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Nest building as an indicator of illness in laboratory mice

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ABSTRACT

Laboratory mice housed at typical temperatures and provided with crinkled paper nesting material build fully enclosed nests, increasing welfare, and reducing cold stress, but complicating daily animal observations by care staff. Anecdotal reports by animal care staff indicate that ill mice are not found within the nest and do not nest build. We hypothesized that both nest shape and whether or not ill mice were found outside the nest could be used as tools to identify ill mice. Forty two female C57BL/6NCrl mice were provided 10 g of nesting material and assigned to a social treatment of either solitary or group housing. Lipopolysaccharide (LPS) injected intraperitoneally at 1 mg/kg was used to induce malaise in 0, 1, 2, or 3 mice/cage; all others received saline. Prior to the study, mice were habituated to handling and injections with positive reinforcement. In order to blind the nest scorer to treatment novel, but experienced, handlers administered the experimental injections. Nest score, number of mice in the nest, and anhedonia measured by sugared cereal consumption were recorded at the following time points: baseline, cage change, saline injection, injection, and injection + cage change and data were analyzed using GLMs with post-hoc contrasts. The number of mice observed outside the nest was not affected by any treatment. Nest score was not significantly altered in group housed mice but LPS-injected solitary mice had significantly lower nest scores than saline-injected solitary mice at the injection + cage change time point. Saline-injected mice also had a significant reduction in nest score from baseline at injection + cage change. It is likely that receiving the injection from novel handlers were likely the cause for this alteration, yielding the unexpected result that nest building in mice is affected by a novel handler. LPS-injected mice, regardless of social treatment, ate ≈2 g less sugared cereal per mouse at both injection and injection + cage change time points compared to their baseline cereal consumption and saline-injected mice at the same time points. Group housing appears to mask changes in nest score if other cage residents are healthy and acutely ill individuals were not observed to have a location bias, in or out of the nest, after LPS injection. However, a reduction in nest score has the potential to be a useful tool to identify acute illness after cage change in solitary mice. Changes in nest complexity may be useful to identify illness earlier for general husbandry and welfare purposes and may be a more robust tool in chronic, rather than acute, disease models.

1. Introduction

In typical laboratory temperatures (20–26 °C), mice are exposed to temperatures resulting in chronic cold stress (Gordon, 2004). However, nesting material can be provided to aid in both thermoregulation (Gaskill et al., 2013a) and provide a biologically relevant enrichment. In the wild, nests aid mice in thermoregulation, provide shelter from predators, and are positively correlated with survival (Brown, 1953). The nests of wild mice are complex structures made from multiple materials, and replenished and manipulated daily (Brown, 1953; Latham and Mason, 2004). Laboratory mice are highly motivated to obtain nesting material (Gaskill et al., 2012; Gross et al., 2011; Van de Weerd et al., 1998) and will construct nests similar to those of wild mice if given the opportunity, but nest quality depends on the material provided (Gaskill et al., 2013b; Hess et al., 2008), and the strain and sex of the mice (Gaskill et al., 2012). Nesting material has been shown to reduce radiative heat loss (Gaskill et al., 2013a), increase feed conversion

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(Gaskill et al., 2013a; Olsson and Dahlborn, 2002) and decrease pup mortality (Gaskill et al., 2013c) in several laboratory strains studied.

Nesting material has one drawback when it is used to enrich laboratory mouse housing. Instructions for routine animal husbandry and care provided in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) stipulate that animals need to be checked for health and well-being daily. For larger laboratory animals, where few are typically housed or used, this can be a relatively simple process. For other animals, where there are often thousands, if not tens of thousands in a small space, this becomes a more complex problem. The solution has been to implement clear caging, which is not preferred by animals (Olsson et al., 2003; Porter et al., 1963; Sherwin and Glen, 2003), and to visually scan each cage for the presence of ill animals. Nesting material can complicate this scanning process, and ill animals may be overlooked. Anecdotally, most animal husbandry technicians and some research technicians report that once they are familiar with how mice behave with nesting material, nests do not impede this process, as mice will be in the nest when the lights are on, but sick animals are found outside the nest. Sick animals might be found outside the nest because they are isolating themselves from conspecifics (Yee and Prendergast, 2012), or because the conspecifics drive them away. The purpose of this study is not to determine the reasons why ill animals are found outside of the nest but instead to test if animals rendered ill would be found outside of the nest, since this would be simple and practical way for animal care staff to identify sick individuals. A general malaise can be induced by administration of a lipopolysaccharide (LPS) which activates the innate immune system through the release of cytokines, causing fever, body aches, and other signs of illness (Kelley et al., 2003) and this is the system we chose to use.

