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TITLE:

Providing Meaningful Environmental Enrichment and Measuring Saliva Cortisol in Pigs Housed on Slatted Flooring

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KEYWORDS:

Pig, environmental enrichment, saliva cortisol, animal welfare, swine industry, food animal production.

SUMMARY:

This protocol demonstrates how to provide practical meaningful environmental enrichment for pigs which are housed on slatted flooring during the different stages of their lives, and how to collect saliva samples in a non-invasive manner for the measurement of cortisol concentrations, as a biomarker for acute stress.

ABSTRACT:

As pork is the most consumed meat worldwide, the welfare of animals in the swine industry has increasingly become a major public concern, which imposes a substantial pressure coming from customers, legislators and other stakeholders, to make management changes to improve the well-being of these animals. Several studies have demonstrated that providing environmental enrichment to pigs allows them to express their natural behavior, such as rooting and exploring, as well as nesting prior to farrowing, and is associated with reduced stress and improved production and welfare. However, many considerations should be taken into account when providing environmental enrichment, such as the type of floor, drainage and sewage systems, the pigs' stage in life, the material, as well as its hanging method, height and location within the pen. The objectives of this paper are (1) to give methodologic information on how to provide a relatively simple and practical meaningful environmental enrichment for pigs which are housed on slatted floors during the different stages of their life, and (2) to demonstrate how to collect saliva samples for the measurement of cortisol concentrations, as a biomarker for acute stress. Protocols include information regarding the use of jute, cotton ropes, straw in racks, as well as chewable silicone sticks devices as environmental enrichment in pens of farrowing and lactation, weaners and finishers. In addition, the use of cotton rope for a non-invasive saliva samples collection for cortisol concentrations analysis is detailed. The protocols provided are relevant for

professionals aiming to improve and monitor animal welfare, in both research and industrial swine farming.

INTRODUCTION:

Pork is the most consumed meat worldwide, with over 1.3 billion pigs being raised and slaughtered annually[1,2]. In recent years, the welfare of animals in the swine industry has increasingly become a major public concern, which imposes substantial pressure, coming from customers, legislators and other stakeholders, to make management changes to improve the well-being of these animals. Several studies demonstrated that providing environmental enrichment to pigs is associated with reduced stress and improved production and welfare, as it allows the pigs to express their natural behavior, such as rooting and exploring, as well as nesting prior to farrowing[3-7].

Pigs are considered to be intelligent animals, and have a highly inquisitive nature; thus, if a suitable environment is not provided, pigs will likely demonstrate stereotypic behavior and direct manipulative social behavior towards pen mates, which may lead to tail biting, as well as other injuries and stress[8,9]. Therefore, providing a meaningful environmental enrichment is advised by professionals, and in some countries even imposed by regulations and legislations, such as the European Union Council Directive 2008/120/EC[5].

Providing meaningful environmental enrichment can be challenging; it should fulfill the natural behavioral needs of the pigs in each stage of their lives, as well as take into consideration practical and technical limitations. Prior to farrowing, providing nesting material is associated with shorter farrowing duration as well as higher survival rate of the newborn piglets during delivery and throughout the lactation period. Moreover, in free pens with environmental enrichment, maternal behavior is improved, as well as the cognitive performance and weight gain of the piglets[3,10-13]. After weaning, grouping pigs (weaners, growing or finishers) from different litters or pens can be stressful and cause aggressiveness towards other pen mates, which may lead to injuries[14,15]. Therefore, when mixing unfamiliar pigs, providing meaningful environmental enrichment can potentially reduce the occurrence of undesired behavior resulting from frustration and aggressiveness or redirection of rooting behavior.

According to the European Union Council Directive 2008/120/EC (established in 2001/93/EC), it is required that pigs have permanent access to a sufficient quantity of material—to enable proper investigation and manipulation activities—such as straw, hay, wood, sawdust, mushroom compost, peat or a mixture of these[5,6]. However, the use of these materials may be unmanageable on many farms, as it may cause blockage of the drainage and sewage systems, particularly on farms with slatted flooring. Still, according to the EU Council Directive, farmers can provide alternative enrichment materials, as long as it allows the pigs to perform proper investigation and manipulation activities.

