavings (refer to Figure 5). There were two choice situations. In one situation one compartment was filled with hardwood shavings and the other compartment was filled with clean pine shavings (an unfamiliar odor). In the other situation one compartment is filled with clean hardwood shavings and the other compartment is filled with soiled nest shavings.

Testing took place under red light illumination because mouse pups have red eyes. When the red light illumination is on the mouse pups are unable to see and the only sense they would have is the sense of smell. The

testing was done in a separate room from where the mouse pups are caged. The mouse was first sexed to see if they are male or female. Males were marked with a black marker on the tail and females were marked with blue marker. Then each mouse (individually) was placed on the screen along the midline about the empty section of the apparatus. Then a video camera was turned on so that the experiment will be recorder. Once the mouse is placed in the apparatus and the camera is turned on the experimenter left the room and shut the door. After the door was shut, a stopwatch was used to record 180 seconds and the time spent over the hardwood and unfamiliar shavings. When 180 seconds passed, the mouse was taken out of the apparatus and placed in the home cage. The apparatus was then cleaned and dried prior to the next subject.

After all the mice were tested and videotaped the experimenter analyzed the tapes. The experimenter turned on the stop watch and then traced the mouse pathway for three minutes. Once the pathway was traced the experimenter used a 48 square grid over the pathway to count the number of squares entered. Then the numbers of boli were counted after the mouse left the apparatus. After this was completed the experimenter recorded how much time the mouse pup spent over each odor.

This design resulted in 12 groups (2sexes x 3 rearing conditions x 2 test ages), with two animals from each group per litter used for each test. After the data were collected it was then analyzed using SPSS. Independent Sample T-Tests were performed to see if there were significant sex or rearing

differences for any of the response variables. One sample t-tests were performed against zero and histograms were created to show if there were similarities and differences. Mean different scores were used to construct the histograms and to find significance. Different scores are calculated by subtracting the familiar odor minus the novel odor. The means were compared to a theoretical mean of zero because this would be the mean expected. If the mean expected is higher than zero this would indicate a preference for familiar odors.

Results

AFR mouse pups at PN 10 (M=40, SEM= 26) did not differ from H mouse pups at PN 10 (M= 23, SEM= 23) in the time spent in Clean Hardwood vs. Pine, t (42) =.480, p>0.05 . AFR mouse pups at PN 10 (M=40, SEM= 26) did not differ from MSc mouse pups at PN 10 (M=19, SEM= 25) in the time spent in Clean Hardwood vs. Pine, t (40) =.591, p>0.05. AFR mouse pups at PN 10 (M=40, SEM= 26) did not differ from MSn mouse pups at PN 10 (M= 22, SEM= 25) in the time spent in Clean Hardwood vs. Pine, t (38) = .509, p>0.05. AFR mouse pups at PN 14 (M=91, SEM= 11) did not differ from H mouse pups at PN 14 (M= 44, SEM= 14) in the time spent in Clean Hardwood vs. Pine, t (42) =2.495, p>0.05. AFR mouse pups at PN 14(M=91, SEM= 11) did not differ from MSc mouse pups at PN 10 (M=31, SEM= 21) in the time spent in Clean Hardwood vs. Pine, t (38) =2.535, p>0.05. AFR mouse pups at PN 14 (M=91, SEM= 11) did not differ from MSn mouse pups at PN 14 (M= 41, SEM= 17) in the time spent in Clean Hardwood vs. Pine, t (40) = 2.398,

$p>0.05$. AFR mouse pups at PN 10 (M=79, SEM= 21) did not differ from H mouse pups at PN 10 (M= 89, SEM= 21) in the time spent in Nest Hardwood vs. Clean Hardwood, t (42) =-.334, $p>0.05$. AFR mouse pups at PN 10 (M=79, SEM= 21) did not differ from MSc mouse pups at PN 10 (M=44, SEM= 24) in the time spent in Nest Hardwood vs. Clean Hardwood, t (40) =1.045, $p>0.05$. AFR mouse pups at PN 10 (M=79, SEM= 21) did not differ from MSn mouse pups at PN 10 (M= 53, SEM= 24) in the time spent in Nest Hardwood vs. Clean Hardwood, t (38) = .789, $p>0.05$. AFR mouse pups at PN 14 (M=28, SEM= 17) showed significant differences from H mouse pups at PN 14 (M= 99, SEM= 8) in the time spent in Nest Hardwood vs. Clean Hardwood, t (42) =-3.955, $p<0.05$. AFR mouse pups at PN 14 (M=28, SEM= 17) showed significant differences from MSc mouse pups at PN 14 (M=94, SEM= 8) in the time spent in Nest Hardwood vs. Clean Hardwood, t (40) =-3.672, $p<0.05$. AFR mouse pups at PN 14 (M=28, SEM= 17) showed significant differences from MSn mouse pups at PN 14 (M= 74, SEM= 16) in the time spent in Nest Hardwood vs. Clean Hardwood, t (38) = -2.017, $p<0.05$ (Please refer to the Appendix).

For mean scores against zero, AFR mouse pup's different scores were not significant for PN 10 $t(19)=1.55$ in Pine vs. Clean Hardwood, t vs. 0 is 2.093. MSn mouse pups were not significant for PN 10 $t(19)=.871$ in Pine vs. Clean Hardwood, t vs. 0 is 2.093. MSc mouse pups were not significant for PN 10 $t(21)=.739$ in Pine vs. Clean Hardwood, t vs. 0 is 2.080. H mouse pups were not significant for PN 10 $t(23)=1.048$ in Pine vs. Clean Hardwood, t vs.

0 is 2.069 (Please refer to Figure 1). AFR mouse pup's different scores were significant for PN 10 $t(19)$=-.333 in Nest vs. Clean Hardwood, t vs. 0 is 2.093. MSc mouse pups were not significant for PN 10 $t(21)$=1.813 in Nest vs. Clean Hardwood, t vs. 0 is 2.080. MSn mouse pups were significant for PN 10 $t(19)$=2.182 in Nest vs. Clean Hardwood, t vs. 0 is 2.093. H mouse pups were significant for PN 10 $t(23)$=1.048 in Nest vs. Clean Hardwood, t vs. 0 is 2.069 (Please refer to Figure 2).

AFR mouse pup's different scores were significant for PN 14 $t(19)$=8.029 in Pine vs. Clean Hardwood, t vs. 0 is 2.093. MSn mouse pups were significant for PN 14 $t(21)$=2.419 in Pine vs. Clean Hardwood, t vs. 0 is 2.080. MSc mouse pups were not significant for PN 14 $t(19)$=1.489 in Pine vs. Clean Hardwood, t vs. 0 is 2.069. H mouse pups were significant for PN 14 $t(23)$=3.011 in Pine vs. Clean Hardwood, t vs. 0 is 2.069 (Please refer to Figure 3). AFR mouse pup's different scores were not significant for PN 14 $t(19)$=1.657 in Nest vs. Clean Hardwood, t vs. 0 is 2.093. MSn mouse pups were significant for PN 14 $t(19)$=4.700 in Nest vs. Clean Hardwood, t vs. 0 is 2.093. MSc mouse pups were significant for PN 14 $t(21)$=2.080 in Nest vs. Clean Hardwood, t vs. 0 is 2.080. H mouse pups were significant for PN 14 $t(23)$=11.535 in Nest vs. Clean Hardwood, t vs. 0 is 2.069 (Please refer to Figure 4).

