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# Housing condition and nesting experience do not affect the Time to Integrate to Nest Test (TINT)

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## Abstract

*Managing and assessing well-being in laboratory mice (*Mus musculus*) is both challenging and necessary. Assessments intended to detect negative welfare states in mice are usually performed via observation of animals in the home cage, but a substantial amount of time and skill may be required to detect subtle behavioural changes. The Time to Integrate to Nest Test (TINT) is a simple, cage-side assessment tool that identifies the presence or absence of a highly motivated normal behaviour in mice. The test is conducted by adding a small amount of new nesting material to a mouse cage. A positive outcome is achieved when this new material is integrated into the home nest within 10 min. This study examined whether housing condition or nesting experience affects TINT outcome. Single or group housing did not influence the TINT outcome, but a significant difference in latency to integration was found; singly housed mice took longer than group-housed mice to integrate TINT substrate. Mice which were raised naïve to nesting material had no significant delays when tested. However, experience with the TINT procedure showed increased speed to incorporate the testing substrate, indicating that previous experience to the paradigm prior to experimental testing may be necessary. These findings help to define the expected outcomes of the TINT, better positioning it for use as an assessment tool in varied research settings.*

**Keywords**: *animal welfare, behaviour, husbandry, mice, nest building, stress*

## Introduction

Although they are the most commonly used mammals in biomedical research today, recognition and management of well-being in mice (*Mus musculus*) has lagged behind that of other species such as primates and canines. Assessment challenges include their typical prey species' stoicism, nocturnal activity period, small size, and a lack of understanding as to what constitutes normal behaviour (Flecknell 1994; Hawkins 2002; Stasiak *et al* 2003; Matsumiya *et al* 2012). The Time to Integrate to Nest Test (TINT) is an assessment tool developed to provide people who work with mice with a simple and effective way by which to measure behavioural homeostasis and, by extension, well-being (Rock *et al* 2014). The TINT is based on the principle that nest building is a highly motivated behaviour demonstrated by all strains of laboratory mice (Latham & Mason 2004; Rock *et al* 2014). It is because of this high level of motivation that, when presented with a novel piece of nesting substrate, mice will collect and integrate that nesting substrate into their home nest within 10 min (Rock *et al* 2014) resulting in a positive TINT outcome. Failure to perform this behaviour within 10 min should be considered abnormal for most group-housed, inbred mice, generating a negative TINT outcome. This should trigger further examination of the mice. To ensure that this assessment tool is useful across a wider variety of laboratory conditions, we have tested its applicability in singly housed mice and in mice that are naïve to nesting material. We also tested two different, commonly utilised nesting substrates to determine how different materials would affect the TINT.

Single housing of mice is a common practice in the United States, despite the fact that mice are a social species. As such, housing them singly should be "justified on experimental requirements or veterinary related concerns about animal well-being" (National Research Council 2011). We know that social housing influences the stress response of mice and is linked to a decrease in post-operative recovery and stress (Pham *et al* 2010). Single housing is still commonly utilised during post-operative recovery periods, however, to provide investigators with the ability to directly monitor individuals, or when animals have implants that might be damaged by conspecifics. Housing conditions are known to affect the outcome of assessment tools that are behaviourally based (Ader *et al* 1991; Sherwin 2003; Spani *et al* 2003; Van Loo

*et al* 2004, 2007), such as the TINT. In pilot work, singly housed mice were more likely to achieve negative TINT outcomes (Rock *et al* 2014). That study, however, did not strictly control for group size to test the effect of housing condition on TINT outcome. Therefore, for Experiment 1, we hypothesise that housing conditions will have an effect on the outcome of the TINT; singly housed mice will be more likely to achieve a negative TINT outcome.

