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TITLE:

The Use of Traditional Fear Tests to Evaluate Different Emotional Circuits in Cattle

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SUMMARY:

Here, we present a protocol to conduct a variety of behavioral tests in cattle that have been designed to evaluate emotions. A battery of behavioral tests (open field test, startle test, bovine zero maze, exit velocity, pen score, and chute score) were conducted to evaluate different components of animal temperament.

ABSTRACT:

Animal temperament is complex and has implications for productivity and economic profitability. Quantifying an animal's response to differing stimuli may facilitate breeding selections and identify animals that are better suited to specific management strategies. Multiple tests have been developed to evaluate cattle temperament (e.g., exit velocity, chute score, pen score, open field test, startle test, bovine zero maze), but each of these tests evaluates the animal's response to different stimuli (e.g., isolation, novel environment, startle, willingness to enter an enclosed area). Cattle temperament has been observed to be relatively stable over time. However, the evaluation of temperament has the potential to be influenced by current conditions, previous experiences, and observer bias. Many of these temperament tests have been improperly categorized as fear tests and have also been criticized for being subjective. This paper provides a framework for standardizing behavioral tests for cattle and suggests that these different evaluations assess different aspects of the animal's overall temperament.

INTRODUCTION:

Animal temperament has been linked to behavioral characteristics such as exploratory behavior and boldness[1,2] and can exhibit consistencies over time and across contexts[3,4]. However, temperament is composed of multiple emotional systems working together. Animals experience

physical and psychological stressors, and evaluating the emotional response to both types is challenging. Emotional state can influence how animals perceive stimuli (e.g., cognitive bias), and is a critical component of animal welfare[5]. Understanding how an individual will behave in response to psychological stressors (e.g., commingling, weaning, change in stockperson) will provide animal managers additional selection criteria when identifying animals that have the skills to cope with psychological stressors.

Emotions are controlled by seven core affective systems within the brain (**Table 1**)[6]. These systems include four that control positive emotions: 1) SEEKING (exploration), 2) LUST (sexual excitement), 3) CARE (nurturance), and 4) PLAY (social joy). Three systems control negative emotions 1) FEAR (anxiety), 2) RAGE (anger), and 3) PANIC/GRIEF (separation distress). These affective systems may be heritable[7], impact profitability, and are a critical component of animal welfare.

A battery of tests has been developed to evaluate cattle temperament (e.g., exit velocity, chute score). However, the evaluation of temperament has the potential to be influenced by current conditions, previous experiences, and observer bias. While many of these behavioral evaluations are commonly referred to as fear tests, they may be quantifying different emotional components of temperament other than FEAR. In addition, the variation in how these tests have been conducted makes comparisons across different evaluations challenging. Thus, there is a need to understand the relationships among these behavioral evaluations as well as have a standardized protocol for these temperament evaluations.

The goal of this article is to visually document the different fear tests used for cattle; present the type of data that were generated from these different tests; evaluate the repeatability, validity, and reliability of these tests; demonstrate how to evaluate the relationships among the behaviors captured from these tests; and suggest which emotional circuit could be evaluated with each test.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M University (IACUC2016-0356).

1. Animal and housing

1.1. House yearling ¼ Bos indicus x ¾ Bos taurus steers (n = 32) from the same herd in two drylot pens (n = 16 steers/pen) for 7 days prior to test commencement. At the beginning of the study, steers weighed 270.9 ± 14.8 kg and were fed the same standard growing ration throughout the study.

1.2. Visually evaluate steers daily as part of routine husbandry practices. No steers received medical treatment throughout the duration of the study.

2. Description of the tests

2.1. Test 1: Exit velocity

2.1.1. Place electronic timers in front of a handling chute so that the distance between the starting and stopping points is 1.8 m. These timers are designed to start timing when the animal breaks the first electronic beam and stop when the animal breaks the second electronic beam.

2.1.2. Move cattle through the handling facility.

2.1.3. Catch each animal in the headgate of the chute and keep it restrained for 10 s.

2.1.4. After 10 s, release the animal from the headgate.

2.1.5. With the electronic timer, record the time it takes for the animal to traverse 1.8 m from the chute.

2.1.6. Calculate the velocity of the animal as it leaves the chute by dividing 1.8 m by the time it took for the animal to traverse the 1.8 m after release from the headgate and chute.

NOTE: Other publications have used this data collection strategy[8-11].

2.2. Test 2: Chute score

2.2.1. Move the cattle through the handling facility.

2.2.2. Catch each animal in the headgate of the chute for 10 s without applying pressure to its body.

2.2.3. Have someone observe the cattle for the 10 s and assign each animal a score according to the 2019 Beef Improvement Federation Guidelines for Uniform Beef Improvement Programs 9th Edition (**Table 2**) based upon its behavior while being restrained.

2.2.4. After 10 s, release animal from the headgate and chute.

NOTE: Other publications have used this data collection strategy[12-14].

2.3. Test 3: Pen score

2.3.1. Place a group of five cattle in a pen (7.3 m W x 7.3 m L x 2.4 m H).

2.3.2. Have a single human observer that is unknown to the cattle enter the pen on foot and close the gate after entering the pen.

2.3.3. Have the observer take two steps towards the group of cattle.

2.3.4. Visually observe each animal's behavior in response to the observer.

2.3.5. Within 30 s of entering the pen, assign each animal a score according to the 2019 Beef Improvement Federation Guidelines for Uniform Beef Improvement Programs 9th Edition (**Table 3**).

2.3.6. Clean the testing arena from urine and feces in between groups of animals.

NOTE: Other publications that have used this data collection strategy[10,11].

2.4. Test 4: Bovine zero maze

2.4.1. Construct a Bovine Zero Maze (BZM).

2.4.1.1. Use cattle panels to create a circular track 1.6 m wide, with the inner and outer diameters measuring 6.6 m and 8.2 m, respectively (**Figure 1**).

2.4.1.2. Divide the BZM into four quadrants of equal length with two opposing open quadrants and two opposing closed quadrants where the panels are covered with shade cloth and the shade cloth is stretched across the inner and outer rings of the maze to make a roof over the closed portions of the maze.

2.4.1.3. If the test is conducted outdoors, to minimize variation due to shadows, orient the BZM such that the closed sections of the maze face north and south and conduct tests at approximately noon each test day.

2.4.2. Mount a video camera(s) to capture the entire arena. Turn the camera on and begin recording.

2.4.3. Using low stress handling practices, move a single animal into an open portion of the maze, and allow the animal to explore the arena for 10 min.

2.4.4. At the end of the 10 min observation period, return the animal to its home pen.

2.4.5. Clean the testing arena from urine and feces in between animals.

2.4.6. Decode the video recordings for frequency and latency of steps, escape attempts, kicks, urinations, defecations, vocalizations, standing bouts, duration of time spent standing, duration of time spent walking, latency to enter closed areas, number of times the animal enters closed areas, amount of time in closed/open portions, number of transitions between open/closed arms. Metrics were identified based upon previously published work[15].

2.5. Test 5: Individual Startle Test and the Group Startle Test

2.5.1. Construct an arena (7.3 m W x 7.3 m L x 2.4 m H) that has a solid, uniform ground surface free of vegetation or manure, and two closed umbrellas at opposite ends of the arena (**Figure 2**). The umbrellas should be designed so that they open suddenly at the push of a button.