Discussion

It was hypothesized that separating the mice from their mother daily for three hours during the first two weeks of life would weaken the mouse

pups preference for familiar odors but separation in soiled nest shavings would show preference for familiar odors. According to the hypothesis the Handled mice would be very attached to their mother and would have an odor preference of the Nest Hardwood. It was also hypothesized that the Maternally Separated Clean would be more likely to branch off to different odors because they less attachment to their mothers.

According to the results, the hypothesis was not supported, but there were lots of apparent findings when Handled, Maternally Separated Clean, and Maternally Separated Nest were compared to the AFR mouse pups. On PN 10 Hardwood vs. Pine the AFR, MSc, MSn, and Handled animals showed no preference for familiar odors (Refer to Figure 1). Since the mouse pups are very young and under developed at PN 10 it would be hard for them to have a preference for a novel and a familiar odor that is not from their nest. On PN 10, for Hardwood vs. Nest odors the AFR, H, and MSn mouse pups showed a preference for familiar Nest odors (Refer to Figure 2). On PN 14, for Pine vs. Clean Hardwood the AFR, H, and MSn mouse pups showed a preference for familiar odors. At this point the mouse pups olfactory system is well developed and they are able to distinguish between the familiar and novel odor (refer to Figure 3). However, on PN 14 for Nest vs. Clean Hardwood the H, MSc and MSn mouse pups showed a preference for familiar odors (Refer to Figure 4).

From the results, AFR mouse pups seem to have normal olfactory development when it comes to interaction with their mother and determining

familiar odors at PN14. Compared to the AFR mice MSc appear to have a retarded course of olfactory development for nest shavings. MS clean mouse pups are not able to generalize the early odors of their mother and nest beyond the environment that they are contained in.

If the Handled, Maternally Separated Clean, Maternally Separated Nest, and Animal Facility Reared mouse pups have an attachment to their mother they are going to spend more time in the odors that they are familiar to as oppose to the unfamiliar odors. This study shows negative developmental effects that may result because of inconsistent caretaking during the first two weeks of the mouse pups life. These effects may even occur long term or have a lasting impact. The Shah et al (2002) study demonstrated that early olfactory has long term effects.

The reason why this study is so important is because it demonstrates that inconsistent care taking has an impact on infants. This correlates to children who are placed in foster homes, or who do not have mothers in their lives. Children that have inconsistent care taking are more likely to try new things and branch out compared to children who are attached to their mothers. Some children who are attached with their mothers will cry if you leave them in a room by themselves or if they are away from their mother. The child will also stay in one spot until the mother comes back and tends to them. This reaction is different for a child who has inconsistent care taking because they could careless if their mother is in the room. When the mother leaves the room they do not care so they will branch off and start to look around and play with

things in the room as if the mothers leaving had no significant impact or affect on them.

To improve on this experiment there should be a better way of letting the mice enter the apparatus because by the experimenter picking the mouse up and putting the mouse back in the cage; the mouse is able to give alert the other mice of what is going on. As a suggestion the mouse should be housed in another cage after the experiment is finished so that the other mice are not alerted about what is going on in the experiment.

