Swine Applied Ethology Methods for a Model of Mild Traumatic Brain Injury

by

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Abstract

Stressors and stress responses are part of everyday life, for both humans and animals. Organisms evolved well-developed mechanisms to cope with most stressors, and to recover from stress responses. Nevertheless, severe acute stressors and chronic stressors lead to health problems. Traumatic brain injury (TBI) is defined as malfunctioning and pathology of the brain caused by external mechanical forces. This physical and psychological stressor may lead to long-term damage on both physiology and psychology mechanisms. Traumatic brain injury becomes a public health issue for millions of soldiers, veterans and general public, who suffer from its aftermath and reduced quality of life. To understand TBI, human patients and rodents models were extensively studied. In recent years, miniswine were utilized to research the histopathology of TBI. They serve as a better human brain model because their nervous system is more anatomically relevant than rodents, their brains have similar white:grey matter ratios as humans, and they have similar cognitive abilities as humans. Despite the progresses in pathology and histology work among miniswine models of TBI, there were not validated behavior tests for this new animal model. This thesis introduced two behavior-tests for Yucatan miniboar models.

The first study was conducted to validate a modified human approach test (HAT) specifically designed for Yucatan miniboars for mild TBI experiments. This test was originally validated and widely used for commercial pigs. The current test was designed around the housing and animal care, with the experimental performing the test outside of pens where pigs were individually housed. Animals were treated with a single blast wave (BLAST) or anesthesia only (control, SHAM), and were tested 3 days before the treatment (baseline) and 3 consecutive days after the treatment. During the test, the spatial positions (Climb, Close, Mid and Far) and structural positions (Stand, Lie) were measured. Climb and Close were collectively named

approach behaviors, and Mid and Far Move away behaviors. Results showed that this test had high reliability, and was sensitive to acute effects of TBI: BLAST-treated pigs showed decreased approach behaviors and increased move away behaviors following the treatment, compared to the baseline.

The second experiment was conducted to develop automated data collection methods to monitor circadian active and inactive behaviors of miniboars. Using the same experimental design as described previously, Fitbit Zip, a commercially available accelerometer with an embedded algorithm (Fitbit, San Francisco, CA), was tested. When attached to ear tags, Fitbit Zip was validated to be recording head movements without locomotion, which were oral-nasal-facial (ONF) behaviors. Results showed that Fitbit Zips best-detected behavior changes following TBI at 2-hour observation intervals. BLAST animals showed decreased ONF behaviors during the day especially around the feeding time, which were also when the pigs were most active.

Both behavior-tests were shown to be reliable and useful in measuring behavior changes following TBI in Yucatan miniboar models. Measures of behavior were shown to be a promising and valuable addition to the biomedical research utilizing large animal models. These advances in knowledge and technology could also benefit farm animal production.

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Chapter 1 - Review of Literature

Introduction

Organisms are faced with and respond to different types of stressors every day. A stress response occurs when homeostasis is disrupted, which then leads to processes of allostasis. Homeostasis, as first defined in early 1930s (Cannon, 1932) ,was the comparatively stable physiological states that are required for survival. Allostasis, first defined in late 20th century (Sterling and Eyer, 1988), is a regulatory mechanism to make serials of changes in responses to homeostasis disruptions and changing environmental factors, and thus to sustain homeostasis (Ramsay and Woods, 2014). Allostasis is reflected in physiological, psychological and behavioral changes (Ramsay and Woods, 2014). Stress is quantified by measuring behavior and physiology (Dantzer and Mormede, 1983): behavior- and hormone-tests are available to measure the stress responses (Grandin, 1997; Sheriff et al., 2011) or the aftermath of stress (Lupien et al., 2009). Behavior measures can also be used as toolsets to evaluate husbandry and welfare of animals (Gonyou et al., 1986; Hemsworth et al., 1987; Hemsworth et al., 1989).

Injury as a type of stressor can also trigger stress responses. With the increase prevalence of traumatic brain injuries in human population, an urgent need of understanding of such injuries leads to the introduction of large animal models to biomedicine as a model for traumatic brain injury (Shultz et al., 2016); and thus an need of understanding and measuring of their stress responses (Bondi et al., 2015). Domestic farm animals may experience many types of stressors, from prenatal stage all the way through slaughter which may influence production and animal welfare (Clark et al., 1997; Beydoun and Saftlas, 2008; Merlot et al., 2013; Miranda-de la Lama et al., 2014; Belhadj Slimen et al., 2016; Hulbert and Mois á, 2016). Therefore, there are many behavior-tests available to be translated into a biomedical environment for rodent species. This

review will discuss current progress of large animal model and behavior-tests available for the use of a large animal traumatic brain injury model.

Stress and Health

Stressors and stress responses

If an animal perceives a stimuli as stressful, homeostasis is interrupted, which changes physiological parameters in response to the stimuli, through allostasis (Fink, 2010). Such stimuli are considered stressors, which can be physical or psychological. The changes of physiological parameters in response to the stimuli are stress responses (Dantzer and Mormede, 1983; Aldwin, 2007). A stress response is not always a negative experience for the animal because and allostatic mechanisms may bring positive effects through changes in physiology, behavior and cognition. Depending on whether the effects caused by a stress response is more destructive or positive, stressors and stress responses were also categorized as "distress" (destructive stress, e.g. predators) and "eustress" (positive stress, e.g. mating; Kupriyanov et al., 2014).

The hypothalamic–pituitary–adrenal axis

Stress responses are regulated by the hypothalamic–pituitary–adrenal (HPA) axis. The main neuropeptide released from the hypothalamus is corticotropin-releasing hormone (CRH), which targets the corticotrophs of the anterior pituitary gland. The primary hormone released by corticotrophs into the periphery is adrenocorticotropic hormone (ACTH), which mainly targets the ACTH receptors on the adrenal cortexes, which causes the release of glucocorticoids (i.e. cortisol, corticosterone). Prior to activation of the HPA axis, the sympathetic nervous system causes release of catecholamines (i.e. epinephrine, norepinephrine, dopamine) from adrenal medulla and at nerve terminals, to stimulate respiration, heart rate, and blood pressure and elicit innate behavior responses. The innate behavior responses to stress include freeze, flight, or fight

(Aldwin, 2007). These responses to stressors are homeostatic mechanisms; glucocorticoids target at hypothalamus and pituitary to inhibit the secretion of CRH and ACTH, respectively, thus providing a negative-feedback mechanism, and the allostatic behavior changes allow the animal to change to remain stable.

When some stimuli trigger repeated stress responses to the point that an animal cannot use allostatic methods to remain stable (Tsigos and Chrousos, 2002), these stressors may be considered chronic. Chronic stress in early life causes a number of stress-related problems during adulthood (Hackman et al., 2010). Chronic stress builds up allostatic load, which is the "tear and wear" of the physiological system (McEwen, 1998; Sapolsky, 2004). Chronic stress can be identified by a dysregulated HPA axis with symptoms of increased circulating glucocorticoids during parasympathetic periods. Allostatic load is an indication that the organism cannot regulate the stress response and recover appropriately; which will then lead to pathology (McEwen, 1998). Hence, chronic stress may lead to the deterioration of the cardiovascular system, immune system, and cognitive functions (McEwen and Seeman, 1999).

Glucocorticoids also play a role in circadian physiological rhythms, even without activation of the sympathetic nervous system (Mason, 1968). In humans and many other diurnal animals, serum cortisol concentrations are low at night during sleep, and increase before waking up. Serum cortisol reaches the peak at the beginning of the day then drops for the rest of the day (Knutson et al., 1997). This trend holds for both females and males, and also holds for difference ages. However, like humans, pigs are diurnal animals with biphasic sleep-cycles (Ingram and Dauncey, 1985), and the same pattern of glucocorticoid secretion observed (Hillmann et al., 2008). Nonetheless, in most species the response to acute stressors, the glucocorticoid response is conserved across species.

Hippocampus and amygdala as part of the stress responses

At least two structures in the limbic system are also involved in the regulation of HPA axis: 1) The hippocampus that is the center of memory and spatial navigation (Bird and Burgess, 2008), and 2) The amygdala which is the center of several emotions including fear and anger (Tovote et al., 2015). An animal's prior experience influences the perception and the severity of the stressor, through the mediation of hippocampus and amygdala. The more novel the stressor, the more the amygdala is involved to respond, whereas, the more experience the animal has with a stressor, the more the hippocampus is involved. As part of the negative feedback, increased binding of glucocorticoids to the GR receptors on hippocampus up-regulates the inhibition of hippocampus over the HPA axis, and thus down-regulates the HPA activation (Hackman et al., 2010). This then decreases the activation of sympathetic nervous system and thus the physiological arousal (Liu et al., 1997). This regulatory mechanism is necessary and vital for the survival of organisms in case of the sudden change of environment or in the event of acute stressors (Aldwin, 2007; Fink, 2010).

On the contrary, the hippocampus could also serve as a target of HPA axis hormones following severe and novel distress, which leads to cognitive impairments. Severe distress during early life as well as chronic stress could damage hippocampus through the CRH-CRHR1 (CRH receptor 1) signaling pathway through elevated CRH, and may influence hippocampus-dependent memories (Wang et al., 2011; Wang et al., 2013). Among highly gregarious animals, social defeat distress caused short-term deficit in learning through mGluR5/Homer1 signaling with elevated glucocorticoids (Wagner et al., 2013).

The amygdala, as part of the network with hippocampus and HPA axis, also serves as both the regulation and target of HPA axis activation (McEwen et al., 2016). Amygdala secretion

of corticotropin releasing hormone increases following restraint as a stressor (Hand et al., 2002). Interestingly, the amygdala released CRH independently of HPA activation and is primarily activated when rats exhibit fight, flight, or freeze behaviors (Makino et al., 1999). Mitra et al. (2009) reported that the down-regulation of amygdala neuronal activities reduced anxiety and stress-induced glucocorticoids secretion in rats. The hippocampus-amygdala network is also a target of HPA axis arousal, which may help reinforce the formation of memories about important experiences, such as exposure to a predator (Adamec et al., 2005). However, rats exposed to repeated, chronic immobilization stress had increased spine density in the basolateral amygdala spiny neurons which are important enhancing fear memories (Mitra et al., 2005).

Acute and Chronic Stress

Acute and mild stressors may not lead to health problems, especially if they are perceived by the animal as a positive stressor (i.e. eustress; McEwen and Seeman, 1999). Eustress increases neurogenesis in hippocampus, thus potentially improving memory, learning, and cognition. Positive stimuli that cause eustress may include exercise or environmental enrichment (Lucassen and Oomen, 2016). However, an environment that never stimulates the HPA-axis can cause dysregulation. For example, gentle handling in early life up-regulated hippocampal GR in neonatal mice. This upregulation of GC made the hippocampus more responsive to stressors, thus improving HPA-axis regulation. As adults, these mice had better HPA-function compared to mice that were not gently-handled as neonates (Meaney et al., 1988). However, no stimulation from handling in early life caused mice to express more anxiety behaviors as adults (Hackman et al., 2010).

Chronic distress can be defined when the HPA axis is repeatedly activated and prolonged glucocorticoid secretion causes down regulation of glucocorticoid receptors in the hypothalamus

and anterior pituitary, thus reducing the effectiveness of the negative feedback signal, which results in increased baseline plasma glucocorticoids concentrations. In addition, the ineffective negative feedback loop can cause adrenal-exhaustion and the release of glucocorticoids following a stress will also decrease. Adrenal exhaustion can be identified through enlarged adrenal cortexes. A dysregulated HPA axis is also identified by a delayed response to stressors and a lessened and prolonged peak of glucocorticoids (Aldwin, 2007).

Traumatic brain injury and animal models

Traumatic brain injury

Physical trauma activates the HPA axis (Aldwin, 2007). If physical trauma is in the periphery, necrotic cells release danger associated molecular patterns (DAMP) and the pro-inflammatory response causes the release of cytokines that can directly activate the HPA axis. In fact, glucocorticoids are released even after sedated animals undergo sterile injury. Glucocorticoids play a key role in regulating inflammation (Dantzer et al., 2008; Garc á-Bueno et al., 2008; Fleshner, 2013). In turn, a severe, psychological stressor without physical trauma can induce an inflammatory response; For example, stress-induced neurodegeneration, as discussed previously, causes the resident macrophage cells (i.e. microglia) to elicit a local inflammatory response within the central nervous system (Emerit et al., 2004).