Lack of environmental enrichment may potentially lead to frustration and stress[3], which may activate the hypothalamic pituitary adrenal (HPA) axis. In pigs, as well as in humans and other animals, stress typically leads to the secretion of adrenocorticotropic hormone (ACTH) from the

pituitary gland. ACTH binds to its receptors on the adrenal cortex and stimulates adrenal release of the glucocorticoid cortisol, which is considered as a major biomarker of stress and can be measured in blood, urine, saliva and hair. Saliva cortisol is a marker for acute stress, since it reflects its biologically active concentration in the blood[4]. It has the advantage of a non-invasive sample collection, without handling of the animals, by means of cotton ropes provided as environmental enrichment[16,17]. Hair cortisol is being used as a marker for chronic stress, since cortisol accumulates in the hair over time and can be extracted and measured[18,19]; however, it requires animal handling, the number of repeated sampling is limited by the hair growth rate, and the analysis is more cumbersome as it require long extraction process. Still, both saliva and hair cortisol may provide complementary information to asses animal welfare.

The objectives of this paper are (1) to give methodologic information on how to provide relatively simple and practical meaningful environmental enrichment for pigs which are housed on slatted flooring during different stages of their lives (farrowing and lactation, weaners to finishers), and (2) to demonstrate how to non-invasively collect saliva samples for the measurement of cortisol concentrations, as a biomarker for acute stress.

PROTOCOL:

The study protocol was ethically approved by the Hebrew University's Institutional Animal Care and Use Committee (MD-16-14754-2). The study was conducted during 2017, at Lahav Animal Research Institute and the Hebrew University of Jerusalem, Israel.

1. Environmental enrichment protocol during farrowing and lactation period

NOTE: In this study, sows were housed in farrowing/lactation pens, under restraint for a limited time, from 3–5 days prior to the expected farrowing date and up to 10 days post-farrowing; thereafter, confinement bars were removed, to allow free movement and interaction between the sow and its piglets (illustrated in **Figure 1A**). However, the following protocol is also suitable for conventional farrowing crates, in which the sow is restrained throughout the entire lactation period.

1.1. Hang the enrichment materials (jute and cotton ropes) in the farrowing pen before the sow is moved into it, in order to allow the sows' nesting-like behavior prior to farrowing.

1.2. For each farrowing pen/crate, prepare two pieces of jute (20 cm wide, one-meter-long), and two pieces of 100% natural, uncolored cotton ropes (2–3 cm in diameter), approximately one-meter-long each.

1.3. Tie the jute and cotton ropes (double overhand knot) to the pen/crates' bars, as illustrated in **Figure 1**, from its middle, allowing the two free ends of each piece to be hung towards the floor. The height of the jute and cotton ropes knots should be just lower than the sows' shoulder, while its free ends are just above the floor (**Figure 1B–C**).

1.4. Sows are typically interested in the jute and cotton ropes; they often pull and chew it.

Therefore, replace or refill materials when dirty or finished.

NOTE: In this study, materials were inspected every 4–5 days, and replaced or refilled when needed.

1.5. After piglets are born, hang the jute and cotton ropes so that they are reachable for both the sow and its piglets, in a safe place. Typically, when the materials are reachable for both, piglets imitate their dams from a very early age.

NOTE: As an addition, shredded paper can also be provided as environmental enrichment for the farrowing sows and piglets, on slatted floor. However, the compatibility of the drainage and sewage systems, should be critically examined on the specific farm.

2. Environmental enrichment protocol for weaners (when straw cannot be provided)

NOTE: In this study, after weaning, each two litters were grouped into one pen of weaners. Optimally, groups should remain static until slaughter, without mixing or introducing new pen mates into the pen, to avoid hierarchy-related struggles and injuries. Space allowance in the pen was according to European Council Directive 2008/120/EC and the Israeli legislation, with approximately 20 pigs per group, and 0.3 m^2 of unobstructed floor area available per pig. For technical reasons, straw could not be provided for weaners, not even in racks. When straw can be provided, use the finishers' protocol from weaning to slaughter, as detailed in protocol section 3.

2.1. For each pen, prepare one-meter-long pieces of 100% natural, uncolored cotton ropes, 2–3 cm in diameter; at least one piece for every 10 pigs. Tie the cotton ropes to a chain hung from the ceiling or to a hanging pole (to distance it from the wall), as demonstrated in **Figure 2B**.

