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ABSTRACT

Background: Use of oral fluid specimens in swine research and diagnostics has become commonplace. Objective of the present study was to evaluate the effect of the number of ropes provided and location in the pen on oral fluid sampling.

Sixty 5-week-old pigs were divided into two groups of 30 (15 gilts, 15 barrows) and housed in two pens identical in size and design. The effect of the number of ropes in the pen on pig oral fluid sampling behavior was evaluated by varying the number of ropes in the pen (1-4). Pig preference for rope location was assessed by observing pigs with 4 ropes in the pen (one at each corner). Four cameras synchronously took pictures at 2 second intervals throughout the sampling period to document pig interactions with the rope. "Pig interaction" was defined as a picture showing a pig's mouth closed around the rope. Oral fluid was collected at the end of each sampling period, aggregated, and volume recorded.

Results: Observations were analyzed at both group and individual level. Mean oral fluid volume and pig interaction increased when more ropes were provided, but 89% of pigs interacted with one rope in the pen. Given a choice of 4 ropes, pigs showed a significant bias toward location.

Conclusions: The data support the interpretation that one rope is sufficient to collect a sample representative of the group. Providing additional ropes increases volume, but this does not increase diagnostic utility. The data likewise suggest that pigs may have a preference for location; this observation will require additional exploration to achieve better understanding.

Keywords: oral fluid, swine, behavior, participation

BACKGROUND

With the ever-increasing global threat of foreign and newly emerging diseases, the need for protecting the U.S. national herd by developing methods for rapid specimen collection and diagnostics testing is more urgent than ever. Consistent with this trend has been the increasing use of oral fluid specimens as a population-based diagnostic approach for endemic diseases of swine. Research has shown that the use of oral fluid specimens in diagnosis provides several advantages compared individual animal specimens, e.g. serum, including easier sample collection and better herd-level sensitivity and specificity (Olsen et al., 2013). Since 2005, a major focus has been placed on development of assays for detecting pathogens or antibody in oral fluids to the extent that, at this point in time, oral fluid-adapted diagnostic assays have been described for essentially all the major pathogens of swine (Bjustrom-Kraft et al, 2018; Rotolo et al., 2018). In contrast, this study focused on the oral fluid specimen, itself. Specifically, the objective addressed was the effect of the number and placement of ropes in a pen on pig interaction with the ropes.

MATERIALS AND METHODS

Experimental design

This experiment was conducted in 60 5-week-old commercial crossbred pigs (30 barrows and 30 gilts) weighing an average of 10 kg at the time they were received at the Animal Resource Station Complex (Iowa State University, Ames, Iowa). Animals were randomly divided into two groups of 30 (15 barrows and 15 gilts) by blindly selecting ear tags from a bag. To expedite individual pig behavioral observations, a subset of ten pigs in each group received large colored ear tags to aid in identification and differentiation from a distance. Thereafter, each group of 30 pigs was housed in a pen (7.3 m by 6.1 m) equipped with 4 nipple drinkers and a bar transecting each corner to hang rope for oral fluid collection (see Figure 1). Animals were provided age-appropriate complete feed throughout the study (Nature's Match®, Purina Mills, LLC, Gray Summit MO USA). Pigs were acclimated to their surroundings for nine days prior to initiating the experiment. During acclimatization, pigs were trained for oral fluid collection by providing 4

ropes, i.e., one in each corner of the pen, for 60 minutes each morning (White et al. 2014). Outcome variables measured included number of pigs interacting with the rope(s), interaction over time, rope location, and total oral fluid (ml) collected. The procedures and experiment were approved and conducted under guidelines established by the Iowa State University Office for Responsible Research (IACUC 6-12-7397-S).