When the injury is in the central nervous system, regulation and recovery are more complex than when the injury is in the periphery. Traumatic brain injury (TBI) is used to refer to damage, malfunctioning or pathology of the brain caused by external mechanical forces (i.e. from direct impact), acceleration or deceleration forces (e.g. automobile crash), blast waves (i.e. pressure changes from explosion), penetrating or blunt force trauma (Iverson, 2005; Taber et al., 2006; White and Venkatesh, 2016). Severe TBI, especially from penetrating forces and extreme

external mechanical forces is marked by extreme changes in central nervous system function, inflammatory response, and neurodegeneration (Kumar et al., 2014; Skolnick et al., 2014; Corps et al., 2015; Wang et al., 2015). However, more subtle injuries (mild TBI) from within the brain manifests in a severe pathology and behavior outcomes are quite apparent and easy to assess (Alam et al., 2005; Halaweish et al., 2015a; Halaweish et al., 2015b). However, mild TBI is widely studied in rodents, and some of the behavior tests capture this type of injury, for example, after low or moderate injury, rats exhibited significant deficits balancing and working on a narrow beam in the first few days after the treatment and then recovered (Lyeth et al., 1990).

Traumatic brain injury research is a significant area of interest because soldiers are exposed to various types of explosive blasts and the explosive devices during their training and during deployment (Taber et al., 2006; Alley et al., 2011). Researchers have tested the hypothesis that TBI contributes to the pathologies of Post-Traumatic Stress Disorder (e.g. Hoffman and Harrison, 2009). Nonetheless, the severe, psychological stress response from the traumatic experience may confound TBI, therefore it is challenging to separate traumatic brain injury from psychological stress among patients with Post-Traumatic Stress Disorder (Hoffman and Harrison, 2009). Long after traumatic brain injury, both children and adults display impulsive, aggressive behaviors that impede their ability to solve social conflicts, even after they appear to be healed neurologically (Greve et al., 2001; Janusz et al., 2002). This often leads to a lifetime of challenges, such as a loss of social support, difficulties of forming new social relationships, decreased leisure activities, and increased anxiety and depression (Morton and Wehman, 1995). The anxiety and depression aspect can be studied in rodent models of brain injury models, where traumatic brain injury induced significantly increased depression-like behaviors during a forced swimming test (Milman et al., 2005).

In addition to the social challenges and mental illnesses, working memory were shown to be imparied in the mild to severe traumatic brain injury human patients. Their performance of high load working memory tests was significantly impaired compared to the control, although their reaction times did not change. Functional magnetic resonance imaging (MRI) revealed that traumatic brain injury patients showed an altered activtion pattern of the hippocampus. Injury early in life appears to heal faster than injuries in adulthood. For example, TBI children acutely showed impaired learning abilities, but the majority of the deficients seemed to heal after 3 months or in the following year, with the help of treatment (Ponsford et al., 2001; Carroll et al., 2004). Nonetheless , as adults, these patients are at higher risks to develop psychiatric disorders (Max et al., 1998).

Neurological pathology and behavior tests are well optimized in rodent models of TBI. For example, spatial memory tests with mice and rats TBI models showed that TBI animals had impaired performances in spatial memory tasks with swim T maze (Milman et al., 2005) and 8-arm radial maze (Lyeth et al., 1990). Morris water maze test showed increased time of finding the platform of TBI rats, also difficulties of retaining memory (Kline et al., 2010; Säljö et al., 2011; Monaco et al., 2013). In tests evaluating balancing abilities, beam balance/beam-walking tests showed rats had difficulties balancing on a narrow beam following TBI; and rotate-rod test showed short time the injured animals could stay balanced on the rotating rod (Kline et al., 2010; Monaco et al., 2013). In passive avoidance tests, mice were trained to avoid an area of aversive stimuli. Injured mice showed decreased abilities of learning to avoid the aversive stimuli (Milman et al., 2005). Forced swimming test showed increased depression-like behaviors of TBI mice (Milman et al., 2005). Fear conditioning tests showed increased fear responses and anxiety-

like behaviors (Rodgers et al., 2014). In addition, during open-field tests, TBI mice spent less time in the open field compared to untreated control (Impellizzeri et al., 2016; Song et al., 2016).

Pig model for traumatic brain injury

Funding agencies (e.g. Department of Defense, NIH, NSF) for traumatic brain injury are seeking out experiments that involve pigs as models for TBI. Pigs are good animal models for human neurological development because they have more similar brain anatomy to humans, more white matter volume and more complex cortex (Friess et al., 2009; Bondi et al., 2015). In addition, their brain growth is very similar to humans (Conrad et al., 2012). Conrad et al. (2012) monitored pigs brain growth from 2 through 24 weeks of age with MRI based volume quantification techinque. They found that had maximum growth in brain volumn at 4 weeks of age in gilts and boars, and 95% at 21-23 weeks of age. They also found sexual dimorphism in pig brain growth, that male hippocampus is larger than females at 24 weeks of age, although the maximum increase of hippocampal volume occurs at 3 weeks of age in females while 8 weeks of age in males. Males showed greater cerebellar growth than females, and showed maximum diencephalon growth earlier than females. These changes are very similar to human brains, indicating that pigs provide a very good brain model for humans. Miniature pigs also successfully provided animal models for effective large scale preclinical studies (e.g. diseases; Whyte and Prather, 2011), and serve as a good model for central nervous system lesions (Dolezalova et al., 2014; Bondi et al., 2015). Given the similarities in brain development, and physiological parameters, pigs may be a very useful model thus far for human traumatic brain injury studies (Bondi et al., 2015).

Behavior tests using large animals

Most neurological behavior-tests were designed for rodents. Given the difference between the behaviors of nocturnal animals like mice and rats, tests designed for rodents may not all be suitable to large animal models. Many of the behavior tests cannot be transferred easily to large animal models. For practicality, larger animals require larger testing areas and testing apparatuses, which is more costly (Shultz et al., 2016). Tests for spatial memory (e.g. multiple-arm mazes and Morris water maze), balancing (e.g. rotarod test, beam walking) and forced swimming test require test apparatuses to match the size of the animals. These tests can be easily done on mice and rats with small body sizes, but are more labor intensity and less cost efficient to be performed on large animal models, especially when space is limited.

Also, the tests were originally designed to match the phylogeny of the rodent. For example, the same tests designed for nocturnal animals may not be easily modified for diurnal animals. Open field test measures the avoidance of the open field in the test arena. Open field and brightness are perceived as aversive by rodents as nocturnal animals, but may be perceived as appetitive stimuli by pigs (Andersen et al., 2000). Although the exact same rodent version is not readily to be used in swine, modified open field test are designed for pigs to measure affective states (i.e. fear; Kornum and Knudsen, 2011), but showed low correlation with other fear tests and was not recommended to study fear in pigs (Forkman et al., 2007). Therefore this requires that we develop new behavior-tests that are suitable to perform on pigs, and can be validated to be sensitive to traumatic brain injury related behaviors of the new introduced large animal models.

Our lab hypothesized that depending on importance to survival, animal behaviors can be categorized into a pyramid (Figure 1), where the most critical behaviors for survival provide the base for all the other behaviors (survival or maintenance), but the less-critical behaviors for

survival may be important for coping and neurological development (e.g. tactile stimulation from exploring the environment) are categorized next. The third category includes behaviors important for social structure, and the final category is the behaviors that are important for reproductive fitness. For example, in most domesticated large animals (e.g. pigs, cattle, sheep, horses), a decrease in reproduction related behaviors is observed in the pre-pathological state of injury or sickness (Johnson and von Borell, 1994; Millman, 2007; Fogsgaard et al., 2012). Then, sick or injured animals may decrease exploratory activities and social interactions with other animals while increasing resting. It is not until a sickness reaches its full pathology that the most basic behaviors for survival, such as feeding and drinking are extinguished (Moberg and Mench, 2001; Mills and Marchant-Forde, 2010; Hulbert, 2015).

Hence, researchers need to choose behavior tests that are sensitive to the specific type and severity of injury. In humans and other animal models, it was already observed that social behaviors were interfered following traumatic brain model (e.g. Janusz et al., 2002), hence the next level of comfort and opportunity behaviors, may be worth investigating in large animal model.

Human approach tests measuring approach and avoidance behaviors

Gieling et al. (2011) proposed criteria for behavior-tasks for pigs: 1) the task the pig needs to perform should not cause distress; 2) the assay needs to be standardized for ease of replication and repeatability, and; 3) the behaviors should be well-defined, non-ambiguous, and as objective as possible. These criteria are important to allow detailed analysis of behavior, that is sensitive enough to detect differences between treatments where a pre-pathological state is induced (Gieling et al., 2011). The human approach test for pigs is a reliable test for testing fear and motivation in pigs (Waiblinger et al., 2006; Powell et al., 2016). Human approach test

(HAT) was first described by Hemsworth et al. (1989). During a 3-minute test, animals' behaviors of approaching humans were observed, including the latency of animals entering an area of 0.5m from the experimenter. This test was used to measure the fear of human in sows in this study, and it result was shown to be correlated to the attitude of the stockperson to the pigs (Hemsworth et al., 1989). In both pigs and cattle, HAT test measures of the paradox of affective states because these species are both fearful and motivated to interact with humans (Waiblinger et al., 2006; Forkman et al., 2007). Hemsworth et al. (1986b; 1987; 1996) reported that pigs are more likely to approach if they experienced pleasant interactions with humans. This means that they associate human interactions with positive stimuli (e.g. stroking, feeding), and are more likely to approach the experimenters during such a test (Hemsworth et al., 1986a; Breuer et al., 2000).

However, aversive treatment causes pigs generalize (Hemsworth et al., 1987); they will not distinguish the experimenter that caused aversive stimuli from an unfamiliar experimenter. Pigs also showed no difference between approach behaviors to familiar or unfamiliar handlers during HAT, regardless of the treatment they received from the familiar handler (positive or aversive; Hemsworth et al., 1994). These results indicated that HAT tests the pigs' overall behavior-responses to humans, rather than individual handling styles; therefore opportunistic generalization helps make HAT a reliable behavior test for pigs.

The human's behavior in HAT can be adjusted for varied outcomes of pig responses. For example, if the human remains stationary during HAT, researchers suspect that HAT measuring curiosity-strength or motivation (Waiblinger et al., 2006). If the human moves in an unpredictable manner, the HAT may be testing fear. However, stationary is preferred because 1) stationary positions are repeatable, and; 2) fear-responses are more variable in pigs than

motivation. Therefore, if the human does not move or approach the animals, the measurement of motivation is less confounded fear responses and between human variations. In addition, Hemsworth and colleagues also observed that pigs were more likely to approach a stationary test-person than a moving test-person during human approach tests (Hemsworth et al., 1986b). The stationary human also facilitates more objective definitions of the pig's spatial behavior.

Hemsworth et al. (1989) designed their HAT test so that commercial pigs were moved one-at-time into an arena, which is similar to open field tests in rodent-models. They defined approach as when the sow entered the 0.5 m radius around the stationary test-person. This test provided two variables to measure: 1) the latency of the sow to approach and, 2) the latency of the sow to make contact with the test person. Short-latencies were interpreted as motivation; long-latencies were interpreted as avoidance. The main concepts behind HAT were repeated, but the assay procedures were modified to match the conditions of the housing and management system of the pigs. Nonetheless, researchers retained in their HAT protocols that the test-person was stationary (stand or squat), they entered the test-area, and the animals' spatial relationship to the human were measured (Waiblinger et al., 2003; Scott et al., 2009; Powell et al., 2016). HAT is a time-efficient and easy-to-standardize test; hence experimenters can be easily trained to follow the protocol. This test measures voluntary approach, which may be used to differentiate curiosity (or the motivation of approaching), from fear of the animals to humans (Powell et al., 2016).

Genetics of animals

Although HAT tests were established and validated for commercial pigs under typical swine husbandry conditions, to the author's knowledge, no researchers modified and validated the test for miniswine. Standard commercial pigs have fairly homogeneous genetic lines, from

mainly a European-boar background (e.g. PIC, Genesus Genetics; Andersson et al., 1994). Large-breed commercial pigs were artificially selected for high growth rates and lean body types (Mrode and Kennedy, 1993; Hermesch et al., 2000; Camerlink et al., 2014).

Genetics could potentially play a role in stress responses and resilience, and thus responses to behavior-tests. Some behavior test phenotypes are heritable and are also been selected with other traits. For example, temperament could be defined in pigs using a measure of resistance to restraint and had high heritability (Spake et al., 2012). Researchers speculated that this particular test indicated that stress-resilience was heritable (Hessing et al., 1993; Bolhuis et al., 2003; Zebunke et al., 2015). Traits like growth rate and lean meat are also related to some temperaments (e.g. van Erp-van der Kooij et al., 2000; Van Erp-Van Der Kooij et al., 2003; Cassady, 2007). Compared with less resistant individuals, more resistant piglets showed significantly higher increase of heart rate in novel object test (Hessing et al., 1994).

However, Velie et al. (2009) reported that novel-object and human approach did not have high heritability. Hence, behaviors during an approach tests may not be as heritable among commercial pigs compared with some other behavior traits mentioned above.