2.5.1.1. Ensure that the sides of the arena are solid or covered with plywood or shadecloth to ensure that the animal cannot see outside of the arena.

2.5.1.2. Cut a hole at approximately cattle head height on opposite sides of the arena for the umbrella to penetrate through.

2.5.2. Mount a video camera(s) to capture the entire arena. Turn the video camera on and begin recording.

2.5.3. Using low stress handling practices, move a single animal into the testing arena. For the group startle test introduce a small group of approximately four animals.

2.5.4. After the animal(s) has been in the arena for 60 s, open the two umbrellas simultaneously.

2.5.5. Leave the animal(s) in the arena for 4 min after the umbrellas have opened.

2.5.6. Clean the testing arena from urine and feces in between tests.

2.5.7. Decode the video recordings for the frequency and latency of steps, escape attempts, touching the umbrellas, kicks, urinations, defecations, vocalizations, standing bouts, duration of time spent standing, steps in the first 60 s of testing, and steps in the 60 s after the umbrellas were opened for each animal. Metrics were identified based upon previously published work[16].

2.6. Test 6: Open field test

2.6.1. Construct a square arena (7.3 m W x 7.3 m L x 2.4 m H) that has a solid, uniform ground surface free of vegetation or manure. The sides of the arena should be solid or covered with plywood or shadecloth to ensure that the animal cannot see outside of the arena.

2.6.2. Mount a video camera(s) to capture the entire arena. Turn the video camera(s) on and begin recording.

2.6.3. Using low stress handling practices, move a single animal to the center of a solid sided open field testing arena.

2.6.4. Leave the animal in the arena for 10 min.

2.6.5. After 10 min, return the animal to its home pen.

2.6.6. Clean the testing arena from urine and feces in between animals.

2.6.7. Decode the video recordings for the frequency and latency to first step, escape attempts, kicks, urination, defecation, vocalization, standing bouts, duration of time spent standing, duration of walking, number of steps taken, number of steps taken during the first 60 s of testing. Metrics were identified based upon previously published work[17-19].

3. Statistical analysis

3.1. Evaluate inter- and intra-test repeatability using a Pearson's Correlation (PROC CORR) and reliability calculated using Cronbach's alpha (PROC CORR). Conduct a validity of response variables with relation to average daily gain (ADG) using a regression analysis (PROC REG).

3.2. After standardizing the variables (PROC STANDARD), use a Cluster Analysis (PROC VARCLUS) to identify relationships among variables from within and among different tests. Many of these variables may be regressed against production metrics to identify production-relevant relationships among cattle behavior during these tests and productivity.

REPRESENTATIVE RESULTS:

The use of these results can help characterize the behavioral responsivity of cattle to different types of stimuli, and this information may influence individual retention and breeding selection decisions. In general, these tests should be conducted when the animals are young to minimize the impact of previous experience on their behavior[20]. The relationships among these different behavioral tests may be predictive of behaviors in other tests and with the animal's productivity. Repeatability of these tests also varies, as some tests are relatively consistent over time, while other tests are not.

For each test, we will present the repeatability, validity, and reliability for the metrics collected in that specific test. We will outline the pros and cons to each test as we see them and discuss what emotional circuit may be evaluated. We will then present a sample principle component analysis on the number of steps performed across all tests.

Exit Velocity (EV)

EV may decrease slightly as animals age, but will remain relatively stable[9,10,21]. There was high repeatability ($R = 0.72$; $p < 0.0001$) and the validity with relation to ADG depended on the circumstances ($R^2 = 0.12$, $p = 0.03$). The reliability was unacceptable (ICC = 0.41). The EV test has a short testing time, an objective response variable, is repeatable and valid, but requires equipment investment, can be influenced by the handling facility and the evaluator's previous experience, and has poor reliability.

Emotional circuit: FEAR

Pen Score (PS)

The PS had low repeatability ($R = 0.35$; $p = 0.05$) and its validity with relation to ADG depended

on the circumstances (R^2 = 0.12, p = 0.03). The reliability was unacceptable (ICC = 0.33). The PS test has a short testing time and multiple animals can be evaluated simultaneously. However, it is subjective. It can be influenced by prior negative experiences to being handled by humans. It can be influenced by the appearance and body language of the evaluator and is risky to the evaluator. There is low repeatability and reliability.
Emotional circuit: PANIC

Chute Score (CS)

CS had slight repeatability (R = 0.15, p = 0.42) and its validity with relation to ADG was unlikely to be useful (R^2 = -0.03, p = 0.67). The reliability was poor (ICC = 0.60). CS has a short testing time (10 s/animal), but it is a subjective response variable. It can be influenced by equipment/infrastructure and the evaluator's previous experience. If the hydraulics are too tight, it may cause a vocalization and change the amount of headgate pulling. Previous negative experiences with the facility may artificially inflate the scores. As the animals become older or heavier, the scores will decrease.
Emotional circuit: RAGE

Relationships among EV, PS, CS, and ADG

Figure 3 illustrates the relationships among these four variables. As ADG increased, EV (FEAR; R = -0.41; p = 0.02) and PS (PANIC; R = -0.42; p = 0.02) decreased. No relationship was observed between ADG and CS (RAGE). A positive relationship (R = 0.45; p = 0.01) was observed between PS (PANIC) and EV (FEAR). No relationship was observed between CS (RAGE) and EV nor between CS (RAGE) and PS (PANIC).

Bovine Zero Maze (BZM)

Behavioral responses while in the BZM (SEEKING, PANIC) are presented in **Table 4**. Because this test is not repeatable[22], cattle behavior during repeated testing may not be an accurate indicator of cattle responsivity to an immediate stimulus, but it may be more indicative of a core affective state (e.g., anxiety).

A number of steps had high repeatability (R = 0.71, p = 0.005). The number of standing bouts (R = -0.61) and latency to the first standing bout (R = 0.61) were valid metrics for EV during only the initial test. The total time standing during the first test was a valid metric for ADG. Several steps had unacceptable reliability (ICC = 0.42). The BZM has several repeatable steps. The duration of time spent standing is a valid metric for ADG and standing behavior can be a proxy for EV and ADG. A wide range of variables are evaluated. Cattle behavior is observed without human interference. Response metrics are objective. However, it is resource, time, and labor intensive to construct the maze and conduct the test (10 min/animal for testing only), and it requires video decoding.
Emotional circuit: SEEKING, PANIC

Individual Startle Test

Although the startle test is repeatable, cattle will behave differently during the startle test when they are evaluated individually compared to when they are in a group[23]. During the individual

startle test, cattle may experience isolation stress; therefore, the activation of the PANIC and SEEKING systems may override any FEAR system activation. The number of steps (R = 0.62, p = 0.0008) and number of steps within the first 60 s after the umbrella opens (R = 0.60, p = 0.001) had moderate repeatability. The validity with relation to ADG was unlikely to be a useful (R^2 = 0.07) indicator of ADG. Several steps (ICC = -0.06) for the entire testing period had unacceptable reliability. However, the number of steps within the first 60 s after the umbrella opens (ICC = 0.70) had acceptable reliability.

