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Identification of Sick or Dead Mice (*Mus musculus*) Housed with 6 Grams of Crinkle Paper Nesting Material

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Although nesting material is beneficial to the welfare of laboratory mice, provision of appropriate amounts may impair visualization of the mice. In anticipation of our academic research institution transitioning to providing 6 grams of nesting material to all mice, we conducted a 2-step prospective epidemiologic study to 1) evaluate whether 0, 2, or 6 grams of nesting material alters the ability to identify sick or dead mice, and 2) evaluate the number and severity of health concerns identified in the presence of 6 grams of crinkle paper nesting material at cage-side health check as compared with cage change. Animal Treatment Reports (ATRs) and death incidences were collected across a variety of research and breeding uses. This information was used to determine if nesting material prevented prompt identification of mice in need of veterinary attention. The clinical health condition category (CHCC) was determined based on the severity of the animal's health condition on initial veterinary exam. Additional assessment determined if the identification of the animal's condition was a success (early-stage or mild illness when first identified) or a failure (late-stage or endstage illness when first identified). Mice that died spontaneously were also assessed with regard to which observation activity was being performed at the time of the animal's identification (daily health check or cage change) and location of the mouse in relation to the nest. The results showed that nesting material did not cause a significant increase in the severity of CHCCs at the time reported for veterinary evaluation. Successful identification of health concerns occurred significantly more often than failures. Death rates were similar between all nesting groups, and dead mice were more likely to be located outside of the nest. In summary, nesting material did not hinder the ability to identify mice in need of veterinary care during routine cage-side health checks and did not critically affect the ability to identify mice that died spontaneously. These results indicate that mice can receive appropriate amounts of crinkle paper nesting material without lowering the ability of staff to recognize mice in need of veterinary attention.

Abbreviations: ATR, Animal Treatment Report; BLR, Binary Logistic Regression; CHCC, Clinical Health Condition Category; GLM, General Linear Model

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In the United States, the 8th edition of *the Guide for the Care and Use of Laboratory Animals*[21] recommends that environmental enrichment programs be implemented for all species of laboratory animals. However, all enrichments are not equally as beneficial to animals. The characteristics of a truly enriching item or substrate are that it 1) is biologically relevant to the specific species, 2) gives the animal some semblance of control over its environment, and 3) shields the animal from perceived stress.[36] For laboratory mice (*Mus musculus*), nesting material is perhaps one of only a few enrichments that meet all of these criteria. The provision of nesting material to mice is now legally required in Europe[7] and has recently become a standard practice in the United States.[21]

Mice exhibit preferences for particular kinds of nesting material, but the amount provided is also important.[20,34] At least 6 grams of nesting material is required to build a dome-like nest,[8] but more may be needed to achieve the adequate insulation required to reduce the cold stress induced by typical laboratory temperatures.[8,9] Providing 8 grams of nesting material increases breeding performance, increasing the number of pups born to dams at the same level of food consumption[13] and also increasing pup weaning weight.[11] Both male and female mice readily build nests and will do so even when not reproductively active.[8,12] Mice are also motivated to find and use nesting material even when it is not necessary for insulation.[8] If an appropriate type and amount of material is offered, nesting material can aid in assessments of mouse health[10] and provide the opportunity to create a preferred temperature microclimate within the cage,[8] thereby promoting behavioral thermoregulation[3,12,15,16,27] and reducing the physiologic impact of ambient cold stress and the energy expenditure needed to maintain normothermia.[8,9,11,13,23]

Providing nesting material to a laboratory mouse gives it the ability to manipulate its microenvironment and perform goal-directed, species-specific behaviors.[35] In addition, how the nesting material is used can provide an indicator of the animals' wellbeing. Studies have shown that mice experiencing pain, distress, or discomfort do not participate in nest building,[30,31] or nest consolidation, resulting in low quality nests.[1,22] Poor nest quality has also been shown to correlate with high levels of wounding from aggressive interactions.[10] In addition, identifying the latency at which nesting material is being manipulated[31] can provide useful information when studying behavioral deficits in mice[6,32] and can indicate diminished welfare.[2] Therefore,

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the quality of a mouse nest can provide insight into the health and status of cage inhabitants.

Mice and research can both benefit from the provision of nesting material. Mice were considered to be poor models for some disease conditions, yet their suitability improved when home cage temperatures were raised to levels equivalent to those made possible by the provision of sufficient nesting material. These include mouse models of Graft Versus Host Disease[25] and western diet induced atherosclerosis.[14] The immunosuppressive effects of cold stress on tumors is also well studied.[5] Cold stress resulted in higher non-shivering thermogenesis[4] and significantly increased chronic stress, as reflected by increased adrenal weight.[5] These factors all contributed to reduce subcutaneous tumor metabolism in immunodeficient mice; an effect ameliorated by the provision of shelter.[5] Nesting material has also been shown to reduce the occurrence of abnormal behaviors in mice, such as stereotypies.[19] Ultimately, these prior studies illustrate how providing nesting material allows mice to cope with thermal stress in the vivarium by being able to build an insulating nest, resulting in a positive impact on mouse model studies and animal welfare.