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Figure 1

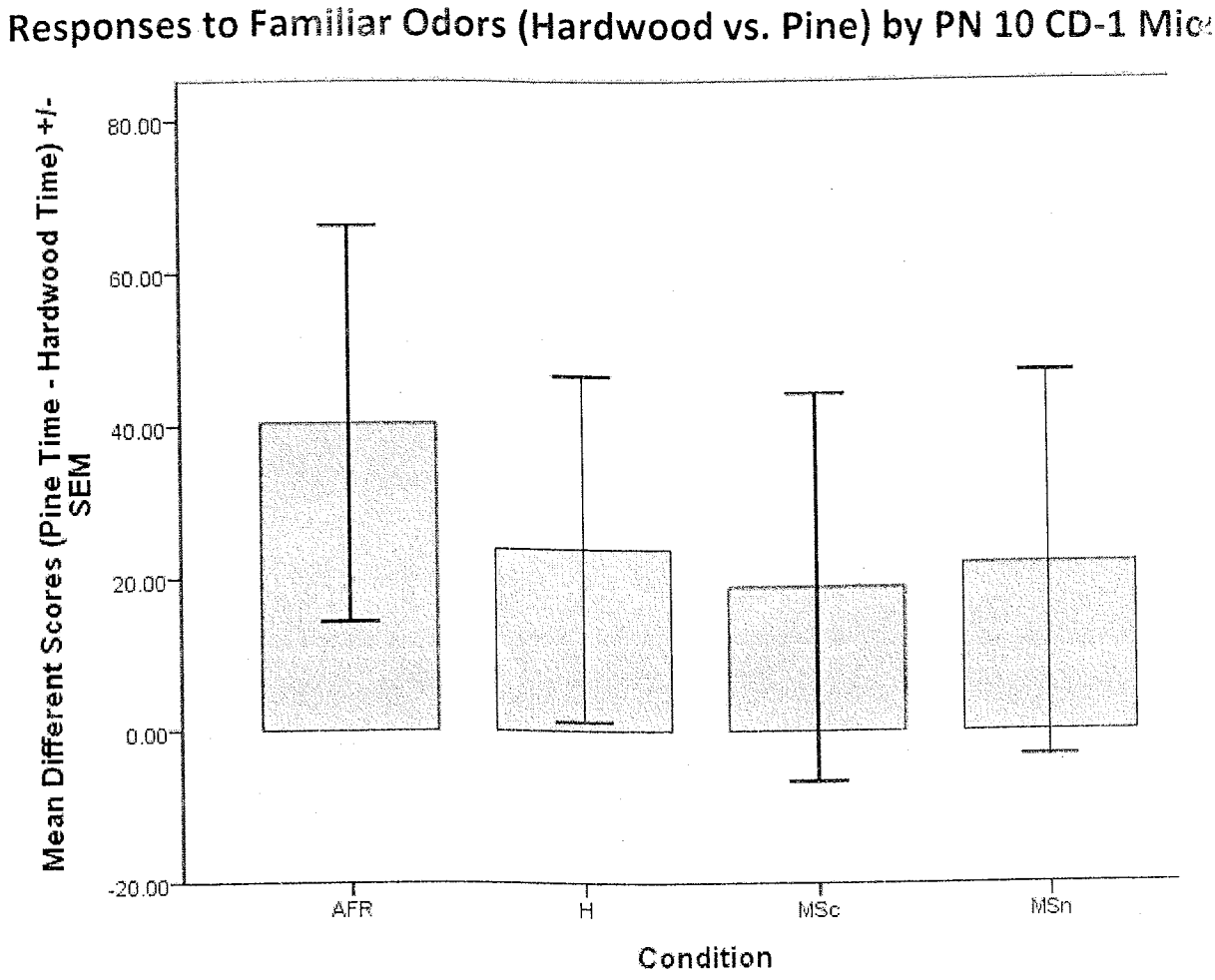
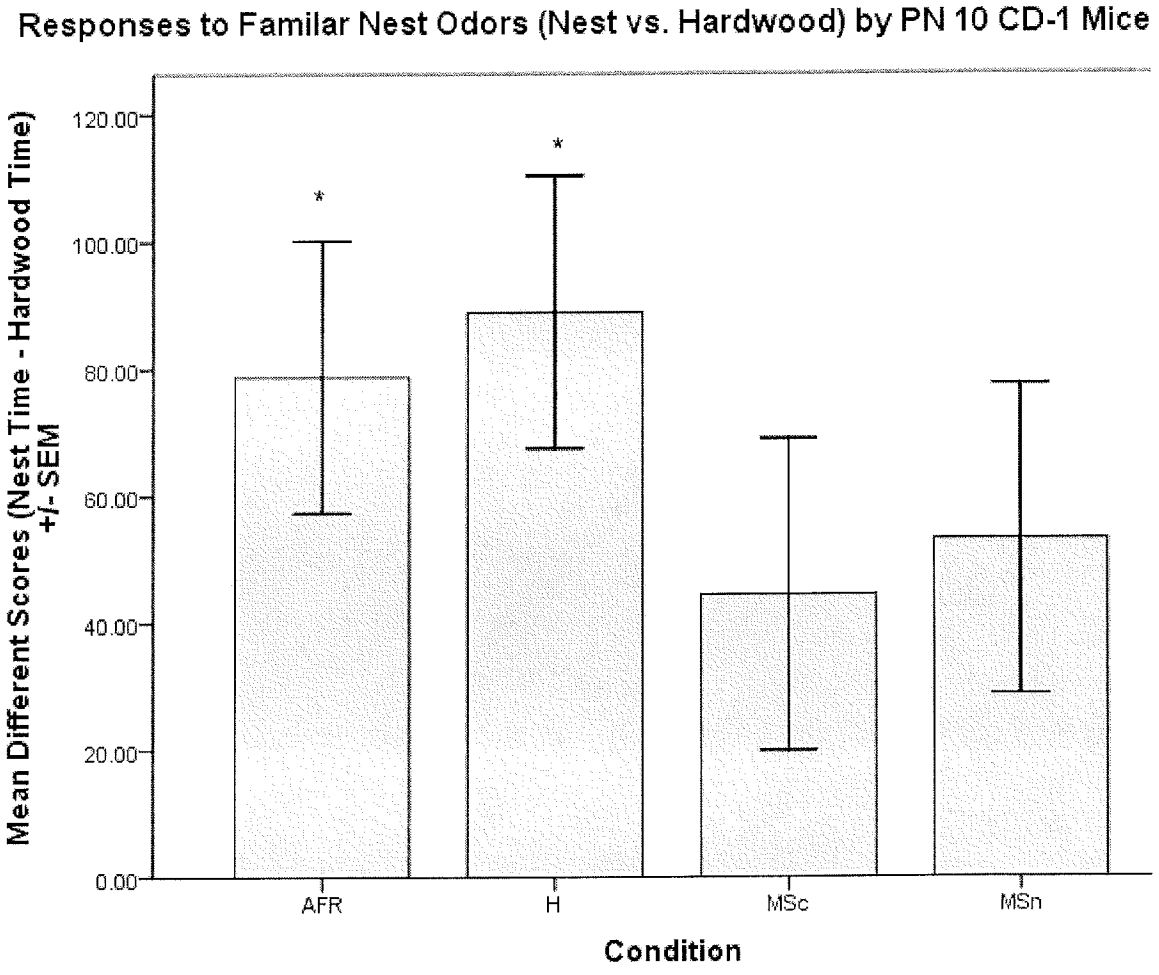
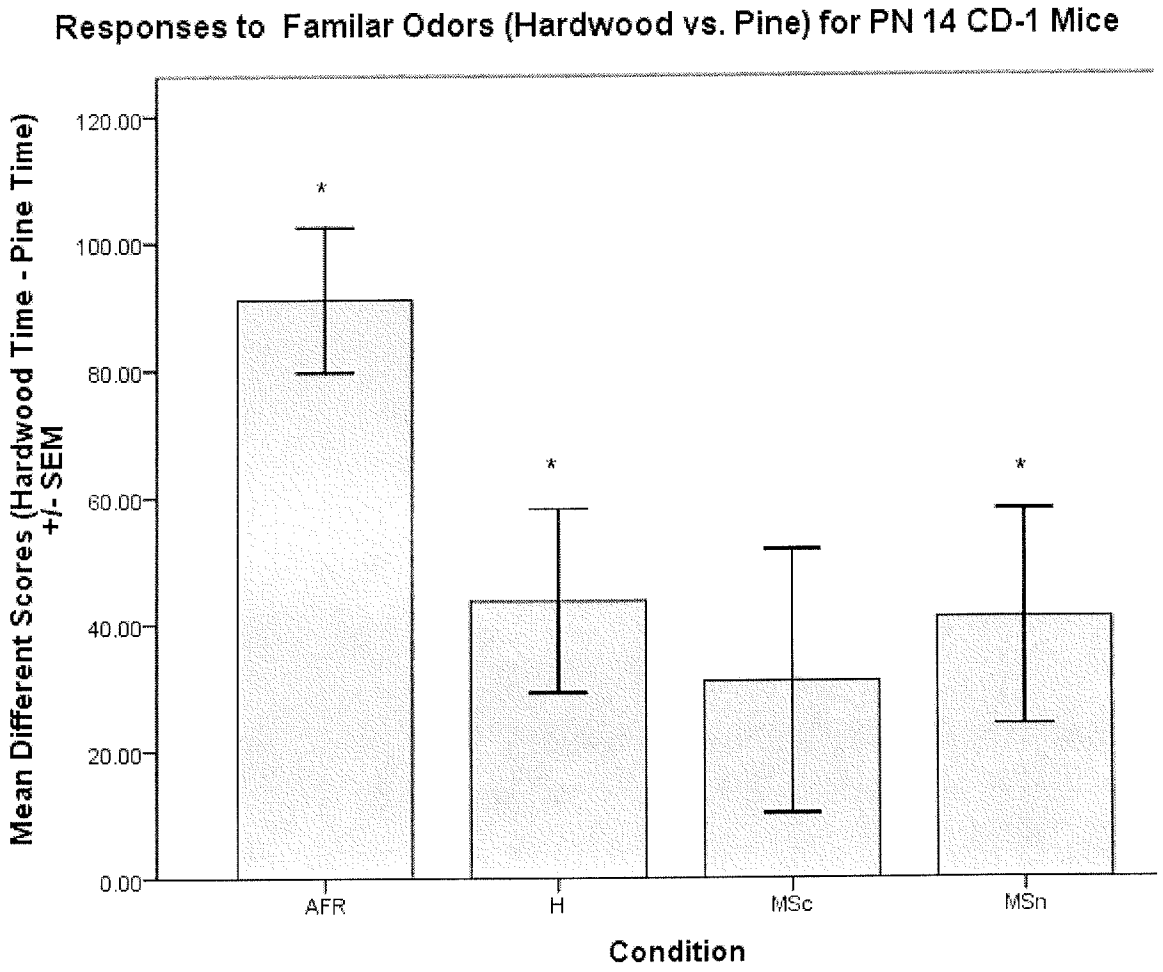