Conclusion

In conclusion, even though miniswine become an increasingly popular animal model among neuroscientists, validated behavior tests to measure effects of mild traumatic brain injuries are needed. Hence, there is need to modify and validate HAT and specifically for miniswine in biomedical research environments. In addition, other measures such as activity and environmental exploration may provide valuable information on these large animal models for traumatic brain injury.

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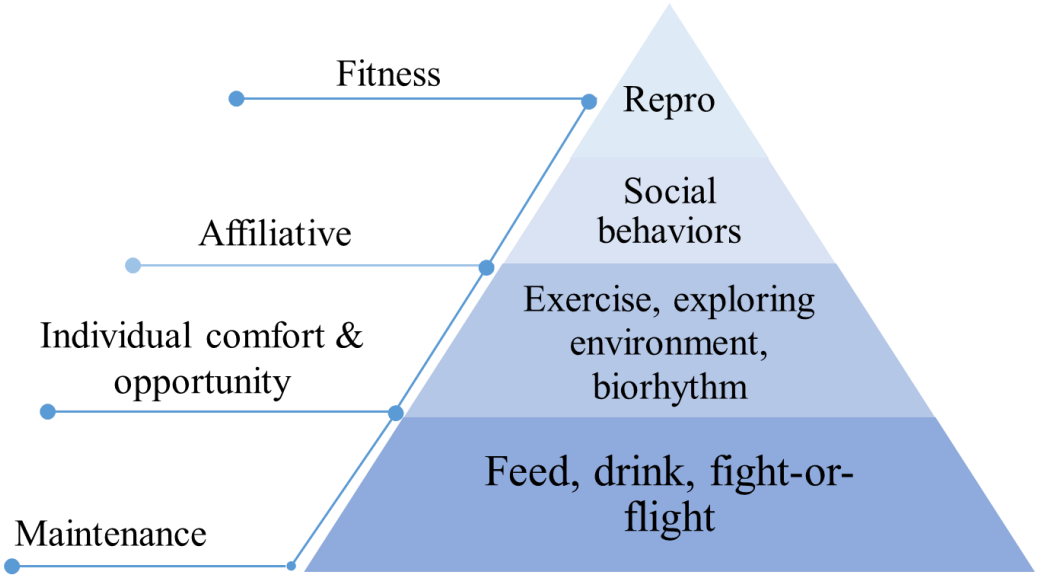
Figures

Figure 1.1. A hypothesized behavior pyramid of animals.



Maintenance behaviors like feeding, drinking and avoiding risks are the behaviors at the bottom of the pyramid. One level above are comfort and opportunity behaviors, include autogrooming, exercise, and exploring the environment. Above these behaviors are social behaviors, which include allogrooming and other interactions between different individuals, both affiliative and antagonistic. At the top of the pyramid is reproductive behaviors, including physiological changes associated with breeding, mating, and talking care of offspring (Moberg and Mench, 2001; Mills and Marchant-Forde, 2010; Hulbert, 2015).

Chapter 2 - Development of a Porcine Human Approach Test for Single Exposure mild Traumatic Brain Injury Model

Abstract

The main objective was to modify a commercial swine human approach test (HAT) for single-housed biomedical miniswine models of traumatic brain injury. Single housed, Yucatan miniboars (n = 46; age = 25.3 wks) were either sham-treated with anesthesia-only (SHAM) or were subjected to single blast wave treatment induced TBI (BLAST). Prior to the morning feeding, HAT was conducted for up to 3 minutes for each pig while video-recorded. The HAT was performed day -3 to -1 (baseline, averaged) and 1, 2, and 3 days after treatment. Trained observers who were blinded to treatments times tamped videos using specialized software (Noldus Observer XT 11.0, Leesburg, VA). The spatial relationships to the human (Far, back of the pen; Mid, middle of the pen; Close, in the front of the pen; Climb, front legs on front-gate fencing) were recorded. The inter-observer reliability for all approach behavior durations and frequency were correlated ($R^2 > 0.98$; $P < 0.01$). Intra-observer reliability was also correlated, although mid-duration, far-duration, and far frequency was less reliable than the other time-stamped behaviors ($R^2 = 0.87$; $P < 0.01$). The first two days after treatment, BLAST pigs decreased approach duration (Climb + Close; and increased Far-duration, $P < 0.01$) after treatment compared to SHAM. However, the first day after treatment, they moved in and out of the mid and far-sections more than SHAM. This HAT was an objective, sensitive, and reliable measure to detect blast wave induced TBI in Yucatan miniswine models, and hence may likely be a repeatable test for other mild TBI models in miniswine.

Introduction

Traumatic brain injury (TBI) is the malfunctioning of brain or other brain pathology caused by external forces, including blast waves (White and Venkatesh, 2016). In human patients and animal models, various cognitive and behavioral changes were observed following mild or severe TBI: increased impulsive aggression (Greve et al., 2001), deficits in solving social conflicts (Janusz et al., 2002), impaired working memory, spatial memory and learning abilities (Carroll et al., 2004; Milman et al., 2005), and development of post-traumatic stress disorder (PTSD; Hoffman and Harrison, 2009). As number of military TBI victims increase, there is an urgent need of understanding TBI pathology and prognosis (Ling et al., 2011). However, given the anatomy, cognition, and ecology differences between rodent models and humans, new animal models are needed for a better understanding of the effects of TBI on human brain pathology. Therefore, biomedical researchers developed swine models to better resemble human neurotrauma (Lind et al., 2007; Kornum and Knudsen, 2011; Conrad et al., 2012; Dolezalova et al., 2014).

Miniature swine were widely accepted as the breeds of choice because biomedical facilities for similar-sized research animals (e.g. canine) could be modified for miniature swine. Although there are many standardized behavior tests for rodent models (Bondi et al., 2015; Shultz et al., 2016), there are few tests available to test miniswine housed and managed in biomedical facilities. Nonetheless, there is a breadth of knowledge on large commercial swine breeds for behavior tests of memory, attention, conditioning, and affective states (e.g. fear and curiosity; Kornum and Knudsen, 2011).

The human approach test (HAT) was developed as a tool measuring approach behaviors of commercial, large breed pigs (Hemsworth et al., 1989). During this test, the test person stands

stationary while the test-animal moves freely in a test-area. Other researchers confirmed the repeatability, reproducibility, and reliability of HAT in commercial pig and dairy cows (Waiblinger et al., 2006; Windschnurer et al., 2008; Scott et al., 2009; Powell et al., 2016). Therefore, the authors chose to start with HAT as a test for miniswine in a biomedical environment. The affective state is a point-of-interest for TBI models because fear, motivation, and avoidance are altered among TBI human patients (e.g. Bryant et al., 2003; Hoffman and Harrison, 2009).

To develop behavior-tests in miniswine in biomedical facilities, the tests should be (1) non-invasive to reduce potential stressors; (2) standardized to reduce variability between observers and laboratories; (3) sensitive to capture the subtle differences between healthy animals and animals in a pre-pathological state; and (4) be customized to match the species, age, animal husbandry, and experimental timeline (Mills and Marchant-Forde, 2010; Gieling et al., 2011). With these guidelines, the authors aimed to develop an in-pen human approach test to measure some of the subjects' affective states before and after treatment. In rodent behavior tests, the subjects are typically removed from their home cages and placed in a testing apparatus (Bondi et al., 2015). However, biomedical facilities may not have the space to scale-up a rodent tests and a novel room would require extra training, handing, conditioning for miniswine. If left as a novel environment, it may be perceived as a stressor, which can add between animal variation to responses (Lewis et al., 2008; Zupan et al., 2016). Therefore, home pens provide a more familiar and thus less stressful testing environment.

Therefore, the goal of this study is to develop a modification of in-pen human approach test in miniswine TBI model. The main objectives were to create and validate a reliable HAT test specific for miniswine in biomedical experiments test by 1) measuring the intra- and inter-

observer repeatability; 2) determining the between and within-animal variation, and 3) determine if our HAT can detect pigs with mild TBI.

Materials and Methods

General animal care and housing

The present study was conducted from February 2016 to February 2017 at Virginia Polytechnic Institute and State University in the Virginia–Maryland College of Veterinary Medicine (Blacksburg, VA). Pigs were housed and managed in accordance to the Guide for the Care and Use of Laboratory Animals (NRC, 2010). All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC; Protocol 15-060).

A total of 46 Yucatan miniswine boars (age = 25.3 wks ± 2.80) were used for this validation experiment. Pigs were transported in groups of 9 to 12 from Sinclair BioResources (Auxvasse, MO) and were provided between 12-39 days of acclimation. Boars were individually housed in climate-controlled biomedical facilities. The pen demensions were 1.90 × 1.12 m; 3 walls of the pen were cement thus ajacent pigs were not able to ineract, but the front wall was chain-linked fencing and gate, therefore, pigs could see across from each other. Rubber mats covered the entire floor exluding the drain area of the back of the pen. Stainless steel buckets were used to provide 350 g commercial pellet feed (Teklad miniswine diet 7037, Harlan Laboratories Inc., Indianapolis, IN) and 2 L of water twice daily (at 830 and 1430 h). At one corner of the back of the pen, a fixed environmental enrichment device on a spring was placed. The device was filled after the morning feeding with 26 g of a treat (a mix of 33.3% raisins, 33.3% unsalted peanut, and 33.3% dog-cookie treat). Animals had 12-hour light-dark schedule (on at 700 h and off at 1900 h). After morning feedings, one pig at a time was allowed to leave the pen and remain in the center isle while its pen was cleaned via high pressure power wash.

Experimental Timeline

After the acclimation period, pigs were randomely assigned their treatments of either SHAM (sedation and anesthesia only) or single-exposure an overpressure device for BLAST treatments. The overpresure technology and anesthesia specificiations are reported elsewhere (Walilko et al., 2017). The anesthesia required a 12 h fasting of feed and water, therefore, the HAT was designed around this constraint (Figure 2.2). Four to five days days prior to treatment, pigs were sedated and anethesized to facillitate baseline magnetic resonance imaging (MRI). On treatment days, 3 to 6 pigs were sedated and transported 5 km to the treatment facility where they were placed under anesthesia. All pigs were placed in the overpressure device. The BLAST treated pigs received a 207-345 kPA overpressure and SHAM pigs received a 0 kPA overpressure. On anesthesthia days (MRI scan or blast treatment), pigs were returned to their homepens and then offered their entire 700 g of feed and 2 L water in the afternoon.

Human approach test

The spatial relationships of tested animals to the human experimenter were taken as HAT measurements: Far, back of the pen; Mid, middle of the pen; Close, in the front of the pen; Climb, front legs on front-gate fencing. Climb and Close were treated as approach behaviors, and Mid and Far as move-away behaviors. Observation started from 3 days before the blast treatment to establish baseline (day-3, Figure 2.1). After treatment during recovery, HAT was performed for 3 more days (day+1 through +3).

This in-pen human approach test was designed around the animal care, housing, and experimental timeline constraints (Figure 2.1). Repeated samples prior to treatment were needed to evaluate between and within animal variation. Therefore, the test was performed prior to the morning feedings for up to four days prior to the treatment day. The treatment day sample was

not taken because pigs were undergoing fasting and received sedation. All pigs received their entire meal at the evening feeding on treatment day, therefore, the test was performed on the three days following the blast treatment (day+1, +2 and +3; Figure 2.2).

A conditioning period was conducted prior to the first MRI to allow the pig to associate a positive reward from a human with a ring tone. On the HAT days, the test person (blinded to the treatments) came close to the fence and offered a piece of treat (peanut, raisin or dog cookie treat) and in the same time play a phone ring tone as a signal. Five seconds later, whether or not the pigs picked up the treat or not, the treat was dropped to the floor in the pen, and the test person stepped back to 0.3m away from the pen, and remained for 3 minutes.

The 3-minute tests were recorded via video cameras. Prior to overhead camera set-up, a GoPro Hero3+ camera (GoPro, San Mateo, CA) was placed on a tripod and placed at a 45 °angle into the pen. However, later in experimental blocks, a video system was installed. The installed recordings were collected using a customized GeoVision GV-1480 16-Channel PC DVR video surveillance system (Points North Surveilannce systems, Auburn, ME) positioned at a 90 °and 2.1 m from floor over 1-2 pens.

The video footage was sent to the Kansas State University applied ethology laboratory to be quantified by trained observers who were also blinded to treatments (Manhattan, KS). Prior to quiantifying videos, spatial behaviors were defined. The pen was divided into 3 different sections and were coded as near, mid and far and climb (Figure 2.1) and the variables for duration and frequency were produced for analyses (Table 2.1). All behaviors were were considered mutually exclusive. Observers quantified behaviors continiously using specialized software (Noldos Observer XT 11.0 Leesburg, VA) on a Dell Precision Tower 5810 workstation (Dell, Lincoln, NE).