Despite all the advantages that nesting material provides to the health and welfare of laboratory mice, it may compromise the ability of care staff to see mice during cage-side health checks. This constraint may result in too little material being provided (< 6 grams) so that mice cannot build a fully domed nest.[8] A smaller, less complete, nest allows easier viewing by care staff, but likely reduces the benefits of providing nesting material to the mice. While the simple provision of material is a legal standard in the European Union,[7] the amount provided is determined by each institution. To determine if 6 grams of crinkle paper nesting material to mice in standard caging would affect the ability of husbandry staff to identify sick or dead mice, we performed a 2-step prospective epidemiologic study. While an epidemiology approach does not have the same structured groupings as more typical controlled studies, it has been used to identify spontaneous disease in laboratory mice[29] and provides an approach to studying the identification of health concerns encompassing a variety of research and breeding purposes. Little published data are available to provide insight into the spontaneous incidence of morbidity and mortality in a large academic institution and little to no published research to support data-driven health monitoring practices as balanced against the provision of environmental enrichment. Therefore, this study aimed to 1) determine whether a disparity in identification of health concerns or deaths occurred when nesting material is present, and 2) determine if the provision of 6 grams of crinkle nesting material impaired the ability of husbandry technicians to identify sick or dead mice during routine cage-side health checks. We hypothesized that the presence of nesting material would not significantly hinder the ability of husbandry or veterinary staff to identify dead mice or mice in need of veterinary intervention.

Materials and Methods

Animals. The animals described in this report were housed at an AAALAC-accredited animal facility at the University of Michigan. A diverse array of strains/stocks, sexes, and ages typical of a large academic setting were included in this study, purchased from a variety of vendors, including Taconic Biosciences (Germantown, NY), Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA). Mouse strains included, but were not limited to, APOE, BALB/c, C57BL/6, CD-1, MYD, NOD SCID, NOD.CB17-Prkdc/J, NOD.CB17-Prkdc^{scid}, Nu/Nu and NSG. The study population included breeding trios and pairs and singly or group housed single-sex experimental mice, with a maximum density of 4 to 5 adults, depending on mouse weight, and ranging in age from 21 d up to 2 y of age. Mouse pups were weaned from the parent cage at 21 d of age unless health concerns delayed weaning. Mice were consistently negative on quarterly surveillance testing involving dirty-bedding sentinels and swabbing of rack plenums for the following pathogenic agents: hepatitis virus (corona, lethal intestinal virus of infant mice), minute virus of mice, mouse parvovirus, epizootic diarrhea of infant mice virus, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus type 3, lymphocytic choriomeningitis virus, mouse adenovirus, polyomavirus, *Mycoplasma pulmonis,* pinworms and fur mites. All procedures and housing were compliant with *the Guide for the Care and Use of Laboratory Animals*,[21] and all animals were included on various protocols approved by the Institutional Animal Care and Use Committee at the University of Michigan.

All mice were housed on ventilated racks in transparent polypropylene cages (Allentown, Allentown, PA) measuring 186 mm × 298 mm × 128 mm and filled with approximately 120 grams of corncob bedding (Bed-o cob, The Andersons, Maumee, OH) from an automated dispenser. The rooms were maintained at 22 °C (± 2 °C) with 30% to 70% relative humidity and a 12:12 h light: dark cycle. Due to Daylight Saving Time, the lights were on during this study from 0500 to 1700 March 1st to March 7th, and 0600 to 1800 March 8th to August 31st. Mice were fed a variety of diets, depending on the primary study protocol, food and water were provided ad libitum. All husbandry and veterinary technicians were trained in rodent care and followed the institution's standard operating procedures. Health checks were performed daily between 0600 and 1600 and cage changes were performed by a husbandry technician every 14 d, or more frequently as needed. As described in more detail below, when a technician identified a mouse needing veterinary attention, an animal treatment report (ATR) was generated and submitted to veterinary staff. If a mouse was found dead in the cage, it was recorded in a room level log.

Census Data. An individual daily mouse census was not performed. An electronic barcode system provided a general statistic of the estimated daily cage census for the rooms used during the 2014 and 2015 study time periods. Based on an average of 3 to 4 mice per cage, we applied similar estimations done in other epidemiologic assessments[29] and approximated the average daily mouse census by multiplying the cage count by 3.5. This provided a conservative estimate for a single day census. To provide a context for the estimated total mouse health observations surveyed, the daily census was then multiplied by the number of days for which data collection occurred (a 6 mo period). To assess the frequencies of ATRs and deaths that occurred during 2014 and 2015, we divided the total number of ATRs and deaths, respectively, by the estimated total animal health observations.

2014 Data. Data were collected from March 1st through August 31, 2014, generating 107 ATRs and 39 deaths, in one room dedicated to a single Principal Investigator focusing on oncology research. On average, 132 cages (approximately 462 mice) were evaluated daily, and approximately 85,008 total animal health observations occurred over this study duration. Animals present in this room were used for breeding and oncology studies. Cages in the study population were located on one ventilated rack and received one of 3 nesting conditions. One-third of the cage population had no nesting material (referred to in the rest

of the paper as 0 grams of nesting material), one-third received a 2 inch square of compressed pulped virgin cotton fiber (2.2 to 2.4 grams: referred to in the rest of the paper as 2 grams of nesting material) (Cotton squares, Ancare, Long Island, NY), and one-third received a small bag, made out of a material used for tea bags, filled with brown crinkle paper nesting material (5.8 to 6.2 grams: referred to in the rest of the paper as 6 grams of nesting material) (Enviropak, Fisher and Son, Sommerville, NJ). The oncology study cages were typically organized in rows across the ventilated rack. To avoid providing only one nesting condition to one study treatment group, we organized the nesting groups in columns on the ventilated rack, so that our nesting conditions were evenly distributed across studies conducted by the oncology lab. Column 1 had 0 grams of nesting material, column 2 was provided with 2 grams of nesting material, and column 3 was provided with 6 grams of nesting material. This pattern was repeated evenly across the ventilated rack. Each nesting group had approximately 28,336 total animal health observations over the 6 mo study in 2014. Death logs for this same group were collected over the same time period. When mice were found dead in the cage, as evidenced by the discovery of any portion of a mouse carcass, the presence or absence of nesting material and the location of mice found dead in or out of the nest was also noted.