Responses to Familiar Odors (Hardwood vs. Pine) by PN 10 CD-1 Mic
Mean Different Scores (Pine Time - Hardwood Time) +/- SEM
80.00
60.00
40.00
20.00
0.00
-20.00
AFR
H
MSc
MSn
Condition

Figure 2



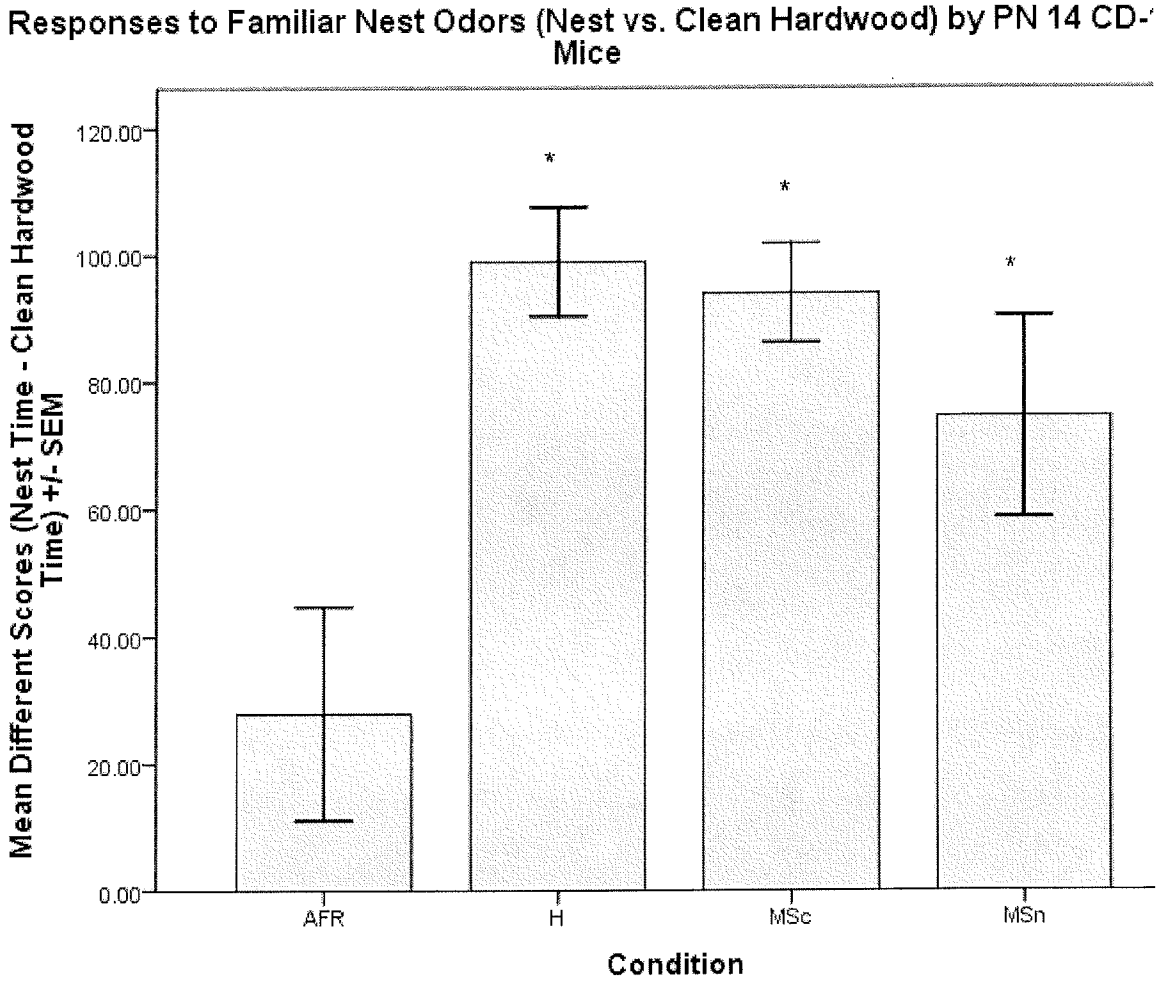
* The asterisk symbolizes significance $p<0.05$ for means against zero. The Animal Facility Reared (AFR), Handled (H), and Maternally Separated Nest (MSn) mouse pups showed a preference for familiar Nest odors on Postnatal day 10.