To evaluate the reliability of HAT test, intra observer repeatability and inter-observer reproducibility were analyzed. Two trained observers (a and b) evaluated the same 57 HAT test recordings from 16 animals (Table 2.1). To obtain intra-observer repeatability, recordings were scored twice by observer a. To obtain inter-observer repeatability, observer b watched and scored the same recordings independently.

Statistical Analyses

For the between- and within-observer variation analyses, Pearson's correlation was calculated with SAS 9.4 (SAS Institute Inc., Cary, NC). Behavior variables were analyzed by restricted-maximum likelihood ANOVA using the PROC MIXED procedure of SAS. All duration of Climb, Close, Mid and Far were converted into percentages over observation time with Observer XT 11.5. A new variable, Approach was calculated as the sum of Climb and Close. Move away as a frequency was calculated as the sum of Mid and Far frequency. Stand and Far were not included in this analysis due to a lack of variance. The model included group, time, treatment, and their interactions. The subject of the repeated statement was pig nested within treatment. Ante-regressive (1) covariance structures were tested for the within pig effect in each model and the most appropriate model was chosen based on the lowest Bayesian Information Criterion. Prior to analyses, normality of the residuals was confirmed by evaluating the Shapiro-Wilk statistic using the Univariate procedure of SAS. In addition, the Univariate procedure was used to calculate the max, min, mean, median, and standard deviation of each behavior-measure within a treatment. The mean and standard deviation were used to calculate the power and potential animal numbers needed to achieve a 20% (day+1), 15% (day+2) or 10% (day+3) difference ($P < 0.01$; Table 2.4). Pair-wise comparisons were performed for each significant effect in a model using Duncan's adjustment to control the familywise Type 1 error.

Least squares means (± SEM) are reported throughout the manuscript. A treatment difference of $P < 0.05$ was considered significant and $0.05 < P < 0.10$ was considered a tendency.

Results and Discussion

Length of acclimation did not show effects on any of Approach, Climb, Close, Mid, Far duration or Move away frequency ($P > 0.05$, linear regression $R^2 < 0.09$). Therefore HAT results do not depend on acclimation length between 12 and 39 days.

Both intra-observer and inter-observer comparison showed high correlation on all measurements of duration and frequencies (Table 2.2). Pearson's correlation coefficients range from 0.849 to 1.000 ($P < 0.0001$). The correlation of frequency of Stand could not be calculated due to a lack of variation, but because all the observation results of both watches of observer a, and the watch of observer b match exactly, this measurement had 100% agreement on intra- and inter-observer reliability. Previous results showed that HAT is a reliable and validated test in both commercial swine (Brown et al., 2009; Scott et al., 2009) and dairy cattle (Rousing and Waiblinger, 2004; Windschnurer et al., 2008); and our results agree with these conclusions and also showed high reliability for Yucatan pigs in biomedical research environment. The correlation coefficients of Mid and Far were numerically slightly lower compared with that of Climb and Close. Between the first and second watch of observer a, the human observer's accuracy also increased, and this contributed to the higher between-observer correlation as observer b was trained later. In addition, observers had to mentally divide the pen up to 3 sections, and as Mid and Far are further away from the observation spot, hence there is more subjectivity in deciding these two positions. To improve this behavior-test, better visual markers that divide up different pen sections, and 90-degree cameras mounted at the ceiling may provide better footage to decrease subjectivity in video watching. Marking the pen floor or wall with a

line to divide these three sections should help in improving correlation of these 2 behaviors as well as reliability of the test.

There were treatment × time interactions for total approach duration, far-duration and the frequency to move away ($P < 0.05$; Table 2.3) and a treatment × time tendency for mid-duration ($P = 0.059$; Table 2.3). One day after treatment, BLAST-treated animals spent less time in total approach than SHAM-treated animals ($P < 0.05$; Figure 2.3). There was a tendency that total Approach of BLAST day+3 is higher than that of BLAST day+1 ($P = 0.064$). Commercial pigs that received aversive treatment showed less approach than gently-handled pigs, suggesting that the avoidance behaviors are associated with fear (Hemsworth et al., 1987; Hemsworth et al., 1996; Waiblinger et al., 2006). In rodents treated with TBI, they were less likely to approach a novel object and remained on the walls of an open-field test than sham-treated mice (Impellizzeri et al., 2016; Song et al., 2016). These researchers suggested that these behaviors in mice indicate fear and anxiety. Nonetheless, the pigs in the current study may have avoided the human simply due to a lack of motivation to approach, as they were only conditioned to approach a human being. Physiological measurements, for example heart rate and saliva cortisol, could be measured to decide if the reduced approach behavior observed in the pig model was also related to fear.

These results showed that total-approach duration measured with HAT is sensitive to mild TBI induced by blast treatment received by these Yucatan miniswine model. The increase of total-approach duration in BLAST pigs from day+1 to +3 may indicate that these pigs were recover from mild TBI or they were returning to more of a pre-pathological state of TBI. BLAST pigs spent more time in the back of the pen (Far-duration) than in total-approach on day+1 compared to their baseline durations, but returned to baseline-measures by day+2 ($P < 0.05$; Figure 2.3). The fact that pigs spent more time in the back of the pen than the middle section,

suggested that their avoidance reached a threshold, which further suggested that avoidance could be an indicator of motivation of avoidance rather than just lethargy. In this experiment, because the total duration of the test is consistent, Total Approach and Far duration are also negatively correlated. Both decreased Approach duration and increased Far duration were avoidance behavior, and the results here agree with previous results in increased anxiety-like behaviors in mice. Not many results of mild TBI are available in rodent models, however with TBI induced by controlled cortical impact, mice showed increased anxiety-like behavior during open-field test and impaired spatial memory for as long as 30 days after the injury when they were euthanized (Impellizzeri et al., 2016). Hence the length of recovery of a behavioral measurement could also be an indicator of the severity of injury.

One day after treatment, BLAST pigs not only spent more time in the back of the pen, they shifted in-and-out of the mid and far section more than their baseline measures. Although animals decreased their approach behaviors, they did not just spend more time in the Far section but also increased activities between Mid and Far These two measures (decreased duration in total-approach, increased move-away frequency) may indicate that they were conflicted with the state of avoidance and the motivation to approach. These findings may also suggest that SHAMS may have been influenced by anesthesia, which happened twice before the treatment and before MRI pre-scan. The evening before treatment, the pigs were also fasted, which could also affect their motivation of approaching following the treatment. Limited results from commercial pigs showed animals' behaviors during HAT did not change over time or after repeated exposure (e.g. Scott et al., 2009). Future research will need to determine with an untreated control group if frequency will increase naturally as they have more exposures to HAT in this testing environment.

In conclusion, this study shows that the in-pen HAT is a promising behavior-test for Yucatan miniswine undergoing mild TBI. This in-pen HAT test was proved to be a reliable test with high reliability and reproducibility. In mild TBI animals it detected blast treatment by decreased approach behaviors and increased avoidance behaviors. As we observed increased avoidance behaviors in mild TBI miniboars and previous research observed increased anxiety-like behaviors in rodent models, other tests like novel object test could also be tested in this TBI animal model. Results in mice humans also showed that severe TBI could induce impaired performances on behavior-tests weeks or even months after the injury (e.g. Impellizzeri et al., 2016; Maas et al., 2006). Nevertheless, if pathology was already caused by more severe injury to inhibit standing and locomotion, then this test based on voluntary approaching would not be as quantitative. In this case, a binomial score of standing and lying would be more appropriate.

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Tables and Figures

Table 2.1. Ethogram of the in-pen human approach test.

Variable	Definition
Spatial duration, %	
Far	
Mid	Amount of time (converted to percent observations) spent in each portion of the pen
Close	
Climb	
Structural duration, %	
Stand	Amount of time (converted to percent observations) spent standing or lying
Lie	
Total Approach Duration, %	The percent duration of close and climb combined
Move-away, no.	The number of times the pig moved into the mid and far section

Total approach duration and move away no. are calculated as the sum of duration of Climb and Close (Approach duration) and sum of frequency of Mid and Far (move away no.).

Table 2.2. Pearson's correlation coefficients of intra- and inter-observer comparison (n = 57).

	Correlation Coefficients			
	Duration		Frequency	
Behaviors	**Within**	**Between**	**Within**	**Between**
Climb	0.997^{***}	0.994^{***}	0.978^{***}	0.978^{***}
Close	0.997^{***}	0.991^{***}	0.942^{***}	0.956^{***}
Approach	0.997^{***}	0.986^{***}	0.967^{***}	0.963^{***}
Mid	0.871^{***}	0.978^{***}	0.933^{***}	0.940^{***}
Far	0.849^{***}	0.992^{***}	0.881^{***}	0.925^{***}
Stand	1.000^{***}	0.999^{***}	1.000^{a}	1.000^{a}
Lie	0.999^{***}	0.999^{***}	1.000^{***}	1.000^{***}

Intra-observer correlation coefficients are calculated with the first and second watch of observer a, and inter-observer correlation coefficients are calculated with observer a's first watch and observer b's watch. [a]The inter-observer Pearson's correlation coefficients of Stand frequency could not be calculated due to a lack of variation. All observed frequencies are the same between the 2 watches of observer a and between that of observer a and b. $^{***}P < 0.0001$.

Table 2.3. Duration of spatial behaviors for miniswine during the daily in-pen human approach test before (baseline) and after being subjected to a mild TBI (Blast; n = 22) or anesthesia only (Sham; n = 8)

	Time Relative to treatment, days												P-values		
	Baseline[1]			1			2			3					
	Sham	Blast	±[5]	Sham	Blast	±	Sham	Blast	±	Sham	Blast	±	TRT	Time	TRT*Time
[2]Duration, %															
[3]Far	2.60[b]	2.21[b]	1.44	0.00[b]	13.35[a]	7.46	4.79[b]	3.02[b]	4.36	8.26[ab]	2.64[b]	2.21	0.900	0.093	0.052
[3]Mid	11.51	3.67	3.41	6.26	10.87	4.63	5.19	14.43	8.66	11.88	7.72	3.16	0.912	0.337	0.059
Close	71.05	80.07	4.33	80.36	67.66	9.07	79.27	73.21	8.85	72.14	81.64	4.30	0.990	0.958	0.122
[3]Climb	14.75	14.40	3.81	13.13	7.57	4.96	10.59	9.69	4.38	7.68	8.22	3.65	0.490	0.067	0.766
[4]Total Approach	85.78[ab]	94.56[a]	4.17	95.13[ab]	74.83[b]	9.84	90.27[ab]	82.99[ab]	9.40	79.87[b]	89.95[ab]	3.78	0.714	0.303	0.032

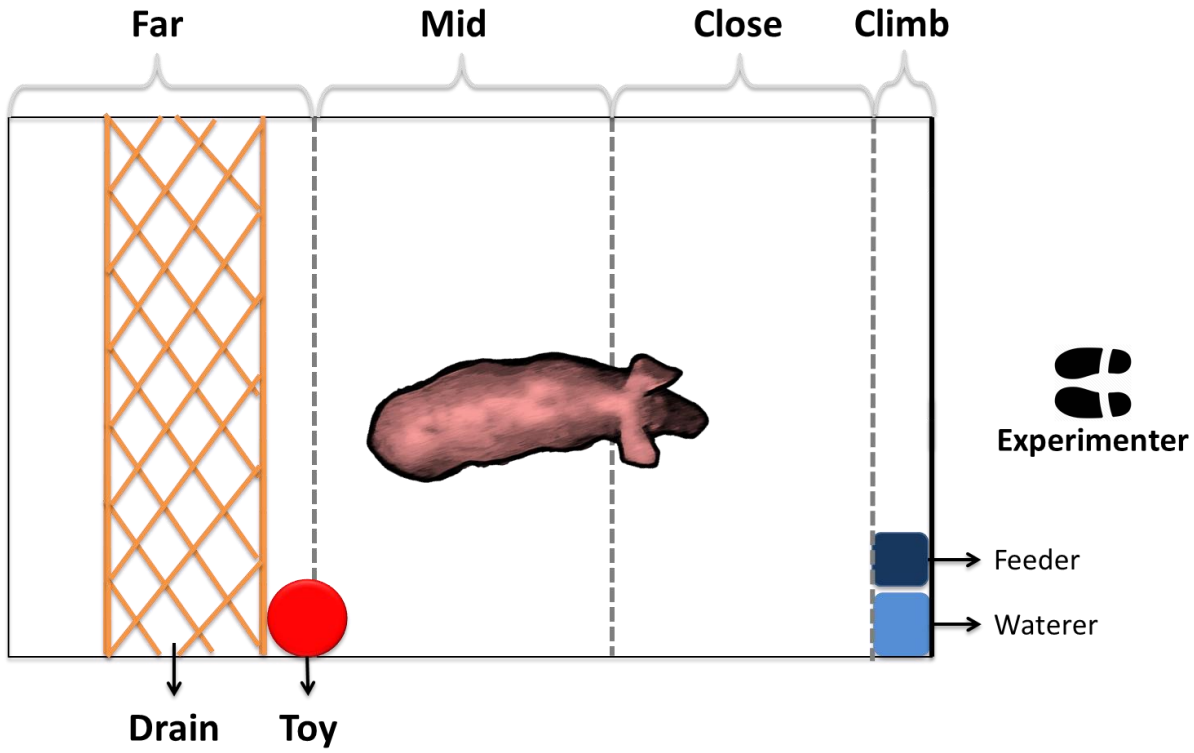
[a,b]LS means differ P <0.05. [1]Days -3 through -1 were averaged before analyses; [2]The % duration of a 180-second sampling period; [3]*P*-values from Log-transformed data; [4]Total approach = Climb + Close; [5] Shown the largest standard error because treatment are not equal.