2015 Data. Data were collected from March 1st through August 31, 2015, which generated 385 ATRs and 238 deaths from 3 rooms in a single animal facility. In the 3 rooms, the average census during those months was 1,053 cages, and approximately 3,686 mice were evaluated daily. The 2015 study duration included approximately 94,024 total animal health observations. Animals in this facility represent a variety of research areas, including cardiology, metabolism and diabetes, muscular dystrophy, oncology, and general breeding. Research areas that represented less than 10% of the studied population were group together into an "other" category. The same oncology laboratory, building, and room studied in 2014 were also part of the 2015 data collection. In contrast to the 2014 data, all mouse cages were provided with 6 grams of nesting material in the form of a small bag made out of a material used for tea bags, filled with brown crinkle paper nesting material (Enviropak, Fisher and Son, Sommerville, NJ).

Over the same time period, death logs from the same 3 rooms were documented. When animals were found dead in the cage, as evidenced by any portion of a dead mouse, the presence of nesting material was confirmed, and the number of animals found dead in or out of the nest was noted. Further, notation was also made concerning whether the dead animals were identified during cage-side health check or at cage change (referred to in the rest of the paper as observation activity).

ATR Data Collection. When clinical health concerns were noted during cage-side health checks or cage changes, an animal treatment report (ATR) was generated and given to the veterinary technicians. Upon initial evaluation, the veterinary technician would assess the clinical health of the animal and determine a clinical condition score. The clinical condition score was classified as a mild, moderate, or severe clinical health condition category (CHCC) to determine the degree of severity at which health concerns were being identified and reported (Figure 1). The clinical condition scoring system is part of a standard of care for which all veterinary technicians are trained. Using this system decreased subjective distinctions in the animals' health scores and improved concurrence between technicians.

Due to the type of research being conducted (that is, oncology or metabolic disease), an animal might be assigned a severe health condition category but was successfully identified as a humane endpoint for that particular project. Therefore, each ATR was also categorized as either a success (correct identification of early-stage illness or at the specified humane endpoint) or a failure (the animal was found at a late-stage or endstage of illness; see Figure 2).

2014 Additional Data collection. During the 2014 study, the individual who identified the health concern would note on the ATR if the cage consisted of 0, 2, or 6 grams of nesting material. The number of ATRs were summed across each category.

2015 Additional Data Collection. During the 2015 study, the individual who identified the health concern would note the observation activity (that is, whether the ATR was generated during a daily cage-side health check or at cage change) and verified that 6 grams of nesting material were present. The number of ATRs were summed across each clinical condition category, research area and observation activity.

Death Log Data Collection. When animals were found dead in the cage, the presence or absence of nesting material, the type of material provided, and whether the dead mouse was found in or out of the nest was noted. In 2015, we also noted whether the death was identified during daily cage-side health check or at cage change. Sex and age were not consistently recorded and therefore could not be analyzed.

Statistics. The 2014 and 2015 data from the same Principal Investigator were analyzed using descriptive statistics. All 2015 ATR data were analyzed as a general linear model (GLM) in JMP 13 (SAS, Cary, NC). The assumptions of a GLM (normality of error, homogeneity of variance, and linearity) were confirmed graphically post hoc[18] and appropriate transformations made if necessary. CHCC data was log10 transformed to meet these assumptions. All cages in this dataset contained nesting material. The number of ATR events were summed for each combination of the following variables: observation activity (that is, at cage change or during health check), clinical health condition category (mild, moderate, or severe), and study type (metabolism/diabetes, oncology, physiology, and surgery); resulting in a count of observations for each of the 24 possible variable combinations. All these variables and the interaction of observation activity and CHCC were tested in the single GLM. If statistically significant variables were identified, Tukey pairwise comparisons were used posthoc to determine differences between levels in the variable (such as between mild and severe CHCCs).

The number of ATRs considered to be successes or failures were summed for each combination of the following variables: observation activity (documentation at cage change or during health check), success or failure, and study type (metabolism/diabetes, oncology, physiology, and other); resulting in a count of observations for each of the 16 possible variable combinations. All of these variables and the interaction of observation activity and success or failure were tested in a single GLM. Data were also log10 transformed for normality. If statistically significant variables were identified, Tukey pairwise comparisons were used posthoc to determine differences between levels in the variable.

A χ^2 analysis was used to determine whether ATRs were reported more often than expected by taking the (total number of ATRs / 184 d data was collected) * the number of observation days per observation activity (cage change or daily health check). Cage change was done every 2 wk over the 184 d data collection, which is approximately every 13 d. Daily health

Mild	0	No significant findings and required no treatment or monitoring.
	1	Bright, alert, responsive, and warrant monitoring but did not require treatment. (Example: small tumor)
Moderate	2	Bright, alert, responsive and required treatment in addition to monitoring. (Example: teeth or nail trim, ointment application, etc.)
	3	Quiet, alert, responsive, and required treatment with heat, fluids, or food supplements and were rechecked within 24 h. (Example: dehydration or difficult recovery postoperatively)
Severe	4	Lethargic or at the protocol endpoint, and the lab was contacted with a 24 h euthanasia notice. (Example: reluctant to move with poor body condition or a tumor endpoint per protocol)
	5	Moribund and requested immediate euthanasia or was found dead.