Figure 3



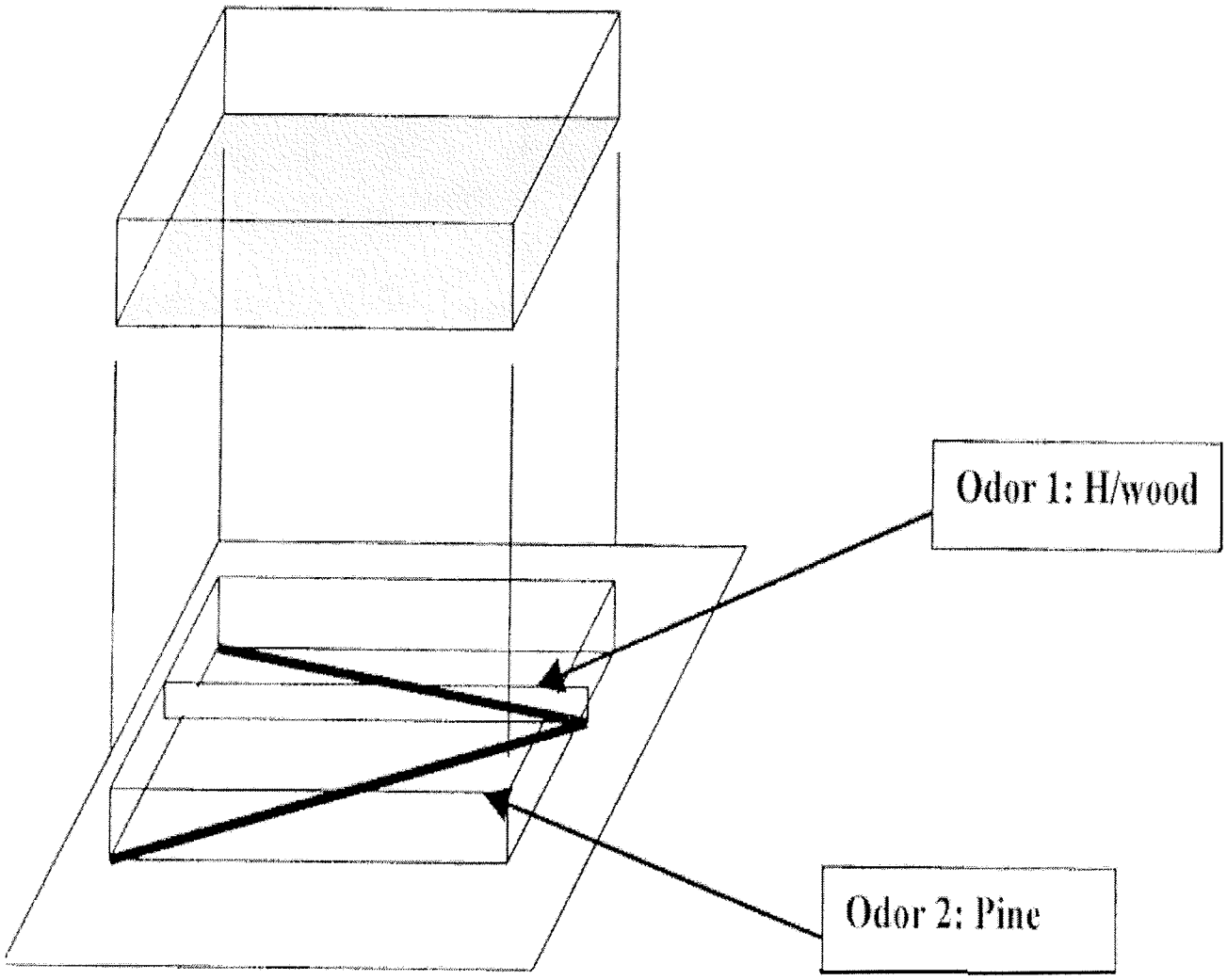
* The asterisk symbolizes significance $p<0.05$ for means against zero. The Animal Facility Reared (AFR), Handled (H), and Maternally Separated Nest (MSn) mouse pups showed a preference for familiar odors on Postnatal day 14.

Figure 4



- The asterisk symbolizes significance $p<0.05$ for means against zero. The Handled (H), Maternally Separated Clean (MSc) and Maternally Separated Nest (MSn) mouse pups showed a preference for familiar odors on Postnatal day 14.

Figure 5



Written Summary

The objective for this research is to measure preferences for nest odors in mouse pups ten to fourteen days old. Early Postnatal stress such as inconsistent care taking from the mother can influence infant mice. This current study examined odor preferences in Maternally Separated mice compared to Handled mice. The study was conducted in the Biology Research Laboratory. The Maternally Separated infant mice are separated for three hours daily in an incubator away from the mother. The inside of the incubator is 25-27 degree Celsius. On Postnatal day 10 and Postnatal day 14 there will be testing for odor preference. There are Handled infant mice, Maternal Separated Clean infant mice, Maternal Separated Soiled infant mice and Animal Facility Reared mice. Animal Facility Reared mice were not touched until testing. The Handled mice will be tested and have odor preferences of Pine vs. Clean Hardwood shavings and Soiled Hardwood vs. Clean Hardwood shavings. The Maternal Separated Clean mice will be tested and have odor preferences of Pine vs. Clean Hardwood shavings, and Soiled Hardwood vs. Clean Harwood shavings. The Maternal Separated Soiled mice will have odor preferences of Pine vs. Clean Hardwood shavings, and Clean Hardwood vs. Soiled Harwood shavings.. During the testing of the different shavings on Postnatal day ten and fourteen there will be two female and two male from each litter (Handled, MS clean, and MS soiled) and they will be placed in an odor maze. The mice will be video tapped to see what odor they preferred. The mice will be taped in a dark room with red light for one hundred and

eighty seconds because this is enough time for them to choose an odor. Once the video taping is finished the tapes will be analyzed. There will be twenty seven litters being tested. The dam will be separated from her infants in a dark room (known as Maternal Separation) and then the infants will be weighed each day and then separated for three hours. The Handled infant mice will not be separated from their mother but they will be weighed each day. When the infant mice reach postnatal day fourteen they are no longer infants and they are done with the study.

Our hypothesis is that the Handled mice will be so attach to their mother that they are going to have an odor preference of the Soiled Hardwood. The Soiled Hardwood is the scent from the Handled mice cage and the scent of the mother. They will have an odor preference of Soiled Hardwood since the Handled mice are very attach to their mother and have never been separated from her. The Maternally Separated Clean will be more likely to branch off to different odors because they have no real attachment to their mother because they are separated for them three hours a day. The Maternally Separated mice are more likely to have an odor preference of Pine or of the Clean Hardwood shavings. The Maternally Separated Soil will also be able to branch out and smell other odors because they are not attached to their mother.

The reason why this study is so important because it demonstrate that inconsistent care taking has an impact on infants. This correlates to children who are placed in foster homes, or who do not have that mother or father

figure in their life. Children that have inconsistent care taking are more likely to try new things and branch out compared to children who are attach to their mothers. Some children who are attached with their mothers will cry if you leave them in a room by themselves away from their mother. The child will stay in one spot until the mother comes back and tends to them. This reaction is different for a child who has inconsistent care taking because they can careless if their mother is in the room. When the mother leaves the room they do not care so they will branch off and start to look around and play with things in the room as if the mothers leaving had no significant impact or affect on them.

This study is important because it will determine whether removing infant mice from their mother for three hours daily during the first two weeks of life will weaken preference of nest odors. This study will also see whether allowing pups to remain in the nest during separation maintain nest nest odor preferences at normal levels. The procedure entails removing the infant mice from their home nest and mother for three hours daily. This procedure has been used to model the effects of rearing human infants in environments that prevent normal boning with a caretaker. Some examples of this are orphanages or successive foster homes. In different literature this procedure has been use by removing the pups from their nest odors and then placed in "clean" bedding during separation. This process does not allow the possible effects of maternal odor cue to be distinguished from the pups being away from their mother's stimulation. This research will address this issue by

adding conditions that have never been used by keeping the pups in their home nest during the period of separation.

The main basis for this study is to see, if taking away the odor of the mother changes or has an effect on the mouse pups. The underlying question that this study will answer: Is the mother's odor important even if she is not present and what effects does it have on the mouse pups?

