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Non-invasive, in-pen, approach test for laboratory-housed pigs

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KANSAS STATE UNIVERSITY | Department of Animal Sciences and Industry

June 4, 2018

Ronald Myers, PhD.
Senior Science Editor
JoVE
1 Alewife Center, Suite 200,
Cambridge, MA 02140

RE: Submission of manuscript "Non-invasive approach test for laboratory-housed pigs"

Dear Dr. Myers,

I am pleased to submit an original manuscript by Hulbert et al., entitled "**Non-invasive approach test for laboratory-housed pigs**" to JoVE's behavior section. These methods were developed through our collaborative work on a porcine model of mild traumatic brain injury. The human approach test (HAT) is conducted in the pigs' home pen, therefore, they are non-invasive, but it still allows for variable housing strategies. This test is reliable and valid; HAT can be used across many laboratories and also for other types of porcine models of injury, sickness, and distress. This test was used originally developed using a scoring system to evaluate behavior-types in pigs and cattle. Our laboratories modified the test to be rapid, non-invasive, quantitative, valid, repeatable, and reliable. The sensitivity of the improved HAT allows for behavior outcomes to be used to distinguish an mTBI-treated subject from a sham-treated subject. Our goal is to create behavior protocols that many researchers can have access to and recreate the tests in their own laboratories, therefore, the medium of visual and written protocols that JoVE provides is perfect for being successful in this objective. We feel that this manuscript will be of interest to researchers that use porcine models for sickness, injury, and distress as well as researchers interested in applied ethology of swine.

I included in my file uploads:
-Manuscript
-Figures
-Table of Materials
- Data collection sheet
-Videos

The videos are currently in normal speed (30 fps) and do not have any sound; we assume your team will aid in the script for the videos. We will not have the animals and facilities readily available to recreate the test, therefore, we hope one of the three play-back videos will be useful. These are unique because the video is timestamped and visually allows the audience to see the accuracy in the pig's behaviors and the adjacent behaviors. The set-up and downloading videos are available. The figures for this manuscript were created to be as visual as possible, and the tables included are also useful for researchers who need to justify experimental units for future projects.

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I thank you for your consideration, and I look forward to working with your team on this manuscript for publication. If it is possible, we would like to publish or be in-press by August because the neurotrauma societies and military research meetings will be held at that time. We are hoping to advertise this article so that researchers can immediately add this simple, effective , non-invasive measure to their research and cite our JoVE article. If you have any questions, please do not hesitate to call me.

Sincerely,

Lindsey Hulbert

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TITLE:

Noninvasive, In-pen Approach Test for Laboratory-housed Pigs

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KEYWORDS:

behavior, appetitive, avoidance, porcine, ethology, mTBI

SUMMARY:

This protocol describes a new behavioral test—the human approach test in the pigs' home pen—to detect functional deficits in laboratory pigs after subconcussive traumatic brain injury.

ABSTRACT:

Traumatic brain injury (TBI) incidences have increased in both civilian and military populations, and many researchers are adopting a porcine model for TBI. Unlike rodent models for TBI, there are few behavioral tests that have been standardized. A larger animal requires more invasive handling in test areas than rodents, which potentially adds stress and variation to the animals'

responses. Here, the human approach test (HAT) is described, which was developed to be performed in front of laboratory pigs' home pen. It is noninvasive, but flexible enough that it allows for differences in housing set-ups.

During the HAT, three behavioral ethograms were developed and then a formula was applied to create an approach index (AI). Results indicate that the HAT and its index, AI, are sensitive enough to detect mild and temporary alterations in pigs' behavior after a mild TBI (mTBI). In addition, although specific behavior outcomes are housing-dependent, the use of an AI reduces variation and allows for consistent measurements across laboratories. This test is reliable and valid; HAT can be used across many laboratories and for various types of porcine models of injury, sickness, and distress. This test was developed for an optimized manual timestamping method such that the observer consistently spends no more than 9 minutes on each sample.

INTRODUCTION:

Human mTBI is often defined by functional deficits despite the absence of global structural changes or significant edema within the brain[1-3]. Indeed, in some mTBI patients, the characteristic feature of this injury is a change in their psychological state in the absence of any neuroanatomical changes[4,5]. We utilized a porcine model of mTBI[6] because pig's brains are both anatomically and physiologically closer to humans than rodents[7], and corresponding measurements could provide a relevant set of common data elements with humans.

In recent years, the porcine model has gained the interest of neurotrauma scientists and mTBI stakeholders for preclinical investigations; however, unlike rodent models of TBI, there are only a few standardized behavioral tests published that allow for the assessment of the laboratory pig's affective state (*i.e.*, psychological state)[7-10]. A long-term goal for our laboratory is to develop several, complementary behavioral toolsets that are sensitive enough to measure when the pigs are experiencing subclinical sickness or when animals are in a prepathological stress-related state.

Repeated behavioral tests that measure the change of affective state in a laboratory pig may be good candidates for distinguishing an animal with a prepathological condition from healthy animals. For example, in-pen HATs were used for the commercial pig production to help farmers select healthy pigs with good temperament or modify management and housing strategies that caused distress, injury, and sickness[11,12]. These tests were used to quantify the motivation and overall affective state of one pig or a group of pigs[13].

Our laboratory and other researchers measured motivation in pigs by quantifying three categories of behaviors: 1) exploratory states that are expressed through nonnutritive oral behaviors (NNOB), where the pig uses its mouth, snout, or face to sniff, lick, chew, and root a substrate, or they chomp without a substrate[14,15]; 2) spatial relationships of the pig to an object or being[16]; 3) nose direction, which is used instead of eye contact because pigs have monocular[17], but near-sighted vision, and they prioritize their sense of smell over vision[18]. If a healthy pig associates humans with rewarding stimuli, they express a high frequency of NNOB, direct their nose towards the human, and seek to gain closer proximity to the human[11,16]. However, after sickness, injury, or a distressful experience, motivation to seek even pleasurable stimuli is reduced, and thus, these measurable behaviors are likely reduced[19]. Swine behavior researchers noted that anhedonia, the lack of motivation to experience pleasurable stimuli, is recognizable and measurable in pigs within their home environments[20]. Thus, repeated HATs (before and after treatment) may serve as a sensitive measure to distinguish laboratory pigs treated with subconcussive mTBI from sham-treated (anesthesia-only) subjects. Anhedonia is one affective state that TBI patients may experience[21]. The HAT used here has the potential in helping to streamline the translation of behavioral findings from an animal model to clinical work. HATs can be administered daily over the course of an experiment, which may also help standardize laboratory pigs' care for optimizing animal welfare and husbandry[22].

Here, using the HAT, the behavioral differences resulting from mTBI in mini-swine are probed. Minimizing behavioral variability was achieved by the utilizing noninvasive measures of the HAT and allowing the pigs to acclimate to their home pens, routine management, and a daily treat. Traditionally, a test arena is used to measure behaviors (*e.g.*, open-field test). An in-pen test can be helpful in laboratories that have limited space. Moving and handling pigs in a test arena can cause a stress response (distress or eustress) and potentially add to the variation of the responses to the test. An in-pen test removes that handling component, and therefore, likely reduces variation from handling-stress[17]. For these reasons stated above, we developed a daily, in-pen HAT for this mTBI model.

Standardized and quantified measures that appropriately define the affective state of the animal are important aspects in developing a new behavior test. In addition, tests should be repeatable across multiple laboratories. Here, for developing this protocol, the HAT was tested in three laboratories' different housing systems. Three subethograms were created to timestamp specific behaviors from sample videos. Next, a weighted formula was created to incorporate the three ethograms and allow for the use of HATs across multiple laboratories. Although this test was developed and used specifically for mini-swine treated with subconcussive mTBI, the methods and protocol developed here will have applications for distinguishing the difference between a subclinically injured/sick or distressed pig and a healthy pig.

Behavior outcomes can be influenced by single *vs.* group housing, free-space allowance, the type of floor used, the type of fence used, the location of feeding and water, the defecation area, and the environmental enrichment location. Therefore, three housing types was examined (**Figure 1**): housing type A was at Kansas State University (Manhattan, KS); housing type B and C were at Virginia Tech University (Arlington, VA). The individual Institutional Animal Care and Use Committee (IACUC) at each location approved the use of the facilities and procedures.

For developing the ethogram of housing type A (**Figure 1A**), Minnesota-cross mini-swine (boars = 7, gilt = 1; National Swine Research Resource Center, Columbia, MO; age = 25.6 ± 3.66 [mean ± standard deviation (SD)] weeks) were housed indoors in single pens with animal-friendly flooring (IACUC #3881). The pigs used for this protocol were in good health did not have treatments applied. For developing the protocol for housing type B, Yucatan mini-pigs (age = 25.3 ± 2.80 weeks [mean ± SD] were single-housed (**Figure 1B**) at Virginia Tech facilities (IACUC #15-060).

Animal treatments are described elsewhere[29] and included the induction of subconcussive mTBI using blast-wave overpressure or sham controls (anesthesia only). For developing the protocol of housing type C, five female Göttingen mini-pigs (age = 23.7 ± 1.18 weeks [mean ±SD]) were pair-housed at Virginia Tech in a large pen (**Figure 1C**; IACUC #15-060). The first two housing environments are typical laboratory housing or contain single-housed pigs. Housing type C is an atypical housing environment that can house two or more pigs and may be considered more of an enriched environment than standard laboratory housing. This protocol can be used across housing types if the following methods are followed.

PROTOCOL:

The individual IACUC at each location (Kansas State University and Virginia Tech University) approved the use of facilities and procedures.

1. Set-up of the Cameras and Pens and the Establishment of a Routine

1.1 Prior to placing the animals in their pens, fix cameras at a 90° angle over each pen (see **Table of Materials** for a suggested camera system).

1.2 Record animals continuously at 30 frames per second (fps), either for the duration of the study or only during the test sessions.

1.3 Fix bowls, waterers, mats, and toys with bolts and chains.

1.4 Place sound machines that continuously play white or pink noise (*e.g.*, the sound of waterfalls) in the pigs' facility.