Figure 1. Clinical Health Condition Categories and corresponding recorded severity score designated by the veterinary technician after evaluating the animals.

Success	**Early Identification:** Alert and responsive mice identified for an issue at a stage of illness when monitoring with or without a treatment intervention could be implemented. **Identification at Protocol Endpoint:** The animal was identified at a stage of illness that met protocol endpoints but did not reach end-stage illness guidelines. Euthanasia performed within 24 h by the lab.
Failure	**Late Identification:** Mice were identified at a stage of significant illness or a condition that was not suitable for treatment. Euthanasia was performed by the lab within 24 h. **Identification at End-Stage Illness:** Mice were identified at end-stage illness by the husbandry staff but upon initial evaluation by the veterinary staff were found dead or require immediate euthanasia.

Figure 2. Success and failure category descriptions.

checks in which the cages were not changed were conducted on 168 d during this time period.

Analysis of the death log data only included adult mice 21 d of age and older. A binary logistic regression (BLR) with likelihood estimation was run to determine whether a difference existed in the likelihood of mice being found in or out of the nest based on the observation activity (cage change or daily health check). The model included the type of study and whether mice were found dead at cage change or not. A χ^2 analysis was also run to determine whether mice were found dead more often than expected. A similar method to the χ^2 analysis used the (total number mice found dead / 184 d data was collected) * the number of observation days per observation activity (cage change or daily health check).

Alpha was set at 0.05 for all analyses, and all data graphed as least square means ± SE (LSM ± SE) and raw counts of data are presented in the tables.

Results

ATR data. ***Comparison of 2014 and 2015 ATRs from a single oncology laboratory.*** A summary of the raw 2014 and 2015 ATRs, collected from the mice of a single oncology Principal Investigator, are shown in Table 1. A low percentage of the total mouse population (approximately 0.13% in 2014 and 0.16% in 2015) received ATRs, based on the estimated total number of animal observation opportunities during the 6 mo data collection period (average number of mice per day * number of days). (Table 1)

In 2014, the daily average total census consisted of 462 mice per day, with approximately 154 mouse deaths and approximately 28,336 total animal health observations in each nesting material group (0, 2, or 6 grams) over the 6 mo study. In 2015, the daily average census consisted of approximately 511 mice per day, equaling approximately 94,024 total animal health observations, with all cages receiving 6 grams nesting material. In 2014, a total of 107 ATRs were reported. Of that total, 38 ATRs were from mice in the 0 gram group, 34 in the 2 grams group, and 35 in the 6 grams group. In 2015, a total of 148 ATRs were reported from mice given 6 grams of nesting material. In 2014, mild CHCCs were numerically more frequent than moderate or severe (see Table 2). In addition, successful ATRs were numerically more frequent in all 3 nesting material groups than were failures (see Table 3). As seen in Tables 2 and 3, respectively, the CHCC and success/failure data from 2015 is similar to the 2014 data.

2015 only ATRs. A summary of the raw 2015 ATR data was used to evaluate CHCC and successes or failures (Table 4 and 5, respectively). A total of 376 ATRs were generated over 6 mo from cages with 6 grams of nesting material. The average census was 3,686 mice per day, totaling an estimated 678,224 animal health observation opportunities over the 6 mo to assess individual mouse health.

Our main question of whether nesting material inhibits the ability of husbandry staff to identify sick mice at the early stages of illness during cage-side health checks was tested with an interaction between observation activity (cage-side health check compared with cage change) and CHCC severity (see Table 4). If nesting material hindered the ability to recognize sick mice, we would anticipate mice displaying the early signs of illness would be missed during daily health checks and as a result, more severely sick mice found at biweekly cage change.

Table 1. A summary of the average mice surveyed, and ATR entries collected from one oncology Principal Investigator in 2014 and 2015.

Year	Average cage census per day	Average number of mice per day	Average number of animal health observations over 6 months	Number of ATRs generated in 6 months
2014	132	462	85,008	107
2015	146	511	94,024	148

Table 2. A comparison of the percentage of animal health observations over the 6 mo period and raw number of ATRs generated in each CHCC category over in 2014 and 2015.

Year - study group	Average animal health observations over 6 months	Mild	Moderate	Severe	Total ATRs
2014 – 0 grams	28,336	0.099% (31)	0.011% (3)	0.014% (4)	38
2014 – 2 grams	28,336	0.099% (28)	0.014% (4)	0.007% (2)	34
2014 – 6 grams	28,336	0.081% (23)	0.021% (6)	0.021% (6)	35
2015 – 6 grams	94,024	0.141% (133)	0.005% (5)	0.011% (10)	148

A total of approximately 85,008 animal observation opportunities occurred in 2014 during those 6 mo and each treatment was applied to 1/3rd of the colony, which would equal approximately 28,336 observations per treatment. In 2015, all cages received 6 grams of nesting material and approximately 94,024 animal observation opportunities occurred in 2015 during the 6 mo study. The raw number of ATRs generated is listed in parenthesis under the percentage of ATRs identified out of the total animal health observations in each category.

Table 3. The raw number of 2014 and 2015 ATRs generated from the same lab over each month period study that were categorized as an identification success (animals were detected at an early stage of illness or at an appropriate humane endpoint) or failure (late identification or at end-stage of illness).