The individual startle test has several metrics that are repeatable and reliable, and a wide range of variables are evaluated. Cattle behavior is observed without human interference. Response metrics are objective. However, it is resource, time, and labor intensive to construct the maze and conduct the test (5 min/animal solely for testing). It requires video decoding and may be confounded by isolation stress.
Emotional circuit for individual startle test: PANIC, SEEKING
Emotional circuit for group startle test: FEAR

Open Field Test
The number of steps (R = 0.67, P = 0.0001) had moderate repeatability. Its validity with relation to ADG is compromised because several steps (R^2 = 0.03) are unlikely to be useful. A number of steps (ICC = 0.26) had unacceptable reliability. The open field test has a wide range of variables evaluated. Some steps during the test are repeatable. Cattle behavior is observed without human interference. Response metrics are objective. However, it is resource, time, and labor intensive to construct the maze and conduct the test (10 min/animal solely for testing), and it requires video decoding.
Emotional circuit: PANIC, SEEKING

Multivariate analyses
Cluster analyses identified three primary clusters (FEAR, RAGE, and PANIC/SEEKING) in the data (**Figure 4**). The number of steps in the Group Startle Test (FEAR) clustered with ADG and EV (FEAR). The number of steps in the BZM (PANIC/SEEKING), OFT (PANIC/SEEKING), and Individual startle test (PANIC/SEEKING) clustered together. CS (RAGE) did not cluster with any of the other variables.

FIGURE AND TABLE LEGENDS:
Table 1: Behavioral evaluations that may identify the activation of different emotional systems within the brain.

Table 2: Description of cattle behavior as evaluated for Chute Scores (Beef Improvement Federation).

Table 3: Description of cattle behavior as evaluated for Pen Score (Beef Improvement Federation).

Table 4: Frequency and latency to perform behaviors observed while cattle are in the Bovine

Zero Maze.

Table 5: Frequency and latency to perform behaviors observed while cattle are in the Individual Startle Test and the Group Startle Test.

Table 6: Frequency and latency to perform behaviors observed while cattle are in the Open Field Test.

Figure 1: Three-dimensional representation of the Bovine Zero Maze.

Figure 2: Three-dimensional representation of the arena for Open Field Test, Pen Score, and Startle Test. Maroon circles indicate placement of umbrellas for the Startle Test only.

Figure 3: Relationships among exit velocity, pen score, chute score, and productivity in *Bos indicus* influenced steers (n = 32).

Figure 4: Representative cluster analysis of behavioral responses of cattle to a variety of fear tests. In this figure, the number of steps performed during the Bovine Zero Maze (BZM), the Individual Startle Test, the Open Field Test (OFT), and the Group Startle Test were evaluated with the Chute Score, Pen Score, Exit Velocity, and Average Daily Gain.

DISCUSSION:

Exit Velocity and Chute Score

The EV and the CS are both evaluated while the animal is being processed through a handling chute. Although cattle behavior for both the EV and the CS are quantified during the same scenario, behavioral responses to these two tests are not related[24]. This suggests that the scenario in which the EV (e.g., escaping from restraint) and the CS (e.g., enduring restraint) are assessed may be perceived differently by cattle, and subsequently evaluate different emotional systems. The EV evaluates the behavior of cattle as they are escaping from restraint and is therefore thought to evaluate the FEAR system while the CS may evaluate RAGE. The CS evaluates the behavior of cattle while being restrained in the handling chute (**Table 2**), and thus may be a good proxy for the RAGE emotional system.

Substantial research has been conducted on the relationship between EV and production, health, and behavioral traits. While EV can be influenced by an animal's previous experience, this objective metric may be effective in quantifying the FEAR system, as substantial relationships between EV and health, productivity, breeding, and behavior have been identified. Cattle with faster EV have reduced growth rates[14], poor carcass quality[11,25], reduced immune function[20], and higher cortisol levels during handling[10]. This measurement can provide information about behavior in the home pen, because EV is positively correlated with step counts in the home pen[13]. From an animal management perspective, cattle with faster EV are more difficult to handle, present greater risk to animal managers, and may influence the behavior of herd-mates. While EV may be a good metric for evaluating FEAR, it does not measure all emotional systems. Therefore, additional tests are required to evaluate all of the emotional systems influencing

production and welfare.

Pen Score

The PS subjectively evaluates the cattle's willingness to be approached by a human (**Table 3**) and may be useful in evaluating the PANIC system. However, the PS has been criticized for lack of objectivity, because different evaluators may have different interpretations of behavior, and several subjective evaluations have suffered from poor inter-rater reliability[26].

Startle Test

Anxiety is highly evolved in all prey species. High levels of FEAR help protect the animal from pain and activates the sympatho-adrenal and hypothalamic-pituitary-adrenal axes as part of the fight or flight and stress response to a perceived danger. The startle test evaluates an animal's response to sudden, novel stimuli, and has been identified as an effective measurement in identifying behavioral differences among different genetic strains of pigs[27]. The startle test may be effective in evaluating the sensitivity and reactivity of the sympatho-adrenal system, which has production-relevant consequences when activated and may provide insight into the FEAR system.

Open Field Test

The OFT is the most commonly used test. The OFT was originally designed to evaluate individual animal boldness, or willingness to enter an open arena, an environment that may be perceived as dangerous and risky to the animal's survival. The OFT has been validated for species that instinctively seek shelter and avoid open spaces, such as rodents, chickens, and turkeys[16].

Cattle evolved to live in open fields, thus the OFT may not induce the behavioral and physiological responses associated with FEAR and may be better suited to evaluate social isolation (PANIC/GRIEF) or exploration (SEEKING). Further, the OFT evaluates individual animals, and because cattle are gregarious herd animals, the experience of the OFT may be eliciting an emotional response other than FEAR. The OFT lacks a strong correlation with other FEAR tests and the results are difficult to interpret (i.e., many factors can lead to the same activity). Therefore, the OFT is not recommended as a general FEAR test for cattle[16] and may not provide a comprehensive understanding of the FEAR systems in cattle. The OFT may, however, be a useful tool in quantifying either the PANIC or the SEEKING systems in cattle.

The SEEKING system is essential for animals to acquire the resources needed for survival. High SEEKING levels provoke intense, persistent enthusiastic exploration, appetitive and anticipatory excitement, and learning. This system can result in forward locomotion as the animal is motivated to explore its surroundings. SEEKING can play a role in both positive and negative emotions; positive SEEKING may engender a sense of purpose while negative SEEKING may result in behaviors associated with safety[28]. Cows that spent more time exploring and explored a larger portion of the range (e.g., stronger activation of SEEKING) ate quicker while in confinement, had calves with heavier weaning weights, higher cortisol concentrations during confinement, and shorter postpartum intervals to estrus[29]. Therefore, the SEEKING system can have production and welfare implications. Identifying animals with high activation of the SEEKING system may be

more successful in extensive ranging environments where individual and reproductive fitness is dependent on the animal's capacity to find resources and shelter. However, animals with high activation of the SEEKING system may experience higher levels of stress and frustration during confinement.

Bovine Zero Maze

Commonly used tests in biomedical research that are designed to evaluate the efficacy of anti-anxiety and anti-depressant drug development in rodents are the elevated plus maze (EPM) and the elevated zero maze (EZM)[30]. These tests exploit the instinctual behavior of the rodent and its natural propensity for dark, closed-in places to quantify their willingness to explore environments that would be inherently fearful or induce anxiety. Metrics from these tests can include the latency to leave the darkened arm of the maze, duration of time in the open and closed arms of the maze, and the number of transitions between the two environments during the testing period as well as the behavior of the animal (e.g., vocalization, urination, defecation, escape attempts) during the test[31].