Note: External sudden noises (*e.g.*, doors opening and shutting) can cause a startle reflex during the sessions[23,24].

1.5 Randomize or stratify treatments by pen across the facility.

1.6 Set up an established animal husbandry routine. This will help the pigs to know when to expect humans to clean the pens, feed and handle them, and perform the test.

1.6.1 Use a familiar treat to allow the pigs to associate humans with a reward.

1.6.2 Use a clicker during the reward to allow the pigs to associate the clicking sound with a reward. Do not use vocalizations and visual signals to familiarize the pigs with the reward (see **Table of Materials**).

1.7 Conduct the sessions before the morning meal or before placing a new feed for *ad libitum* fed pigs.

2. Identification of the Pigs

2.1 Make subjects identifiable on the video feed, even if they are single-housed.

2.2 Make sure observers remain blind to treatments and unbiased during timestamping with a marking system that is not related to treatments. Use a medical grade tape (see **Table of Materials**) that is adhered to a strip of duct tape of a specific color, round shape, and pattern.

2.3 Use one round patch to mark the top of the pig and one down each side (see **Figure 1**, green and blue markers).

2.4 Smear tag cement (less than 0.35 g) on the tape corners to help increase the adherence longevity.

Note: Too much tag cement will not dry very quickly, causing the tape to fall off prematurely.

2.5 Troubleshoot and customize the marking strategy during the acclimation period so that official tests are performed efficiently and without added stress to the pigs.

2.6 Use data collection sheets to keep track of the markings and subject identification (see **Supplementary File 1** for an example data sheet).

Note: To remove tape, do not rip the tape off because it will cause pain if any hair is pulled out. It can either slough off on its own, a water-based lubricant can help dislodge it, or it can be shaved off with clippers. If the tape comes off at unwanted times, prepare extra marking tape and reapply it while the pig is eating a meal rather than restraining the pig.

3. HAT Sessions

3.1 Have the test-humans wear the same hat, coveralls, boots, odors, *etc.* every time the session is conducted.

3.2 Conduct sessions daily, at least 3x, before the treatment and, then, daily thereafter.

3.3. The pig can be in any area in its pen before starting the session. To start the session, ask the test-human to drop the treat in the bowl or in front of the pen and click the clicker 3x.

3.4. The test-human needs to place their hands out of the pig’s sight and stand stationary during the test. Have another researcher mark the start of the session on the data sheet and start a timer. After 120 s, the researcher silently signals to the test-human to move to the next subject and restart the test.

4. Establishment of HAT Ethograms for Software

4.1 Construct ethograms (see **Figures 1** and **2** and **Supplementary Video 1**) under one project using specialized software.

Note: **Spatial behaviors** are the location of the animal relative to the human. In the ethograms, the spatial relationships must be customized to the animal's pen and published every time a novel pen set-up is used (**Figure 1**). Within this category, behaviors are considered mutually exclusive. The space is divided into four areas, with varying levels of approach (**Figure 1**). The areas are standardized across housing types. Closest, or climb (Cl) means that pigs may climb on the fence to gain access to the human; therefore, climbing is considered a spatial behavior that indicates the pig is most actively seeking human contact. Close (Co) denotes the area within 61 cm of the human. Mid (M) is the area within 61 - 122 cm from the human. Far (F) is the area of 123 cm or more from the human.

4.2 **Structural behaviors** focus on the orientation of the whole body or parts of the body (**Figure 2**). Create two structural categories: 1) the pig-nose position and 2) the active state of the pig. Use **Figure 2A** to identify directionality (direction 1 denotes the pig directs its nose towards the human; direction 2 means the pig directs its nose away from the human).

4.3 Divide **activity behaviors** (**Figure 2B**) into three mutually exclusive states of approach: NNOB, stand or walk without NNOB, and resting without NNOB (see the color coding in the ethograms in the video).

Note: NNOB is used to describe when a pig is using its mouth, snout, or face to lick, sniff, chew, bite, rub, or root a nonnutritive object to seek out either familiarity or novel opportunities. Therefore, when it is in this active state, it is interested in the human, and in an approach-state. If overhead cameras are used at 90° angles, the pig's nose position and head movement are indicators of NNOB. Occasionally, pigs will chomp or sham-chew; the nose can be seen but the head moves up and down. Stand or walk without NNOB is used to the describe when the pig is in the upright position, the head is still, and the nose is not touching a substrate or chomping, which means it is in less of an approach state. Resting without NNOB is used the describe when the pig is resting by lying or sitting, which is the least approach state under this category of behaviors.

5. Timestamping of the Videos for Efficiency and Reliability

5.1 Based on the start-times recorded by the data collector, edit the footage into exact, 3 min sessions. The method for timestamping will take 9 min per session.

5.2 Only use up to two trained observers to timestamp the videos.

Note: If two observers are used, the intra-observer variation should be quantified, evaluated, adjusted, and then reported as a Pearson correlation coefficient after the observers have timestamped the same sample video (for methods, see Martin and Bateson[25]).

5.3 Set the playback speed at 1x regular speed (*i.e.*, 30 fps). Do not pause, rewind, or timestamp frame by frame.

5.4 Timestamp each category of mutually exclusive behaviors separately.

5.4.1 Timestamp **spatial behaviors**. Restart the video.

5.4.2 Timestamp **structural behaviors**. Restart the video.

5.4.3 Timestamp for **structural/nose position behaviors**.

5.5 Use the duration of each behavior outcome (see **Supplementary Videos 2** and **3**) for summarizing data. The duration measurements need to be converted to a percentage of time per category.

6. Approach Index

6.1 Apply the formula (**Figure 3**) so that each structural and the spatial behavior is combined to create an AI (**Figures 3** - **4**). The AI is used in addition to reporting behaviors and categories separately as figures (**Figure 5**) or in tabular form.

Note: See **Table of Materials** for software details. Within each category, behavior durations are first converted to percentages (the duration of the behaviors divided by the total duration of the test session). Each behavior is weighted based on the level of approach (**Figure 3**). **Avoidance behaviors** (the percentage of time in the far area, resting, with the nose turned away) are multiplied by 0. **Moderate-approach behaviors** (the percentage of time in the middle area, turned towards the human, and standing with the head still) are multiplied by 1. The **greatest level of approach behaviors** (the percentage of time in the close and closest area and showing NNOB) are multiplied by 2. Then, each category is further weighted 3, 2, and 1 for spatial, nose orientation, and activity, respectively. A constant (0.10) is applied to create a percentage scale. For example, if the pig faces the human, is in the close/closest area, and performs NNOB during the entire test session, note that the AI is 100% (the red pig in **Figure 3**). In contrast, if the pig is in the far area, is not facing the human, and remains in the rest position during the entire test session, the AI is 0% (the black pig in **Figure 3**). Pigs in the close zone can have the same AI as a pig performing NNOB in the mid area if they are turned away from the human and standing with their head still (the orange pigs in **Figure 3**).

REPRESENTATIVE RESULTS:

Three housing types (A, B, and C; see **Figure 1**) were used in three different laboratories for HATs. Structural behavior categories and head and body orientation were used in HATs across all laboratories and experiments as presented in **Figure 2**. **Table 1** represents the data collected from all three housing types and descriptive statistics that were performed using the data obtained from pretreated healthy pigs during the HAT. Next, a formula was developed to calculate an AI from the data obtained during HATs (see **Figure 3**). The results indicate that the use of an AI reduced variation (**Table 1**) in the data collected. This is an important finding because less variable data will allow for the use of less experimental animals to detect smaller differences.

For **Table 1**, descriptive statistics were obtained using the PROC UNIVARIATE procedure (see **Table of Materials** for specific software information). To compare the outcome of each lab's housing type behavior, the MIXED procedure with a repeated measures model was used. The pig was treated as a random variable. The first-order autoregressive type was selected as the covariance structure. Least square means were separated using the Tukey-Kramer adjustment method. The significance was defined as $P \leq 0.05$.

Next, it was determined if the same person or a different human experimenter should be used for every test session of the HAT. To compare the unfamiliar behavior responses to the familiar responses, the MIXED procedure with a repeated measures model was used. The pig was treated as a random variable. The first-order autoregressive type was selected as the covariance structure. Least square means were separated using the Tukey-Kramer adjustment method. The significance was defined as $P \leq 0.05$. We found that there was no difference in the AI when a familiar human was used compared to when an unfamiliar human was used during the testing (see data in **Table 2**). If the pigs never had any adverse interactions with people, they typically generalized and associated all people positively with food[26].

The acclimation period for the HAT protocol was determined from the approach indices of the pigs from housing type A (336 test sessions). The HAT started on day 8 after arrival and was performed twice by the familiar human (who spent the previous week handling the pigs) and by an unfamiliar human (who had had no previous contact with the pigs). To determine the effect of time on the outcomes of the HAT, the MIXED procedure with a repeated measures model was used. The pig was treated as a random variable. The first-order autoregressive type was selected as the covariance structure. Least square means were separated using the Tukey-Kramer adjustment method. The significance was defined as $P \leq 0.05$. As stated above, the data did not detect a difference between the responses from familiar or unfamiliar human exposure (**Table 2**). However, the acclimation period was determined based on days 9, 11, and 13 displaying AIs that were significantly lower than all other days. The baseline should include a minimum of three measurements after acclimation, but we recommend six sessions to calculate an AI mean as a covariate in the models.