Year - study group	Average animal health observations over 6 months	No significant findings	Success	Failure	Total ATRs
2014 – 0g	28,336	0.011% (3)	0.109% (31)	0.014% (4)	38
2014 – 2g	28,336	0	0.113% (32)	0.004% (2)	34
2014 – 6g	28,336	0	0.109% (31)	0.014% (4)	35
2015 – 6g	94,024	0.001% (1)	0.147% (139)	0.009% (8)	148

The raw number of ATRs generated is listed in parenthesis under the percentage of ATRs identified out of the total animal health observations in each category.

Table 4. The raw number of ATRs generated at health check or cage change, in each CHCC category and study category, over the 6 mo study in 2015. A total of 376 ATRs were documented from cages with 6 grams nesting material.

		2015 CHCC found at health check (193)			2015 CHCC found at cage change (183)		
Study category	Average animal health observations over 6 months	Mild	Moderate	Severe	Mild	Moderate	Severe
Metabolism/ Diabetes	117,944	0.011% (13)	0.001% (1)	0.002% (2)	0.001% (1)	0	0
Oncology	203,504	0.056% (114)	0.005% (10)	0.008% (17)	0.062% (126)	0.009% (18)	0.004% (8)
Physiology	327,152	0.006% (18)	0.002% (6)	0.002% (5)	0.006% (18)	0.002% (7)	0.001% (2)
Other	29,624	0.017% (5)	0	0.007% (2)	0.007% (2)	0	0.003% (1)
Total Data Summary	678,224	0.022% (150)	0.003% (17)	0.004% (26)	0.022% (147)	0.004% (25)	0.002% (11)

The raw number of ATRs generated is listed in parenthesis in addition to the percentage of ATRs identified out of the total animal health observations in each category.

However, the severity of the CHCC did not differ between daily health check or at biweekly cage change ($F_{2,15} = 1.12$; $P = 0.35$). Furthermore, the main effect of observation activity (daily health checks or biweekly cage change) also did not alter the overall number of ATRs generated ($F_{1,15} = 4.48$; $P = 0.051$). Because the number of observations for the 2 activities was not equal (13 cage-side: 1 cage change observation), a χ^2 analysis comparing the actual observed ATRs per observation activity with the expected rate of ATRs (see statistics section in the methods) was conducted. Based on this analysis, the rate at which ATRs were reported was higher at cage change due to the lower frequency of observations ($\chi 2 = P < 0.001$). Regardless of whether the mice were first identified during health check or cage change, there was a significant difference in the number ATRs between the 3 CHCCs ($F_{2,15} = 19.20$; $P < 0.001$). Overall, mild CHCCs were documented statistically more often than moderate (Tukey; $P < 0.05$) or severe (Tukey; $P < 0.05$). Further, the type of study conducted significantly affected the number of ATRs produced ($F_{3,15} = 32.03$; $P < 0.001$). Oncology studies produced significantly more ATRs than any other study category (Tukey; $P < 0.05$). Physiology studies produced more ATRs than metabolism/diabetes studies and the "other" research category (Tukey; $P < 0.05$) but there was no difference between metabolism/diabetes and other (Tukey; $P > 0.05$). Table 4.

Table 5. The raw number of 2015 ATRs generated over 6 mo that were categorized as an identification success (animals were detected at an early stage of illness or at an appropriate humane endpoint) or failure (late identification or at end-stage of illness).

		2015 ATR found at health check (193)		2015 atr found at cage change (183)	
Study category	Average animal health observations over 6 months	Success	Failure	Success	Failure
Metabolism/ Diabetes	117,944	0.012% (14)	0.002% (2)	0.001% (1)	0
Oncology	203,504	0.061% (125)	0.008% (16)	0.071% (144)	0.004% (8)
Physiology	327,152	0.007% (24)	0.002% (5)	0.008% (27)	0
Other	29,624	0.017% (5)	0.007% (2)	0.010% (3)	0
Total Data Summary	678,224	0.025% (168)	0.004% (25)	0.026% (175)	0.001% (8)

A total of 376 ATRs were produced from cages with 6 grams of nesting material. The raw number of ATRs generated is listed in parenthesis in addition to the percentage of ATRs identified out of the total animal health observations in each category.