Oral fluid sampling and collection "treatments"

In brief, the oral fluid sampling procedure consisted of hanging a 100% 3-strand cotton rope (1.6 cm diameter) approximately 1 m length such that the end of the rope was at pig shoulder height (Figure 1). After allowing the pigs to interact with the rope, oral fluids were recovered by placing the chewed end of the rope in a plastic bag, cutting the chewed end from the dry portion of the rope, and then passing the bag with the rope inside slowly through a chamois wringer. As the rope was compressed, oral fluid had pooled in a corner of the bag, the bag was pierced with a sterile needle, and the fluid drained into a 50ml tube.

The four rope exposure treatments consisted of placing 1, 2, 3, or 4 ropes in the pen for 20 min, during which time pig behavior was recorded with cameras and after which aggregate oral fluid collection volume (ml) was determined. The 5 replications of each of the 4 treatments were randomly ordered (Random.org) over the 20 day observation period (Table 1 and Figure 1). After the initial 20 min collection, 4 ropes (one in each corner) were placed for an additional 20 minutes to avoid habituating the pigs to any specific presentation pattern and to evaluate pigs' bias toward location. In each case, the investigator left the room immediately after hanging the ropes to avoid affecting the pigs' behavior.

Behavioral data acquisition

One camera (PC900 Hyperfire Professional High Output Covert IR camera, Reconyx®, Holmen WI USA equipped with SanDisk ultra 32GB SDHC class 10 memory card) was mounted in each corner of the room and placed to best capture images of the pigs interacting with the rope closest to them (Figure 1). The 4 cameras operated synchronously at 2 sec intervals throughout the 20 min sampling period using an external computer trigger. The remote triggering hardware

consisted of 4 5VDC electromechanical relays, one for each camera, initiated with a logic 1 digital pulse from a microcontroller (Arduino Uno, Somerville MA USA). Each relay was fitted with an up-front transistor and matched pull-up resistor to safeguard the digital signals from the microcontroller. A program was written using open-source software (Arduino IDE) that utilized four of the available 13 digital pins to initiate camera triggering for the duration of the study. Each image was time-stamped by each camera.

All images were reviewed and interpreted by one individual (first author). "Interaction with a rope" was defined as a pig with its mouth open around, or closed on, a rope. Time-stamping and simultaneous imaging by all 4 cameras avoided double-counting of pigs. Behavioral data for the group of 30 pigs was collected by reviewing the images at one minute intervals and recording observations for the rope treatment effect (initial 20 min observation period) and for rope location effect (second 20 min observational period). Behavioral data for the 10 individual pig subset was based on identifying each pig in the images using large colored ear tags. For the individual pig data, any interaction(s) with a rope during a 1 min period counted as an interaction, regardless of the duration of the interaction. Notably, the color recording capability of the cameras failed sporadically. Therefore, location effect for the individual pigs was based on rope treatments in which 4 ropes were deployed.

Statistical analyses

Statistical analysis was performed using *R* 3.5.2 (R core team, 2019). At the group level (30 pigs per pen), a generalized linear model with mixed effects was used to model the logit-transformed probability of the pigs' rope interactions as a function of number of ropes and rope location. To study the effect of number of ropes, the fixed effects were number of ropes, study day, observation minute, and their interactions; pen was a random effect. For the analysis of rope location data, corner 1 was used as a baseline, fixed effects were rope location, study day, observation minute, and their interactions; pen was a random effect.

At the individual pig level, i.e., the subset of 10 well-identified pigs in each pen, a logistic regression model with mixed effects was utilized to model the average estimated odds of pigs'

interaction with the rope. Because the response variable was whether a tagged pig interacted with any ropes within a minute interval (binary response), a logistic regression with mixed effects was used to model the odds of a pig's interaction with rope. The fixed effects were the same as the pen level model, while the random effect contained individual pigs nested within a pen.

The effect of number of ropes and rope location on the volume of oral fluid collected was evaluated using a linear mixed-effects model balanced design with repeated measurements. Fixed effects were number of ropes, study day, and their interaction. Oral fluid volumes obtained from the same pen over time were not independent, so pen was a random effect. The difference in the analysis for rope location was replacing number of ropes with rope location as a fixed effect using rope corner #1 as the baseline.