To determine if HAT methods could distinguish mTBI-treated pigs from sham-treated pigs, HAT data from 12 pigs 1 day before (-1) and 3 days after they were treated with either anesthesia[29] only (sham) or anesthesia and blast-wave exposure using a shockwave tube[29] to a peak psi of 47.4 ± 13.6 SD for a length of 4.7 ± 0.9 ms SD (blast). The data were analyzed by restricted likelihood ANOVA using the mixed model procedure in a statistical software program. This analysis determined differences between treatment, time, and their interactions. The maximum SEM from the model is reported, and $P < 0.05$ was considered significant. The AI encompasses all behaviors (**Figure 3**). On days 1 and 2 after treatment, the AI measure distinguished mTBI pigs from sham pigs ($P < 0.05$; **Figure 4**). Behaviors can be analyzed and presented within their mutually exclusive categories (**Figure 5**). The close spatial behavior measure distinguished blast pigs from sham pigs on days 1 and 2 after treatment ($P < 0.05$; **Figure 5A**). Likewise, nose direction, resting time, and NNOB measurements distinguished sham pigs from blast pigs on days 1 and 2 after treatment (**Figures 5B** and **5C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Spatial category of behaviors of three different laboratory housing types. The ethogram is set up in relation to the human (footprints), and in relation of pig size to the amount of free space. The greatest level of approach for this category of behaviors is when the pig attempts to climb on the panel closest to the human (Cl; closest or climb). A trained observer timestamps "close" (Co; 0 - 61 cm from the human), "mid" (M; 61 - 122 cm from the human), and "far" (F; 123+ cm from the human) when the pig's ears or more are in those spatial areas. Each laboratory pen was set up with one or two bowls for twice-daily feedings, waterer (W), and a toy. (**A**) Each 50 kg boar was single-housed in 190 x 114 cm pens with grated flooring. (**B**) Each 50 kg boar was housed on a black mat with grated flooring and a drain in the back of the pen. (**C**) Boars of approximately 10 kg were pair-housed in 274 x 366 cm pens, with concrete flooring, a mat, a drain, and fixed bowls and toys. The color strips (*e.g.*, green and blue) represent the marking strategy. All pigs in the diagram are marked with green or blue patches as examples for marking and identifying pigs on videos.

Figure 2: Structural behavior categories employed in all three laboratories and experiments. (**A**) For the head orientation category, the pig was facing either towards or away from the human or moving object. (**B**) For the body orientation category, the pig's head was either down, performing nonnutritive oral behaviors (NNOB); in the upright position, standing or walking, but the head not moving or down; in a resting-state, which includes sitting or lying down.

Figure 3: Approach index formula and diagram. The approach index was developed to place all combinations of behaviors on a scale of 0 to 100, with 0 being the pig in the least approach state (in the far section, nose turned away, lying with its head still) and 100 in the close section, nose pointing towards the human, performing NNOB. A software program (see **Table of Materials** for more information) was used to measure the behaviors so that they aligned linearly. Each pig structure represents a data point that linearly aligns from each category. Within each category, the behavior durations are first converted to percentages (the duration of the behaviors divided by the total duration of test session). Then, each behavior is weighted based on the level of approach. Avoidance behaviors (the percentage of time in the far area, resting, with the nose turned away) are multiplied by 0. Moderate approach behaviors (the percentage of time in the middle area, turned towards the human, and standing with the head still) are multiplied by 1. The greatest level of approach behavior (the percentage of time in the close and closest areas, performing NNOB) is multiplied by 2. Then, each category is further weighted 3, 2, and 1 for spatial, nose orientation, and activity, respectively. A constant (0.10) is applied to scale the data across the full range from 0 to 100 percent. A heat-map-like color scheme is used to represent the pig in the most approach state *vs.* the pig in the least approach state (black).

Figure 4: Approach index of pigs treated with blast-wave exposure. This figure shows the approach index of pigs 1 day before (-1) and 3 days after they were treated with either anesthesia only (sham, *n* = 6) or anesthesia and blast-wave exposure to a peak psi of 47.4 ± 13.6 SD for a length of 4.7 ± 0.9 ms SD. The error bars represent SEM. The *P*-values for treatment = 0.032, for time = 0.033, and for treatment x time = 0.012. The data were analyzed by restricted likelihood ANOVA using the mixed model procedure in a statistical software program. This analysis determined differences between treatment, time, and their interactions. The maximum SEM from the model is reported, and $^*P < 0.05$ is considered significant.

Figure 5: Stacked bar method of displaying behaviors. These panels show a stack-bar method of displaying behaviors for (**A**) the spatial behavior, (**B**) the nose direction, and (**C**) the activity of pigs 1 day before (-1) and 3 days after they were treated with either anesthesia only (sham, *n* = 6) or anesthesia plus blast-wave exposure to a peak psi of 47.4 ± 13.6 SD for a length of 4.7 ± 0.9 ms SD. Housing type B was used for this experiment. All mutually exclusive behaviors can be represented in each stacked bar chart. (**A**) The treatment x time *P*-values for spatial behaviors are far = 0.060, mid = 0.110, *close = 0.014, closest = 0.557; (**B**) the treatment x time *P*-values for nose direction are < 0.001*; (**C**) the treatment x time *P*-values for the activity are > 0.10; the treatment *P*-values were rest = *0.046, stand = 0.584, and *NNOB = 0.042. The pooled SEMs were (**A**) 7.5%, (**B**) 9.6%, and (**C**) 9.7%. Each behavioral outcome was analyzed by restricted likelihood ANOVA using the mixed model procedure in a statistical software program (see **Table of Materials** for the specific program) and, then, were combined in a chart. The analyses determined the differences between treatment, time, and their interactions. The maximum SEM from the model is reported, and $^*P < 0.05$ was considered significant.

Table 1: Baseline HAT measurements were examined for all housing types to create this data set. Behavior outcomes were analyzed by restricted likelihood ANOVA using the MIXED procedure of a statistical analysis software. These analyses determined the differences between the behavior duration and approach index of each laboratory housing type. The maximum SEM from the model is reported, and $P < 0.05$ was considered significant. In addition, the UNIVARIATE procedure of the statistical analysis software was used for descriptive statistics. The confidence value (CV) % was then entered into an experimental unit calculator[27] and the conditions for the expected differences between two treatments were examined.

Table 2: An experiment was performed on seven pigs from housing type A. Two sessions were performed each day. For each session, one familiar (female) or one of seven (three males and four females) unfamiliar humans was used in HATs. The same familiar person went first, and seven unfamiliar people were used. An ANOVA model for statistical analysis software was examined for treatment (familiar or unfamiliar), time (day), and their interactions.

Supplementary Video 1: Observer software set-up with subtitles.

Supplementary Video 2: Data export with subtitles.

Supplementary Video 3: Data analysis with subtitles.

Supplementary File 1: Example data collection sheet.

DISCUSSION:

Mild injuries to the brain that do not result in overt anatomical and structural changes detectable with state-of-the-art imaging can be difficult to identify and treat[28]. However, patients with mTBI are especially vulnerable to additional insult that can cause significant damage to the brain, and therefore, it is important for this population to be identified. Behavioral tests developed in a mini-pig model of mTBI are especially relevant to human mTBI patients because pigs have a similar physiology as humans and express similar affective states, such as anhedonia[8-10,20]. Here, we have developed a noninvasive, in-pen behavioral test (the HAT), and have shown that it is sensitive enough to distinguish mTBI pigs from sham pigs. In addition, a weighted index (the AI) was developed for behaviors observed during the HAT that are ubiquitous across housing and pig types.

Modifications and Troubleshooting:

The methodologies for the HAT were established based on ethology guidelines[25] and several trial-and-error strategies for improving the reliability, repeatability, and validity of the test[6]. Reliability measures helped identify the strengths and limitations of the test. Reliability defines the extent to which the measurement is repeatable and consistent and free from random errors[28,29]. We have previously reported on the intra- and interobserver reliability of the HAT, and with the additional structural ethograms, reliabilities are similarly high (Pearson's $R^2 > 0.90$) for duration[6]. Frequency and latency measures require trained observers, whereas duration measures are less observer-dependent, and therefore, more reliable across laboratories[30].

Reliability within a laboratory and repeatability across laboratories is dependent on methods. In our laboratory, the video system recorded continuously, the files were initially stored as 5-minute files, and some HAT sessions occurred over two files. Fewer mistakes were made when the exact time from the data sheet was used to clip and combine the videos. Before developing the ethogram, observers were allowed to pause, stop, and rewind the video footage to timestamp all of the behaviors in the entire ethogram. This method not only caused variation in timestamping each sample, ranging from 3 minutes to 20 minutes, but the between- and within-observer reliability was also poor for most behaviors. Therefore, we set the playback speed, and had observers timestamp each category at a time. Therefore, when reliability was low in just one category, observers independently retimestamped just the category rather than the entire ethogram, after they consulted the definitions and footage together. The set playback and category methods allowed for a consistent prediction of how much time was needed to timestamp each sample. For projects that span longer than a month, routine reviewing of the coded videos and within-observer reliability is important to measure.

Another factor that reduces reliability and repeatability is the video set-up. Initially, a handheld camera and a tripod were used, which were moved from pen to pen. When this method was used, the pigs needed to be introduced to the tripod and camera before the HAT; otherwise, the pigs appeared to react to the tripod and movement more than to the test-human. In addition, nonoverhead camera angles limited the observer's view during timestamping and the depth perception of space increased the within- and between-observer variation in the spatial behavior measures; therefore, we developed the protocol with fixed cameras. When this method is used, extra care is needed to make sure the camera is placed correctly before each test, and more time is needed for the set-up in between each pig's session. However, we learned that the continuous video overhead system needed to start its initial recording at midnight at least 24 hours before the first HAT. The timestamp display for many video systems are not accurate and in sync down to the frame; therefore, we no longer rely on the display times. The midnight starts allowed for the exact frame-capturing and video editing, and the timestamp display was not used.

In addition, the acclimation of the pigs and setting up a routine was important when troubleshooting this test. In footage of pigs that were not well acclimated to their environments, pacing was observed during the HAT. This is an indicator that the pig may be in an agitated state[31] rather than in an exploratory state[32]. Acclimation periods of three or more weeks may reduce the number of pigs that pace in an experiment. However, if pacing persists throughout all the sampling periods, this ethogram may need to be adjusted to include walks and standing still.