Despite evidence available that suggests that nesting materials are beneficial to mice in laboratory settings (Bult & Lynch 1997; Van de Weerd *et al* 1997; Gross *et al* 2011; Gaskill *et al* 2013; Jirkof *et al* 2013), nesting material is not consistently provided to all research animals in the United States. For example, despite updated welfare legislation which directs its use, the amount and type of nesting material used is not consistent across laboratories worldwide. Adoption of the TINT assessment method could be problematic if mice required an acclimation period to different nesting materials. There are many different nesting substrates available to laboratories (Enviro-dri®, Nestlet™, facial tissues, etc) which result in varied nest quality (Hess *et al* 2008) and should be considered as a factor which could affect the TINT. We hypothesise that the type of nesting material provided in Experiment 2 will not affect the TINT outcome in healthy, unmanipulated mice.

There is a genetic and anatomic basis by which mice learn how to utilise nesting substrate to achieve a functional nest (Lee 1972; Lynch & Hegmann 1972; Lynch 1981; Deacon *et al* 2002). Mice with limited nesting experience and intact hippocampi have demonstrated the ability to readily recognise and utilise nests (Kuang *et al* 2010). We deliberately chose mice that were reared by dams with no access to nesting material from pregnancy to weaning. Thus, mice from Experiment 2 were naïve to substrates specifically intended for nesting and were not exposed to adults that had experience building with these materials. Despite this, our hypothesis is that all mice, regardless of their lack of exposure to nesting material during fetal development and rearing, will achieve positive TINT outcomes.

Overall, the goal of this group of experiments is to develop a better understanding of how varying animal housing and rearing conditions affect the TINT. The usefulness of this test in the wider laboratory environment is contingent on understanding its limitations.

## Materials and methods

All experiments were conducted at Charles River (Wilmington, MA, USA). The facility is AAALAC accredited, and all work was approved by Charles River's IACUC. Animals were free of common pathogens; details may be found at: http://www.criver.com/files/pdfs/rms/hmsummary.aspx.

In both experiments age- and sex-matched, virgin Crl:CD1(ICR) mice were originally housed in stable groups of five. Mice were housed in clear plastic cages (37.3 × 23.4 × 14.0 cm; length × width × height) with static lids (Innovive Inc, San Diego, CA, USA). Cages were bedded with 100 g of aspen shavings (NEPCO, Warrensburg, NY, USA). Mice in Experiment 1 were provided with 10 g Enviro-dri® (SSP, Watertown, TN, USA) as nesting material at the time of birth; mice in Experiment 2 were nesting naïve, thus never exposed to nesting material during fetal development or rearing. Food and water were available *ad libitum* (Lab Diet 5L79, Purina Mills, Richmond, IN, USA). The average temperature in the room was 20 (± 2)°C. The room was on a 12:12 h light:dark cycle.

Nests were scored using a naturalistic scoring method described by Hess *et al* (2008). Briefly, manipulated material with no central nest site received a score of 1; 2 was a flat nest; 3 was a cupped nest; 4 was a nest that had an incomplete dome; and 5 was a complete and enclosed dome with an internal cavity. Nests were scored immediately prior to TINT administration.

For the TINT, either four individual strips of 3-ply Enviro-dri® (SSP, Watertown, TN, USA) crumpled into a cohesive ball or one-quarter of a 5-cm square Nestlet™ (Ancare, Bellmore, NY, USA) was used as the testing substrate. The cage top was opened and TINT substrate was placed into the corner of the home cage by hand. A timer was used to note the time of the mouse's first integration of the TINT substrate into the home nest. First integration was defined as carrying a piece(s) of the TINT substrate to the main nest site. Mice were observed for 10 min by a single female observer positioned approximately half a metre from the cage front. All testing observations were made within the first 3 h of lights on, which corresponds to a natural peak of nest-building behaviour (Jirkof *et al* 2013; Rock *et al* 2014). A positive TINT outcome was recorded if the TINT substrate was integrated into the home nest within 10 min while a negative TINT outcome was recorded if the latency to integration was greater than 10 min.