Appendix

Figure 1 T-Test for AFR compared to Handled (Harwood vs. Pine) PN 10 CD-1 Mice >

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	98.7940	58.96914	13.18590
	H	24	81.4975	61.79513	12.61388
Pinetime	AFR	20	58.2630	60.03270	13.42372
	H	24	57.6317	59.28188	12.10086
Squares	AFR	20	23.3000	9.46517	2.11648
	H	24	19.2083	11.06101	2.25782
Differentscores	AFR	20	40.3810	116.33072	26.01234
	H	24	23.8658	111.53757	22.76751

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	.048	.828	.944	42	.351
	Equal variances not assumed			.948	41.190	.349
Pinetime	Equal variances assumed	.007	.931	.035	42	.972
	Equal variances not assumed			.035	40.392	.972
Squares	Equal variances assumed	.000	.983	1.303	42	.200
	Equal variances not assumed			1.322	41.960	.193
Differentscores	Equal variances assumed	.065	.800	.480	42	.634
	Equal variances not assumed			.478	39.912	.635

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	17.29650	18.32729	-19.68947	54.28247
	Equal variances not assumed	17.29650	18.24768	-19.55027	54.14327
Pinetime	Equal variances assumed	.63133	18.05167	-35.79842	37.06108
	Equal variances not assumed	.63133	18.07283	-35.88418	37.14684
Squares	Equal variances assumed	4.09167	3.13954	-2.24418	10.42751
	Equal variances not assumed	4.09167	3.09471	-2.15388	10.33722
Differentscores	Equal variances assumed	16.51517	34.43375	-52.97495	86.00528
	Equal variances not assumed	16.51517	34.56879	-53.35577	86.38611

T-Test for AFR compared to MSc (Harwood vs. Pine) PN 10 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	98.7940	58.96914	13.18590
	MSc	22	77.3964	68.05411	14.50919
Pinetime	AFR	20	58.2630	60.03270	13.42372
	MSc	22	58.5623	62.93438	13.41766
Squares	AFR	20	23.3000	9.46517	2.11648
	MSc	22	19.0909	8.99158	1.91701
Differentscores	AFR	20	40.3810	116.33072	26.01234
	MSc	22	18.8341	119.52899	25.48367

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	1.164	.287	1.084	40	.285
	Equal variances not assumed			1.091	39.918	.282
Pinetime	Equal variances assumed	.015	.905	-.016	40	.988
	Equal variances not assumed			-.016	39.898	.987
Squares	Equal variances assumed	.149	.701	1.478	40	.147

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	21.39764	19.74237	-18.50318	61.29845
	Equal variances not assumed	21.39764	19.60573	-18.22956	61.02483
Pinetime	Equal variances assumed	-.29927	19.02346	-38.74713	38.14858
	Equal variances not assumed	-.29927	18.97972	-38.66176	38.06322
Squares	Equal variances assumed	4.20909	2.84845	-1.54785	9.96603

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Squares	Equal variances not assumed			1.474	39.133	.148
Differentscores	Equal variances assumed	.001	.982	.591	40	.558
	Equal variances not assumed			.592	39.802	.557

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Squares	Equal variances not assumed	4.20909	2.85559	-1.56626	9.98444
Differentscores	Equal variances assumed	21.54691	36.46332	-52.14821	95.24202
	Equal variances not assumed	21.54691	36.41509	-52.06217	95.15599

T-Test for AFR compared to MSn (Hardwood vs. Pine) PN 10 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	98.7940	58.96914	13.18590
	MSn	20	73.6600	70.42810	15.74820
Pinetime	AFR	20	58.2630	60.03270	13.42372
	MSn	20	51.7155	55.50032	12.41025
Squares	AFR	20	23.3000	9.46517	2.11648
	MSn	20	21.3500	13.63152	3.04810

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Differentscores	AFR	20	40.3810	116.33072	26.01234
	MSn	20	21.9445	112.59228	25.17640

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	1.813	.186	1.224	38	.229
	Equal variances not assumed			1.224	36.862	.229
Pinetime	Equal variances assumed	.363	.550	.358	38	.722
	Equal variances not assumed			.358	37.768	.722
Squares	Equal variances assumed	4.602	.038	.525	38	.602
	Equal variances not assumed			.525	33.866	.603
Differentscores	Equal variances assumed	.001	.974	.509	38	.613
	Equal variances not assumed			.509	37.960	.614

Independent Samples Test

		t-test for Equality of Means			
		Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
				Lower	Upper
Hardwoodtime	Equal variances assumed	25.13400	20.53957	-16.44618	66.71418
	Equal variances not assumed	25.13400	20.53957	-16.48839	66.75639
Pinetime	Equal variances assumed	6.54750	18.28143	-30.46131	43.55631
	Equal variances not assumed	6.54750	18.28143	-30.46877	43.56377
Squares	Equal variances assumed	1.95000	3.71085	-5.56222	9.46222
	Equal variances not assumed	1.95000	3.71085	-5.59245	9.49245
Differentscores	Equal variances assumed	18.43650	36.20073	-54.84805	91.72105
	Equal variances not assumed	18.43650	36.20073	-54.85061	91.72361

Figure 2 T-Test for AFR compared to H (Nest vs. Hardwood) by PN 10 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	109.7770	55.46877	12.40319
	H	24	126.1592	55.31385	11.29089
Nesttime	AFR	20	30.9925	44.52301	9.95565
	H	24	37.1787	50.52614	10.31361
Squares	AFR	20	23.2000	12.13781	2.71410
	H	24	24.3333	12.92341	2.63798
Differentscores	AFR	20	78.7845	95.73230	21.40639
	H	24	88.9804	104.92767	21.41827

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	.017	.896	-.977	42	.334
	Equal variances not assumed			-.977	40.540	.335
Nesttime	Equal variances assumed	.323	.573	-.427	42	.672
	Equal variances not assumed			-.432	41.848	.668
Squares	Equal variances assumed	.240	.627	-.298	42	.767
	Equal variances not assumed			-.299	41.362	.766
Differentscores	Equal variances assumed	.312	.579	-.334	42	.740
	Equal variances not assumed			-.337	41.623	.738

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	-16.38217	16.76833	-50.22204	17.45770
	Equal variances not assumed	-16.38217	16.77270	-50.26700	17.50267
Nesttime	Equal variances assumed	-6.18625	14.50357	-35.45564	23.08314
	Equal variances not assumed	-6.18625	14.33476	-35.11808	22.74558
Squares	Equal variances assumed	-1.13333	3.80700	-8.81616	6.54950
	Equal variances not assumed	-1.13333	3.78487	-8.77501	6.50834
Differentscores	Equal variances assumed	-10.19592	30.54044	-71.82901	51.43718
	Equal variances not assumed	-10.19592	30.28161	-71.32307	50.93123