Table 2.4. Descriptive statistics of human approach test results, and number of replications needed.

	Max[b]	Min[b]	Mean[b]	±SD[b]	CV%	Δ%	Reps needed for α=0.05 ß=0.20[c]
Climb, %							
Baseline[a]	53.4	0.0	15.2	13.1	86.05	--	
+1	31.9	0.0	11.5	13.6	118.40	20	566
+2	21.3	3.3	10.5	6.6	63.10	15	278
+3	16.9	0.0	7.6	5.7	74.48	10	861
Close, %							
Baseline[a]	100.0	14.9	77.1	14.9	19.34	--	
+1	67.3	95.7	79.7	12.3	15.48	20	10
+2	87.0	71.2	79.2	6.6	8.30	15	6
+3	86.9	61.2	72.1	11.2	15.48	10	39
Mid, %							
Baseline[a]	55.6	0.0	5.4	9.5	175.60	--	
+1	26.2	0.0	6.7	11.1	166.72	20	1082
+2	8.0	2.7	5.2	2.2	42.58	15	127
+3	24.3	6.0	11.9	6.8	57.08	10	512
Far, %							
Baseline[a]	29.4	0.0	2.3	5.6	243.72	--	
+1	6.4	0.0	2.1	3.0	141.31	20	781
+2	18.9	0.0	5.2	8.0	155.32	15	1684
+3	20.3	2.3	8.4	6.4	76.32	10	915
Total Approach, %							
Baseline[a]	100.0	36.3	92.3	12.9	14.02	--	
+1	100.0	67.3	91.2	13.6	14.94	20	10
+2	76.0	96.8	89.7	9.0	10.05	15	8
+3	91.70	65.10	79.8	9.8	12.26	10	25
Move-Away, no.							
Baseline[a]	17	0	2.4	3.4	138.11	--	
+1	3	0	2.2	1.6	74.55	20	219
+2	8	1	4.5	2.3	50.22	15	177
+3	12	4	7.5	3.3	43.60	10	299
Stand, %							
Baseline[a]	100.0	98.5	100.0	0.2	0.18	--	
+1	100.0	100.0	100.0	0.0	--	20	--
+2	100.0	100.0	100.0	0.0	--	15	--
+3	100.0	100.0	100.0	0.0	--	10	--
Lie, %							
Baseline[a]	1.5	0.0	0.0	0.2	--	--	
+1	0.0	0.0	0.0	0.0	--	20	--
+2	0.0	0.0	0.0	0.0	--	15	--
+3	0.0	0.0	0.0	0.0	--	10	--

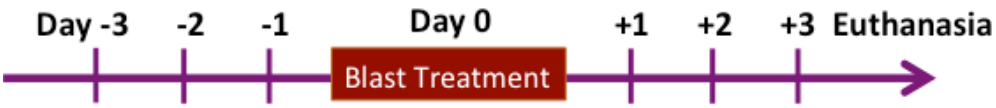
[a]Baseline was the average of 3 pre-treatment days (day -3 through day 0). [b]Values were taken from all SHAM days (SHAM day-3 through +3) and pre-treatment BLAST days (BLAST day-3 through -1). [c]Number of replications needed for α=0.05, ß=0.20. Formulas used for this calculation were presented by Berndtson, 1991; the calculator was kindly provided by Dr. James Drouillard at Kansas State University.

Figure 2.1. Pen set up of the experiment and division for human approach test



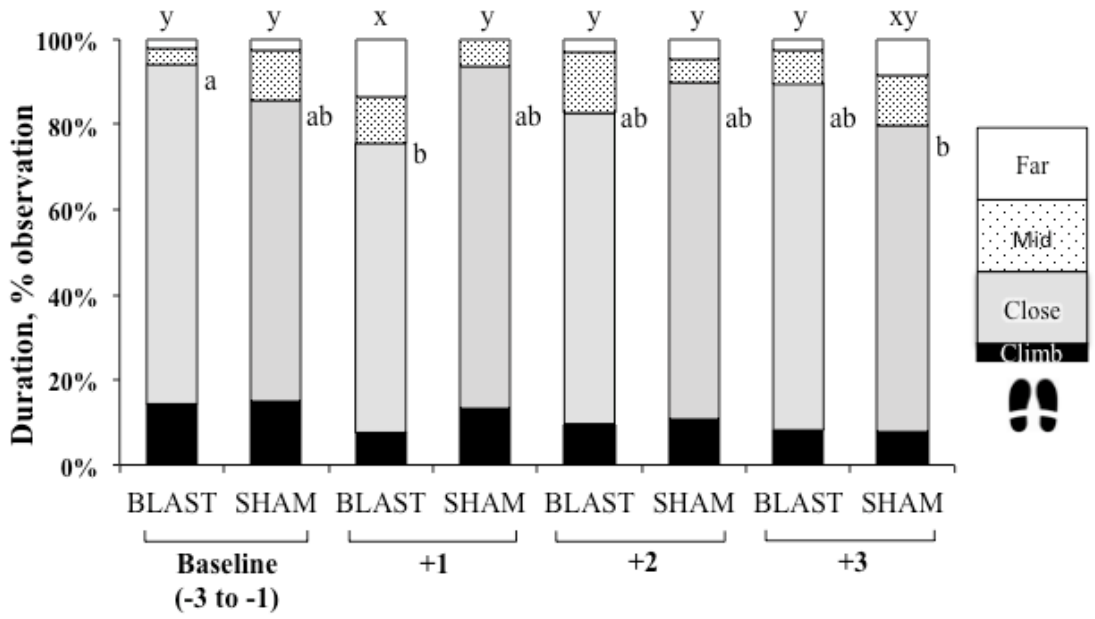
In each pen, the animals were provided a feeder and a waterer fastened to the fence, and an envrinmental enrichment toy mounted to the floor mat. Each pen had a drain at the back of the pen in the far-section. During the human approach test, the experimenter remains stationary outside the pen at 30 cm from the fence. When watching and scoring the positions, observers mentally divided pens into 3 sections, and the pig's position at each time point is decided by the position of its head. In the example of this figure, the pig's position is Close.

Figure 2.2. Timeline of experiment.



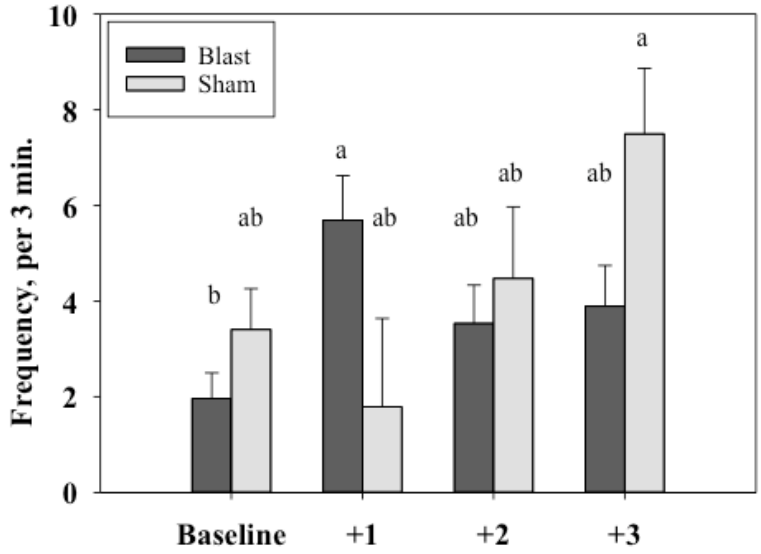
Once animals are delivered to the experimental facility, they had a quarantine and acclimation period with various lengths. Five or four days before behavioral observation, animals are pre-scanned with MRI under anesthesia. Treatment day is day 0. Human approach tests started on the third before the treatment (day-3) and end on the third day after the treatment (day+3). Then later on day+3 or +4 animals are post-scanned with MRI again and then euthanized while still under anesthesia.

Figure 2.3. Ethogram chart of human approach test.



Stacked bars show average percentages of Climb, Close, Mid and Far of BLAST and SHAM animals during each 3-min human approach test. Baselines are averages of all available days from day-3 through day-1. [x,y]Duration of Far as percentage of total test duration (data was log transformed). [a,b]Duration of Total Approach (sum of Climb and Close). There is a tendency that total Approach of BLAST day+3 is higher than that of BLAST day+1 (P = 0.064).

Figure 2.4. Results of human approach test Move away frequency.



Move away frequency (sum of frequency of Mid and Far) in 3 minutes of observation. Baselines are averages of all available days from day-3 through -1.

Chapter 3 - Validation of a commercially available accelerometer for detecting a non-nutritive oral state as a biomarker for mild brain trauma injury in Yucatan Miniswine

Abstract

The objective of this study was to validate an automated activity monitoring method for Yucatan miniswine model of traumatic brain injury (TBI) using a commercially available accelerometer and software (Fitbit Inc., San Francisco, CA). Yucatan miniboars were individually housed in an indoor biomedical research facility with 12-hour light:dark periods and devices were fastened to the ear-tag 4 days prior to treatment. Animals were treated with blast waves under anesthesia (BLAST) or only anesthesia as control (SHAM). Observations started 3 days before BLAST treatment (day-3) and ends at 0700 of the third post treatment day (day+3). Three pigs (age = 25.86 wks) were used for validation and showed that when installed on ear tag, Fitbit Zip activity arbitrary units (AU) did not record locomotion of the animals but head movements when pigs were standing. Activities were divided up into 6-hour periods or 2-hour periods. Using Fitbit Zips on ear tag to monitor daily activities, it was shown that pigs are more active during light periods than dark periods ($P < 0.01$). Six-hour periods were not sensitive enough to detect changes in behavioral following TBI. In 2-hour periods, acute effects of TBI were detected; BLAST pigs were less active compared to SHAM pigs during certain light periods ($P < 0.05$). Although the Fitbit Zip installed on ear tag detects all movements, not just locomotion, is a sensitive toolset for detecting head movements which related to the animal's motivation to explore their environment and find food. In our initial TBI-model, this toolset

appears sensitive during the light periods to identify animals in a pre-pathological state of mild TBI.

Introduction

Traumatic brain injury is a public health problem affecting quality of life of millions of people in the United States, and demands more understandings and solutions (Langlois et al., 2006; Maas et al., 2008). Traumatic brain injury (TBI) is amount one of the main sources of mobility in military services and also become common even outside of battlefields, and can be caused by many types of external forces (Warden, 2006; Ling et al., 2011). These forces may damage both the structure and function of the brain (Iverson, 2005), and cause non-penetrating and diffused injury of the brain (Taber et al., 2006). In human patients, aftermath of TBI affects many aspects of daily life, from attention problems and aggression to post-traumatic stress disorder (Rosenthal et al., 1998; Trudeau et al., 1998; Hoffman and Harrison, 2009; Rosema et al., 2014).

In order to better understand TBI and its neuropathology, and hence measure the injury and come up with treatment, a number of animal models have been utilized and being developed (Bondi et al., 2015). Many behavior tests had been utilized in TBI rodent models (Bondi et al., 2015). Researchers used these Behavior tests measures motor and cognitive functions (e.g. learning and memory) in rodents models (Bondi et al., 2015; Shultz et al., 2016). Nonetheless, despite the large volume of research results from rodent models, new animal models are still in need with greater similarities of brain anatomy, function and behavioral patterns to humans. In recent years, large animals are becoming popular models for mild TBI (Bondi et al., 2015; Shultz et al., 2016). Pigs have very similar brain development to humans (Conrad et al., 2012), as well as a number of neural anatomy and functioning, and have been wildly used in biomedical

research as a model for human brain (Whyte and Prather, 2011; Dolezalova et al., 2014; Shultz et al., 2016). Miniswine are preferred because of their smaller body size and similar brain size (Watanabe et al., 2001). Also they were bred in a standardized laboratory environment and thus have clear genetic background and controllable microbial status (Kornum and Knudsen, 2011). Hence a lot of progress has been made in replacing rodents with Yucatan miniswine in histology and neuroanatomy. Nevertheless, common behavior-tests that are available in rodent models are not always appropriate for the porcine species, because of both the nature of the test and the cost associated with testing animals with greater body size (Bondi et al., 2015; Shultz et al., 2016).