2.1.1. Position the ropes preferably at the center of the “active” area of the pen, close to the feeder and drinkers, and far from the pens’ wall (as marked in **Figure 2A**) to allow 360˚ access, which is expected to prevent aggressive behavior due to competition.

2.2. Hang the ropes at a height which allows the pigs to reach it with their mouths easily. Typically, the knot should be lower than the pigs’ shoulder, while the free ends of the ropes should be just above the floor, without touching it (**Figure 2**).

2.3. Replace or refill ropes when dirty or finished.

NOTE: In this study materials were inspected every 4–5 days, and replaced or refilled when needed.

2.4. In addition to the cotton ropes, provide chewable silicone sticks or similar chewable devices (**Figure 2**). The device used (see the **Table of Materials**) contains four chewable sticks which allows at least four pigs to interact with the device simultaneously. Provide one device for every

10 to 15 pigs.

2.5. Hang the chewable device with a chain from the ceiling (included in the kit) to allow the device swinging movement; this increases the activity and challenge to the pigs, and also allows more pigs to participate at once.

2.5.1. Position the device at the center of the active area of the pen, far from the pen's walls to allow 360˚ access, which is expected to prevent aggressive behavior due to competition. The end of the silicone sticks should be at a height of 10 to 20 cm above the floor.

2.6. Change the silicone sticks when finished or if dirty.

NOTE: In this study silicone sticks required to be changed only when moved from the weaners' pens to the finishers' pens.

3. Environmental enrichment protocol for finishers

NOTE: When straw can be provided during the whole period, start using this protocol from weaning until slaughter, instead of in the finishers' pens only.

3.1. Provide each pen with straw placed in a rack, in a hanged net, or in a tower, as illustrated in **Figure 2C**. When provided in such devices, only small amounts of the straw are available for the pigs at a time, and most of it is being chewed. Therefore, this enrichment can be used on slatted floors, when straw cannot be provided as bedding.

NOTE: In this study, straw was provided in commercial racks (**Figure 2C**).

3.2. Hang the straw racks at a height which is reachable for all pigs, usually just below the shoulder level. Attach them to the wall with the easiest access for as many pigs as possible, preferably within the "active area". Make sure the racks are properly and safely secured to the wall to avoid device breakage and injuries.

3.3. Make sure racks are completely filled with straw at the beginning; an addition small amount of fresh straw can be added every 1–3 days. Alternatively refill when the rack is empty, typically every 7–10 days.

NOTE: Long straw is better than chopped straw to reduce aggressiveness in the group and to meet pigs behavioral needs for rooting and exploring[20].

3.4. When applicable, consider placing a collecting tray or a mat underneath the rack, when the floor is slatted, to avoid straw from being scattered and blocking the drainage and sewage systems (**Figure 2C**). Since straw is provided for pigs mainly to fulfill their rooting and exploratory behavior, a mat can allow the presence of some free straw to be explored at the ground level.

3.5. In addition to the straw, provide chewable devices, as detailed in section 2.

3.6. When cotton ropes are used for saliva collection, as detailed in the next section, leave the ropes in the pens after saliva collection, as an additional enrichment. Other cotton ropes can also be provided, as detailed in section 2.

4. **Saliva samples collection for cortisol concentrations analysis**

NOTE: This section provides information how to non-invasively collect saliva for cortisol concentration analysis, as a biomarker for acute stress; however, saliva samples can also be used for analysis of other biomarkers and even for screening of potential pathogens. In addition, hair cortisol should be measured when applicable.

4.1. Plan saliva sampling for the same time of the day, to avoid bias, as cortisol secretion changes along the day due to physiologic circadian rhythm, or due to feeding[21]. Sampling is typically easier before feeding, preferably before the second feeding of the day.

4.2. Use uncolored, 100% cotton rope (2–3 cm in diameter). One rope is typically needed per 12–15 pigs.

NOTE: In this study, commercial, ready to use kits were used for collection of saliva samples.

4.3. Tie the rope close to the center of the pen, far from the pens' wall, with the knot at the height of pigs' shoulders. Make sure that the cord which is used for hanging (between the cotton rope and the bar) is not reachable for chewing by the pigs, and that the free ends of the cotton ropes do not touch the floor, for hygiene reasons (**Figure 3A**).