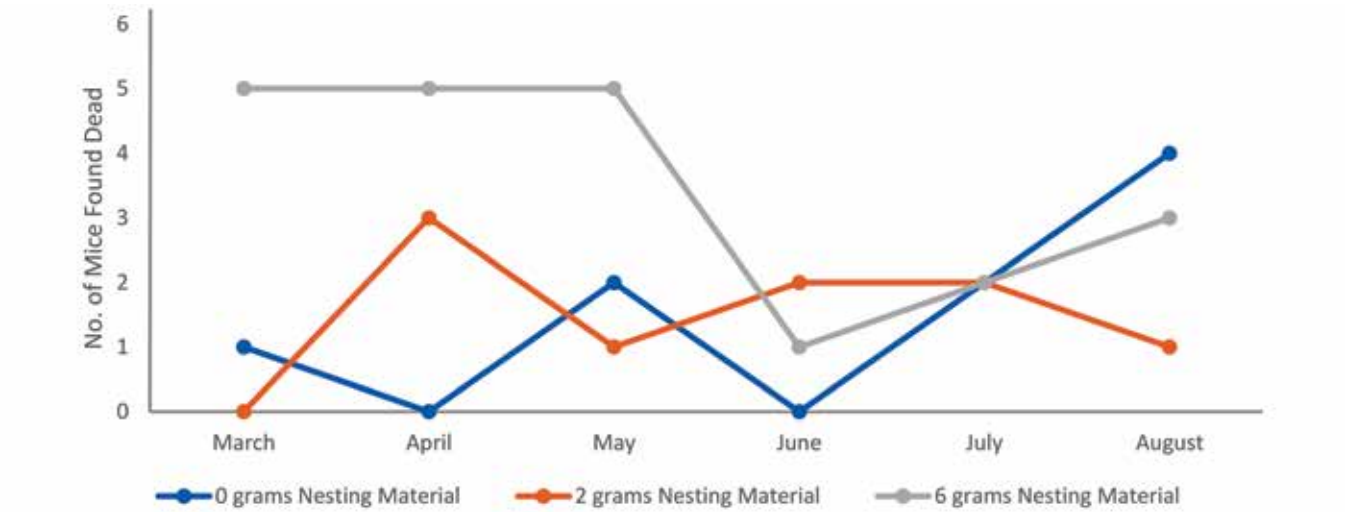

Figure 3. The raw number of mice found dead per month in 2014 compared between nesting groups, from one Principal Investigator, housed in one room. March had a total of 6 deaths, (1 to 0gram/0 to 2gram/5 to 6gram), April had a total of 8 deaths, (0 to 0gram/3 to 2gram/ 5 to 6gram), May had a total of 8 deaths, (2 to 0gram/1 to 2gram/5 to 6gram), June had a total of 3 deaths, (0 to 0gram/2 to 2gram/1 to 6gram), July had a total of 6 deaths, (2 to 0gram/2 to 2gram/2 to 6gram) and August had a total of 8 deaths, (4 to 0gram/1 to 2gram/ 3 to 6grams). The study population was divided evenly between the 3 nesting groups. On average, 132 total cages (approximately 462 mice) were evaluated daily and approximately 85,008 total animal health observations occurred over this 6 mo period.

The analysis of the ATR success and failure is similar to the CCHCs. Overall, more ATRs were reported during daily health check ($F_{1,9} = 7.3$; $P = 0.024$). However, because the number of observations of the 2 activities was not equal (13 cage-side:1 cage change observation), more ATRs were reported at cage change due to the lower frequency of observations ($\chi 2 = P < 0.001$). Reporting rates were equivalent at daily cage-side exams and biweekly cage change for both successful identification and failure (see Table 5), ($F_{1,9} = 0.95$; $P = 0.35$). Thus, the risk of failure was not higher when animals were observed during daily cage-side health check as compared with biweekly cage change. Whether the mice were observed at cage-side or cage change, successful identifications were more common than failures ($F_{1,9} = 30.70$; $P < 0.001$). Study type was again significant ($F_{3,9} = 32.03$; $P < 0.001$), with oncology studies accounting for significantly more ATRs than the other areas (Tukey; $P > 0.05$). Table 5.

Death Logs. ***2014 and 2015 Death Logs from a single oncology laboratory.*** In 2014, a total of 39 deaths were reported out of a daily census of approximately 462 mice, about 8% mortality, during the 6 mo study. Over the study duration in 2014, approximately 85,008 total animal health observations were made. The cage population was divided into thirds, resulting in a total of 28,336 opportunities for care staff to identify any dead mice over this 6 mo period, from cages with 0 grams of nesting material, 2 grams of nesting material and 6 grams of nesting material. Of 39 mice reported on the death logs over the 6 mo observation period , 9 were from cages with 0 grams of nesting material (0.03% of the total animal health observations), 9 were from cages with 2 grams of nesting material (0.03% of the total animal health observations) and 21 (0.07% of the total animal health observations) were from in cages with 6 grams of nesting material. The number of mice with 6 grams of nesting material that were found dead each month in 2014 decreased numerically over the time period during which mice had the nesting material (Figure 3).

In 2015, a total of 42 deaths were reported out of a census of approximately 511 mice per day, resulting in about 8% mortality over the entire 6 mo study. This represents a total of 94,024 opportunities for care staff to identify any dead mice in cages with 6 grams of nesting material over this 6 mo period. In 2015, the percent found dead out of the total number of health observations made over the 6 mo period was approximately 0.04%.

2015 Death Logs. A summary of the raw 2015 complete data set is found in Table 6. A total of 230 adult death log entries were made from a census of approximately 3,686 mice per day,

Table 6. The raw number of mice found dead over 6 mo in 2015. A total of 230 adult death log entries were documented from cages with 6 grams of nesting material.

		2015 found dead at health check (156)		2015 found dead at cage change (74)	
Study category	Average animal health observations over 6 months	In nest	Out of nest	In nest	Out of nest
Metabolism/ Diabetes	117,944	0.003% (4)	0.007% (8)	0.002% (2)	0.003% (3)
Oncology	203,504	0.016% (32)	0.029% (60)	0.011% (22)	0.014% (28)
Physiology	327,152	0.001% (3)	0.012% (38)	0.002% (5)	0.003% (8)
Other	29,624	0.030% (9)	0.007% (2)	0.017% (5)	0.003% (1)
Total Data Summary	678,224	0.007% (48)	0.016% (108)	0.005% (34)	0.006% (40)

The raw number of deaths identified is listed in parenthesis in addition to the percentage of deaths reported out of the total animal health observations in each category.

or about 6% mortality over the 6 mo period. This represents a total of 678,224 opportunities for care staff to identify any dead mice over 6 mo, from cages with 6 grams of nesting material. The percentage of total animal health observations in which dead mice were found was approximately 0.034%. Dead mice were significantly more likely to be found outside of the nest during cage-side health check than at cage change (BLR = 3.88; $P = 0.048$). Of the 230 total dead mice, 148 (64%) were located outside of the nest and 82 (36%) were found dead with some portion of their body inside of the nest. Regardless of where the mice were found, 156 were identified on cage-side health check and 74 were found dead at biweekly cage change. The study type again influenced the likelihood of mice being found outside the nest (BLR = 26.71: $P < 0.001$).