Beyond simply observing the animals, assessing sickness in mice is another problem, as their small size, unfamiliar body language, quick movements, nocturnal tendencies, prey stoicism, and sheer number usually limits detailed assessments unless part of a research protocol. Some clinical signs are universally recognized as mouse sickness behavior, such as hunched posture, reluctance to move, piloerection, lethargy, lack of grooming, unsteady gait, and reduced food consumption. Nest building in the context of illness has been studied, where gathering behavior is restored in sick lactating mice at cold temperatures (Aubert et al., 1997). Other studies have tested alteration of nest conformation when mice are in pain but the observations were not statistically evaluated (Arras et al., 2007) or results were not completely clear (Jirkof et al., 2013b).

Mice and other animals assess sickness and parasitism in their own species using behavioral and olfactory cues unavailable to humans. Sickness can lead to avoidance by conspecifics (Arakawa et al., 2010), changes in social interactions (Renault et al., 2008; Yee and Prendergast, 2010, 2012), disinterest from potential mates (Penn et al., 1998), and even aggression (Hart, 1990). The anecdotal reports from caretakers and researchers, the literature on sickness behavior, and our own experience led us to hypothesize that nest quality would be reduced with an increasing number of sick mice, making it easier for care staff to observe mice in a cage and that any sick animals in a group would be found outside the nest during routine health checks. We also predict that cages receiving LPS would be readily apparent after cage cleaning because animals experiencing general malaise would be less likely to gather material and build a nest.

2. Materials and methods

2.1. Animals and husbandry

All work was conducted at Charles River's AAALAC-accredited Wilmington, MA, facility and was approved by the IACUC. Animals were free of a list of common mouse pathogens; further details may be found at http://www.criver.com/files/pdfs/rms/hmsummary.aspx. For all tests, female C57BL/6NCrl (B6) were used. Mice were housed in disposable, transparent, individually ventilated cages (Innovive, San Diego, CA; LxWxH: 37.3 × 23.4 × 14.0 cm). The cages were bedded with irradiated aspen shavings (NEPCO, Warrensburg, NY), and mice were provided with 10 g of fresh nesting material at every cage change (Enviro-Dri, Shepherd Specialty Papers, Watertown, TN). Food (5L79, LabDiet, St. Louis, MO) and hyperchlorinated water via water bottle were provided *ad libitum*. The light cycle was 12:12 light:dark (on at 06:30, off at 18:30), temperature was maintained at 21 °C ± 1 °C, and humidity was maintained between 30 and 70%. Animals were observed daily for appearance and general health; if animals had appeared ill more than 48 h after experimental onset, they would have been euthanized and the cage replaced. All randomization of animals and cages was accomplished through the use of the random integer generator at random.org.

Table 1
Number of cages (n) assigned to each treatment.

Social treatment		LPS treatment			
		Saline	1LPS	2LPS	3LPS
	Group (3 mice)	3	3	3	3
	Solitary (1 mouse)	3	3		

Forty two 8 week old female mice were randomly assigned to a social treatment (single or group housing) using a Latin square experimental design (N = 3 per combination). The authors intended to conduct this study in both sexes, however due to a large amount of injurious aggression in the group housed males prior to the study start, too many data points were lost and we could not include males in the analysis. Handling rats at the time of LPS administration alters their hyperthermic response to LPS (Romanovsky et al., 1998). Therefore, before any tests were performed, all mice were habituated by an investigator (B. Gaskill) to handling and injections using a reward of several microliters of chocolate milk for three weeks, with this habituation consisting of 3–4 sessions per week lasting approximately 10–60 s per animal. Solitary cages of mice were randomly allocated to either saline or LPS treatments (1 mg/kg IP, from *E. coli* 0111:B4, Sigma-Aldrich, St. Louis, MO; see Table 1). Group housed cages were also randomly assigned an LPS treatment: all 3 mice injected with saline (Saline); 1 mouse injected with LPS and the other 2 mice injected with saline (1LPS); 2 mice injected with LPS and the other injected with saline (2LPS); or all 3 mice injected with LPS (3LPS). Different numbers of mice were injected in group housing to determine if nest scoring or observing mice outside the nest site would change as a function of the number of ill mice. All animals received intraperitoneal injections at approximately 17:00, one hour before the start of the dark cycle. Mice were injected at this time of day because it corresponds to the end of the daily inactivity period, meaning it would be more likely to disrupt overall activity as well as nest building peaks found toward the end of the dark and beginning of the light period (Jirkof et al., 2013a).