RESULTS

Number of ropes in pen

The initial sampling was based on varying the number of ropes in the pen. At the group level, each additional rope placed in the pen was associated with a 65.6% ($p < 0.01$) increase in the odds of pig interaction with any rope (Fig 2, top). Likewise, the mean aggregate volume of oral fluid collected from the pen increased by 15.7 ml ($p < 0.001$) with each additional rope placed in the pen (Table 2, Figure 3, top). At the individual pig level, each additional rope placed in the pen was associated with an increase both in the odds of pig interaction by 1.33 ($p < 0.01$) and in the cumulative participation (Table 2, Figure 4, top).

Additionally, an effect of time was seen both at the group and individual level. Specifically, each additional minute was associated with a 9.7% ($p < 0.01$) decrease in the average estimated odds of pig interaction at the group level (Figure 2, top) and a 6.3% decrease ($p < 0.05$) in the average estimated odds of pig interaction when analyzed at the individual pig level.

Rope location effect

Rope location effects were tested under the condition that one rope was available in each of the

four corners. At the group level and using corner 1 as the baseline, the odds of pig-rope interaction was 1.2 times higher at corner 4 ($p < 0.001$); 0.4 times lower at corner 2 ($p < 0.01$); not different at corner 3 (Fig 2, bottom). The mean volume of oral fluid collected at corner 4 was 6.4 ml ($p < 0.01$) greater than corner 1 (Table 2; Figure 3, bottom).

At the individual pig level and using corner1 as the baseline, the odds of pig participation was 1.13 times higher at corner 4 ($p = 0.053$) (Figure 4, bottom). Overall cumulative participation of the individual pigs and aggregate oral fluid volume was highest at corner 4 (Table 2).

DISCUSSION

On the farm, integration of surveillance data with herd records can provide the means to: (1) detect and/or identify the spatiotemporal pattern of specific pathogens; (2) quantify their effects on pig health and productivity; (3) target interventions to the correct pathogen and population; and (4) time the intervention for maximum effect. At the regional level, oral fluid-based testing makes real-time surveillance feasible and affordable. A large number of research publications have reported the adaptation of oral fluid specimens to contemporary diagnostic technology for the detection of pathogens in swine populations to meet this need; even in low disease prevalence situations (Olsen et al, 2013). The current study focused on pig behavior in the context of collecting oral fluid samples.

There are relatively few papers with which to compare the results of this study. Graage et al. (2019) evaluated oral fluid sampling by pig age (4-5, 7-8, 12 weeks of age) and group size (16-20, 21-25, 26-30 pigs) and reported that a mean of 78.5% of pigs interacted with the rope, with fewer interactions as group size increased. White et al. (2014) observed the behavior of 6-to-12-week-old pigs with an average group size of 25-28 pigs per pen and found that prior experience with the rope ("training") increased interactions from 44% of the group within 20 minutes to 70% participation within 20 minutes. Seddon et al. (2011) observed better oral fluid collection from pigs housed on fully slatted floors versus straw bedding, but found no difference in collection when varying the number of ropes presented (1, 2, 3, 4). However, the rope were placed at close (~18 inch) intervals. There are no previous studies evaluating the location of

ropes within pens and its effect on oral fluid collections.

The observation most consistent across all studies to date is the fact that ~80% of pigs will interact with a rope placed in the pen in 20-30 minutes. In the present study, the estimate was ≥ 90%. It may be conjectured that the reason for higher pig interaction in the current study was the placement of the rope so as to allow up to 8 pigs access at one time. In contrast, many or most oral fluids are collected by hanging the rope from a gate or panel. The observation of pig "preference" for a particular rope location, corner #4 in this case, raises the question of whether this was unique to this environment or whether "preferred locations" could be identified or created in the field setting. Ultimately, understanding pig behavior in the context of oral fluid sampling is important because sampling is the first step in the surveillance process. Less than optimum sampling can only result in less than optimum surveillance data; for which reason additional work in this area is justified.