A χ^2 analysis was used to compare the actual observed deaths per observation activity with the expected rate of death (see statistics section in the methods). Based on this analysis, the rate at which dead mice were reported was higher at cage change due to the lower frequency of observations compared with daily health checks ($\chi 2 = P < 0.001$). Table 6.

Discussion

Optimal amounts of nesting material should be supplied to support animal care, health, and wellbeing.[8,9,17,34] In addition, care staff must verify adequate provisions of food, water, and enrichment and perform comprehensive daily animal health assessments to ensure that veterinary care will be provided as needed. Typically, animal wellbeing is confirmed by visual inspection of the animals themselves, although other signs of health and wellness in the cage environment can also be used. Increasingly complex and complete nests make it harder to observe mice during cage-side exams, particularly while the lights are on and mice are naturally inactive.[33] Thus, animal care programs must weigh the relative risks of providing optimal amounts and types of nesting material with the consequent decreased visibility of the animals at cage-side health check. This can potentially delay identification of health concerns until the cage is changed.

In the 2014 study of the single oncology Principal Investigator's mouse colony, we compared mouse health concerns reported over a 6 mo period in each of the 3 groups: 0, 2, and 6 grams of nesting material. The overall occurrence of health conditions and the stage of illness when reported was assessed. During the 2014 study, we found a similar rate of ATRs across the 3 nesting conditions. Providing up to 6 grams of nesting material did not result in health conditions being identified at a later stage of illness. These results indicate that provision of nesting material did not prevent the ability to identify health concerns. In all 3 groups, more mild clinical condition scores were found than either moderate or severe. In addition, more mice with health concerns were successfully identified in all 3 groups. As a result these mice could be monitored and treated rather than euthanized. The mouse colony of the same oncology lab was studied in 2015, with all cages given 6 grams of nesting material. In this 2015 data set, the ATR data from cages with 6 grams of nesting material was consistent with the 2014 ATR data from all 3 nesting material groups. Again, mild clinical health condition scores were numerically more frequent than moderate or severe, confirming that mice displaying early signs of illness were successfully identified. These results support that 6 grams of nesting material does not hinder the ability of husbandry or veterinary staff to identify mice in need of veterinary intervention.

In 2015, we conducted the same assessment of mouse health and death incidences as in 2014, but with 6 grams of nesting material provided to all cages, and further evaluated the observation activity at the time of identification. The observation activity performed at the time of the health observation was included to evaluate whether the identification of sick or dead mice was influenced by whether the animal health observation was made during a daily cage-side health check or biweekly cage change. We anticipated that if 6 grams of nesting material did interfere with the ability to recognize mice in need of veterinary attention, earlier signs of illness would not be identified during daily cage-side observations, and the data would show an increase in the number of severely sick mice first identified at cage change. The study findings showed that, based on the frequency of observations at health checks compared with cage change, health conditions were identified at a higher rate during cage change. However, severe health concerns, or failures, were not higher at cage change as compared with health check. This finding confirms that the presence of 6 grams of nesting material did not increase the risk of more severe health outcomes for the mice. While this may imply that large nests are reducing the ability of staff to identify health concerns during daily observations, cage-side health checks overall have previously been shown to identify fewer health concerns than cage change[24] regardless of the presence of nesting material. At cage change, staff must pick up animals, often inciting ambulation and other active behaviors, and they may take more time to observe the condition of the cage and animals than during the cage-side health check. We found no significant difference in the number of mice identified for veterinary attention at cage change, compared with daily health checks in each of the 3 clinical health

Figure 4. Visual guide created as a training tool for health check procedures in cages with 6 grams of crinkle paper nesting material.

categories. Regardless of whether a cage-side health check or cage change observation was made, significantly more animals were successfully identified during the early stages of illness, allowing monitoring with or without treatment intervention or at protocol-specific endpoints. Based on the frequency of observations no statistically significant differences were detected in success or failure of identifying mice in need of veterinary care between daily cage-side health check and biweekly cage change, indicating health concerns can be successfully identified without opening the cage. This should allay the fear that mice may experience extended periods of unalleviated pain or distress before receiving veterinary care in cages with robust nests even though these mice may be more difficult to visualize directly. These results support the concept that husbandry staff can identify sick mice during routine daily cage-side health checks and are providing timely recognition of mice in need of veterinary attention even when 6 grams of nesting material is provided.

The presence of nesting material did not increase the number of animals reported for severe health issues, however, the type of research affected the type of CHCCs documented. Oncology research had the largest reported number of animal health concerns; but the vast majority were reported at the mild stage of clinical illness. Taken together, our results indicate that husbandry staff can readily identify mice in need of veterinary care, regardless of the presence of nesting material, at early stages of disease across the wide variety of mouse models.