4.4. Unravel the free end of the rope, to increase the available chewable surface (**Figure 3B**).

4.5. Leave the rope hanging for 15–30 min. Typically, pigs are interested in newly provided ropes and would chew it during this time. Typically, the vast majority of the pigs will approach the rope.

4.6. To extract the saliva from the rope, put the free part of the rope into a sterile plastic bag (as illustrated in **Figure 3D**) and squeeze the rope by applying pressure from the outside of the bag, from top to bottom, until enough liquid (suggested at least 2 mL) accumulates at the bottom of the bag (Figure 3E), and then remove the rope from the bag.

NOTE: In this study a 30 cm x 40 cm bag was included in the kit. If possible, this step should be performed in the pen without cutting the hanging cord. In this way, the saliva extraction is usually easier, and the ropes can be left in the pen as an enrichment. If there is not enough saliva, a wringer can be used (was not required in this study).

4.7. To transfer the saliva from the bag into a sterile tube, pull the rope out and carefully cut the bottom corner of the bag above a 50 mL sterile plastic tube (although only 400 µL is usually

required for each analysis in duplicates, at least 2 ml are recommended for collection; **Figure 3F**). Seal the tube, and mark it with a permanent marker or a pre-prepared sticker (animals/pen, date, time, farm, etc.).

4.8. Store saliva samples in an insulated container with ice packs until delivered to the lab.

4.9. In the lab, centrifuge saliva samples until dirt can be separated (for 8 min at 3000 x *g*). The clean saliva should be transferred with a clean disposable Pasteur pipette into pre-marked sterile tubes of smaller volume (e.g., 2 mL screw cap freezer tubes) for convenience. Samples can be analyzed immediately, or can be kept frozen at a temperature of -20 °C or below until analysis.

4.10. Prior to cortisol concentration analysis, the relevant samples identity should be confirmed, and samples should be thawed at room temperature if frozen. Gently mix each sample, and then centrifuge it again before the analysis to remove possible leftover dirt that may interfere with the assay (8 min at 3000 x *g*).

4.11. Perform cortisol concentrations analysis with a validated assay, according to the specific kit protocol.

NOTE: In this study, saliva cortisol samples were measured in duplicates by enzyme-linked immunosorbent assay according to the manufacturer guidelines. For pigs that were previously provided with ropes as enrichments, collecting saliva would be very easy as pigs show high interest in the ropes. However, for pigs that are unfamiliar with the ropes, it might take time for training; thus, collecting adequate saliva may not be possible from the first time. If needed, ropes can be soaked in apple juice for training (obviously, in such case, saliva cannot be used for analysis). If saliva samples are needed from individually housed pigs, the collection may be more challenging than from a group, and training sessions with apple juice-soaked ropes are typically required. Chewing of collection ropes may also be encouraged by sampling individuals one next to each other due to mimicking.

REPRESENTATIVE RESULTS:

In the current study, 16 litters (170 piglets) were allocated randomly into two treatment groups; in one group, environmental enrichment was provided to eight litters ("Enriched Group", as described in protocol sections 1-3), while it was not provided to the other eight litters ("Non-enriched Group"). After weaning, each two litters were grouped into one group of about 20 pigs. Saliva samples were collected and analyzed for cortisol concentrations every two weeks in each pen, as described in protocol section 4.

In this study, when provided properly, sows, piglets and pigs showed interest in all enrichment devices. Sows used the jute and cotton ropes prior to farrowing, when provided. During the lactation period, when jute and cotton ropes were reachable for both the dam and her piglets, piglets imitated their dams from the age of 2–3 days old; however, when materials were not reachable for the sow, piglets started using the material later, typically after more than one week of age. In lactation and weaners' pens, the jute and cotton ropes were usually replaced or refilled

every 4–5 days, while the chewable silicone sticks were replaced only once, when pigs were moved from weaners' pen to finishers' pen, at the age of 70 days. In the finishers' pens, no drainage and sewage blockages were recorded.