Measures collected from the cage were: individual mouse weights (averaged per cage), nest score, food consumption, and the number of mice in-or-out of the nest. Illness is often accompanied by a depressive state, which may be assessed by a decrease in enjoyment of pleasurable experiences (Aubert, 1999). In rodents, this is often evaluated by the consumption of sweet solutions. Sugar solution could not be used since cage design did not allow for a second water bottle. Therefore we added a test that determined the change in consumption of a sweetened cereal as a measure for anhedonia. In this test, 5 g of FrootLoops® (Kellogg's, Battle Creek, MI; a fruit-flavored cereal with approximately 12 g of sugar per 29 g of cereal) per mouse are placed in the cage at 17:00, at the start of the activity

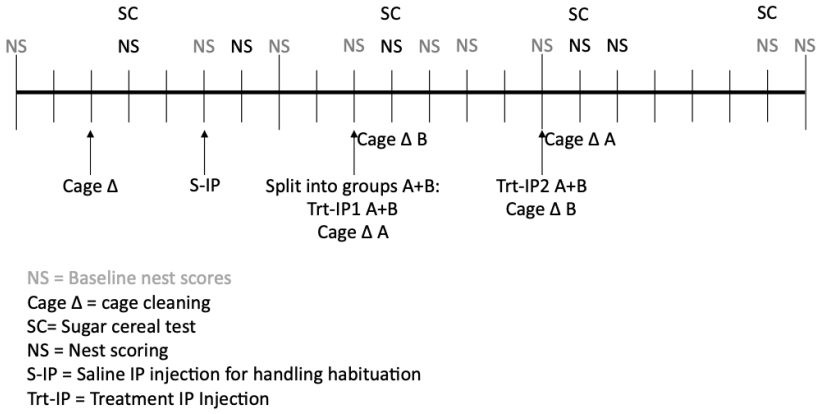

Fig. 1. Experimental timeline where each tick mark on the timeline indicates one 24-h period. NS (gray): nest scoring for baseline or evaluation of normal building, Cage Δ: cage cleaning, SC: sugared cereal test, NS (black): nest scoring post-injection, S-IP: last handling habituation saline IP injection, Trt-IP: treatment IP injection. SC and NS indicate the day when measurements were recorded, following a time point or treatment.

period, and the amount remaining is weighed the next morning at 09:00. All measures were collected after the following events: cage change, where a new cage and fresh bedding were provided and new nesting material was scattered around the cage to determine the extent of gathering behavior and nest building that occurred; injection of saline for all animals during a handling habituation session (saline injection), animals receiving their assigned treatment injections of LPS or saline (injection), and treatment injections combined with a cage change (injection + cage change). A timeline of experimental manipulations is provided in Fig. 1.

For the injection and injection + cage change time points, B. Gaskill recorded animal weights and changed cages, but did not administer injections. The injections for these time points were administered by one of two experienced laboratory animal veterinarians in order to allow blinding of B. Gaskill for nest scoring the following day. At the time of the treatment IP injections (Trt-IP1 and Trt-IP2; see Fig. 1) cages were split into groups A and B, balanced across social and LPS treatments. At Trt-IP1, group A received their treatment injection and were immediately placed into a clean cage. At Trt-IP2, group A received the treatment injection but their cages were not changed until the following day. For group B, injection with cage change occurred at Trt-IP2 and injection with delayed cage change at Trt-IP1. Dividing the cages into 2 groups allowed us to evenly distribute variability of the novel handler across all treatments and time points. All injections at Trt-IP1 were administered by K. Pritchett-Corning and by C. Winnicker at Trt-IP2.