Despite the various social behavior-tests available for rodents model and potentially for large animal models, not many behavioral measurements are available for comfort and opportunity behaviors (e.g. autogrooming, exploring environment; Bondi et al., 2015; Shultz et al., 2016). Injury and sickness results in a collective and organized change of multiple behaviors as an adaptive strategy, rather than changes in individual behaviors (Hart, 1987; Hart, 1988; Johnson, 2002). In addition, pre-pathological behaviors may require more frequent sampling so that changes in behavior patterns can be identified (e.g. Fu et al., 2005; Zhu et al., 2009). Common behaviors affected by illness include locomotion, appetite, thirst, and non-nutritive oral behaviors (Johnson and von Borell, 1994; Millman, 2007; Weary et al., 2009; Ahmed et al., 2015). Therefore, in our pig models we are interested in investigating the daily rhythm of non-nutritive oral behaviors in pigs. Over 70% of a pig's active activities are rubbing, licking, sniffing, and chewing using their snout, mouth and face. Researchers combined these activities as one state of oral-nasal-facial (ONF) behaviors (Dailey and McGlone, 1997; Hulbert and McGlone, 2006).

Traditional methods of observing behaviors are either in person, scan sampling, or by reviewing video recording systems, which is time and labor consuming. Therefore, there is demand and search for a more efficient and accurate way of monitoring behavior. Automated loggers were validated as reliable in the detection of motion in domestic animals, including dogs (Clarke and Fraser, 2016), dairy cows (Ledgerwood et al., 2010; Nielsen, 2013; Beer et al., 2016), turkeys (Dalton et al., 2016), and even wild animals (Dell et al., 2014). For swine, there are various validated behavior accelerometer monitoring systems (e.g. Escalante et al., 2013; Conte et al., 2014; Pastell et al., 2016). Advanced imaging system for agouti miniswine for behavior tests (e.g. Kulikov et al., 2014), but such . Also most of the techniques focus on commercial swine (Matthews et al., 2016), and need validation in miniswine in biomedical research environment.

Because of this, this study aimed to develop a cost and labor efficient way to monitor activities of Yucatan miniboars in biomedical environment, and find the best observation interval for this purpose. Fitbit Zips™ (Fitbit, San Francisco, CA), which are commercially available accelerometers with imbedded algorithm and reasonable cost. Fitbit Zips and its algorithm were deemed accurate for logging long-term human physical activities and locomotion (Diaz et al., 2015; Schneider and Chau, 2016), but very little research has been conducted for their use and accuracy in animals. Therefore, the objectives of this study were (1) to determine if the commercially available activity monitor attached to the ear tag captures movement, true-locomotion behavior or ONF behaviors; (2) to determine the optimum time intervals of activity-monitors in a model of mild brain trauma using Yucatan miniswine.

Materials and Methods

This study was conducted at the Virginia–Maryland College of Veterinary Medicine at Virginia Polytechnic Institute and State University in (Blacksburg, VA). The Institutional Animal Care and Use Committee (IACUC) approved all procedures in this study (Protocol 15-060). Animals were housed and managed in accordance to the Guide for the Care and Use of Laboratory Animals (NRC, 2010).

General animal care and housing

Twenty-six Yucatan miniature boars (Age = 24.09 wks ± 2.26) were collected from Sinclair BioResources (Auxvasse, MO). Pigs were transported 1,210 km to the facility. Three Yucatan miniswine boars (age 25.86 wks ± 0.29) were used in the validation of Fibit Zip, and 24 (age 24.09 wks ± 0.62) pigs were used for daily activity observation. Pigs were transported in groups of 9 to 12 from Sinclair BioResources (Auxvasse, MO) and were provided between 3-37 days of acclimation. Boars were individually housed 1.90 × 1.12 m pens, with cement walls on three sides and fencing and gate on one side. Pigs could see across from each other but could not see animals next to it. A drain area locates at the back of the pen, and rubber mats covered the rest of the floor. An environmental enrichment device was placed at the inside edge of the floor mats. Once a day the device was filled with 26 g of a treat (a mix of 33.4% raisins, 33.3% unsalted peanut, and 33.3% dog cookie treat). Twice a day around 830 and 1430 h, 350 g commercial pellet feed (Teklad miniswine diet 7037, Harlan Laboratories Inc., Indianapolis, IN) and 2 L of water were provided with stainless steel feeders and water buckets. Animals were on 12-h:12-h light-dark cycle (lights on at 700 h and off at 1900 h). Between 830 and 900h, pens were cleaned daily via high pressure power wash, and meanwhile one pig at a time was allowed to leave the pen and move freely in the center isle of the room.

Data Collection

Automated data collection. Loggers (Fitbit zip; Fitbit, San Francisco, CA) were installed on ear tags on the left ears, with the screen facing away from the ear tag and top of the device facing cranial. Each pig had a Fitbit Zip attached to their ear tag, that was wrapped in plastic and taped securely to avoid destruction (Figure 3.1). Fitbit Zips were synchronized at the end of the observations, and then Fitbit activity arbitrary unit (AU; "Steps" in Fitbit system) were presented in 1 min intervals with a commercially available program Fitabase (Small Steps Labs LLC, San Diego, CA). Fitbit algorithm only provide data at 1-min as the lowest intervals.

Behavior Observations. Video footage was collected using a surveillance video recording system (IP Camera: LT-CMIP7233-S; Recording Software: GV-1480; Geovision, Points North Surveillance Systems, Auburn, ME). Cameras were placed at a 90-degree angle in each pig's housing unit, approximately 2 m from the floor. The camera resolution was 1920 × 1080 and recording rate was 30 frames per second.

Experiment 1 A: Locomotion

Two 12-hour intervals (0700-1900, 1900-0700; Figure 3.2B) of each pig's video footage were observed and analyzed, giving a total of 24 hours per pigs. Two trained observers who were blinded to treatments continuously time-stamped behaviors (Table 3.1) at an average speed of 60 frames per second (Observer XT 11.5; Noldus, Leesburg, VA). The videos were time-stamped for Locomotion (Figure 3.1; citation). T Inter-observer variation for was 93.66%. Using the software package (Observer XT) data were divided into 1-min intervals to be aligned with automated logger data. Automated logger data intensity was maximized at 137 AU per minute (2.3 Hz).

Experiment 1 B: Head movement (ONF behaviors)

From experiment 1A's videos, nine subsamples of 15-minute intervals with high, medium and low percentage of locomotion duration were selected. A third trained observer the total of 135 minutes of footage at an average playback speed of 15 frames per second. The video were time stamped for structural states of the head (Table 3.1), the structural frequency of the back two legs (counted steps) and a structural state of the front two legs (pivot; Table 3.1). Structural states were considered mutually exclusive. Using the software package (Observer) data were divided into 1-min intervals to be aligned with automated logger data. Duration data were maximized at 60 seconds and the maximum step frequency was 137 per minute (2.3 Hz).

Experiment 2: Using Fitbit AU as an activity measurement

Experimental Timeline

A total of 21 pigs are used in experiment 2. Pigs were randomly assigned to either Sham treatment or BLAST treatment, and treated with previously reported procedure (Walilko et al., 2017). Loggers were placed before day -3 and removed on day+3 from 800 h (Figure 3.2). The BLAST treated pigs were sedated and anesthetized and received one 207-345 kPA overpressure and SHAM pigs received a 0 kPA overpressure (sedation and anesthesia only). Loggers were removed before treatments and re-installed after the treatment. The treatment day was (day 0) excluded from observations because anesthesia and treatments were implemented at various times of the day and loggers were temporarily removed for treatments. Data were synchronized during treatment and at the end of the experiment. All data were summarized to 2–hour and 6-hour intervals as two separate data sets, using the PivotTable function in Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA). The three days prior to treatment (day -3 through 0) were treated as subsamples for baseline.

Statistical Analysis

All data were analyzed with SAS (version 9.4, SAS Institute Inc., Cary, NC). Data were first evaluated with Shapiro-Wilk test in UNIVARIATE function for normality. Data that were not normal were log transformed prior to analyses. For experiments 1A and 1B, Pearson's correlation coefficients were calculated with PROC CORR function, and regression models were built with PROC REG function. For experiment 2, the automated data intervals (2- or 6-hour) were analyzed separately with PROC MIXED function with Treatment, Time, and Day (relative to treatment) and their interactions.

Results and Discussion

Experiment 1

In human subjects, the same loggers were shown to be accurate in measuring number of steps (Diaz et al., 2015; Schneider and Chau, 2016), therefore, out first experimented tested if true pig locomotion could be detected by the logger fastened to the ear tag. Although duration of locomotion vs. Fitbit AU was positively correlated ($P < 0.01$), Locomotion only represented a small amount of the logger data ($r = 0.162$). Additional regression analysis (before log transformation) yielded similar results ($R^2 = 0.09$).

This finding lead to our second objective (Experiment 1B). The logger was fastened to the ear tag, therefore, hypothesized that head movement may represent a larger portion of the automated data. Head movement was still of interest because it represents oral-nasal-facial behaviors, which can make up about 70% of a pig's active behaviors (Hulbert and McGlone, 2006). For experiment 1 B, 4 out of 135 1-min data points were removed from the analyses because observers could not view pigs on the footage. Head movement was positively correlated ($P < 0.001$; Table 3.2) with the logger AU and represented a larger portion of the data ($r = 0.647$)

than Locomotion in the previous experiment. Steps were also correlated ($P < 0.001$) with Fitbit AU, but to a lesser degree ($r = 0.343$) than head movements. These results suggested that both steps and head movements accounted for most of the Fitbit AU data. Therefore regression analyses (Table 3.3) were performed.

Duration of head movement and steps as predictors could both individually explain the variance of Fitbit au (head movement: $R^2 = 0.418$, $P < 0.01$; step: $R^2 = 0.118$, $P < 0.01$; Table 3.3). With both predictors, they can explain 71.8% of the variance in head movement duration ($R^2 = 0.457$, $P < 0.01$; Table 3.3).

From correlation and regression analysis, Fitbit AU was measuring activities involve head movements. For Fitbit AU 42% of variation was explained by duration of head movement, and only 12% of the variation was explained by Step (Table 3.3). The correlation coefficient between Step and Fitbit AU was also not as high. Using both Head movement duration and Step, about 46% of the variation in Fitbit AU could be explained. Adding Step as a predictor does not contribute much to the regression model. Hence head movement duration was the best predictor to Fitbit AU among the measurements tested, explained about half of the variation. Therefore, Fitbit AU in this experiment was recording head movements (i.e. performing ONF behaviors at one spot). Because most of the head movements are ONF behaviors (Hulbert and McGlone, 2006; Daily and McGlone, 1996), we could use this valuable behavior assay to measure daily activities of these animal models.

ONF is an important behavior for pigs to investigate their environments; and previous results showed that ONF behaviors show daily rhythm (e.g. Hulbert and McGlone, 2006). Therefore, this ONF behaviors measured by Fitbit AU is a great candidate for observation of behavior changes following mild TBI in the current miniboar model. With the easy and efficient

data collection, large amount data could be collected over several days for biorhythm analyses. Although Fitbit AU was shown to be a promising measurement of ONF behavior in the current design, it could still be improved as the R^2 value was only around 0.5. Further validation could compare Fitbit AU to ONF behaviors scored by human observers, and with ONF behaviors with and without locomotion of the whole body.

Experiment 2

Because the Fitbit AU data represent an active, and exploratory state, we sought to determine at what intervals and what time of the day the loggers would detect a difference between SHAM-treated and mild-TBI pigs. There was a large difference between dark and light periods activities; therefore, the dark 2-hour intervals were analyzed separately from the light 2 h intervals. When light and dark periods were analyzed separately for 2-hour intervals, BLAST pigs showed decreased activity intensity during some light 2-hour intervals (Intensity Treatment*Time*Day $P = 0.037$; Table 3.4). Specifically, higher ONF intensities were observer in SHAM pigs on day+1 at 0900 and 1100, as well as 0700 on day+2 ($P < 0.05$), and a tendency at 0700 of day+1 ($P = 0.06$), 0900 and 1100 of day+2 ($P = 0.07$). SHAM pigs were more active around the morning feeding on day+1 and +2 after treatment than BLAST pigs. A similar trend was also shown in the early dark periods of day+2 (time 300 h, $P = 0.06$; 500 h, $P = 0.09$). This finding suggested that if data were broken into 2-h intervals, the loggers could identify the time of day that SHAM pigs were more active than BLAST pigs.

In commercial pigs that are limit-fed, the ONF-state peaks in the intervals around feeding (e.g. Hulbert & McGlone, 2006). Therefore, we sought to determine if our loggers would detect differences between treatments in the 2-hinterval that includes feeding. The automated data from the dark 2-hour intervals tended to detect a difference between treatments at day+2, at the 500 h

(Table 3.4); SHAM-pigs tended to be more active at this time than BLAST-pigs. This was not expected, but we speculate that the fasting period on the day of treatment may have caused SHAM-pigs to be motivated at an earlier time to perform ONF.