T-Test for AFR compared to MSc (Nest vs. Hardwood) by PN 10 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	109.7770	55.46877	12.40319
	MSc	22	94.7768	63.38100	13.51288
Nesttime	AFR	20	30.9925	44.52301	9.95565
	MSc	22	50.3073	55.01515	11.72927
Squares	AFR	20	23.2000	12.13781	2.71410
	MSc	22	22.9545	10.57165	2.25388
Differentscores	AFR	20	78.7845	95.73230	21.40639
	MSc	22	44.4695	115.01741	24.52180

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	1.612	.212	.813	40	.421
	Equal variances not assumed			.818	39.950	.418
Nesttime	Equal variances assumed	2.560	.117	-1.243	40	.221
	Equal variances not assumed			-1.255	39.499	.217
Squares	Equal variances assumed	.584	.449	.070	40	.945

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	15.00018	18.46126	-22.31143	52.31179
	Equal variances not assumed	15.00018	18.34222	-22.07228	52.07265
Nesttime	Equal variances assumed	-19.31477	15.54209	-50.72650	12.09696
	Equal variances not assumed	-19.31477	15.38475	-50.42081	11.79127
Squares	Equal variances assumed	.24545	3.50437	-6.83714	7.32805

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Squares	Equal variances not assumed			.070	37.924	.945
Differentscores	Equal variances assumed	2.675	.110	1.045	40	.302
	Equal variances not assumed			1.054	39.712	.298

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Squares	Equal variances not assumed	.24545	3.52793	-6.89694	7.38785
Differentscores	Equal variances assumed	34.31495	32.84034	-32.05786	100.68777
	Equal variances not assumed	34.31495	32.55076	-31.48745	100.11736

T-Test for AFR compared to MSn (Nest vs. Hardwood) by PN 10 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	109.7770	55.46877	12.40319
	MSn	20	100.5665	65.85462	14.72554
Nesttime	AFR	20	30.9925	44.52301	9.95565
	MSn	20	47.3795	50.80830	11.36108
Squares	AFR	20	23.2000	12.13781	2.71410
	MSn	20	19.4500	8.63576	1.93101

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Differentscores	AFR	20	78.7845	95.73230	21.40639
	MSn	20	53.1870	109.00889	24.37513

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	2.427	.128	.478	38	.635
	Equal variances not assumed			.478	36.933	.635
Nesttime	Equal variances assumed	.760	.389	-1.085	38	.285
	Equal variances not assumed			-1.085	37.356	.285
Squares	Equal variances assumed	3.503	.069	1.126	38	.267
	Equal variances not assumed			1.126	34.312	.268
Differentscores	Equal variances assumed	1.046	.313	.789	38	.435
	Equal variances not assumed			.789	37.377	.435

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	9.21050	19.25307	-29.76531	48.18631
	Equal variances not assumed	9.21050	19.25307	-29.80231	48.22331
Nesttime	Equal variances assumed	-16.38700	15.10593	-46.96735	14.19335
	Equal variances not assumed	-16.38700	15.10593	-46.98467	14.21067
Squares	Equal variances assumed	3.75000	3.33094	-2.99313	10.49313
	Equal variances not assumed	3.75000	3.33094	-3.01701	10.51701
Differentscores	Equal variances assumed	25.59750	32.44041	-40.07469	91.26969
	Equal variances not assumed	25.59750	32.44041	-40.11068	91.30568

Figure 3 T-Test for AFR compared to H (Hardwood vs. Pine) by PN 14 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	121.0775	35.79270	8.00349
	H	24	96.9496	43.09164	8.79604
Pinetime	AFR	20	33.8660	31.54741	7.05421
	H	24	53.2879	30.06240	6.13646
Squares	AFR	20	39.2500	6.64811	1.48656
	H	24	39.2917	13.46002	2.74751
Differentscores	AFR	20	91.1160	50.75122	11.34832
	H	24	43.7267	71.12127	14.51757

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	1.193	.281	1.995	42	.053
	Equal variances not assumed			2.029	42.000	.049
Pinetime	Equal variances assumed	.103	.750	-2.087	42	.043
	Equal variances not assumed			-2.077	39.806	.044
Squares	Equal variances assumed	4.592	.038	-.013	42	.990
	Equal variances not assumed			-.013	34.825	.989
Differentscores	Equal variances assumed	1.979	.167	2.495	42	.017
	Equal variances not assumed			2.572	41.113	.014

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	24.12792	12.09705	-.28492	48.54075
	Equal variances not assumed	24.12792	11.89228	.12833	48.12751
Pinetime	Equal variances assumed	-19.42192	9.30793	-38.20608	-.63776
	Equal variances not assumed	-19.42192	9.34976	-38.32137	-.52246
Squares	Equal variances assumed	-.04167	3.30565	-6.71274	6.62941
	Equal variances not assumed	-.04167	3.12389	-6.38465	6.30132
Differentscores	Equal variances assumed	47.38933	18.99275	9.06041	85.71826
	Equal variances not assumed	47.38933	18.42672	10.17893	84.59974

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T-Test for AFR compared to MSc (Hardwood vs. Pine) by PN 14 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	121.0775	35.79270	8.00349
	MSc	20	91.4765	46.25656	10.34328
Pinetime	AFR	20	33.8660	31.54741	7.05421
	MSc	20	60.4685	48.60236	10.86782
Squares	AFR	20	39.2500	6.64811	1.48656
	MSc	20	39.9000	10.91546	2.44077
Differentscores	AFR	20	91.1160	50.75122	11.34832
	MSc	20	31.0080	93.12024	20.82232

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	1.843	.183	2.263	38	.029
	Equal variances not assumed			2.263	35.748	.030
Pinetime	Equal variances assumed	2.937	.095	-2.053	38	.047

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	29.60100	13.07820	3.12557	56.07643
	Equal variances not assumed	29.60100	13.07820	3.07069	56.13131
Pinetime	Equal variances assumed	-26.60250	12.95652	-52.83160	-.37340

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Pinetime	Equal variances not assumed			-2.053	32.597	.048
Squares	Equal variances assumed	3.538	.068	-.227	38	.821
	Equal variances not assumed			-.227	31.391	.822
Differentscores	Equal variances assumed	4.500	.040	2.535	38	.015
	Equal variances not assumed			2.535	29.372	.017

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Pinetime	Equal variances not assumed	-26.60250	12.95652	-52.97514	-.22986
Squares	Equal variances assumed	-.65000	2.85784	-6.43539	5.13539
	Equal variances not assumed	-.65000	2.85784	-6.47566	5.17566
Differentscores	Equal variances assumed	60.10800	23.71399	12.10154	108.11446
	Equal variances not assumed	60.10800	23.71399	11.63413	108.58187

T-Test for AFR compared to MSn (Hardwood vs. Pine) by PN 14 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	121.0775	35.79270	8.00349
	MSn	22	92.7345	45.04144	9.60287
Pinetime	AFR	20	33.8660	31.54741	7.05421