Nests were scored daily between 09:00–10:00 using a 0–5 scale (Hess et al., 2008). Briefly, a score of 1: was manipulated material but no central nest site was evident; 2: was a flat nest; 3: was a cup nest; 4: was an incomplete dome; 5: was a complete and enclosed dome. Nest scores from the 5 days prior to the first injection were averaged to determine the particular cage's baseline nest building.

2.2. Statistical analyses

Analyses were performed as split plot ANOVAs using GLM, in JMP v 9 (SAS, Cary, NC). The assumptions of GLM (normality of error, homogeneity of variance, and linearity) were confirmed *post-hoc* (Grafen and Hails, 2002). Cage was considered the experimental variable. Significant effects were then analysed using *post-hoc* Tukey tests or Bonferroni corrected planned contrasts using custom contrasts or Test Slices in JMP. Nine days throughout the study, scored no fewer than 2 days after a treatment injection occurred (see Fig. 1), were averaged to determine normal nest scores for a cage (baseline). Difference in nest scores (time point nest score − mean baseline score), sugar cereal consumption, food consumption (total cage food consumption/number of mice in the cage), and the number of mice in the nest were all tested with the following model:

Dependent variable = cage [social treatment LPS treatment] + LPS treatment [social treatment] + time point + time point × LPS treatment [social treatment] + social treatment + social treatment × time point

To avoid pseudoreplication, and accommodate repeated measures, analyses were blocked by cage, nested within social treatment and LPS treatment. LPS treatment was also nested within social treatment, since solitary mice would only be assigned to Saline or 1 LPS injection. Time point was treated as a categorical variable. All values are provided as least squares means and standard error. Effects were significant at $P < 0.05$.

3. Results

General sickness behavior was observed in animals during the light cycle after the injection, as was expected from the dose of LPS administered, and no animals died or were removed during the course of the study. Animals resumed normal behavior, as measured by nest building and maintenance, within 24 h after the injection. The mean body weight of the animals was 23.5 ± 1.5 g (mean ± standard deviation). Nest scores were significantly altered by the interaction of time point and LPS treatment [social treatment] ($F_{12,54} = 2.54$; $P = 0.01$). There were significant differences in nest scores for the singly housed females (test slice: $F_{8,54} = 14.50$; $P < 0.001$; Fig. 2) but no differences were found in the group housed mice (test slice: $F_{11,54} = 1.51$; $P = 0.15$). The only significant difference between LPS and saline treatments was found at the injection + cage change time point (contrast: $P < 0.001$). Nest scores were significantly different from zero (baseline) for both saline and LPS treatments at the injection + cage change time point (*t*-test: $P < 0.025$). Food consumption was significantly altered by the interaction of time point by LPS [social treatment] ($F_{12,36} = 2.19$; $P = 0.03$; Fig. 3). However the only significant differences found was that all mice ate less after injection and injection + cage change compared to the other two time points, regardless of LPS treatment (Single contrast: $F_{1,36} = 699.0$; $P < 0.001$; Group contrast: $F_{1,36} = 776.4$; $P < 0.001$). Sugared cereal consumption was significantly altered by the interaction of time point and LPS treatment [social treatment] ($F_{12,36} = 14.7$; $P < 0.001$). In singly housed mice, the amount

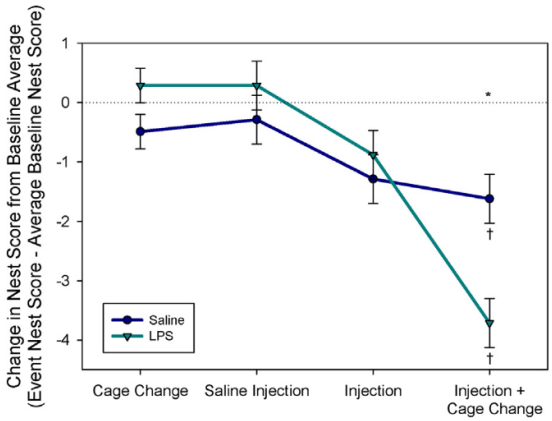

Fig. 2. Difference in nest score from mean baseline measurement in singly-housed mice. A negative value indicates a reduction in nest quality compared to mean baseline values before experimental manipulation. LSM and SE are plotted and significant *t*-tests (value different from zero; α corrected for the number of comparisons) are indicated by†. Asterisks indicate a significant custom contrast (α corrected for the number of comparisons) between LPS and saline treatments.