In Experiment 1, we utilised a 2 (sex) × 2 (housing treatment) × 2 (replicates or cages per variable combination) factorial design for a total of eight cages. Mice were originally weaned into same sex groups of five mice and housed until eight weeks of age (a total of six cages). At eight weeks of age, one cage of males and one cage of females was randomly selected and two mice from this cage were, again, randomly selected, and housed singly (creating two cages of solitary females and two of solitary males). Separations were made the day of cage change at 0700h. The remaining four cages of five group-housed mice plus the four cages of individually housed mice resulted in a total of eight experimental cages. TINT scoring began the next morning between 0700–1000h and was repeated for the next three consecutive days (a total of four data points per cage). Mice in Experiment 1 were tested with Enviro-dri® as the TINT substrate.

To examine the effect of prior nesting experience and TINT substrate on the TINT outcome in Experiment 2, we used 2 (sex) × 3 (nesting treatments) × 2 (TINT substrate treatments) × 2 (replicates or cages per variable combination) factorial design = 24 total cages tested. Nesting-naïve mice were housed for two weeks with one of three nesting treatments: no nesting material, Nestlet™ nesting material (10.6 g, equal to three 5-cm squares), or Enviro-dri® nesting material (10.6 g). This amount of nesting substrate was chosen because it has been documented that it



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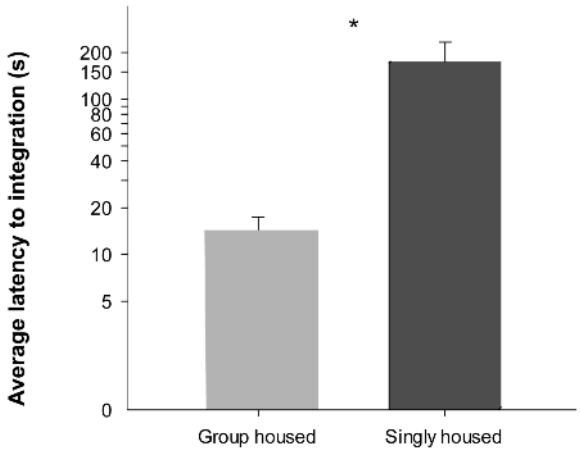
adequately provides mice with enough material to make a naturalistic nest that provides essential thermoregulatory support (Gaskill *et al* 2013). After a seven-day acclimation period to their nesting treatment, the TINT was administered with either Enviro-dri® or Nestlet™ as the testing substrate. TINT was evaluated for four consecutive days after cage change between 0700 and 1000h.

All statistical analyses were performed using JMP v10 statistical software for Mac (SAS Institute Inc, Cary, NC, USA). In Experiment 1, repeated TINT outcomes were analysed as a binary logistic regression. To avoid pseudoreplication and accommodate repeated measures, analyses were blocked by cage of mice, nested within sex and housing treatment. A full factorial of sex, housing condition, and day were investigated. The time to first integration was also analysed in Experiment 1, using a GLM to highlight any differences in motivation to collect TINT substrate between single- and group-housed mice. The time to first integration is defined as the time it takes a mouse to collect the newly deposited TINT substrate and integrate it into their home nest. Analyses were blocked by cage of mice, nested within sex and housing condition. Cage was also treated as a random variable. In this model, main effects of sex and housing condition and their interaction were investigated. and its interactions with sex and housing conditionwere but not included in the final model. The blocking factor day and its interactions were all found to be insignificant, and due to reasons of marginality (Grafen & Hails 2002) in a non-orthogonal model (some data were missing due to animals failing the TINT), it was removed from the final model. Nest score was also originally included in the model as a covariate but it did not significantly explain changes in the dependent variable, therefore it was removed from the final model. The assumptions of GLM (normality of error, homogeneity of variance and linearity) were confirmed *post hoc* graphically and the time to first integration values were log-transformed to meet these assumptions (Grafen & Hails 2002).