The EPM and the EZM are both well validated tests for quantifying FEAR/ANXIETY in rodents[15,31]. A modified EPM has been used to quantify the FEAR response in swine[32] but has not been utilized in ruminants. However, the EPM has been criticized for its ambiguity of interpretation regarding behavior in the central square of the maze. Therefore, the EZM was designed to evaluate the same metrics as the EPM but allows uninterrupted exploration without ambiguity. When identifying a test to evaluate FEAR/ANXIETY and SEEKING in cattle, the EZM was a logical model. The EZM is conducive to the natural behavior of cattle, as they instinctively move in circular patterns and have a propensity for returning to the areas from which they came.

By applying principles similar to the EZM with an inverse of interpretation, a Bovine Zero Maze[33] has been developed to evaluate the FEAR, PANIC/GRIEF, and SEEKING systems in cattle. Cattle evolved to live in open spaces; therefore, cattle with reduced activation of the FEAR and PANIC/GRIEF systems will be more willing to spend time in the open portions of the BZM than the darkened portions of the maze, will be less likely to enter the closed portions of the maze, and will perform more escape attempts.

Quantifying cattle behavior across multiple evaluations may identify complex emotional relationships that can have economic significance, are easily measured, and can be included in breeding selection efforts. The emotional circuits of PLAY, LUST, and CARE were not evaluated in this study.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1 Bovine Maze.ai