Validity is the extent to which a measurement represents the intended scope of the question being asked[25]. When first developing the HAT, we used only a spatial ethogram. Definitions of spatial ethogram behavior describe accurately and specifically the proximity to the human subjects and they tell the observer directly how much space the pig leaves between itself and a human. However, once these methods are needed to be applied to a new laboratory set-up, we recognized that spatial ethograms are laboratory specific. Pen dimensions and the placement of other objects influence the outcome of the spatial ethogram; therefore, a diagram with measurements and specifics of the pen will need to be published if the pen set-up has not been previously reported. In addition to reporting on the pen environment, the structural behaviors were added to the ethogram. Unlike spatial behaviors, structural behaviors can be evaluated more easily across laboratories; these behaviors have validity because they specifically describe the pig's level of active state. When a pig is resting, it is likely not motivated to approach and is unable to change positions to approach as quickly as a standing pig. Similarly, a pig displaying NNOB is in an exploratory state, but a pig with its head still while standing is more likely in a catatonic state. The nose orientation helps with validity because the nose, ears, and then eyes are what the pig uses to gather information about the human.

Limitations of the Technique:

A potential concern with this technique is the variability in the pigs' responses to the test-human. In addition, pigs will look at the test-human's hands, which can cause unintentional cueing by that person. Therefore, these limitations were expressed through the experimentally testing of 1) the pigs' responses to a familiar human and unfamiliar humans, and 2) standardizing that, after the pellet is dropped, the test-human stands still and places their hands out of the pig's sight. Data showed that there were no treatment or treatment x time differences during the HAT (**Table 2**), suggesting that the HAT could be administered by either familiar or unfamiliar humans. Other researchers suggest that pigs tend to generalize about humans based on previous interactions[11-13]; therefore, a pig's previous experiences with humans need to be positive. This challenge can also be remedied with a vigilant experimental design; for each block, a sufficient number of experimental units represented for each treatment of interest is needed.

In this study, although there were only two experienced observers timestamping all the videos for all three housing types, there were differences among the housing types for specific behavior outcomes (**Table 1**). For example, pigs in housing type B entered the closest area more often than those in housing types A and C. This is likely due to a difference in the pen material; in housing type B, the front of the pen was a chain-linked gate with horizontal bars that allowed the pig to climb the gate during the HAT. Housing types A and C, on the other hand, had vertical bars and fewer horizontal surfaces for the pigs to climb on. This variability can be remedied by adding the duration spend in the close and closest areas before comparing them across housing types (**Table 1**; $P > 0.10$). However, pigs in housing type C spent more time in the far area than those in housing types A and B (**Table 1**; $P < 0.05$), which was likely due to the placement of waterers in the back of the pen rather than at the front of the pen. This is a limitation that can be remedied if laboratories choose to standardize the placement of waterers, bowls, and toys and make sure that they are fixed so that the pig does not move the object into another area.

This test has great accessibility for laboratories of all types, but, as mentioned previously, the manually stamped spatial ethogram and measurements will vary more across laboratories. Nonetheless, the body- and head-structural ethograms are ubiquitous. Laboratories that have access to validated, automated tracking for pigs may benefit by having the spatial ethogram tracked automatically rather than manually because the distance moved and the rate of movement may be additional outcomes of behavior measures from the HAT. The limitations from the in-pen set-up and traditional technologies rather than test areas and automatic tracking technologies may be remedied by adapting the AI formula. The AI provides standardized measurements and terminology for how individual pigs use their pen space and express interest in a human. This calculation, derived from common behavior measures, is sensitive to porcine models of subconcussive mTBI and, possibly, other states of subclinical injury or sickness. In addition, the AI reduces random variations during experimentation and may be more easily compared across experiments and laboratories than methods that rely on more experiment-specific measurements. The structural behaviors provided the foundation for this formula, because these behaviors are standard measurements across treatments, whereas the spatial behaviors are dependent on the pen set-up, number of pigs in a pen, and tracking system. For example, we observed that when two healthy pigs are tested in a pen, they will perform similar spatial behaviors by approaching together, but the pig that follows the first may orient its nose more towards its pen mate than towards the human and express more NNOB, because the pig who leads serves as the sentinel. Nonetheless, the AI aids in reducing this variation even from paired behaviors.

Although the AI is an excellent toolset for standardizing the test across laboratories, researchers may still want to examine test-specific behavior outcomes within a laboratory or experiment, especially if they have enough power (*i.e.*, experimental units and repeated tests) in a single experiment. Therefore, **Table 1**, containing all behavior outcomes, the variance, the distribution, and a calculated test for the number of animals for each specific behavior, was included here. For example, if researchers have pen environments that allow pigs to consistently climb during the HAT and they know their treatment causes over a 75% difference in climbing behaviors, then they can justify animal numbers based on the variance measured. If new behaviors are added to the ethogram, scientists will need to justify which behaviors are indicative of approach or withdrawal before incorporating them into the index. For example, if most of the animals within an experiment pace along the walls of the pen (*i.e.*, thigmotaxis)[32], the duration of this behavior could be incorporated into the body-structural ethogram category. The behavior could be represented in the detailed stacked bar graph (*i.e.*, **Figure 5**) or tabular form, and then, it can be summed up with stand-still before applying the index calculation. The AI, therefore, can represent behaviors that are ubiquitous across laboratories, but additional unique behaviors can still be represented separately.

Significance with Respect to Existing Methods:

The existing methods for the HAT were established for pigs on commercial farms to assess animal welfare. Here, a protocol for laboratory pigs has been established, which can help researchers to assess animal welfare and distinguish mTBI pigs from sham-treated pigs. An alternative traditional test could be to use an open-field test. This test was previously used to assess pig emotionality and welfare[33]. Open-field tests were originally designed for testing the affective states of rodents by measuring their natural aversion to open space and light. In contrast, healthy pigs may view the same stimuli as appetitive[10], and after a sickness, injury, or stress treatment, they likely express fear. This test requires more laboratory space and will require pigs to acclimate to being handled and placed in an open-field arena. If laboratories have space and protocols for handling the pigs are in place, repeated HAT sessions, in addition to one open-field test, may help further distinguish treated animals from sham-treated animals.

Critical Steps Within the Protocol:

The first three steps in the protocol are the most critical for successful HAT measures. The sessions per pig only take 3 minutes; however, adequate preparation will help make this test reliable. As stated above, the camera location and recording set-up is crucial for clarity and replication. Improper camera angles can limit the observer's vision, which will add errors to the measurements. Another often overlooked step is fixing the objects in the pen. The pig will move unfixed objects, and this may affect its motivation to approach the human. The set-up and managing system are important because pigs need to be acclimated to their environments before they can perform the test consistently. Pigs that are not well-acclimated to their home pens or routine or are experiencing stress will defecate in other areas rather than at the back of the pen[34]. The defecation area may affect their motivation to approach. From the camera view, the observer should be able to identify individual pigs; however, it is important that the marking scheme does not provided information about the animal's treatment, as this will bias the observer[25].

Identifying pigs is very important for obtaining the correct behavioral data for the right pig, even when they are single-housed. Pigs are often moved for their treatments, and a marking reinsures the observer that they are watching the same pig after it has been removed and placed back in its pen. Pigs can be housed in pairs, as in housing type C, and therefore, it becomes very important to identify the pigs. Livestock marking paints and markers require daily application; therefore, this protocol requires the use of a medical grade tape and a smear of tag cement. The tape sticks best to pigs with longer hair. Pigs with short hair and dry skin will slough off the tape more often than pigs with longer hair.

Future Applications:

In summary, the noninvasive in-pen HAT test described here is sensitive enough to detect mild and temporal dependent changes in pigs after mTBI. Moreover, we have developed a weighted index called the AI to evaluate changes in pigs housed in different pen types, as well as in different types of pig. Although the HAT has been used to detect changes in pigs exposed to mTBI, this behavioral test can be useful for detecting measurable behavioral changes in animals experiencing stress or prepathological conditions.

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DISCLOSURES:

The authors have nothing to disclose.

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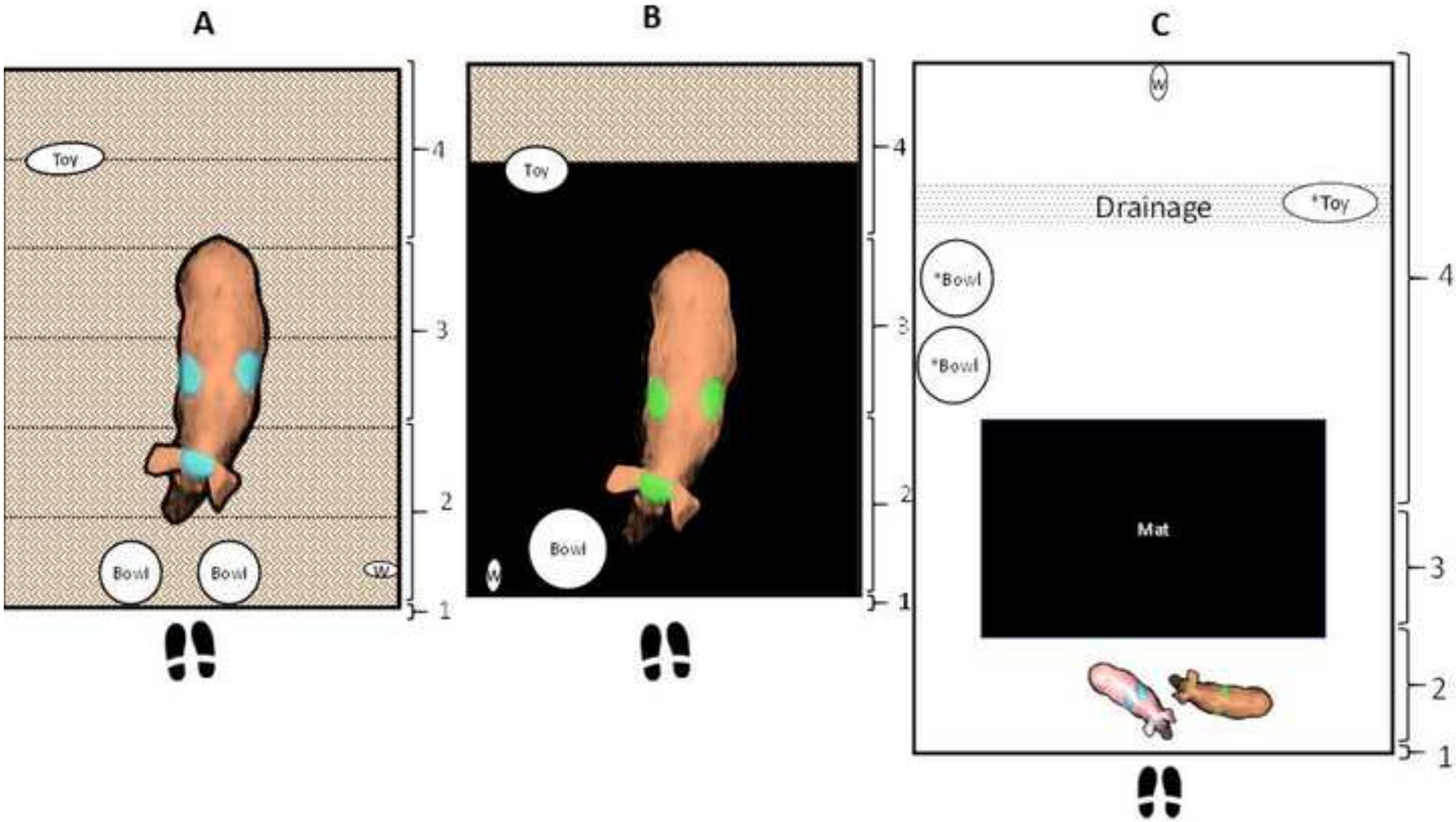