All mice in Experiment 2 achieved a positive TINT outcome. Therefore, statistical analysis could not be performed on this binary measurement. Instead, time to first integration of the TINT substrate was analysed as a repeated measures General Linear Model (GLM). Again, the assumptions of GLM (normality of error, homogeneity of variance and linearity) were confirmed *post hoc* graphically and the time to first integration values were log-transformed to meet these assumptions (Grafen & Hails 2002). Analyses were blocked by cage of mice, nested within sex, prior nesting condition, and TINT substrate. Cage was also treated as a random variable. The main effects and all second order interactions of sex, prior nesting condition, TINT substrate, and day, plus the third order interaction of sex, prior nesting condition, and TINT substrate were tested. Day was included in the analysis as an ordinal variable due to TINT experience historically affecting outcomes. Nest score was originally used as a covariate to determine if nest shape might affect latency to incorporate the material, however it did not explain significant amount of variation and was dropped from the model. Significant effects were analysed *post hoc* using Bonferroni-corrected planned comparisons, or Tukey tests were used to further evaluate significant differences. These comparisons were corrected to a family a level of 0.05. All data are represented as least square means (± SEM).

**Figure 1**




Comparison of time to TINT substrate integration between individually housed mice and group-housed mice. The average time to first integration for each given housing condition is represented on the y-axis as a log-transformed scale. Least square means (± SEM) are plotted and the asterisk represents the effect of singly housed mice taking significantly longer to integrate TINT substrate than group-housed mice ($P < 0.05$).

## Results

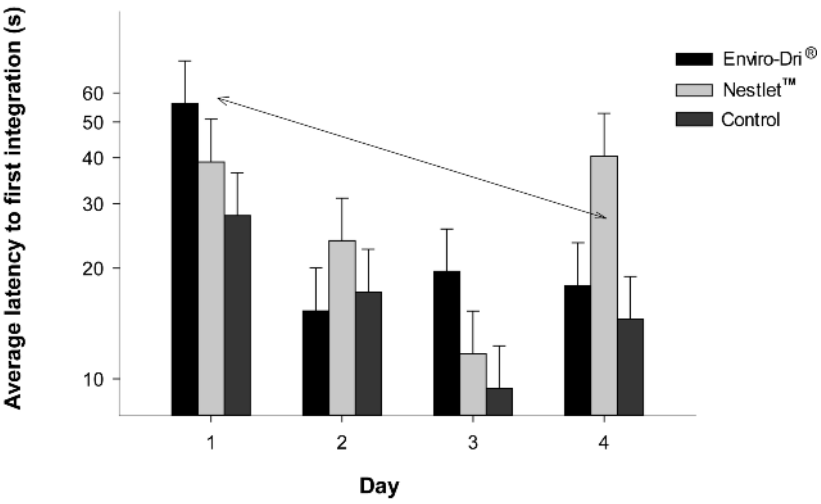
### Experiment 1: Effect of individual versus group housing conditions

There was no statistical difference in achievement of a positive TINT outcome between mice that were housed in groups and mice that were housed singly (LR $\chi^2 = 0.000009$; $P = 0.99$). Although not statistically significant, there were six failed tests over the four days. All cages that failed contained singly housed mice. Singly housed mice took 174.5 (± 1.3) s, on average, to integrate TINT substrate, whereas group-housed mice took 14.1 (± 1.23) s, which was significantly different (GLM: $F_{1,3.48} = 49.1$; $P = 0.004$; Figure 1).

### Experiment 2: Effect of previous nesting experience and testing substrate

The only significant variable that altered the latency to integrate TINT substrate was the interaction between day of test administration and prior nesting substrate (GLM: $F_{6,57} = 2.62$; $P = 0.0258$; Figure 2). Alpha-corrected test slices did not reveal any significant differences of prior nesting conditions within each day. However, a linear contrast did identify a decrease in the average time it took mice to integrate the TINT substrate over the subsequent testing days ($F_{1,57} = 14.8$; $P < 0.001$).

**Figure 2**




Comparison of average latency to integration of previous nest condition by day of testing. Data are represented as least square means (± SEM). The arrowed line indicates a significant ($P < 0.05$) linear decrease in average time to TINT substrate integration.