On both 6-hour and 2-hour level, intensity of activities also showed circadian rhythms that these pigs were more active during the day ($P < 0.01$, Table 3.4; 3.5). To the authors' knowledge, not very many studies reported activities rhythm of individually housed commercial boars and we could not find reports of Yucatan miniswine biorhythm behaviors. Observation on commercial sows showed that ONF behaviors peaked around feeding time, which agreed with our results (Hulbert and McGlone, 2006). Similar studies showed that Göttingen miniswine showed higher heart rate during the day (Kuwahara et al., 2004). Also in commercial growing pigs and sows, Pedersen et al. (2015) measured heat production from gas exchange with respiration chambers, and showed heat production was the highest during the day and lowest at night, and peaked around feeding time. This heat production was converted into activity level and showed that activities were also higher during the day than at night. Hence the increased activity intensity as measured by Fitbit AU/min. agree with elevated heat production and hear rate and during the day.

Intensity analysis showed that the highest average intensity reported by Fitbit Zip is less than 140/min, and the highest intensity measured in 1 min is 2.3 Hz. Nonetheless, ONF could happen at a higher frequency, hence it is possible that the frequency of ONF behaviors were limited by precision of Fitbit Zip. The accelerometers could be recording data at higher frequency, but is limited by the algorithm of Fitbit Zip. Therefore, with more sensitive algorithm, it is possible that greater differences in circadian rhythm and TBI effects on ONF intensity could be observed.

In conclusion, this study showed with Fitbit Zip that automated accelerometers could be used as activity loggers to measure activities of Yucatan miniswine TBI models. When installed on ear tags, these loggers were most likely measuring states of ONF behavior without locomotion. Two-hour periods observation provided higher sensitivity than 6-hour periods, and observation during the day, especially around feeding time were the most efficient observation window. The accuracy of measurement was somewhat limited by the algorithm of the devices, hence more advanced algorithm could provide even more precise measurement with the accelerometers.

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Tables and Figures

Table 3.1. Experiment 1 Ethogram.

Behavior	Definition	Type
Experiment 1A		
Locomotion	Walking or climbing the fence, leg movements	State
No Locomotion	Standing still, sitting, lying	State
No Data	Pig cannot be seen in the video	State
Experiment 1 B		
Step, no.	Numbers of back leg steps (back leg movement)	Event
Pivot	Front leg movements, back legs are still, duration	State
Head-move	Head movements, oral-nasal-facial behavior, in the stand-position	State
Head-still	Standing still, no head movements	State
Lie-move	Animal lying with head movements	State
Lie-still	Animal lying still with no movements	State

For Experiment 1A, six 12-hour light periods footage from 3 pigs were observed. For Experiment 1B, nine 15-min high, medium and low activity periods were selected from footage scored with ethogram 1A to make a total of 135 1-min intervals were scored with this ethogram and 4 were excluded from analysis due to a less of 60 seconds total duration of usable data. Steps are counted as numbers. States were recorded as mutually exclusive. Lie-move was later dropped from further analysis because it had only 4 occurrences in all 135 observation windows.

Table 3.2. Correlation coefficients of head movement duration, pivot duration, Fitbit AU and back leg movement (Step).

	Fitbit AU	Head-move	Pivot	Step	Head-Still
Fitbit AU		0.647***	0.173*		
Pivot		0.294***			
Step	0.343***	0.736***	0.397***		
Head-Still	-0.210*	0.079	0.209*	0.269**	
Lie	-0.415***	-0.836***	-0.316***	-0.629***	-0.482***

Behaviors and definitions (except for step and pivot) were adapted from Hurnik et al. (1985). Back leg steps and pivot were two behaviors we noticed and worth differentiating when observing these animals. Data were analyzed at 1-min interval. Correlation coefficients of duration or frequency of behaviors in Experiment 1 B were calculated. Data are log transformed. N = 131. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Table 3.3. Regression analyses on Fitbit AU with different predictors.

Predictors	β	SE	t	P	R^2
Model 1					
Head-move	0.7126	0.0740	9.63	<.0001	0.4184
Model 2					
Step	0.5128	0.1234	4.15	<.0001	0.1179
Model 3					
Head-move	0.9470	0.1059	8.93	<.0001	0.4568
Step	-0.4316	0.1436	-3.00	0.0032	

Fitbit AU and Step showed high correlation to duration of head movement and were tested with regression models. Three models are tested with Head-move, Step and these two predictors together to explain variance of duration of Fitbit AU. Head-move was reported as the best predictor, and Step did not predict Fitbit AU very well. Data are log transformed. β – standardized regression coefficient; SE – standardized error of standardized regression coefficient; t – value of t-test.

Table 3.4. *P* values of activity AU and Intensity break data down into 6-hour and 2-hour intervals.

	***P* values**			
	6-hr interval		**2-hr interval**	
Effects	Activity[a]	Intensity[a]	Activity[a]	Intensity[a]
TRT	0.5899	0.6992	0.5899	0.6992
Day	<.0001	0.0018	<.0001	0.0018
Time	<.0001	<.0001	<.0001	<.0001
TRT*Day	0.0428	0.3752	0.0428	0.3752
TRT*Time	0.3354	0.2692	0.3354	0.2692
TRT*Day*Time	0.2874	0.1275	0.2874	0.1275

Activity (measured as Fitbit AU) and intensity of ONF (measured as average Fitbit AU/min.) were summed into 6-hour intervals. Effects of treatment (TRT), periods and time were analyzed with ANOVA. *P* values of the main effects and interactions between these effects are shown.

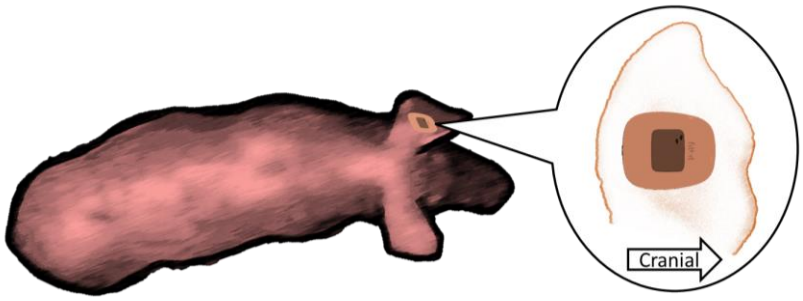
[a]Data are log transformed.

Table 3.5. *P* values of activity AU and Intensity break data down into 2-hr intervals, dark and light periods separated.

	***P* values**			
	Light Periods		**Dark Periods**	
Effects	Activity	Intensity	Activity[a]	Intensity[a]
TRT	0.2011	0.2008	0.2011	0.2008
Day	<.0001	<.0001	<.0001	<.0001
Time	<.0001	<.0001	<.0001	<.0001
TRT*Day	<.0001	<.0001	<.0001	<.0001
TRT*Time	0.3930	0.3925	0.3930	0.3925
TRT*Day*Time	0.0368	0.0369	0.0368	0.0369

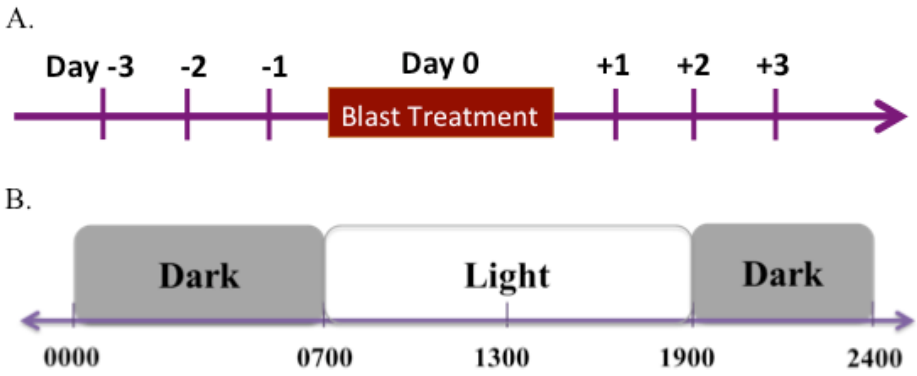
Activity (measured as Fitbit AU) and intensity of ONF (measured as average Fitbit AU/min.) were summed into 6-hour periods. Data are separated into dark periods (1900-0700, six 2-hour periods) and light periods (0700-1900, six 2-hour periods) for analysis. Intensities are measured as AU/min. On the third post-treatment day (day+3) observation terminated after the first dark period (ended at 0700). Effects of treatment (TRT), periods and time were analyzed with ANOVA. *P* values of the main effects and interactions between these effects are shown. [a]Data are log transformed.

Figure 3.1. Position of Fitbit Zip on the ear tag.



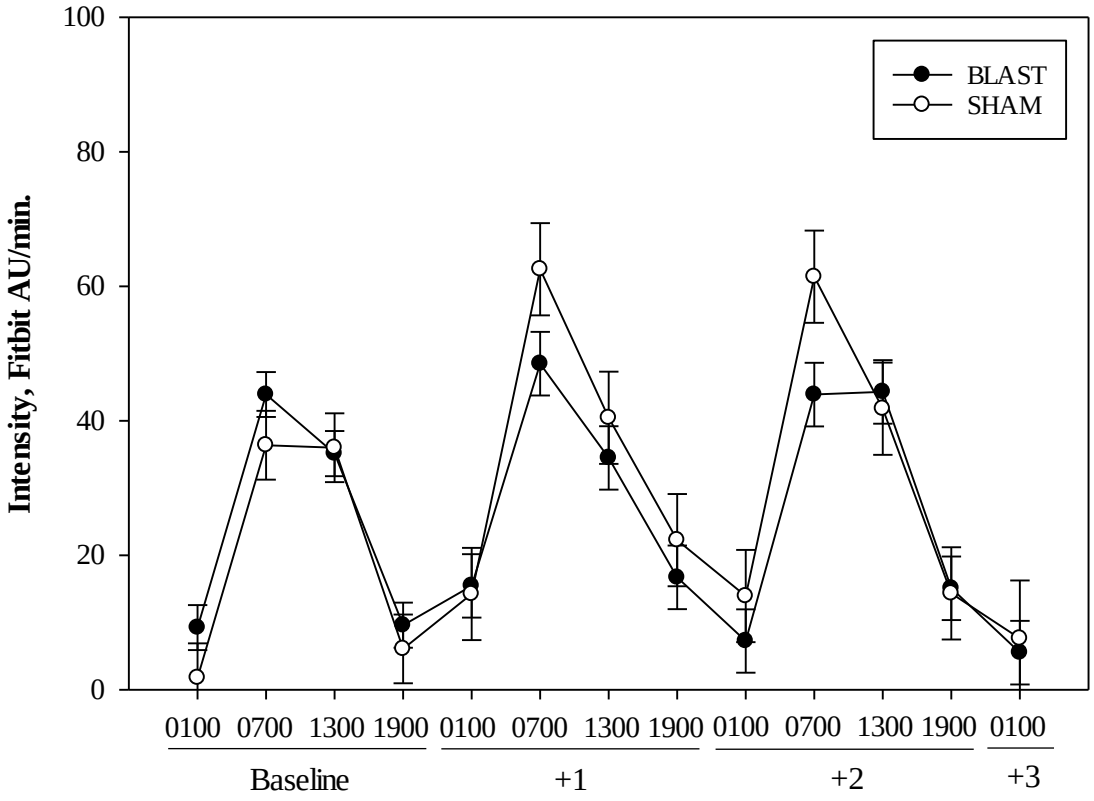
Fitbit Zips were installed on the ear tags on the left ears of the pigs. The screens faced away from the ear tags, and the top of the devices face cranial. All devices were wrapped with plastic wrap, stabilized on ear tags with Velcro, and then wrapped with tapes together with the ear tags.

Figure 3.2. Experimental timeline of the study and within a day.