Dead mice should ideally be quickly identified and removed from the home cage. A peer academic institution with a similar size and diversity of rodent research types reported a roughly 10% spontaneous mortality rate[29] of adult mice. The mortality rates in our study ranged from 6 to 8%. An animal care program considering the provision of optimal nesting material may be concerned that mice will be more likely to die inside of the nests, making it difficult to quickly identify them, and thus increasing the number of mice found dead at biweekly cage change. The comprehensive death log data from 2014 showed comparable deaths for mice with 0 and 2 grams of nesting material, with a small numeric increase in the number of mice found dead in cages with 6 grams of nesting material. These results may suggest that 6 grams of nesting material is contributing to more animals being found dead, but when the data was broken down by month, a decline was observed in the percentage of mice given 6 grams of nesting material and found dead as the study progressed. A larger number of deaths occurred during the first 3 mo in cages with 6 grams of nesting material, but during the last 3 mo deaths were similar in number between all 3 nesting groups. While many factors could lead to animals being found dead, these results imply that a transition period occurs when introducing nesting material, and the ability to identify mice prior to death improves with time and practice. This transition may be due to both human and animal factors. The mice may be learning how to use the new material effectively, and technicians are likely adapting a different approach to assessing cages with large nests. Although low to begin with, the overall percentage of deaths in cages with 6 grams of nesting material decreased numerically from 2014 to 2015. The percentage of mortality in cages with 6 grams of nesting material in 2015 was similar to the 2014 groups with 0 and 2 grams of nesting material. This provides some support to the idea that both mice and humans require an adjustment period when new amounts of nesting material are provided.

The 2015 death log data showed an overall higher likelihood of mice being found dead outside of the nest, suggesting the nests did not significantly hinder the discovery of dead animals. The reason for commonly finding dead adult mice outside of the nest is still unknown. They may leave the nest when they are moribund, be exiled from the nest by cage mates, or removed from the nest by their cage mates postmortem. Dead mice located outside of the nest were more likely to be found during daily cage-side health checks than during cage change. The ratio of mice found outside the nest during a routine cage-side health check was over twice the number found inside the nest, while the ratio of mice found dead during a cage change was approximately equivalent. This could be a result of dead mice that were located outside of the nest and therefore removed from the cage at a higher rate during daily health checks, prior to cage change, resulting in a higher ratio of mice remaining inside the

nest found dead at cage change. When the number of observations is factored in (χ^2 analysis), the number of mice found dead at daily cage-side health check is less than the expected rate. Much like the identification rate of health conditions,[24] this may be a result of increased examination and time spent to observe the condition of the cage and animals during cage cleaning. Mice were not found outside the nest equally across the type of research being conducted. Thus, more vigilant observation may be necessary for certain types of research. Unfortunately, due to unbalanced data, we could not test interactions between where mice were found dead and the nest and study type.

Together with previously published literature,[8,9,10,12,15,16,27,35] the current studies have led to 6 grams of crinkle paper nesting material becoming a standard provision for our 49,000 daily mouse cage census. Our standard operating procedure for daily cage-side health checks has been updated to include evaluation of the shape and complexity of the nest and cage environment as indicators of mouse health. A visual guide of the process is depicted in Figure 4. We recognize that environmental enrichment may inhibit full visualization of rodents during daily cage-side health checks, so other factors such as nest building, cage organization, and movement within the nest are now being used to inform the health assessments of the mice. The presence of fresh feces, urine, and an organized cage (a well-formed nest located away from the urine site)[28] is indicative of healthy mice inside the cage. Movement within the nest, even if not fully visualized, is an indication of active animals. Healthy social behavior and interaction with the nesting material are also key components to assess. The lack of cage organization or a flat poorly formed nest may indicate poor welfare and the need for close monitoring of the mice in this cage. Unusual social behavior in which animals, or a single animal, are nesting separately from the group may also warrant increased monitoring. Figure 4.

One limitation of this study is the amount of nesting material provided to the cages. The recommended amount of nesting material needed to achieve sufficient insulation to reduce cold stress is 8 to 10 grams.[8,9] We did not evaluate how providing 8 to 10 grams would affect the ability to observe health conditions or deaths. Furthermore, only crinkle paper was provided in the larger 6 gram quantity, and our results may not be applicable to all other varieties of nesting material. However, nests made with crinkle paper have been shown to be larger and more dome-like than nests made with tissues or compressed cotton.[20] Thus, if evaluation of visibility is a concern when providing nesting material, crinkle paper provides the best model. Another limitation is that additional factors are likely to affect the quality of nests, including background strain or stock or genetic manipulation of the mouse, sex, unique environmental factors in specific facilities and other unknown factors. We attempted to include strain or background strain into our analyses, but data were not consistently recorded. However, we were able to verify how the mice were used in research. Health conditions warranting an ATR are more common in mice used by an oncology lab compared with animals used purely for behavioral studies. This information was included to account for variability in the data but was not meant for extrapolation to other research in these areas. However, these data may provide information to husbandry staff about what kind of research may need increased vigilance. An additional limitation to this study is that we evaluated correlational data and did not assess any direct affect nesting material had in relation to causing health conditions or death incidence.

Implementing enrichment programs for all laboratory animals species is recommended by the eighth edition of the *Guide For The Care And Use Of Laboratory Animals*.[21] Multiple studies have shown the health and welfare benefits of providing optimal nesting material to mice.[19,26,34,35] However, some institutions may refrain from providing the recommended type and amount of nesting material due to concerns that larger and more complete nests will hinder the identification of sick or dead mice. Our results indicate that providing up to 6 grams of nesting material does not critically inhibit the ability of animal care staff to adequately identify sick or dead mice in a standard mouse cage, suggesting that this concern should not be an impediment to providing mice with an appropriate amount of nesting material.

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