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Table 1. Randomly ordered "rope exposure" treatments by day

Day	Pen One					Pen Two				
	No. ropes	Rope location (corner)				No. ropes	Rope location (corner)			
		1	2	3	4		1	2	3	4
1	2			✓	✓	3	✓		✓	✓
2	3	✓		✓	✓	3	✓		✓	✓
3	4	✓	✓	✓	✓	4	✓	✓	✓	✓
4	3	✓		✓	✓	2			✓	✓
5	2			✓	✓	3	✓		✓	✓
6	2			✓	✓	4	✓	✓	✓	✓
7	3	✓		✓	✓	1				✓
8	4	✓	✓	✓	✓	2			✓	✓
9	1				✓	3	✓		✓	✓
10	1				✓	1				✓
11	1				✓	4	✓	✓	✓	✓
12	4	✓	✓	✓	✓	2			✓	✓
13	4	✓	✓	✓	✓	2			✓	✓
14	1				✓	1				✓
15	3	✓		✓	✓	3	✓		✓	✓
16	3	✓		✓	✓	1				✓
17	1				✓	2			✓	✓
18	2			✓	✓	4	✓	✓	✓	✓
19	2			✓	✓	4	✓	✓	✓	✓
20	4	✓	✓	✓	✓	1				✓

Table 2. Summary of pig interaction by number of ropes in pen and rope location (4 ropes in pen) over 20 minute observation period with 95% confidence intervals

	Number of ropes in pen			
	1	2	3	4
Aggregate sample volume (mean, ml)	29.9 (25.0, 33.4)[a]	39.0 (30.7, 47.3)[a]	57.3 (46.9, 67.7)[b]	79.2 (68.7, 89.7)[c]
Overall mean percent of individually identified pigs that interacted with rope	89% (81, 97)	91% (84, 98)	92% (86, 98)	97% (94, 100)
	Location effect (4 ropes in pen)			
	1 (baseline)	2	3	4
Mean volume oral fluid (ml)	13.7 (11.7, 15.7)	11.7 (9.6, 13.9)	12.9 (11.2, 14.6)	17.5 (15.9, 19.1)
Overall mean percent of individually identified pigs that interacted with rope	78% (70, 86)	55% (36, 74)	65% (48, 82)	82% (72, 92)

[abc] Superscripts within a row indicated significantly different values ($p < 0.001$)