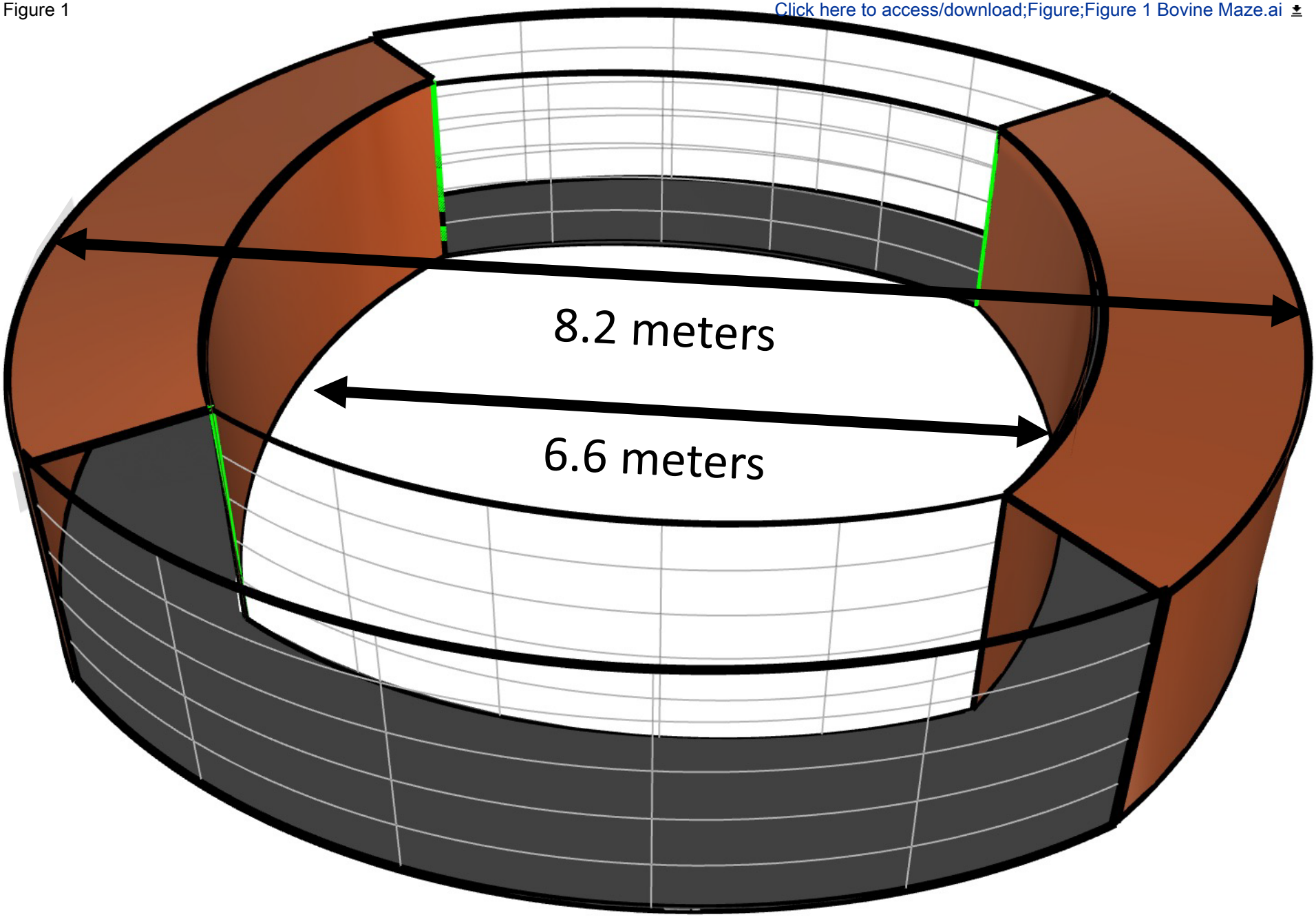


Figure 2

Click here to access/download;Figure;Figure 2 Startle test diagram (002).ai

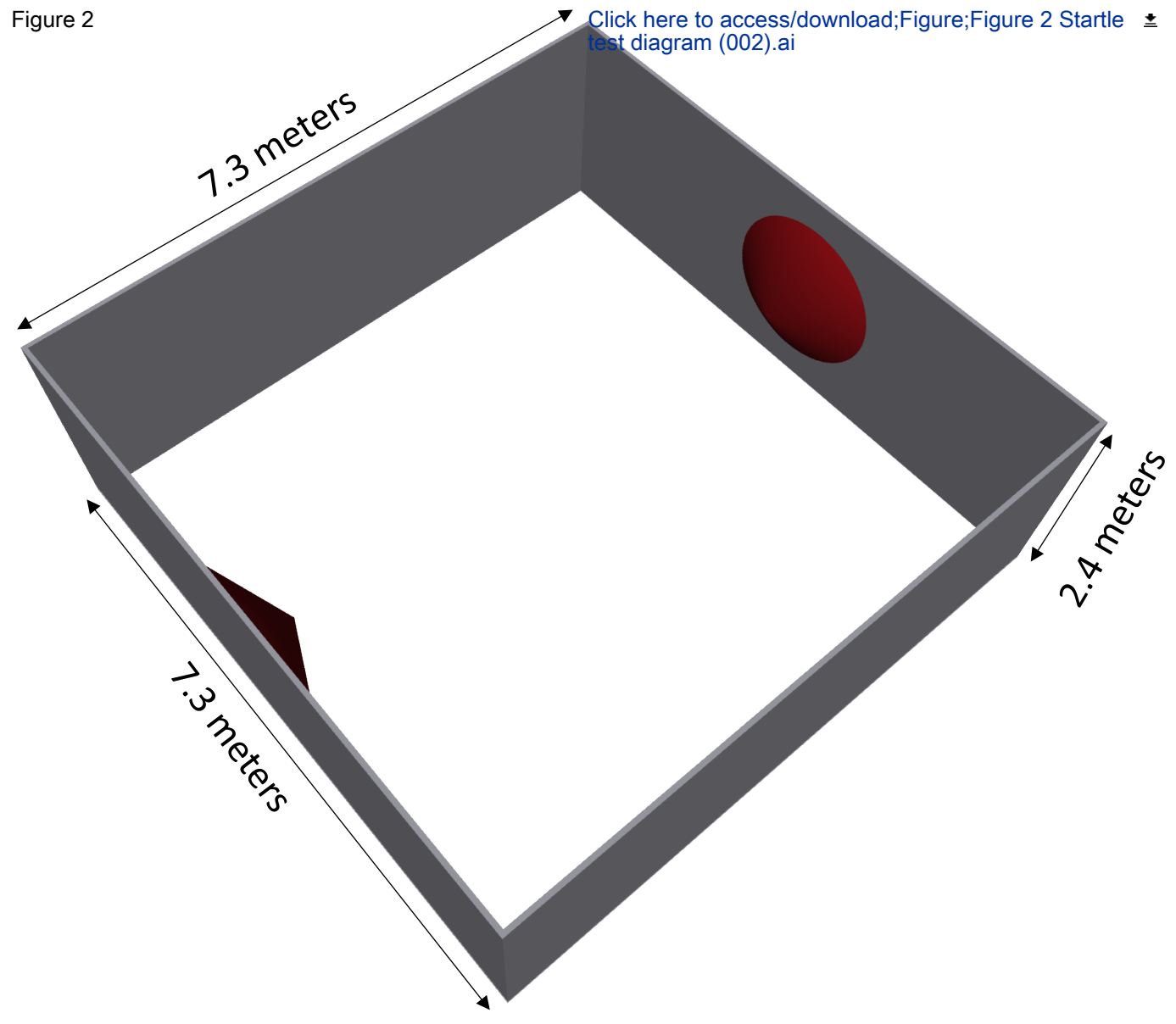


Figure 3

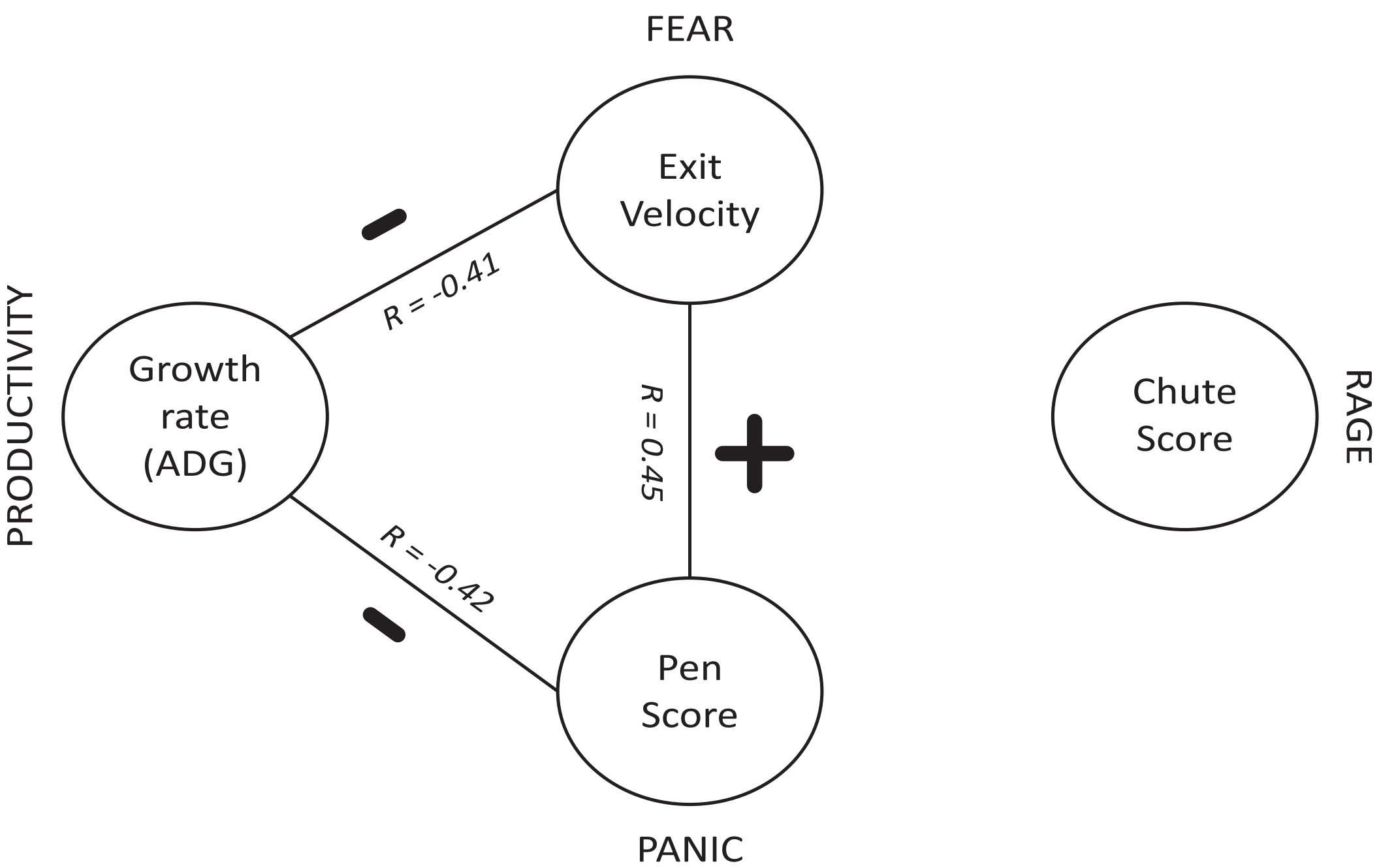
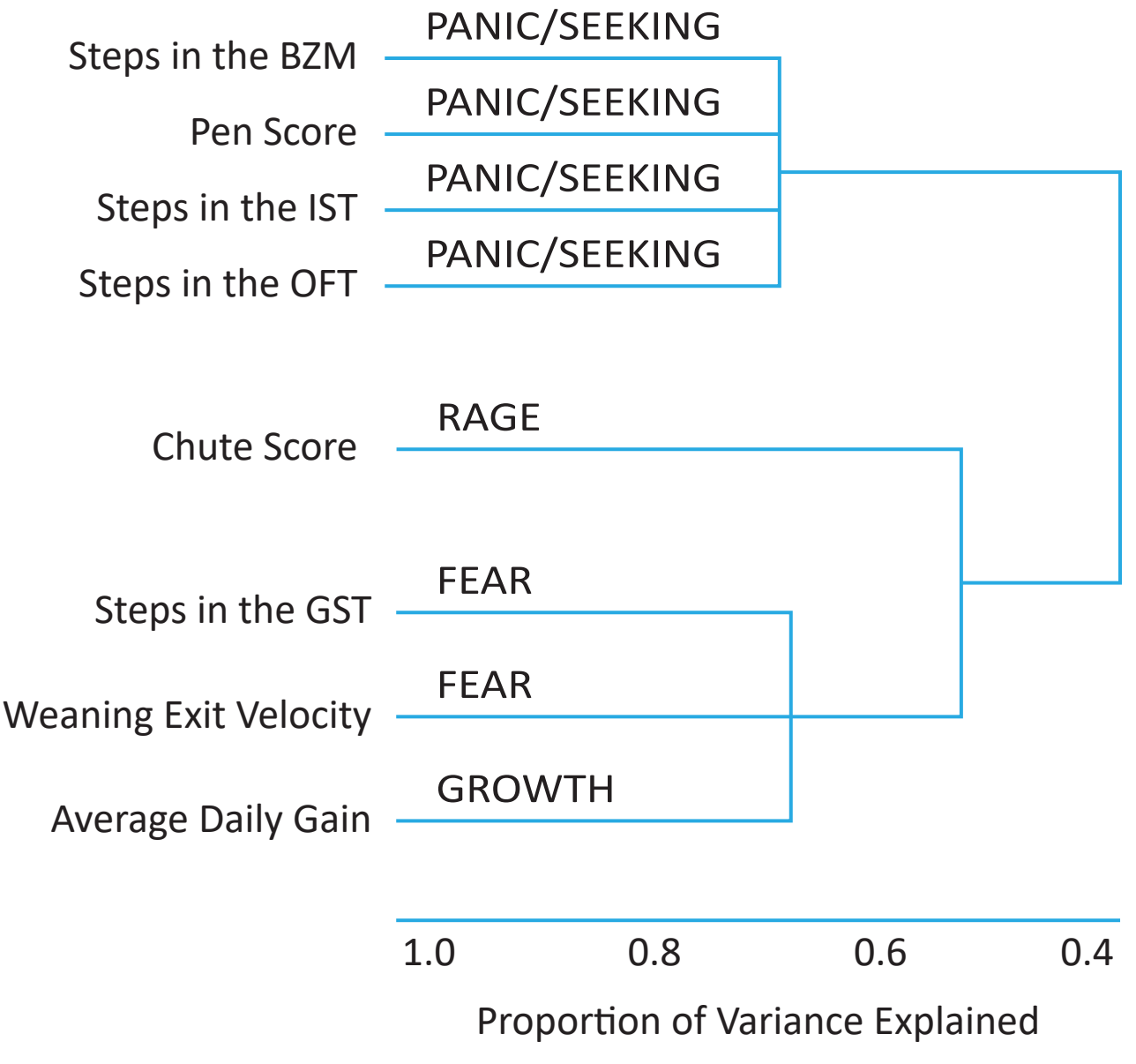