Figure 2

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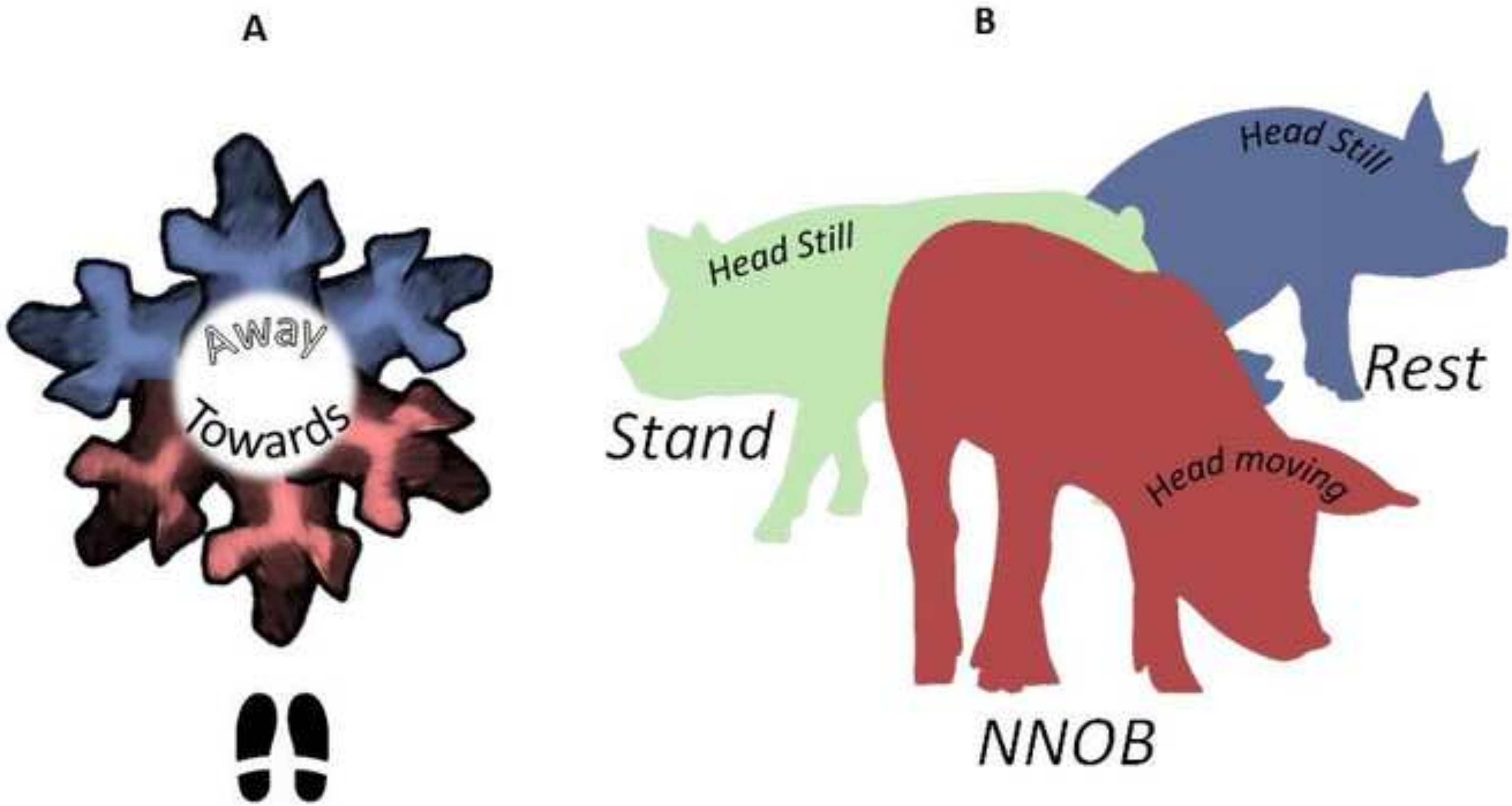

Figure 3

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IF: f = far, m = mid, c = close, C = closest, w = away, t = towards, r = rest, s = stand, N = NNOB:

Approach-Index, % =

$$0.10\{3[0f + 1m + 2(c + C)] + 2[0w + 1t] + 1[0r + 1s + 2N]\}$$

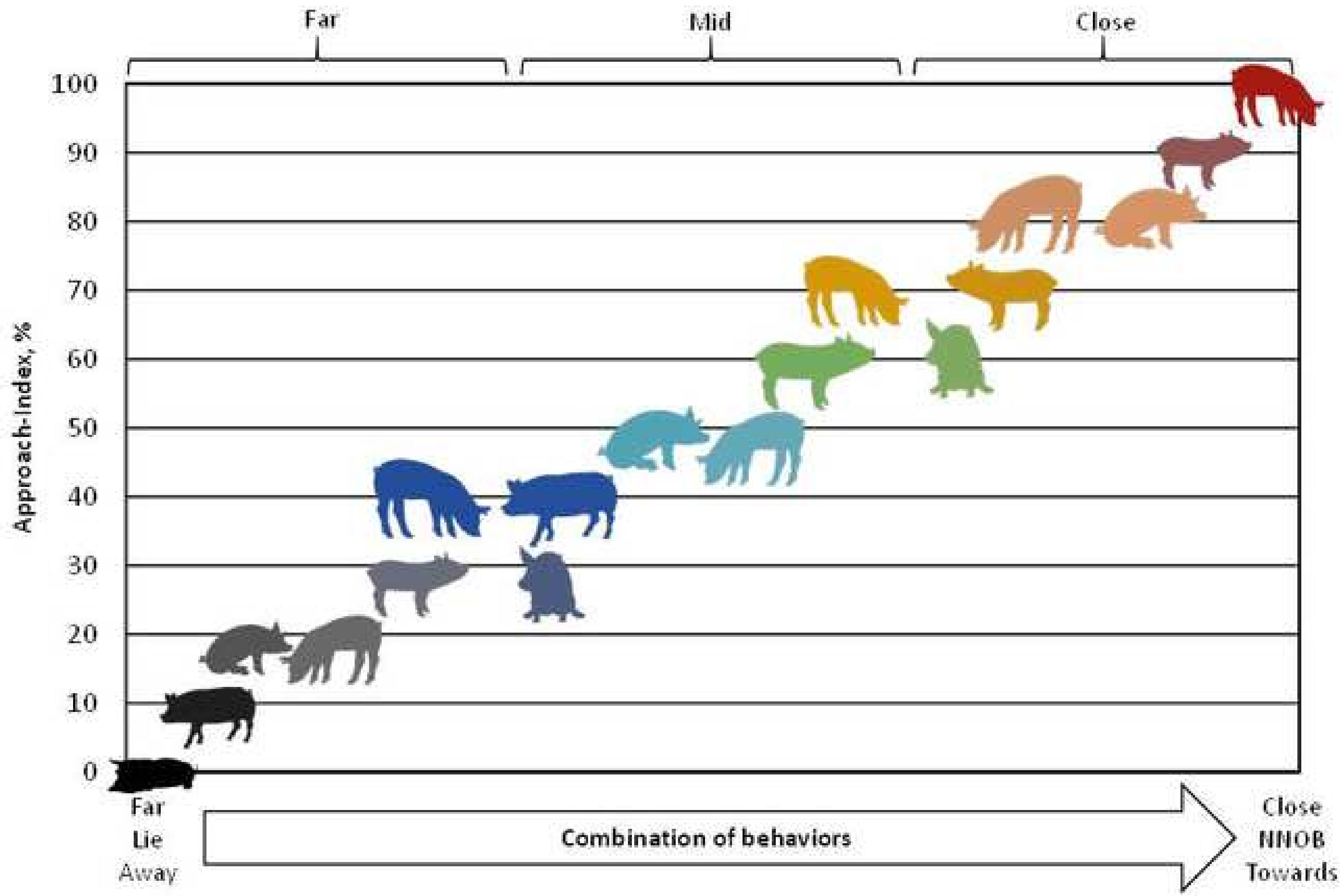

Figure 4

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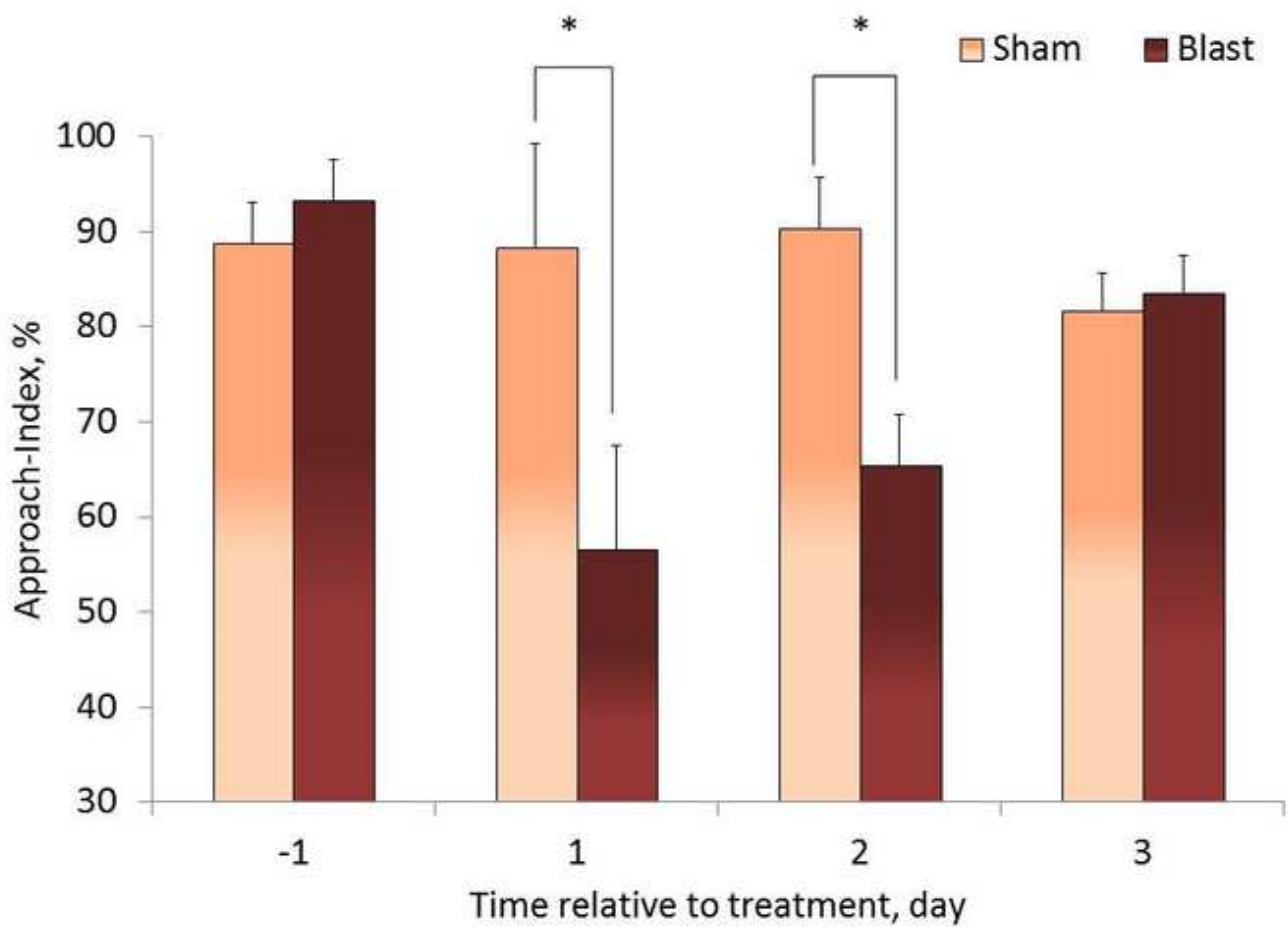

Figure 5

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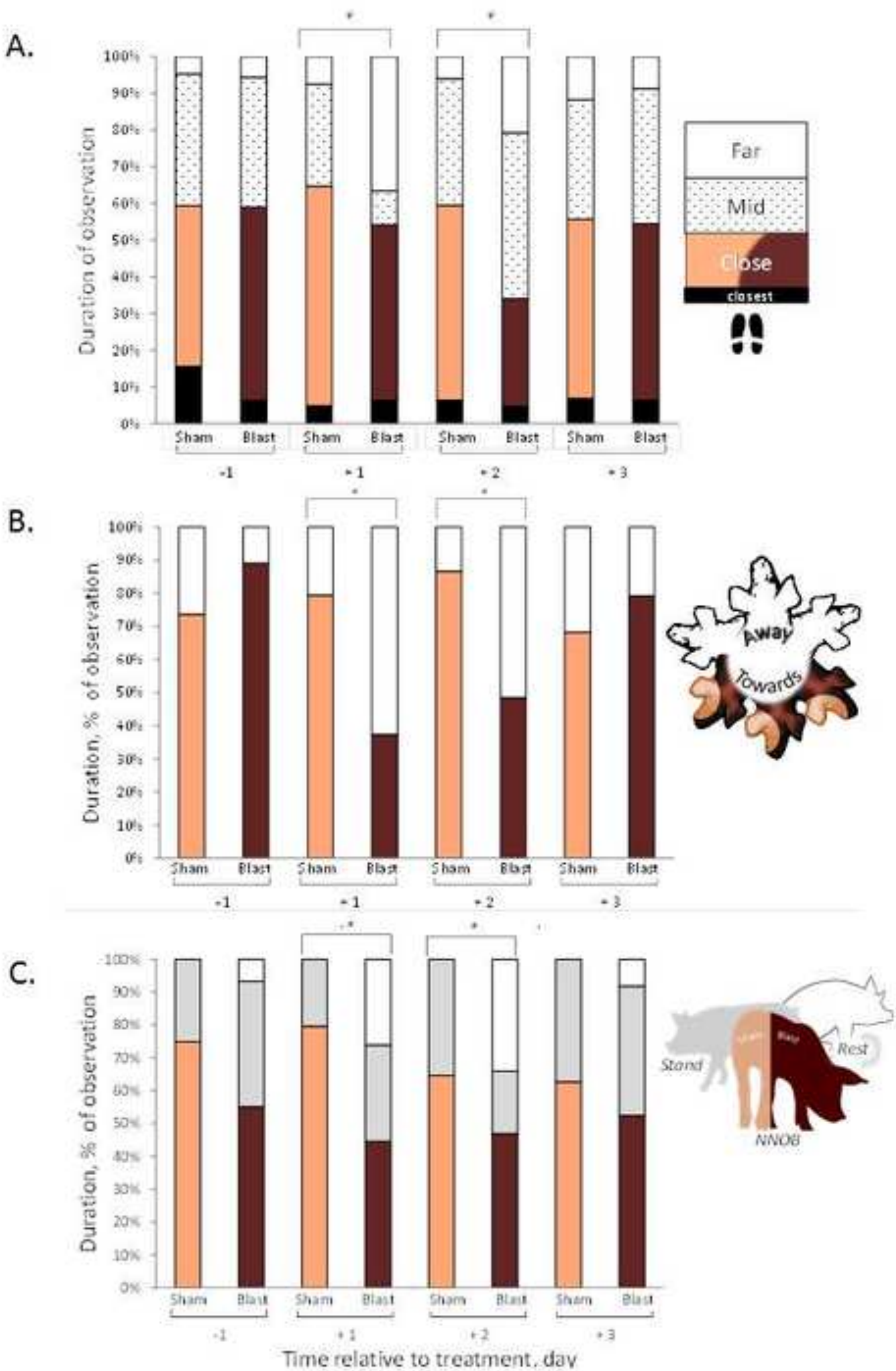

	ANOVA stats for across housing types					
	Housing Type				*P*-values	No. Obs.
	A	B	C	SEM		
Approach-Index, %	75.4	69.2	76.6	5.5	0.318	288
Pen location duration, %						
Far	8.5[a]	5.3[a]	23.0[b]	4.8	0.008	288
Mid	18.9	46	13.75	16.2	0.066	288
Close	72.4[a]	43.9[b]	62.4[a]	12.8	0.032	288
Closest (climb)	0	8.9	0	3.1	0.001	288
Close + Closest	72.5	53	62.3	14.9	0.311	288
Activity duration, %						
Lie/sit with head still	5.6[a]	0.0[b]	17.0[c]	1.8	0.001	288
Stand/walk with head still	48.3[a]	63.6[a,b]	83.5[b]	10	0.006	288
NNOB	81.5	57.7	71.1	13.3	0.109	288
Head direction, duration, %						
Away	24.7[a]	17.4[a]	50.7[b]	4.5	0.001	288
Towards	75.3[a]	82.6[a]	49.3[b]	4.5	0.001	288

Descriptive stats for all healthy pig data								
					Quantiles, %			
μ	SD	CV%	SEM	Min	25	50	75	Max
74	17	23	1	0	70	80	84	99
10	20	200.1	1.2	0	0	0	10	100
23	29	125	1.7	0	2	10	32	100
23	29	123.9	1.7	0	47	78	93	100
67	32	47.8	1.9	0	46	80	95	100
68	32	46.6	1.9	0	46	78	93	100
1.3	5	393.7	0.3	0	1	3	7	59
52	43	82.1	2.5	1	7	47	100	100
77	28	36.4	1.7	0	66	91	98	100
26	21	79.3	1.2	0	10	22	39	92
74	21	27.8	1.2	8	62	79	90	100

No. experimental Units to detect Δ% from control[1]				
25	50	75	100	200
18	4	3	3	3
--	336	149	84	21
526	131	58	33	8
509	127	57	32	8
74	19	8	5	3
74	19	8	5	3
--	--	579	326	82
226	57	25	14	4
44	11	5	3	3
212	53	24	13	3
26	6	3	3	3

	Treatment			*P*-Values		
	Familiar	Unfamiliar	SEM	TRT	Time	TRT*Time
Approach-Index, %	84.8	84.4	3.06	0.766	0.002	0.661
Pen location duration, %						
Far	10.7	10.1	3.49	0.844	0.008	0.522
Mid	18.7	17.6	3.38	0.717	0.014	0.918
Close	70.4	72.3	5.25	0.617	<0.001	0.895
Activity duration, %						
Lie or sit, No NNOB	5.8	5.8	0.8	0.995	<0.001	0.901
Stand or walk, no NNOB	5.5	5.5	1.4	0.995	<0.001	0.524
NNOB	82.1	83.3	4.12	0.722	0.0029	0.617
Head direction, duration, %						
Away	23.9	23	2.81	0.725	<0.001	0.329
Towards	76.1	77	2.8	0.725	<0.001	0.329