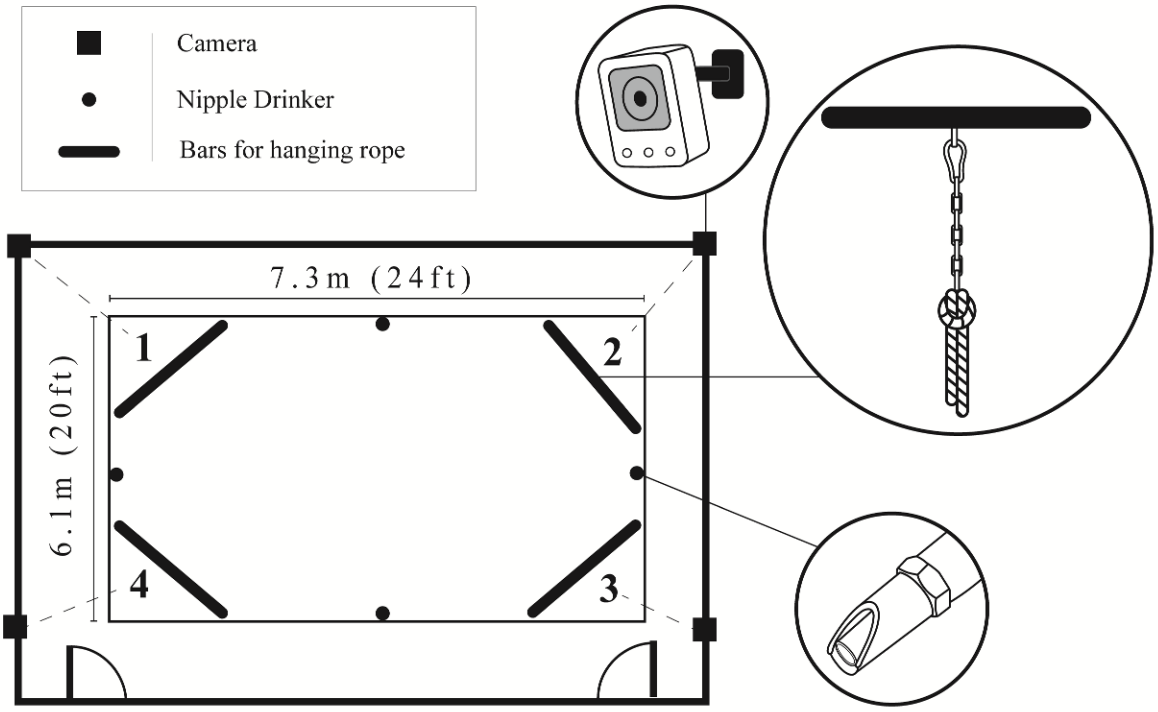

Figure 1. Pen layout showing placement of cameras, nipple drinkers, and ropes

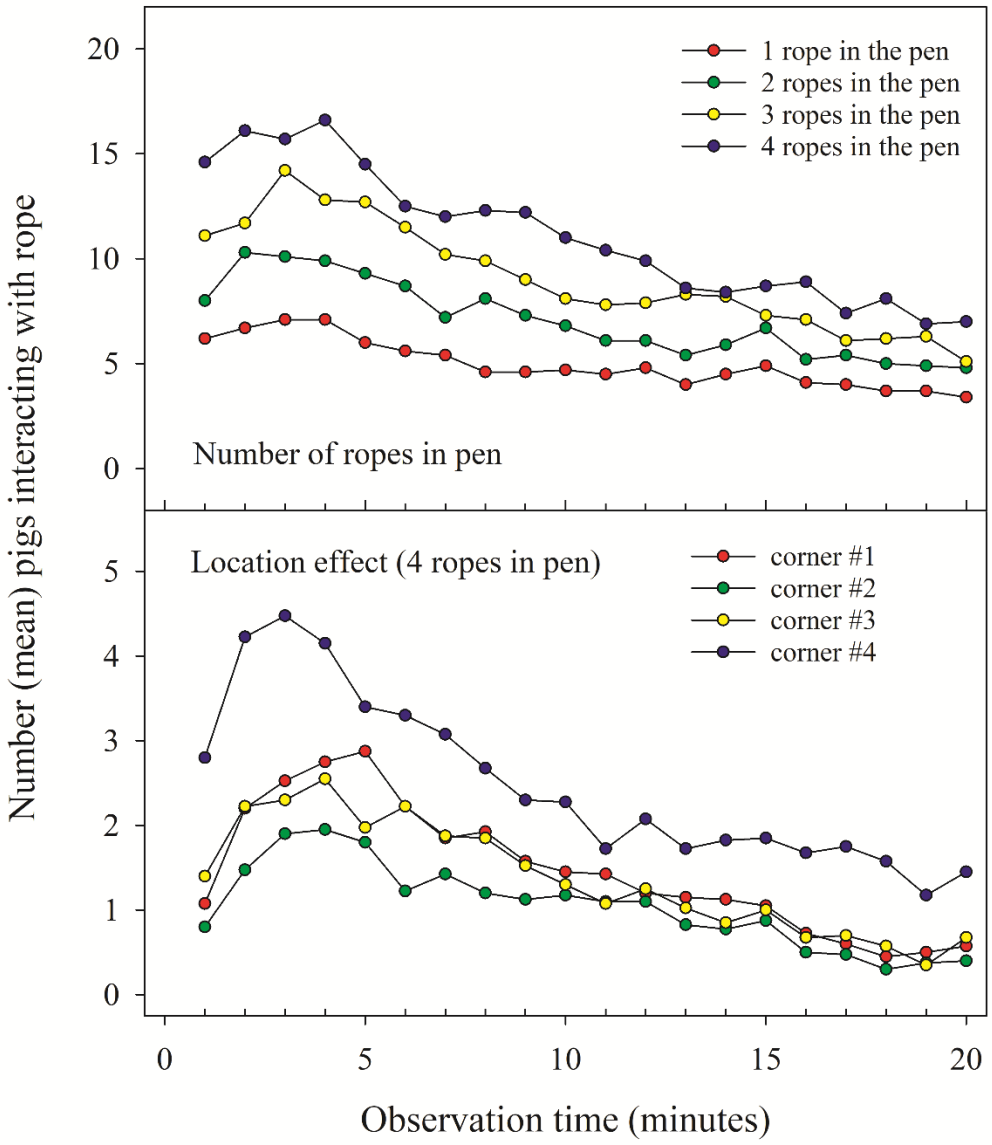

Figure 2. Group level analysis: mean number of pigs interacting with rope over time by number of