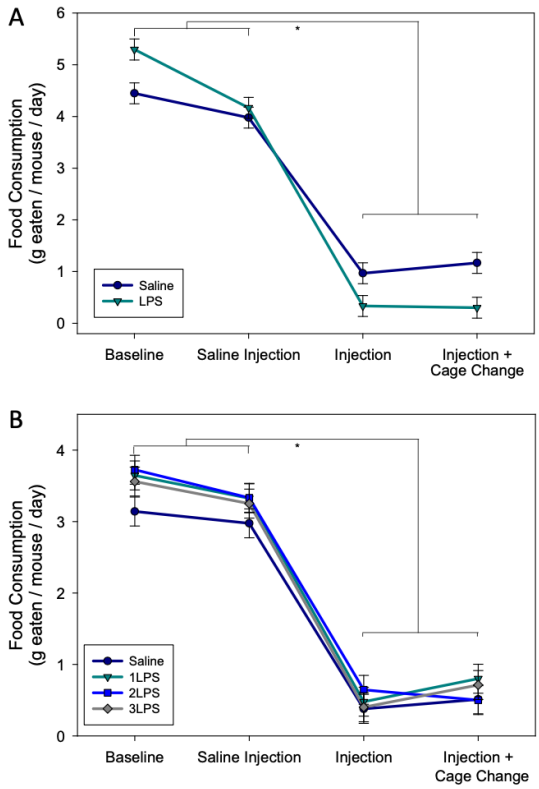

Fig. 3. Food consumption in singly (A) and group-housed (B) mice at different time points. LSM and SE are plotted and an asterisk indicates a significant custom contrast ($P < 0.05$).

of sugared cereal consumed was significantly decreased by the administration of LPS at both the injection and injection + cage change time points (Tukey; $P < 0.05$; Fig. 4A). In group-housed mice evaluated with the sugared cereal test, saline only cages never differed significantly from their baseline consumption (Tukey: $P < 0.05$; Fig. 4B). In cages where only 1 mouse was injected with LPS, the amount consumed was not different from any other treatment (Tukey: $P > 0.05$). The mean amount of sugared cereal

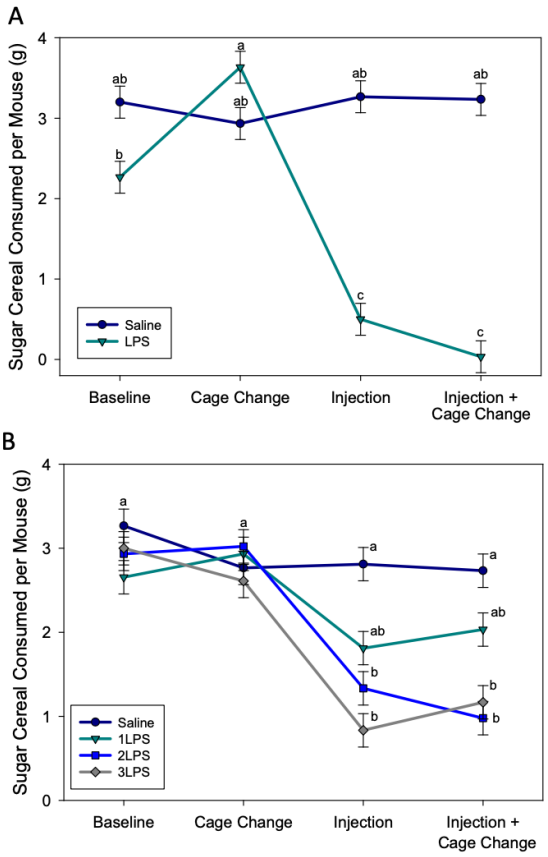

Fig. 4. Sugar cereal consumption in singly (A) and group-housed (B) mice at different time points in the study. Consumption for all group housed mice at the baseline and cage change time points were similar and are indicated by a single letter due to space. LSM and SE are plotted and different letters indicate significantly different Tukey comparisons ($P < 0.05$).

consumed per mouse in cages where 2 or all 3 animals were injected with LPS was significantly less than saline control cages (Tukey: $P < 0.05$). The number of mice found inside or outside the nest was not significantly altered by time point *LPS [social treatment] ($F_{12,53} = 1.55$; $P = 0.13$). However, more animals in group housed cages were found inside the nest than in single cages ($F_{1,53} = 111.1$; $P < 0.001$). This is expected as there were more animals in those cages.