Figure 4

Click here to access/download;Figure;Figure 4
Cluster analysis diagram.ai



Emotional System	Behavioral test proposed to detect system activation
SEEKING	open field test, novel object test, bovine zero maze, pen score
LUST	libido evaluation
CARE	maternal behavior, distress surrounding weaning
PLAY	TBD
FEAR	startle test, exit velocity
RAGE	chute score, offspring protection
PANIC/GRIEF	social isolation test, bovine zero maze, pen score

Score	Label	Description
1	Docile	Mild disposition. Gentle and easily handled. Stands and moves slowly during processing. Undisturbed, settled, somewhat dull. Does not pull on headgate when in chute. Exits chute calmly
2	Restless	Quieter than average, but may be stubborn during processing. May try to back out of chute or pull back on headgate. Some flicking of tail. Exits chute promptly.
3	Nervous	Typical temperament is manageable, but nervous and impatient. A moderate amount of struggling, movement and tail flicking. Repeated pushing and pulling headgate. Exits chute briskly.
4	Flight (wild)	Jumpy and out of control, quivers and struggles violently. May bellow and froth at the mouth. Frantically runs fence line and may jump when penned individually. Exhibits long flight distance and exits chute wildly.
5	Aggressive	May be similar to Score 4, but with added aggressive behavior, fearfulness, extreme agitation, and continuous movement which may include jumping and bellowing while in chute. Exits chute frantically and may exhibit attack behavior when handled alone.
6	Very aggressive	Extremely aggressive temperament. Thrashes about or attacks wildly when confined in small, tight places. Pronounced attack behavior.

Score	Label	Description
1	Non-aggressive (docile)	Walks slowly, can be approached closely by humans, not excited by humans or facilities
2	Slightly aggressive	Runs along fences, will stand in corner if humans stay away, may pace fence
3	Moderately aggressive	Runs along fences, head up and will run if humans move closer, stops before hitting gates and fences, avoids humans
4	Aggressive	Runs, stays in back of the group, head high and very aware of humans, may run into fences and gates even with some distance, will likely run into fences if alone in pen
5	Very aggressive	Excited, runs into fences, runs over humans and anything else in path, “crazy”

Frequency of behavior performance	Mean ± SEM
Steps (count)	244.11 ± 29.19
Escape attempts (count)	9 ± 1.48
Kicks (count)	8.67 ± 1.17
Urinations (count)	0.32 ± 0.13
Defecations (count)	1 ± 0.29
Vocalizations (count)	0.96 ± 0.3
Standing bouts (count)	10.61 ± 1.06
Duration of time spent standing (s)	200.23 ± 22.59
Steps (count) during first 60 seconds of testing	32.18 ± 5.31

Latency to perform behavior after entering the Bovine Zero Maze	Mean ± SEM
Latency to first step (s)	18.32 ± 8.36
Latency to first escape attempt (s)	165.67 ± 38.31
Latency to first umbrella touch (s)	76.05 ± 14.43
Latency to first kick (s)	520.31 ± 31.64
Latency to first defecation (s)	325.63 ± 52.13
Latency to first vocalization (s)	437.03 ± 45.69
Latency to first standing bout (s)	68.72 ± 23.6

Max-Min
594 - 34
29 - 0
25 - 1
3 - 0
6 - 0
6 - 0
25 - 0
456.32 - 0
106 - 0

Max-Min
228.7 - 0.03
600 - 1.6
290.96 - 2.87
600 - 42.3
600 - 0
600 - 1.7
600 - 0.54

Table 5

	Individual Startle Test	
Frequency of behavior during test	Mean ± SEM	Max-Min
Time at which umbrellas open	63.27 ± 0.35	68.34 - 60.09
Steps (count)	318.5 ± 37.52	948 - 65
Escape attempt (count)	0 ± 0	0 - 0
Touches umbrella (count)	2.27 ± 0.53	Nov-00
Kicks (count)	0.16 ± 0.09	Mar-00
Urinations (count)	0.19 ± 0.07	Jan-00
Defecations (count)	0.72 ± 0.12	Mar-00
Vocalizations (count)	0.44 ± 0.29	Oct-00
Standing bouts (count)	7.91 ± 0.56	15 - 0
Duration standing (sonds)	140.87 ± 13.77	316.25 - 0
Steps in the first 60 seconds of testing (count)	62.44 ± 8.92	248 - 6
Steps in the 60 seconds after umbrellas opened (count)	72.52 ± 10.1	295 - 6

	Individual Startle Test	
Latency to perform behaviors	Mean ± SEM	Max-Min
Latency to first step (s)	4.14 ± 1.46	36.98 - 0.11
Latency to first escape attempt (s)	-	-
Latency to first umbrella touch (s)	94.79 ± 14.74	282.84 - 11.64
Latency to first kick (s)	137.29 ± 16.78	167.2 - 93.47
Latency to first urination (s)	135.47 ± 38.38	293.79 - 29.74
Latency to first defecation (s)	104.18 ± 23	271.98 - 3.35
Latency to first vocalization (s)	67.32 ± 41.27	226.89 - 3.83
Latency to first standing bout (s)	26.52 ± 7.1	193.48 - 0.44
Latency to first step after umbrella opens (s)	63.2 ± 1.77	84.19 - 6.36
Latency to first escape attempt after umbrella open (s)	-	-
Latency to first touches of the umbrella after the umbrellas open (s)	110.2 ± 16.38	282.84 - 11.64
Latency to first kick after umbrella opens (s)	137.29 ± 16.78	167.2 - 93.47
Latency to first urination after umbrella opens (s)	152.34 ± 40.79	293.79 - 29.74
Latency to first defecation after umbrella opens (s)	160.57 ± 26.49	271.98 – 1
Latency to first vocalization after umbrella opens (s)	100.91 ± 44.77	226.89 - 11.47
Latency to first standing bout after umbrella opens (s)	85.59 ± 10.32	297.33 - 1.27