ropes in the pen (top) and, with four ropes in the pen, by rope location (bottom)

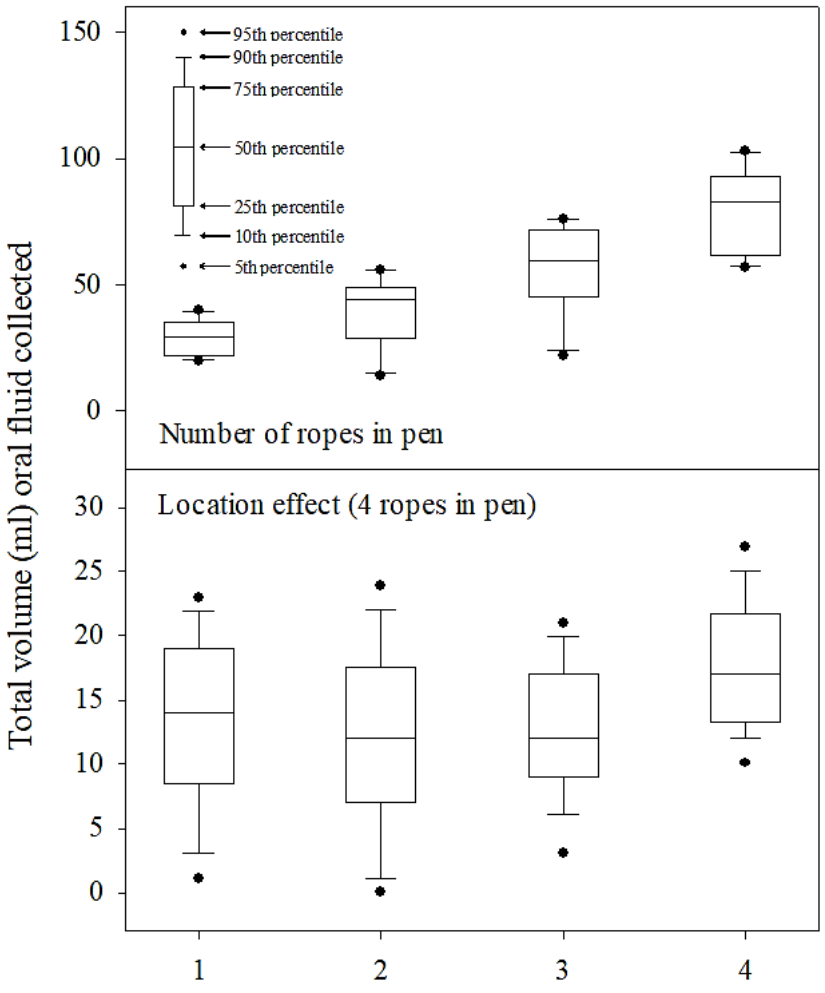

Figure 3. Group level analysis: oral fluid volume by number of ropes (top) and, with four ropes in the pen, by rope location (bottom).