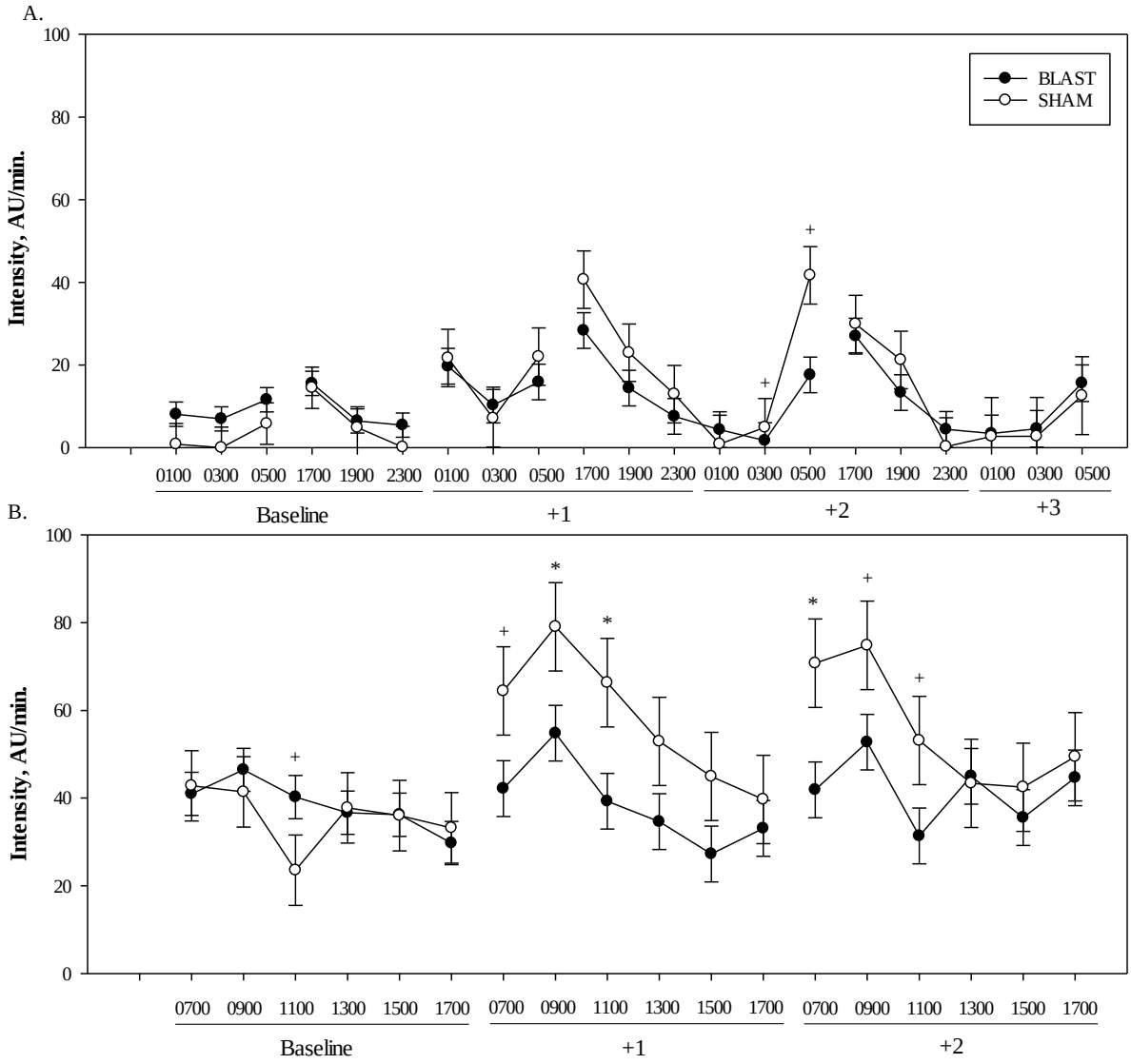
A. Observation last for 7 days, from 3 days before the treatment day (day0) through the end of the first dark period on the third day after treatment (at 0700 on day+3). Pigs were pre-scanned and post-scanned with magnetic resonance imaging. B. In each day pigs had a 12hr:12hr light:dark schedule. Lights turn on at 0700 and turn off at 1900 every day. Six-hour observation periods start at 0100, 0700, 1300, and 1900. For 2-hour observation periods, each 6-hour periods then are divided into three 2-hour periods, start at 0100, 0300, 0500, 0700, 0900, 1100, 1300, 1500, 1700, 1900, 2100 and 2300. For Fitbit analysis, 0000 to 0100 of each day counts toward the last periods of the previous observation day.

Figure 3.3. Intensity of activities measured as Fitbit AU/min. in 6-hour interval.



Intensity of activities shown as Fitbit AU/min. in 6-hour periods. Each time stands for the 6-hr interval starting from the time point stated. On the last day of observation (day+3), pigs were removed from observation room start 0800, thus observation terminated after the first 6-hour period (0100-0700). No significant effects of treatment were observed at 6-hour periods.

Figure 3.4. Intensity of activity measured as Fitbit AU/min. in 2-hour interval.



Intensity of activities are showed in 2-hour periods. Each time stands for the 6-hr interval starting from the time point stated. Baseline is the average of day-3 through -1. A. Activities as Fitbit AU during dark periods (1900-0700 of the next morning). B. Activities ad Fitbit AU during light periods (0700-1900). *Treatment effects on intensity of activities, P value from log transformed data, $P < 0.05$. $^{+}$Tendency of treatment effects on intensity of activities ($0.05 < P < 0.10$).

Chapter 4 - General Thoughts and Future Directions

Behavioral observation could be a very promising and efficient way to collect information from animals. In the first study, the human approach test, developed for commercial swine; was adapted to Yucatan miniswine in a biomedical research environment. Approach behavior as a type of appetitive behavior, can reflect the affective states of animals. Behaviors measured with this test reflected the competition and balance between curiosity and fear. In the second study, daily activities were monitored with commercially available accelerometers and algorithms and were used as behavioral measurements. This measurement was shown to be sensitive to the acute activity pattern changes following distressor like brain injury, sensitive at a 2-hour interval.

Human approach test was first developed for commercial pigs and could be modified for miniboars in biomedical research environment. This test was also modified for diary cattle and was shown to be reliable and sensitive in measuring fear to humans, and thus plays an important role in the monitoring and improvement of animal welfare. One problem with this test was that animals need to be tested individually or in a small group, and thus is not very time and labor efficient for large commercial farms. Efforts are being made to modify this test so that it could be easily used for group housed animals and more friendly for large production system. This test will be a promising tool to measure approach and avoidance behaviors for various purposes.

Compared with a short test like the human approach test, monitoring behaviors over an extended period of time could provide more information on animals of interest. Before one challenge of such measurements was that it was labor consuming – observers either record behaviors and spend hours and hours watching these recordings; or utilize the scan sampling method. Scan sampling could also be time consuming, and has the risk of losing valuable

information. The second study in this thesis provides thoughts on both problems. First, automated loggers are currently being tested and employed in various research projects, providing more accurate, reliable and efficient data recording. One challenge faced by many devices and researchers is how to convert raw data recorded by loggers into meaningful behavior measurements. Commercially available algorithm could be one of the solutions; however in the case of the current study, it was limiting the precision that could be reached by the loggers. Also these software packages add extra cost. Hence the whole field of animal behavior research would be largely benefited my more user-friendly software package to translate automated logger data into various behavior measurements.

The second challenge is the observation interval. Even if loggers provide precise measurements, it may not make sense to analyze all the data on the millisecond level. Here in the current study we took into consideration other factors like human activities and experimental schedule in addition to the animals and behaviors of interest. Future work with automated loggers could also be using loggers to pinpoint intervals valuable for observation. For example, our lab is currently working on using automated pacifier loggers in dairy calves to find time points when pacifiers were used. Then human observers only need to watch and score the windows selected by the loggers if the pacifier-related behaviors are the behaviors of interest. This method would be way more efficient then watching all the footages then analyzing only the pacifier related behaviors, which may be only 5% of the total time.

In addition to research environment, behavioral observations could also be applied into commercial productions as a way to monitor the herd. Behavioral observation could be a tedious work that requires trained eyes. Nonetheless, in this era of artificial intelligence, more and more automated monitoring system were being developed and tested. As mentioned in this thesis,

many accelerometer, imaging and acoustic monitoring system were developed, validated and being utilized to promote both productivity and animal welfare in commercial farms. The knowledge we learned from laboratory animals, domestic animals, zoo animals and even wild animals, will all add into our modern technology, to benefit both animals and human beings.

Appendix A - Standard Operation Procedures (SOP)

Human Approach Test (HAT) Protocol

1. **Timing of the tests (Figure A.1)**
    1) Testing happens in the morning before the morning feeding.
    2) Each human approach test (HAT) last at least for 3 minutes (180 seconds).
    3) Repeat the test on the same order of pigs every time (assuming that pens/treatments are randomly assigned)

2. **Pen and test set up**
    1) The pen will be divided up into three sections: Far, Mid, and Close (Figure A.2). The feeder and waterers are installed in the Close section, and the inside section is the defecate area, and the mat will be put in the mid section.

    2) The test person stands in front of the fence, on the midline of the pen and 30 centimeters from the pen door. Test person stands at the same place during every approach test.

    3) If the test will be recorded with a handheld camera, the camera needs to be stabilized with a tripod. Before the tests, adjust the tripod to optimize the view, making sure the whole animal and his behaviors can be recorded. The camera view needs to be consistent and stable for all of the tests.

3. **Auditory stimulus association** (see also Figure A.1)
    1) Conditioning: before the day of the first HAT test, stand in front of the pen like during a HAT test, and then play the ring tone. When pig approaches, offer him a piece of treat and let it smell and investigate it.
    2) During conditioning, the pig will learn to associate a ring tone with a reward (treat). Therefore, prior to performing the HAT tests, play the ring tone to gain the pig's interests. As soon as the pig approaches, offer the treat and hold it for 5 seconds. If the pig does not pick it up from the test person, drop the treat into the pen.

4. **Test procedure**
    1) Morning test
        i. Every test day at the same time in the morning before feeding, the test person (only one person) stands in front of the pen of the test pig at the stand line.
        ii. The recording of the test pig starts as soon as the test starts (the test is considered start when the test person stands in front of the pen).
        iii. At the beginning of the test, play the ring tone once, at the same time hold a piece of treat so the pig could smell it if he decides to smell. Hold the

treat for 5 seconds, and then drop it to the floor inside of the pen whether the pig picked it up or not.

iv. After dropping the treat, the test person will walk back to 30 cm from the fence, and stand there for the rest of the test.

Please do not use any other vocal, physical or visual signals except for the clicker, and keep the tests as consistent as possible within and between tests.

Fitbit Zip Protocol

1. Use the Fitbit Zip
    1) All the Fitbit zips are already registered and ready to use. Simply put them on the animals, and they will start recording activities.

2. Data Collection
   Although Fitbit Zip automatically record activity data, there are two important pieces of information need to be written down in the experiment diary when using them:
    1) The Fitbit Zip ID and ID of the animal that has this Fitbit Zip put on. So the data can be matched to the individuals.
    2) The time when the Fitbit Zip is put on the animal and removed from the animal.

3. Installing Fitbit Zip on Ear Tag
    1) Wrap the Fitbit Zip with plastic wrap, to protect the device from water and dirt;
    2) Stabilize the Fitbit Zip (in plastic wrap) on the ear tag with Velcro (already attached to the Fitbit zips);
    3) Wrap the Fitbit Zip and ear tag with tape. Fitbit Zip will block the animal ID on the ear tag, so use another piece of tape to write the animal ID, and put it on top;

4. Removing Fitbit Zip from Ear tag
    1) Cut off the tape from the space between the Fitbit Zip and the ear tag;
    2) Free Fitbit Zip from the tap;
    3) Remove Fitbit zip from the ear tag by separating the Velcro (so they can be put back later).

   Note: Fitbit Zip can be synchronized when wrapped with plastic wrap and tape. It does not need to be removed if more data needs to be collected and synchronization is the only purpose.

5. Synchronization
    1) Since there are more than one Fitbit zips in use, the best way to do the synchronization will be using a computer with Fitbit Connect app installed. Please download and install the app from here: https://www.fitbit.com/setup
    2) To synchronize a Fitbit zip, open Fitbit Connect, plug in the dongle of the Fitbit zip you are synchronizing, then click “Sync now”. The app will search of the zip and upload data.
    3) A few important notes:
        i. Please make sure to match the dongles with the zips. Each of them have an ID number, on the metal part of the dongle and the back of the zip. This is important in making sure that the data will be uploaded into the correct account.

ii. When synchronizing one zip, please keep other zips at least 3 feet away from the computer. If the computer cannot synchronize when it finds more than one zip nearby.

For the best data quality, Fitbit zips need to be synchronized every week.

Figures

Figure A.1. Timeline of behavior observations.

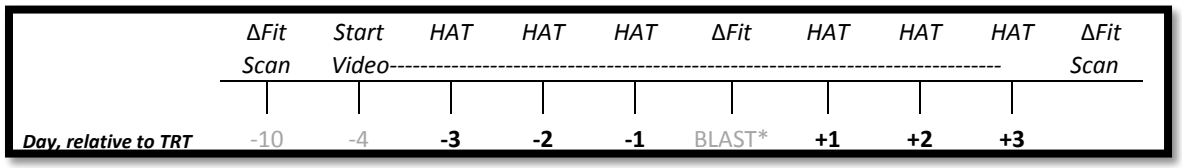


Figure A.2. Pen set up and approach test diagram.

Figure A.3. Diagram of installing Fibti Zip on ear tag.