The odds to die during the whole period until slaughter was significantly higher among piglets/pigs in the non-enriched group as compared to the enriched group (OR = 3.38, 95% CI = 1.05–10.83; P = 0.004). Saliva cortisol samples were successfully collected during the study every two weeks, just before the second feeding of the day. The pigs in the enriched group showed high interest in the saliva collecting cotton ropes from the beginning; however, in the non-enriched group saliva volumes in the initial collection were typically lower (2–10 mL), but from the second or third collection sessions it was similar between the groups. Typically, a volume larger than 30–40 mL was easily extracted from each rope. Samples were stored frozen (-80 °C) until analysis. Cortisol concentrations, as measured by ELISA, were overall significantly lower in the enriched pigs, as compared to the non-enriched pigs (P = 0.0044; **Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Environmental enrichment during farrowing and lactation period. (**A**) Designed farrowing pen illustration which demonstrates two stages of the confinement bars, either when the sow is restrained (stage R) or after confinement bars are removed (stage F) to allow free movement and interaction between the sow and her piglets. Suggested locations for the jute and cotton ropes before and after farrowing are marked in green and blue, respectively. (**B**) Images of a sow and her piglets interact with cotton ropes (CR) and jute (J) provided as environmental enrichment in a farrowing/lactation pen.

Figure 2: Environmental enrichment for weaners and finishers. (**A**) A schematic illustration of a pen demonstrating the suggested locations for hanging the environmental materials in the "active area" (blue X). (**B–C**) Images of pigs enriched as suggested. (**B**) In the weaners' pen, cotton ropes (CR) provided as environmental enrichment or for saliva samples collection hung from the ceiling or from a pole (P) to distance it from the pen's wall, chewable silicone sticks device (BR) hanged (hung) by a chain from the ceiling (HC). (C) In the finishers' pen, straw is provided in a rack (SR), a straw tower (ST) or a straw basket (SB); a mat (M) can cover the slatted floor underneath each of these straw devices.

Figure 3: Saliva sampling process using cotton ropes. (**A–F**) The six main stages for the saliva sampling are illustrated: hanging the rope, unraveling the free end of the rope, allowing the pigs to chew it, saliva extraction from the rope into a plastic bag and then transferring it to a plastic tube.

Figure 4: Saliva cortisol concentrations in enriched and non-enriched pigs. Saliva samples were collected every two weeks using cotton ropes, at the pens' level, from weaning to slaughter, from enriched and non-enriched pigs; cortisol concentrations were measured by ELISA. For this analysis, all samples were clustered together per group, without taking the sample date into consideration. Cortisol concentrations were significantly lower in the Enriched Group, as

compared to the Non-enriched Group (Mann Whitney test; P = 0.0044). Results are presented as mean ± SEM.

DISCUSSION:

Herein, we describe protocols detailing how to provide simple and practical environmental enrichment for pigs which are housed on slatted flooring during different stages of their life, and how to non-invasively collect saliva samples for the measurement of cortisol concentrations, as a biomarker for acute stress. Straw as a bedding is considered as one of the most suitable environmental enrichment for pigs, but may be impractical on slatted floors. However, relatively inexpensive alternatives such as jute, cotton ropes, straw in racks, and chewable devices can practically and safely be applied.

The range of saliva cortisol concentrations in this study are comparable to previous publications[17,22,23]. The significant differences in saliva cortisol concentrations between enriched- and non-enriched animals, is in accordance with the hypothesis that environmental enrichment may reduce acute stress in pigs at a point sample, as has been shown in previous studies, at least up to a certain age, as long as the circadian rhythm is not damaged by the prolonged chronic stress[24,25]. As noted, cortisol can be measured in blood, urine, and saliva as markers for acute stress, and in hair as a marker for chronic stress. A protocol for measuring hair cortisol was previously described by Meyers et al. and may be relevant when assessing animal welfare[19]. However, when looking for markers for acute stress, one should consider that any sampling, by itself, may increase cortisol release due to animal handling or pain caused during the procedure[16,26]. Hence, non-invasive saliva sampling, as suggested in the current study, is a relevant method, which can be used for cortisol analysis, as well as for the analysis of other biomarkers and even for screening of potential pathogens[27].