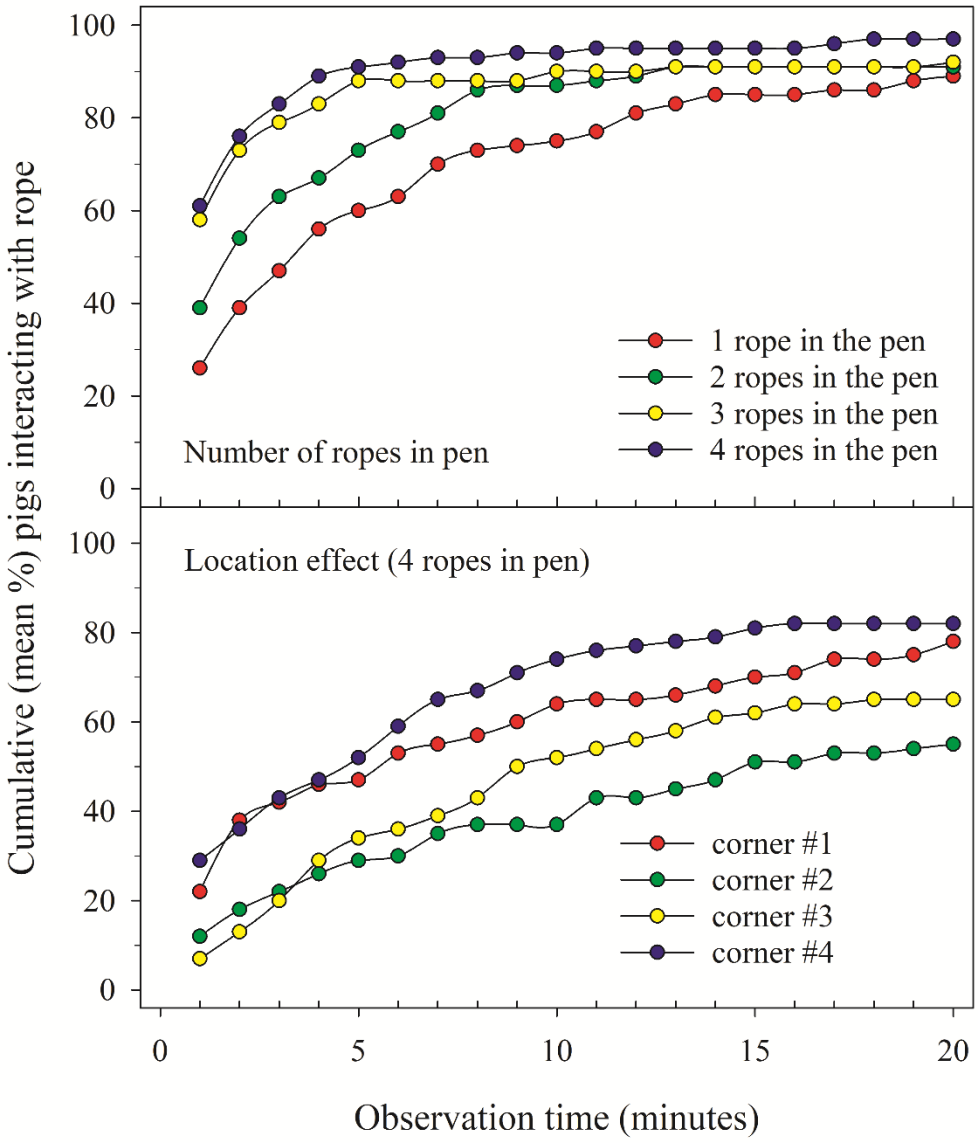

Figure 4. Individual pig level analysis: mean number of pigs interacting with rope over time by number of ropes (top) and, with four ropes in the pen, by rope location (bottom)