Group Startle Test

Mean ± SEM	Max-Min
61.2 ± 0.08	62.16 - 60.33
126.72 ± 12.68	312 - 25
0 ± 0	0 - 0
0.03 ± 0.03	1 - 0
0 ± 0	0 - 0
0.13 ± 0.07	2 - 0
0.72 ± 0.15	3 - 0
0.03 ± 0.03	1 - 0
8.66 ± 0.52	14 - 3
188.94 ± 9.91	299 - 64.74
33.84 ± 3.11	81 - 6
27.09 ± 3.76	92 - 0

Group Startle Test

Mean ± SEM	Max-Min
2.61 ± 0.88	28.65 - 0.11
-	-
157.76 ± 157.76	157.76 - 157.755
-	-
52.87 ± 9.39	69.66 - 37.17
62.44 ± 13.74	196.76 - 15.11
68.15 ± 0.00	68.15 - 68.15
11.43 ± 1.76	45.4 - 1.12
65.94 ± 5.09	167.34 - 6.96
-	-
-	157.76 – 0
-	-
67.94 ± 1.72	69.66 - 66.21
90.03 ± 21.26	196.76 - 17.39
-	68.15 – 0
76.91 ± 5.33	182.69 - 15.47

Frequency of behaviors during test	Mean ± SEM	Max-Min
Steps (count)	464.28 ± 42.65	1607 - 91
Escape attempts (count)	0.06 ± 0.04	2 - 0
Kicks (count)	0.16 ± 0.06	2 - 0
Urinations (count)	0.14 ± 0.04	1 - 0
Defecations (count)	0.44 ± 0.08	2 - 0
Vocalizations (count)	1.91 ± 0.7	32 - 0
Standing bouts (count)	13.75 ± 0.84	40 - 4
Duration of time spent standing (s)	294.94 ± 17.85	562.98 - 48.72
Steps (count) during first 60 seconds of testing	69.36 ± 7.72	297 - 0

Latency to perform behavior	Mean ± SEM	Max-Min
Latency to first step (s)	5.9 ± 2.42	148.18 - 0.11
Latency to first escape attempt (s)	357.81 ± 158.26	563.23 - 45.56
Latency to first kick (s)	355.95 ± 53.7	584.58 - 66.51
Latency to first defecation (s)	135.38 ± 31.51	486.29 - 1.98
Latency to first vocalization (s)	162.67 ± 49.87	742 - 8.8
Latency to first standing bout (s)	28.11 ± 6.06	255.97 - 0.35

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Electronic timers	FarmTek, Wylie, TX		
Priefert Cattle Panels	Priefert Rodeo & Ranch Equipment, Mount Pleasant, TX, USA		
Shade Cloth	Windscreen4less, San Bernardino, CA, USA	Heavy Duty Privacy Screen Fence in Color Solid Black,	
SILENCER Commerical Pro	Silencer Hydraulic Chutes, Stapleton, NE		
Umbrella	WinCraft	Model# A04852, Winona, Minnesota	
Video Camera	Canon	Canon VIXIA HF R800 HD, Mellville, NY, USA	

TEXAS A&M AGRILIFE

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AGRICULTURE & LIFE SCIENCES
DEPARTMENT OF ANIMAL SCIENCE

Dr. Courtney Lynd Daigle
Assistant Professor

October 1, 2019

Dr. Nam Nguyen
Manager of Review
Journal of Visualized Experiments

Dear Dr. Nguyen and anonymous reviewers,

We would like to extend our deepest gratitude to you and the anonymous reviewers for the feedback and genuine effort to enhance the quality of our Manuscript JoVE60641 "Fear tests don’t always measure fear: the use of traditional fear tests to quantify different emotional circuits in the brain."

We are thankful for your input and recommendations as they have enhanced the quality of information provided and clarity of message to the reader. Should any part of this manuscript require further alteration, please let us know and we will make the necessary changes.

Below are our responses to the concerns raised by the editor anonymous reviewers.

Should you have any questions or concerns, please do not hesitate to contact us directly.

Sincerely,

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Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

AU: we have provided as much information regarding the products as is available to the authors

3. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg).

AU: Corrected

4. Please upload each Figure individually to your Editorial Manager account.

AU: Corrected

5. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

AU: Corrected

6. Please use SI abbreviations for time in the Tables: s instead of sec, etc.

AU: Corrected

7. Please revise the title to be more concise. Additionally, please do not use contractions.

AU: the title has been revised to "The use of traditional fear tests to evaluate different emotional circuits in cattle"

8. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

AU: The revised summary is as follows: "Here, we present a protocol to conduct a variety of behavioral tests in cattle that have been designed to evaluate emotions. A battery of behavioral tests (Open Field Test, Startle Test, Bovine Zero Maze, Exit Velocity, Pen Score, and Chute Score) were conducted to evaluate different components of animal temperament."

9. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

AU: Corrected

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Farmtek, SILENCER, etc.

AU: Corrected

11. Please insert a one liner spacer between each protocol step.

AU: Corrected

12. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

AU: Corrected

13. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

AU: Corrected

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The summary should be more specific to give a complete idea of the different tests covered in the article.

AU: The summary now contains reference to all of the tests conducted.

Minor Concerns:
In the introduction no reference should be made to the work tables. In this case it would be pertinent to make a state of the art on the main themes of the article.

AU: New Lines: "The goal of this article is to visually document the different fear tests used for cattle, present the type of data that is generated from these different tests, evaluate the repeatability, validity and reliability of these tests, demonstrate how to evaluate the relationships among the behaviors captured from these tests, and suggest which emotional circuit could be evaluated with each test."

Line 173: should be test 6 and not test 3.

AU: Corrected

In the results chapter line 198 and following - in the body of the text, reference should be made to the tables for the respective results. And the table results should have a larger description in the body text.

The inclusion of the main conclusions of the work is recommended.

AU: corrected

Reviewer #2:
Manuscript Summary:
The manuscript describes the protocol for a set of tests for "fear" or temperament in cattle, with some general discussion of how these are analysed and can be interpreted. Some data from the tests is presented.

AU: the format for this journal indicates that the data used here can be hypothetical in nature. In order to preserve the novelty of information that has been generated from this specific research trial, we are using hypothetical data to demonstrate how the tests could be conducted, decoded, and analyzed.

The set of tests described is appropriate, and includes some less frequently used tests that may be of use for other researchers to consider. There is some good critical discussion of the interpretation of certain tests. The protocols described are fairly simple and easy to follow.

Major Concerns:
My biggest concerns with this manuscript are that there is little discussion of the rationale behind the detailed protocols, and the discussion of statistical analysis is so general that it provides little guidance. The clear benefit of describing all of these tests together would be to discuss how to use them in conjunction with one another, which is not really discussed. The scoring system referred to for some tests may not be the ideal scientific method.