## Discussion

Housing-related behavioural differences are well documented between mice housed in groups and mice housed singly (Ader *et al* 1991; Sherwin 2003; Spani *et al* 2003; Van Loo *et al* 2004, 2007). We speculated that this could potentially alter the final outcome of the TINT under our experimental conditions. However, the final TINT outcome achieved by both singly and group-housed mice was statistically similar. A difference between these groups was observed, though, when latency to integration of TINT substrate was compared. Specifically, the latency to integration was significantly longer for singly housed than for group-housed mice. Singly housed mice took, on average, 160 s longer than group-housed mice to integrate the TINT substrate. Anecdotal observations during testing revealed noticeable behavioural differences related to the handling of and interaction with the TINT substrate between these two housing conditions. For example, when mice were housed in groups, some or all of the mice in the cage would engage in nesting behaviour when TINT substrate was introduced. Furthermore, nesting behaviour also started immediately in the group cohort upon provision of the TINT substrate in all cases, and integration was typically complete in less than 20 s. In contrast, singly housed mice were never observed to display the full set of nesting behaviours in a prolonged manner. Instead, singly housed mice were seen alternating between short, small bursts of nesting behaviour and non-nesting related behaviours, such as investigating other parts of the cage or standing still. The exact function of these non-nesting behaviours was not specifically uncovered in this experiment. However, it seems likely that they are related to increased awareness of surroundings and the threat of predation. Despite the reason, our study shows that this increased vigilance does not interfere directly with TINT outcome or the usefulness of the TINT in unmanipulated mice. Regardless, our data indicate that singly housed mice have a different experience when compared to group-housed cohorts and adjust their behavioural repertoire to respond. Motivation to perform behaviours to protect against threat and maintain homeostasis becomes altered. This highlights the importance of treating single housing of mice as an independent variable in biomedical and behavioural research. Given that TINT relies on motivation to obtain nesting material, it is valuable to note that singly housed, manipulated mice could have a reduced desire to obtain this resource, thereby failing the TINT.

Experience with nesting materials or nesting behaviours during development had no effect on the ability to achieve a positive TINT outcome under our study conditions. However, a significant influence of day was again found but in this study it only affected the average latency to first integration, and not the overall result of TINT. These results draw attention to the importance of maximising motivation and preference when designing a behavioural assay that assesses behavioural homeostasis. We know that mice will use different substrates to accomplish different nest construction goals, if given the opportunity (Hess *et al* 2008). In the wild, the exterior of mouse nests are composed of long-fibre materials with softer, short fibre materials lining the interior (Latham & Mason 2004). However, given that the TINT substrate did not alter the final outcome of the TINT, our results support the use of either Eviro-dri® or Nestlet™ as TINT substrate. The nesting material that is most readily available and easy to use at a given vivarium can be utilised to administer the TINT. In our experience, administering the TINT using Nestlet™ as TINT substrate was easier to judge due to the cohesive nature of the Nestlet™ square. However, care should be taken to observe any subtle differences in strains not tested in our preliminary experiment as it relates to handling of nesting material



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or utilisation of TINT substrate. Specifically, we recommend for each vivarium to administer the TINT using unmanipulated mice under normal laboratory husbandry conditions to obtain baseline TINT outcome values before conducting the TINT in experimental conditions.

The TINT continues to show promise as an easy, useful assessment tool that is of potential use to detect an abnormal behavioural state in laboratory mice. It should be emphasised that the testing procedure does not require constant observation of an individual cage for 10 min; rather, the nesting material could be placed in a series of cages and the assessment conducted 10 min later, to score mice as positive or negative. In addition, it does not depend on a set of behavioural assessment skills, thus proving useful in the absence of trained observers. An important consideration is that TINT positive status may not necessarily indicate a condition of *good* welfare in mice. Rather, it could detect a threshold below which further need for monitoring, treatment or humane endpoint decision-making should be considered. The strength of this rapid screening test as a welfare assessment may lie in its simplicity to detect when welfare is of significant concern, even by untrained observers.

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