Name of Material/ Equipment	Company	Catalog Number
Dome 3.0 Megapixel Cameras with 2.8-12mm lens set between 2.8-3.2 mm	Points North Surveillance, Auburn, ME	CDL7233S
Manfrotto 244 friction arm kit	B&H Photo	B&H # MA244; MFR # 244
Video Recording System	Points North Surveillance, Auburn, ME	NVR-RACK64
Colored and patterned duct tape attached to a double-sided medical grade tape	MBK Tape Solutions, Chatsworth, CA	3M 1522H
Approach Index Formula generator	Dinasym, Manhattan, KS	Approach Formula
Geovision Software	Points North Surveillance, Auburn, ME	Geovision
Clicker	Petco	Good2Go Dog Training Clicker
Reward treat (feed pellet, carob chip, raisin, marshmallow)	Variable	N/A
Statistical Analysis System (SAS)	SAS Institute, Cary, North Carolina	SAS 9.0
Observer 11.5 software	Noldus Information Technology, Leesburg, VA	Observer 11.5

Comments/Description
Lower mm lenses are needed for low-profile pens
To mount and secure cameras at a 90 degree angle
NVR is customized
Sustainable marking of pigs
Company will customize macros for specific lab needs
Software to edit video time into 180 second clips
Depending on previous exposure, adult pigs are very neophobic when new food is introduced. Limit-fed pigs can be fed a few pellets of feed.
Our laboratories preference for analyzing mixed models and repeated measures
Software to manually timestamp video clips

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Institution: Kansas State University
Article Title: Non invasive in pen approach test for laboratory pigs

Signature: Lindsey Hulbert Date: 6/8/18

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First, we would like to thank the editors as well as the reviewers for their thoughtful comments. We feel strongly that their comments and suggestions have improved the revised manuscript's quality and readability. Because we had significant alterations to the entire manuscript document, we did not highlight specific sections. Below, we have included detailed responses to each of the comments we received.

Editorial Comments:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
Response 1: Thank-you, we proofread the document before submitting.

Comment 2: **Protocol Language:** The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please re-write your ENTIRE protocol section accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
Response 2: We rewrote the protocol into short steps and reincorporate the extra information into the intro and discussion.

Comment 3: Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? (e.g. button clicks for software actions, numerical values for settings, etc). Alternatively, for steps that will not be filmed, add references to published material specifying how to perform the protocol action.
Response 3: We included video with to show the many, many steps for analyzing video. There was not a voice over, but there is text instead within the video. Please let us know if the new uploaded supplementary files will work.

Comment 4: **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
Response 4: We have now altered the numbering system to comply with the suggestion above.

Comment 5: **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when

calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.
6) Please match the videos provided (along with specific time stamps) to steps in the manuscript (the time stamps can be provided as a supplementary document). Please upload un-edited footage here -> http://www.jove.com/files_upload.php?src=17872133
Response 5: Completed just before submission and all other highlights removed The supplementary videos now have closed-captioning. The timestamps for behaviors are included in the sample video of the test.

Commented [ZK1]: Lindsey: This is an important step that I want to point out to you. I am sure you and your students did this already but just making sure.

Comment 6: **Results:** Please mention all statistical tests and sample sizes. It is unclear which test was used in Fig 4, 5
Response 6: We have now added this information into the figure legends.

Comment 7: **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
Response 7: The discussion section has been edited to include all the subsections (1-5) listed above.

Comment 8: **References:**
1) Missing citation on Line 115.

2) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

Response 8: Citation was added as # 14 and others following adjusted accordingly. In addition, we have now complied with the JoVE suggested format for citations.

Comment 9: **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Ellegaard, (CDL7233S Dome, Points North Surveillance, Manfrotto 244 friction arm kit; B&H photo, NVR-RACK64 Points North Surveillance, 3M 1522H, MBK Tape Solutions, Nasco's, Microsoft Excel-, Dynasim, Tenderfoot, SAS (version 9.3; SAS Institute Inc, etc.

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2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
Response 9:
1) We have now removed all commercial sounding language in the manuscript.
2) We have now removed the registered trademark symbols from the materials and reagent table.

Comment 10: Please define all abbreviations at first use.
Response 10: We have now looked through the entire manuscript and have defined all abbreviations at first use.

Comment 11: Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
Response 11: We have edited the manuscript to comply with this comment.

Comment 12: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
Response 12: The tables and figures are original and from pilot data collected specifically for testing the methodologies in the manuscript.

<u>Reviewer's comments</u>

Reviewer #1:

Comment 1: Manuscript Summary:
The authors adapted a common, non-invasive behavioral test to detect changes in pigs with mTBI. The authors did not, but should, discuss other potential applications for HAT; such as using this tool to evaluate general welfare state of laboratory pigs without treatment (negative affective state given barren environment) when performing routine health checks. All the materials and equipment needed listed in the table and are described well in the main text.
Response 1: We would like to thank the reviewer for this really good point. We have added more information about other applications such as animal welfare and sickness detection.

Comment 2: There is no explanation given as to why this procedure begins with using operant conditioning to allow pigs to associate clicker with human/food reward.
Response 2: The conditioning discussion was added to the new section in the discussion "trouble-shooting". The treats and clickers were incorporated when we were using a tripod and the test-person(s) would change their body posture and vocalize to call the pigs. By adding a treat, the pigs learned that the human was there for the treat, and not to provide feed, water, clean, etc. By adding the clicker, the human no longer needs to use a signal (i.e. vocal) that is human-dependent. The clicker signal can be used across laboratories. We learned that depending on the pig's experience, some treats are less motivating than others, so we believe that this portion needs to be up to the laboratory to decide what is most motivating for their pigs.

Comment 3: Lines 97-103: The number of Yucatan minipigs for housing type B and Göttingen minipigs for housing type C are not given (boars and gilts).
Response 3: The age + SD were added or the 5 female Göttingen minipigs.

Comment 4: Lines 120-126: Item "D: Defecation area should be used as an indicator that pigs are acclimated to their environment". No reference is provided to defend this statement.

Response 4: Reference is now provided and explanation was moved to the discussion.

Comment 5: Lines 191-193: "If pigs never had any adverse interactions with people, they typically generalize and associate all people positively with food" Again, no reference is given to defend this statement.
Response 5: Reference is now provided and a better clarification was added in the discussion.

Comment 6: Lines 241-241: How inter-observer reliability is calculated is not described. This is an essential step in any animal behavior study.
Response 6: Thank you for pointing this out. We cited Martin and Bateson for various methods for measuring calculating both inter- and intra-observer reliability. In our results, we added a statistical description that specifies and cites previous calculations.

Comment 7: Minor Concerns:
Authors did not provide explanation of "Why" rodent models are not sufficient
Authors did not define "Affective states"
Response 7: We modified these statements extensively in the introduction; this was the second reviewer's concern as well.

Comment 8: Lines 114-115: This statement is not a general fact for swine behavior. It is a reference to one study that is not listed. Coffin et al., 2018 is cited in text, but not listed in references (also, other in-text citations are numerical).
Response 8: This section was moved into discussion and the citation is now provided.

Comment 9: Lines 293-295: This report of intra and inter-observer reliability has not been published.
Response 9: This was published as a master's thesis report and is accessible online: Luo, Y. Swine Applied Ethology Methods for a Model of Mild Traumatic Brain Injury (Master's Thesis). Retrieved from http://hdl.handle.net/2097/35760 (2017).

Comment 10: Lines 282-291/Figure 3: This graph is not intuitive. There is no justification/rationale for color-coded pigs in the graph. Axis labels are needed on figure 3 (it is not clear why there is a secondary axis as well; it is assumed that both y-axis represent the approach %).
Response 10: The figure legend has been updated. This graph is a visual representation of how the formula and weighting was composed and will be explained in protocol-script. The colors are formulated similar to a heat map (red = most approach to black = least approach).

Reviewer #2:

Comment 1: Manuscript Summary:
The authors have created a behavioral task wherein the piglets behavior when approaching a human can be used to quantify the effects of mTBI and possibly other conditions (injury, sickness, or stress). First, the authors have embarked on a momentous task, that of creating and validating a new behavioral paradigm. However, significant revisions should be made.
Response 1: We thank the reviewer for their thoughtful comments.

Comment 2: The authors state there is a need to develop and standardize functional tests specific to somatic, cognitive, and affective symptoms under study (lines 49-51), but continue to describe a behavioral test that has no known relation to any specific cognitive domain or brain region. Of course, some flexibility should be allowed as behavioral tests that have been in use for decades still see debate in

academic literature on what these tests truly measure. However, it should be acknowledged that the underlying biology of the HAT test is unknown, and in its current state, it is more of a global rather than specific measure of approach behavior.
Response 2: The introduction was extensively changed to better reflect a general affective state of motivation vs. anhedonia and motivation rather than a focus on cognition and specific brain regions.

Comment 3: The authors suggest that as there is no need to physically handle or train animals, thus reducing the subjectivity of the test. While this is true, the authors should also acknowledge the range of characteristics which make HAT impossible to standardize, e.g., the experimenter's height, gender, temperament, odor, relationship to the animal, etc. This limitation is addressed in table 2, however can the authors clarify the sample size? I see 7 pigs were used with two sessions each, but how many different familiar and unfamiliar humans were used, one each?). These are also limitations with other behavioral paradigms, but where other tasks make an object or food reward the stimulus and the human an auxiliary function of the task, here the human is the stimulus. Thus, it is impossible to standardize the stimulus of interest across labs, whereas the use of an object or food reward can easily be standardized. A comment on this limitation would be helpful.
Response 4: We agree with the reviewer’s comments and have now added more to the discussion, plus our additional studies reflecting familiar vs. unfamiliar humans. As previous studies in using hat for evaluating commercial pig production, we did not find differences between a familiar person and an unfamiliar person. Objects and food to be introduced early in life for standardization, and most biomedical researchers do not have direct access to developing pigs. In addition, we have developed another test that uses an object that moves rather than a human and compared the human approach behaviors to the object approach behaviors. The pigs behave very consistently to the human, but variable to the object, therefore, we think both tests (HAT followed by Object test) will be useful toolsets. We are currently preparing a manuscript detailing the protocol and findings from the object-approach test.