4. Discussion

For a mouse to build a nest, a complex series of behaviors must occur. Mice must first gather nesting materials, then sort them into groups. After sorting, mice process materials into a more useful form and finally begin to build. Although many of these steps can be accomplished by a relatively stationary mouse, gathering nesting material and building the nest cannot. Animals housed in groups may have an advantage in that the unaffected animals in the cage may perform these behaviors and benefit the affected animals. Our findings indicate that the nest score, which describes the relative complexity of the nest, but not the presence of animals outside the nest, can indicate malaise or illness in solitary animals, especially when the animals are forced to build a new nest when the old nest is removed and fresh material provided after cage cleaning. The strong motivation to nest in healthy animals seems to result in a well-built nest, regardless of the health of the other animals in the cage, at least over the short period of illness we evaluated. If

a nest is present in the cage, unwell animals will apparently seek refuge within the nest. Our findings seem to indicate that when all of the animals in a group are acutely ill, group nest building masks changes to the nest score. A singly-housed ill animal forced to build a nest, however, is not capable of the task, although if nesting material does not require gathering or processing, it may huddle beneath it. Assessing gathering behavior, the portion of the patterned nesting behavior involving collecting materials with which to build a nest, may be a better means of evaluating a mouse's health and welfare since it requires movement of both animals and materials (Rock et al., 2014a; Rock et al., 2014b).

Administration of LPS results in the activation of the innate immune system through the release of cytokines, which induce systemic effects including fever, body aches, and other signs of illness, generally known as malaise (Kelley et al., 2003). LPS induces an acute malaise which resolves within 12–24 h (Berg et al., 2004; Burton et al., 2011; Leon et al., 1999; Rudaya et al., 2005). We used C57BL/6 mice since they are one of the most commonly used strains in research. The aim of this experiment was to determine if nest building could be used as a daily task to evaluate animal health, so behavior other than presence or absence from the nest was not observed or recorded. It is likely that more severe prostration or illness-related changes in nesting behavior may have been evident earlier in the progression of the cytokine storm. An unexpected result of this study was that LPS-treated mice were not found outside of the nest. Recent work by Yee and Prendergast (2012) with LPS-treated rats may provide insight into the behavior of mice after LPS treatment. The motivation to remain in contact with conspecifics, perhaps for heat conservation reasons such as huddling (Yee and Prendergast, 2010), and the inability to move far enough away in order to isolate themselves from other animals (Yee and Prendergast, 2012) due to the size of standard caging resulted in a high amount of variation in cage location causing no significant effect of treatment in this variable. The untreated mice may have had ambivalent social responses to the LPS-treated mice, but been unable to express them fully. Since behavior throughout the period of illness was not directly documented and our binomial nest occupation observation occurred approximately 16 h after injection, it is possible that by the time we made our observations, the LPS-treated mice had recovered sufficiently to begin engaging in social behaviors. The point of this study was to mimic normal laboratory procedures and determine if this technique could identify animals that had become sick overnight. Previous literature that identified changes in social behavior after LPS injection made their observations at 2 (Renault et al., 2008), 4 (Arakawa et al., 2009), and up to 10 h (Yee and Prendergast, 2010, 2012) post-injection. This study was designed to mimic observations made by animal caretakers, not to carefully document behavior of mice after LPS injection. Anecdotally, only a few mice appeared ill (indicated by hunched postures and lethargic movement) at the time of observation.

Mice rely heavily upon olfactory stimuli for communication and olfaction is considered a primary sense in this species (Brown, 1985; Latham and Mason, 2004). Health status is one of the many pieces of information that can be detected by other mice through the use of odorant communication (See review by Arakawa et al., 2011). Scent marks left by male mice challenged with nonreplicating bacteria were found to be less attractive to females (Zala et al., 2004). Males infected with influenza virus or parasitized were also less attractive to females (Kavaliers and Colwell, 1995; Penn et al., 1998). LPS appears to be sufficient to produce avoidance-eliciting odor cues at 4 h post injection from the soiled bedding of an adult male rat (Arakawa et al., 2009). However odor cues from female mice may not be as strong a signal as those produced by adult males (Arakawa et al., 2008; Arakawa et al., 2011; Renault et al., 2008). Results from Renault et al. (2008) suggest that healthy female mice do not avoid other LPS injected females, but does not rule out that the ill animals might actively avoid healthy ones. This may also explain why we did not find location differences between saline and LPS injected mice. Since LPS malaise is short-lived, animals with a more chronic or severe condition resulting in greater discomfort may show greater differences in nest scores and cage location than the mice given LPS in this experiment.