The main challenges in collecting saliva according to this protocol may be when collecting it from pigs that are unfamiliar with the ropes, as they may avoid chewing the ropes, and therefore, training may be needed. Training can be performed by using ropes which are soaked in apple juice (in such cases, those saliva samples cannot be used for analyses). If saliva samples are needed from individually housed pigs, the collection may be even more challenging than from a group, and training sessions with apple juice-soaked ropes are typically required. Chewing the ropes may also be encouraged by sampling individuals next to each other due to mimicking. For the training of young piglets, ropes should be hung to be reachable for their mother to encourage learning and early chewing. Another possible obstacle when using cotton ropes for enrichment or for saliva collection is that it can be caught in ear tags of certain brands. If it occurs, either the ear tags or the enrichment method has to be replaced.

Using cotton ropes allows measuring saliva cortisol concentration at the group level, which provides applicable screening for this hormone. A possible disadvantage of this collection method is that it may not be a representative measurement of all individuals in the group, as it is affected by the amount of saliva absorbed in the rope from each individual pig. In order to minimize this disadvantage, it is advised to provide adequate number of ropes, to allow sufficient collection time, as well as to perform several repeated collections. As compared to the existing

method of individually sampling, the collection itself is not stressful for the pigs, since it is given as an environmental enrichment, without handling of the pigs.

In summary, pigs can be environmentally enriched at all life stages, even on slatted flooring, using jute, cotton ropes, straw in racks and chewable devices. The type of enrichment, the hanging method, as well as the height and location within the pen, are important for their effectiveness. Regarding saliva collection by cotton topes, the timing and the hanging recommendations are crucial for successful samplings. These protocols are relevant for professionals aiming to improve and monitor animal welfare, in both research and industrial swine farming.

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DISCLOSURES:
The authors have nothing to disclose.