Comment 4: On line 61 it is stated that changes in the HAT measures should signify symptoms of mTBI rather than excess stress or fear, why? Is it not plausible that mTBI disrupts emotional processes (e.g., the amygdala) or the HPA axis, and the HAT is truly measuring the disruption of those specific processes? There have not been adequate controls to suggest that the severity of mTBI is the true measure. Proper habituation to a testing environment will still not remove the effect of stress during testing, although it will mitigate it. The human is most likely a source of stress, whether that be distress or eustress. Indeed, the authors go on to state on line 81 that HAT can be used to measure injury, sickness, and stress. These are competing hypotheses that should be reconciled or at least addressed prior to publishing.
Response 4: We thank the reviewer for this insightful comment. We have modified our introduction extensively to address these points. The stress response will indeed be activated whether the stressor causes distress or eustress, but pigs responses to leaving the pen and handling is variable, therefore, this test reduces that variation. There were sham-controls (received anesthesia-only) that were compared to Blast-treated pigs. Other than exposure to Blast, shams were treated identical to Blast-treated pigs. The wording in the manuscript now says “HAT was able to *distinguish* Sham-pigs from mTBI-pigs.”

Comment 5: Overall, there are questions that remain unanswered and claims put forth without relevant data for backing. However, it is unfair to judge new behavioral paradigms with those that have existed for decades. Based on the present data, the HAT appears to be a sensitive marker of mTBI, however it is recommended that the authors revise the manuscript with a more conservative attitude.
Response 5: The introduction was edited extensively and we have ensured that we did not over-reach. The main objective of this manuscript is to develop one new protocol for lab pigs, and it is not our intention to replace rodent models or replace HAT with other published behavioral tests.

Comment 6: On lines 90-91, it is stated that the approach index mitigates variation caused by confounding factors between labs. As the approach index is created by using the behaviors that are

affected by confounds between labs, isn't it impossible for the AI to not also be subject to those confounds?
Response 6: We thank the reviewer for this comment and this sentence has been deleted.

Comment 7: Step 4: What is the pig acclimating to? The human? The reward? The use of a food reward turns this into an operant task that further complicates the behavior being tested. Is HAT supposed to be a measure of sociability? Of learning? Of exploratory behavior?
Response 7: The purpose of this step is further explained in the introduction and in the discussion. The food reward was added during our troubleshooting of the test. Pigs are exposed to humans daily for various purposes (feeding, cleaning, handling before treatments). The specific routine and treat allows the pig to anticipate what the test-human will do next so that it behaves similarly (when untreated or recovered) for every session.

Comment 8: Close, mid, and far are arbitrary distinctions. Why not measure distance from the human using automated tracking software? This is more expensive, but less arbitrary and removes the possibility of binning errors. The author notes the use of such software in the manuscript (Step 6C).
Response 8: We wanted to make the first protocol using manual timestamping of the spatial positions of the pigs, so that all labs have access to this test. The new troubleshooting section and limitations to the test now include further explanation with citations.

Comment 9: There are two nose directions (away and towards human), what approach should be taken when the head is neutral to the human? Pigs may orient their head to the side, but still look at a human.
Response 9: Figure 2A and the accompanying legend describes what was considered away and towards. The neutral positions were highly variable within- and between-observers, so the definition was reduced to two states rather than three.

Comment 10: Why is it suggested that pigs standing or walking without NNOB are in an agitated state (Line 232)? There is no data or reference for this behavior.
Response 10: We added more explanation and references in the introduction. NNOB are expressed in pigs ubiquitously, and were once considered stereotypies or even abnormal. Pig ethologists are now reaching a consensus that NNOB are an important functional behavior when expressed at normal time-budgets in a 24-h period. Pacing is often seen in captive exotic animals (i.e. zoo). Even among other species, the lack of NNOB during pacing is considered abnormal because the animal is expending energy without a purpose. NNOB has a clear purpose- it allows the animal to gather information about its environment, with the likely motivation to seek out a substrate to chew on.

Comment 11: Step 6: Why edit footage into three-minute sessions? Data can be binned post analysis.
Response 11: This is now better explained in the trouble-shooting section discussion.

Comment 12: Step 6D: The assertion that even trained observers do not need to pause, rewind, or time-stamp frame by frame is based on opinion and fosters an assumption that trained observers are not subject to their own internal biases. Pausing and re-winding is a valuable tool for accurately assessing behavior. It is recommended to omit or revise this section as is based on opinion.
Response 12: We have provided additional insights under the discussion as why we ended up with these methods. These methods were proposed based off of experience, but using guidelines for measuring behavior and our own experiences for improving the repeatability and reliability of the test.

HAT data collection sheet   Experiment:________________   Year_______________

Event	Day DD	Date MMDDYYYY	Start Time HH:MM	End Time HH:MM	Pen #	PinkPigID ####	GreenPigID ####	Initials	Video File Name HAT_Date_EndTimeh_Pen##_Green####_Pink####
Acclimation	-11	03082018	6:30	6:33	1	8831	8838	EM	HAT_3082018_6:33_01_Green8831_Pink8838
Acclimation	-11	03082018	6:40	6:43	2	8850	8834	EM	HAT_3082018_6:43_01_Green8850_Pink8834
Baseline	-1								
Treatment	0								
1post	1								
2post	2								
3 post	3								
4post	4								

HAT data collection sheet Experiment:________________ Year_______________

Notes

Step	Folder	Filename		Start time
1.2 and 1.3	Hat Test Steps	Behavior with side bar pig 1		0:00:00
1.6	Routine Set up	Cleaning (Rufus)_01		0:00:00
1.6	Routine Set up	Feed Evening (Kevin)		0:00:00
1.6	Routine Set up	Feed Morning (Kevin)		0:00:00
1.6	Routine Set up	Feed Evening (Rufus)		0:00:00
1.6	Routine Set up	Feed Morning (Rufus)_01		0:00:00
1.6	Routine Set up	Moving (Rufus)		0:00:00
1.6	Routine Set up	Moving with the board (Kevin)		0:00:00
2.3		Figure 1		--
2.6		Supplementary File 1_example data collection		--
3.3	Hat Test Steps	Dropping the pellet		0:00:00
3.4.2	Hat Test Steps	Behavior with side bar pig 2		0:00:00
4.1	Supplemental videos	1_Observer set up with subtitles		0:00:00
4.2		Figure 1		--
4.3		Figure 2A		--
4.4	Ethogram descriptors	Chomp 1		0:00:00
4.4	Ethogram descriptors	Chomp 2		0:00:00
4.4	Ethogram descriptors	Rooting1		0:00:00
4.4 to 4.4.3		Figure 2B		--
5.5 to 5.5.3	Hat Test Steps	Behavior with side bar pig 1		0:00:00
5.6	Supplemental videos	2_Export data Hat Subtitiles		0:00:00
5.6	Supplemental videos	3_Open_Excel_Subtitles		0:00:00
6.1		Figure 3		Example, ~15 seconds
6.1		Figure 4		Example, ~15 seconds
6.1		Figure 5A		Example, ~15 seconds
6.3		Figure 3		--

End time
0:00:30
0:03:05
0:00:15
0:00:25
0:00:18
0:00:12
0:00:14
0:00:54
--
--
0:00:59
0:01:20
0:00:20
--
--
0:00:18
0:00:24
0:00:11
--
0:01:20
0:00:15
0:00:15
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Folder	Filename	Filetype
Hat Test Steps	Behavior with side bar pig 1	.wmv
Hat Test Steps	Behavior with side bar pig 2	.wmv
Hat Test Steps	Dropping the pellet	.wmv
Supplemental videos	1_Observer set up with subtitles	.mp4
Supplemental videos	2_Export data Hat Subtitiles	.mp4
Supplemental videos	3_Open_Excel_Subtitles	.mp4
Ethogram descriptors	Chomp1	.mp4
Ethogram descriptors	Chomp2	.mp4
Ethogram descriptors	Rooting1	.mp4
Routine Set up	"Cleaning (Rufus)_01"	.mp4
Routine Set up	"Feed Evening (Kevin)	.mp4
Routine Set up	Feed Morning (Kevin)	.mp4
Routine Set up	Feed Evening (Rufus)	.mp4
Routine Set up	Feed Morning (Rufus)_01	.mp4
Routine Set up	Moving (Rufus)	.mp4
Routine Set up	Moving with the board (Kevin)	.mp4

Description	Purposes for presentation	Entire video for supplementary materials
At the start of the test, the pig is in the lie-position. The behavior time-stamps are represented with bars	Can be used to show video set up and starting position	Yes
At the start of the test, the pig is in the stand-position	Used to show that animal can start in different spatial and stuctural behaviors	Optional
At the start of the test the researcher drops pellets and place his hands out of pig's sight	Usen to show the begining of the test	Yes
A step-by-step protocol that is specific to the software	A short duration to show availability	Main purpose is for supplemental steps
A step-by-step protocol that is specific to the software	A short duration to show availability	Main purpose is for supplemental steps
A step-by-step protocol that is specific to the software output	A short duration to show availability	Main purpose is for supplemental steps
To describe behaviors included in NNOB	Short durations to show behaviors including in NNOB	Yes
To describe behaviors included in NNOB	Short durations to show behaviors including in NNOB	Yes
To describe behaviors included in NNOB	Short durations to show behaviors including in NNOB	Yes
To demonstrate how pens were cleaned everyday	To emphasize the importance of cleaning the pens in a daily basis	
To demonstrate how evening feeding	To demonstrate husbandry routine	
To demonstrate how morning feeding	To demonstrate husbandry routine	
To demonstrate how evening feeding	To demonstrate husbandry routine	
To demonstrate how evening feeding	To demonstrate husbandry routine	
To demonstrate moving the miniboar for pen cleaning	To demonstrate husbandry routine	
To demonstrate moving the miniboar for pen cleaning using a board	To demonstrate husbandry routine	