AU: Substantial effort has been made to increase the clarity of direction regarding statistical analysis. Because these tests are typically conducted in conjunction with physiological or other behavioral testing, the ultimate analysis of these results will depend on the hypothesis of the researcher using this test. That was the motivation for the author's choice to keep the description of the statistical analysis general. However, in lieu of the reviewer's comments, we have added information regarding the repeatability, validity, and reliability of some of the metrics in these tests. This particular article is not a hypothesis-driven manuscript as this is a methods paper that seeks to demonstrate and standardize methodology that is used within the field.

The scoring systems were developed in the early 1990s, and while we agree that they may be subjective, they have been widely used in the literature and are therefore important to include in this video documentation of methodology.

Minor Concerns:
It is not entirely clear that these data are novel, and although data on repeatability are given, no information about the repeated testing is given so these results are not very informative.

AU: As this is a methods paper, the results are not intended to make a novel contribution to the scientific literature. The purpose of this article is to demonstrate how to conduct the tests and what an analysis of the data could look like. However, the way in which the data is analyzed can vary greatly depending upon the design of the hypothesis-driven research in which these tests are used.

Specific comments follow:
l. 113: Why was a group size of 5 chosen? The discussion could address whether group sizes are likely to have an impact on the results.

AU: The group of 5 was chosen because that is what is standard in the literature and previous studies.

l. 117-119 "Observe the behaviour" is not specific enough for others to follow this protocol. The scores described in Table 3 are about "aggression", not "fear", so their fit with this paper is unclear. However, the lower scores that are labelled as reflecting some level of aggression actually seem to describe behaviour more associated with fear. This scoring system may not be appropriate, and should be critically discussed.

AU: this was addressed in the above comment.

l. 121 Why is this cleaning not recommended for the chute tests?

AU: this is impractical based upon the realities of cattle handling.

l. 171 No such protocol for cattle seems to be described in the review paper cited. Where did this come from?

AU: this paper describes the zero maze that is regularly used in biomedical research. We are the first group to conduct this test in cattle, therefore, there are no other references with which to cite. Further, we used the metrics that were collected in the rodent maze to guide the metrics we chose to evaluate in the maze designed for cattle.

l. 210: Is there justification for stating that this will remain stable over time, based on one study?

AU: Additional references have been added to support this statement.

l. 226-8: The logic behind this statement is not clear.

AU: The research that evaluated this test has recently been accepted for publication.

l. 320: Justification for this claim is also unclear.

AU: Explicit explanation of this phenomenon can be found in "The Archaeology of Mind" by Panksepp and Biven. A reference to this has been added to the text.

l. 354-55: Is there a source for this statement about cattle's tendencies?

AU: this section has been revised.

Table 2, "restless": I think the word "management" is incorrect but am not sure what this is supposed to mean.

AU: Thank you. This was a typo. "management" has been changed to "manageable"

Reviewer #3:

Manuscript Summary:
I was not entirely sure how to review this. It is a great idea to visual present each of the different fear tests and to talk about their pros and cons. There have been a lot of questions raised about what emotions/motivations each of the test actually assessed, and using the Panksepp categories as a framework is a good idea. I am unsure what results should be presented. Is the purpose to present the different tests and their pros and cons? If so, do we need the stats on repeatability and relation to growth? Or is it a paper on the relationship between the tests? Then we need more correlations between the tests presented, and more comprehensive stats on repeatabilities etc. for the BZM, Pen Score, OFT or startle test.

I would be tempted to go for the 'what are the different tests, pros and cons and discussion of whether they all measure fear or other emotions' approach.

AU: the results have been substantially revised to provide pros and cons, repeatability, validity (regarding ADG), and reliability for all tests and what emotional circuit we propose is being evaluated. A sample cluster analysis has been conducted on results from multiple tests to demonstrate how the data could be evaluated synergistically.

Major Concerns:
As above, either go for a 'discussion of what these test actually tell us' approach or a 'what is the statistical relationship between these tests' approach

AU: we took the approach of how repeatable, reliable, and valid indicators of growth are each of these tests, pros and cons of each test, and what these tests actually tell us. We also included sample analysis for evaluating the interrelationships among the different tests. However, as this is a methods paper, we did not seek to address any specific hypothesis but more demonstrate how you could use the data generated from these tests to address a specific hypothesis-based research study.

Minor Concerns:
Authors: it says Amanda Hubbard in the opening piece and Amanda Mathias further down
L54.

AU: Thank you for this. During the period of manuscript preparation, Ms. Mathias became Mrs. Hubbard.

I think you are following the Panksepp model here? I think it provides a good framework to consider temperament tests, ie separating fear from panic/grief (separation) is clearly important, but that model is not universally accepted. It would be good to acknowledge this here. Also acknowledge Panksepp in Table 1 ('after Panksepp' is how you do it I think??).

In the results, the Pankseppian emotional systems that each test might involve are linked. It would be good to formalise this in a hypothesis here in the introduction. What elements do you envisage are involved in each test? What will you do to test this?

AU: This is not a hypothesis-driven research paper. We have added language at the beginning of the discussion explaining that these circuits are not mutually exclusive and will influence one another, so researchers should be aware that they may be evaluating more than one emotional circuit during a single test. A listing of what emotional circuits are most likely activated during each of these tests is included in the results section.

L74: Description of animals and housing. Some more details of the testing procedure are needed here. What order were the tests done in? Did all animals experience all tests in the same order? Over how many days were the tests run? One per day on consecutive days or were there rest/wash-out days as well? ADG is mentioned in the results. How/when was this assessed?

AU: A general description of how the animals were housed and the order in which tests were conducted has been included.

A lot of other authors have used flight score and exit velocity. Flight speed/flight time was originally designed by Heather Burrow in Australia in 1988 and has been used extensively in that country. Are you just giving a few examples? If you want to be comprehensive, you could look wider. Also for the chute test. Outside of the US, it is generally called a 'crush score'. However, this could become a review in its own right! I think I would acknowledge Burrow, but then suggest that you are giving examples of where these tests have been used. However, acknowledging work outside the US would be diplomatic.

AU: Our intention is to show that exit velocity is one of the stalwarts of cattle temperament evaluation. Because this metric is so easily captured, our hope, in future studies is to evaluate the efficacy of this behavioral metric in evaluating other components of cattle temperament beyond fear. We have added language to acknowledge that chute score and crush score are evaluating the same thing. We have also added references to highlight the wide use of exit velocity across the cattle temperament literature.

What about the docility test as pioneered by Le Neindre et al. in France? This is used in France extensively in Limousin breed programmes. Why was this not included? Focus on procedures used in the US?

AU: We attempted to avoid using any breed-specific evaluations

L209. See comments above, but here the emotional systems are linked to the tests. However, it is not clear what the rationale for this was. Exit velocity is certainly 'fear', but as they are isolated, isn't there an element of 'panic' as well? I think it would be good to be clearer about how you assigned the tests to the elements.

AU: Additional language has been added throughout the manuscript to address this and clarify the text to the reader.

L225: 'seking'

AU: Corrected

Table 2. In definition of nervous - Typical temperament is management? Manageable?

AU: Corrected

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Author(s):	Courtney Daigle, Amanda Hubbard, and Temple Grandin

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