A further unanticipated outcome of this experiment was the magnitude of the effect that the novel handler had on the mouse's nest building response. As part of our study design, animals were acclimated to handling, since this is known to affect immune system parameters (Romanovsky et al., 1998), such as their response to LPS. Only one investigator performed the initial training, however. Despite the other investigators having extensive experience handling mice and the same reward being offered, the mice did not generalize the positive handling experience with one handler to being handled by others. Thus the amount of training received by the individual mouse was not sufficient to alleviate stress in this situation. The contribution of the experimenter themselves to the overall variability of the data is quite substantial in behavioral testing, even under highly standardized testing protocols (Chesler et al., 2002; Crabbe et al., 1999). Although the effect of handling on experimental parameters is not a novel finding as this has been documented in rodents (Andrews and File, 1993; Romanovsky et al., 1998; Rudaya et al., 2005; Ryabinin et al., 1999), birds (Lemaho et al., 1992), cattle (Hemsworth, 2003), rabbits (Swennes et al., 2011) and pigs (Hemsworth, 2003), the effect of handling on nest building behavior in mice has not been previously reported.

The strong response by the mice to novel handlers calls into question how mice perceive the way we interact with them. It is common for humans to confuse the way we experience the world with the way that other animals do. We generally do not have the same primary senses as other animals and ecology has shaped our behavior for survival. One study by Pajor et al. (2000) found that dairy cattle disliked being yelled at as much as being shocked by a cattle prod, a very unexpected result, as yelling was not expected to be highly aversive to the cattle at all. The way we handle rodents may be equally as aversive as being yelled at is to cattle. Hurst and West (2010) have investigated the impact of different handling techniques on mice. They found that mice handled by being cupped in a hand or encouraged to enter a tube, which was then picked up, were more likely to voluntarily interact with the experimenter and were less anxious than the mice handled by the tail, the traditional method. Results from the tunnel handling technique were significant after 9 sessions lasting only 60 s and the "taming effect" was not reversed by scruffing the mice. Simply handling the animals differently from birth might result in less stressed animals and only minimal habituation would be needed for novel procedures.

The response of the control mice to the new handler resulted in a significant reduction in nest scores at the time of injection + cage change, similar to animals injected with LPS, indicating the significant effect of a novel handler on mouse behavior. Food consumption for all the mice, whether injected with LPS or saline, was significantly reduced at time points where the animals encountered novel handlers. As food consumption is frequently used as an indicator of illness, pain, or as an end of life measure, these measures may be confounded by the fact that habituation to handling in mice is uncommon. However, our sugared cereal test for anhedonia was best at distinguishing whether 1 or all the mice in the cage were ill. While useful in some circumstances, this measure is difficult to apply as part of a daily monitoring routine and has the potential to interfere with dietary studies. Most tests for anhedonia rely on isolation of mice for testing (Henry et al., 2008; Mateus-Pinheiro et al., 2014) or animals do not respond reliably to stress with anhedonia (Strekalova et al., 2011). Further refinements of this test, while still allowing for group housing, might involve RFID-triggered feeding or drinking apparatuses.

In mice housed alone, nest building after cage disruption can be a useful indicator of illness. However, in group housed mice, the status of the nest does not necessarily indicate animal health or welfare in relation to an acute illness. The status of nest construction may be adversely affected by more chronic illnesses and this was not addressed by this experiment. Further work to elucidate the effects of models of human disease resulting in chronic illness in mice, such as collagen induced arthritis or tumor induction, on nest building would be valuable additions to the literature. Mice do seem to be strongly affected by unfamiliar handlers as shown by nest scores and food consumption in both LPS and saline injected mice. What remains unknown is how quickly mice would adapt to novel handlers; how much familiarity do mice need with their handlers to be unstressed?

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