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Pinetime	MSn	22	57.4018	39.91976	8.51092
Squares	AFR	20	39.2500	6.64811	1.48656
	MSn	22	40.6818	10.10604	2.15461
Differentscores	AFR	20	91.1160	50.75122	11.34832
	MSn	22	41.1009	79.68325	16.98853

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	1.596	.214	2.242	40	.031
	Equal variances not assumed			2.267	39.332	.029
Pinetime	Equal variances assumed	2.484	.123	-2.105	40	.042
	Equal variances not assumed			-2.129	39.277	.040
Squares	Equal variances assumed	1.765	.192	-.537	40	.595
	Equal variances not assumed			-.547	36.588	.588
Differentscores	Equal variances assumed	2.505	.121	2.398	40	.021
	Equal variances not assumed			2.448	36.000	.019

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	28.34295	12.63939	2.79780	53.88811
	Equal variances not assumed	28.34295	12.50084	3.06444	53.62147
Pinetime	Equal variances assumed	-23.53582	11.17969	-46.13082	-.94082
	Equal variances not assumed	-23.53582	11.05431	-45.89024	-1.18140
Squares	Equal variances assumed	-1.43182	2.66874	-6.82554	3.96190
	Equal variances not assumed	-1.43182	2.61768	-6.73775	3.87412
Differentscores	Equal variances assumed	50.01509	20.85609	7.86336	92.16682
	Equal variances not assumed	50.01509	20.43023	8.58066	91.44952

Figure 4 T-Test for AFR compared to H (Nest vs. Hardwood) by PN 14 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	95.1165	44.51051	9.95285
	H	24	129.7429	25.45461	5.19590
Nesttime	AFR	20	67.2320	33.36675	7.46103
	H	24	30.7412	18.46262	3.76867
Squares	AFR	20	42.2000	7.40270	1.65529
	H	24	40.8333	7.96005	1.62484
Differentscores	AFR	20	27.8845	75.22345	16.82048
	H	24	99.0017	42.04615	8.58264

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	5.349	.026	-3.233	42	.002
	Equal variances not assumed			-3.084	28.989	.004
Nesttime	Equal variances assumed	4.552	.039	4.587	42	.000
	Equal variances not assumed			4.366	28.405	.000
Squares	Equal variances assumed	.151	.699	.585	42	.562
	Equal variances not assumed			.589	41.458	.559
Differentscores	Equal variances assumed	4.328	.044	-3.955	42	.000
	Equal variances not assumed			-3.766	28.581	.001

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	-34.62642	10.70896	-56.23796	-13.01487
	Equal variances not assumed	-34.62642	11.22750	-57.58960	-11.66323
Nesttime	Equal variances assumed	36.49075	7.95483	20.43725	52.54425
	Equal variances not assumed	36.49075	8.35882	19.37947	53.60203
Squares	Equal variances assumed	1.36667	2.33520	-3.34595	6.07929
	Equal variances not assumed	1.36667	2.31950	-3.31609	6.04943
Differentscores	Equal variances assumed	-71.11717	17.98320	-107.40874	-34.82559
	Equal variances not assumed	-71.11717	18.88359	-109.76306	-32.47127

T-Test for AFR compared to MSc (Nest vs. Hardwood) by PN 14 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	95.1165	44.51051	9.95285
	MSc	22	126.0659	23.31140	4.97001
Nesttime	AFR	20	67.2320	33.36675	7.46103
	MSc	22	32.0755	14.81030	3.15757
Squares	AFR	20	42.2000	7.40270	1.65529
	MSc	22	42.0455	8.52155	1.81680
Differentscores	AFR	20	27.8845	75.22345	16.82048
	MSc	22	93.9905	36.71125	7.82686

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	7.717	.008	-2.861	40	.007
	Equal variances not assumed			-2.782	28.077	.010
Nesttime	Equal variances assumed	8.592	.006	4.484	40	.000
	Equal variances not assumed			4.339	25.670	.000
Squares	Equal variances assumed	.301	.586	.062	40	.951

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	-30.94941	10.81950	-52.81643	-9.08238
	Equal variances not assumed	-30.94941	11.12476	-53.73462	-8.16420
Nesttime	Equal variances assumed	35.15655	7.84041	19.31048	51.00261
	Equal variances not assumed	35.15655	8.10168	18.49289	51.82020
Squares	Equal variances assumed	.15455	2.47463	-4.84686	5.15595

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Squares	Equal variances not assumed			.063	39.927	.950
Differentscores	Equal variances assumed	7.445	.009	-3.672	40	.001
	Equal variances not assumed			-3.563	26.974	.001

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Squares	Equal variances not assumed	.15455	2.45780	-4.81313	5.12222
Differentscores	Equal variances assumed	-66.10595	18.00288	-102.49112	-29.72078
	Equal variances not assumed	-66.10595	18.55231	-104.17384	-28.03807

T-Test for AFR compared to MSn (Nest vs. Hardwood) by PN 14 CD-1 Mice

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Hardwoodtime	AFR	20	95.1165	44.51051	9.95285
	MSn	20	111.4440	40.30741	9.01301
Nesttime	AFR	20	67.2320	33.36675	7.46103
	MSn	20	36.9250	35.68827	7.98014
Squares	AFR	20	42.2000	7.40270	1.65529
	MSn	20	38.1000	8.29013	1.85373

Group Statistics

	Condition	N	Mean	Std. Deviation	Std. Error Mean
Differentscores	AFR	20	27.8845	75.22345	16.82048
	MSn	20	74.5190	70.90550	15.85495

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means		
		F	Sig.	t	df	Sig. (2-tailed)
Hardwoodtime	Equal variances assumed	.110	.742	-1.216	38	.231
	Equal variances not assumed			-1.216	37.632	.232
Nesttime	Equal variances assumed	.098	.756	2.774	38	.009
	Equal variances not assumed			2.774	37.829	.009
Squares	Equal variances assumed	1.807	.187	1.650	38	.107
	Equal variances not assumed			1.650	37.523	.107
Differentscores	Equal variances assumed	.360	.552	-2.017	38	.051
	Equal variances not assumed			-2.017	37.868	.051

Independent Samples Test

		t-test for Equality of Means			
				95% Confidence Interval of the Difference	
		Mean Difference	Std. Error Difference	Lower	Upper
Hardwoodtime	Equal variances assumed	-16.32750	13.42735	-43.50974	10.85474
	Equal variances not assumed	-16.32750	13.42735	-43.51847	10.86347
Nesttime	Equal variances assumed	30.30700	10.92473	8.19105	52.42295
	Equal variances not assumed	30.30700	10.92473	8.18777	52.42623
Squares	Equal variances assumed	4.10000	2.48522	-.93106	9.13106
	Equal variances not assumed	4.10000	2.48522	-.93316	9.13316
Differentscores	Equal variances assumed	-46.63450	23.11510	-93.42857	.15957
	Equal variances not assumed	-46.63450	23.11510	-93.43393	.16493

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