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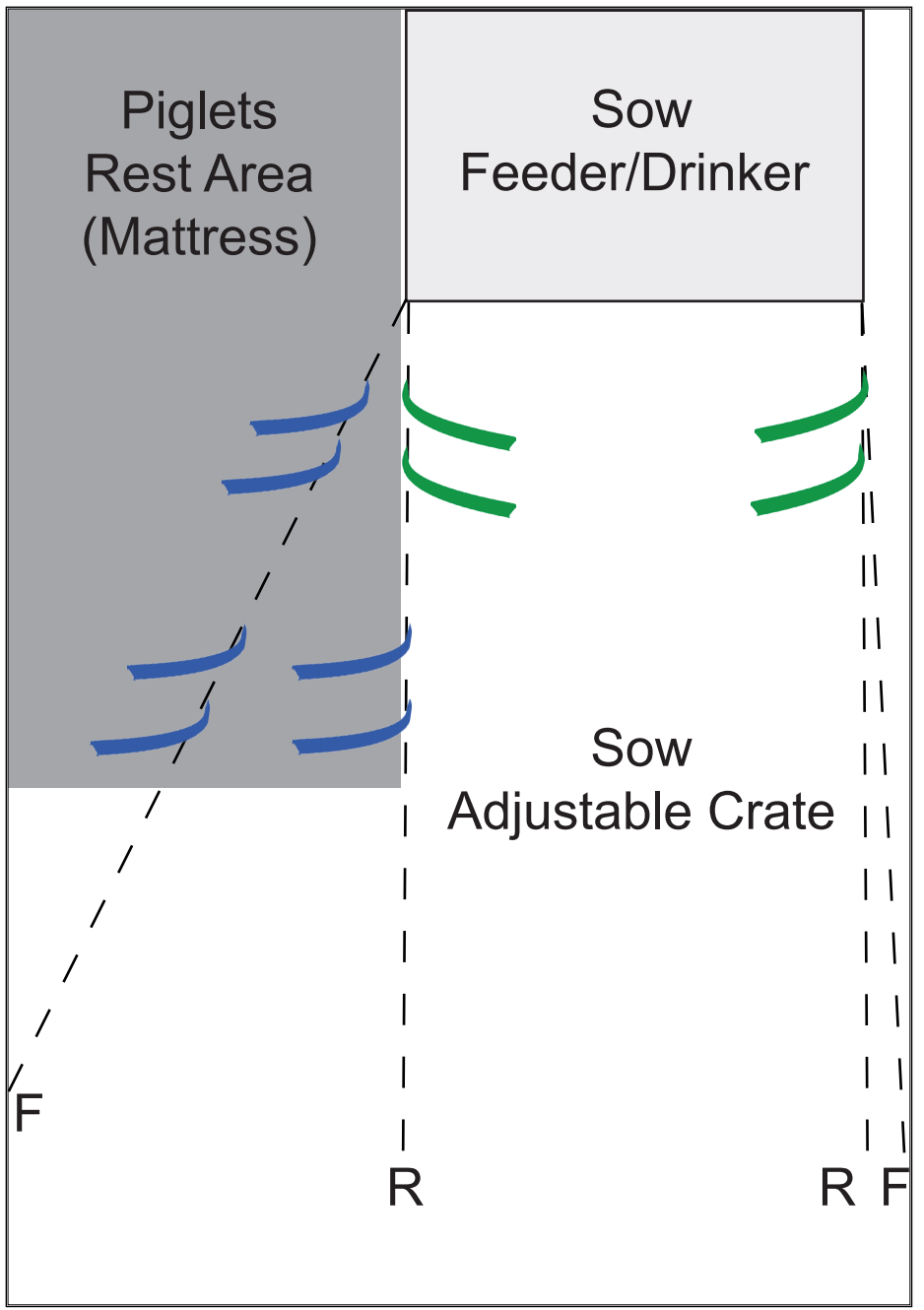
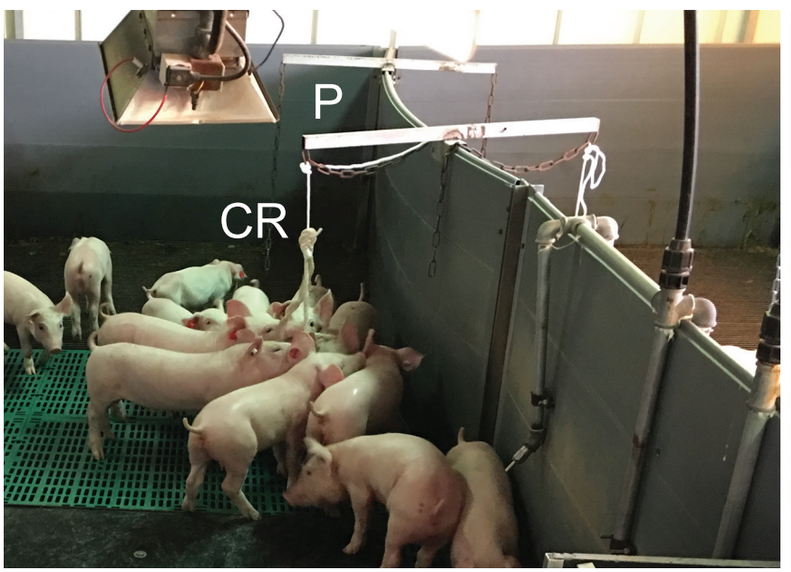
Fig. 1

A.



B.

C.



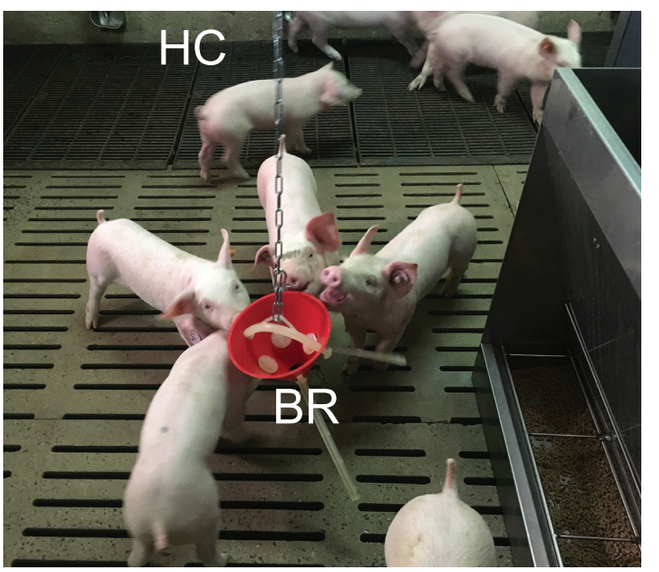


Fig. 3

Click here to access/download;Figure;Figure3_Moragn and Raz.pdf

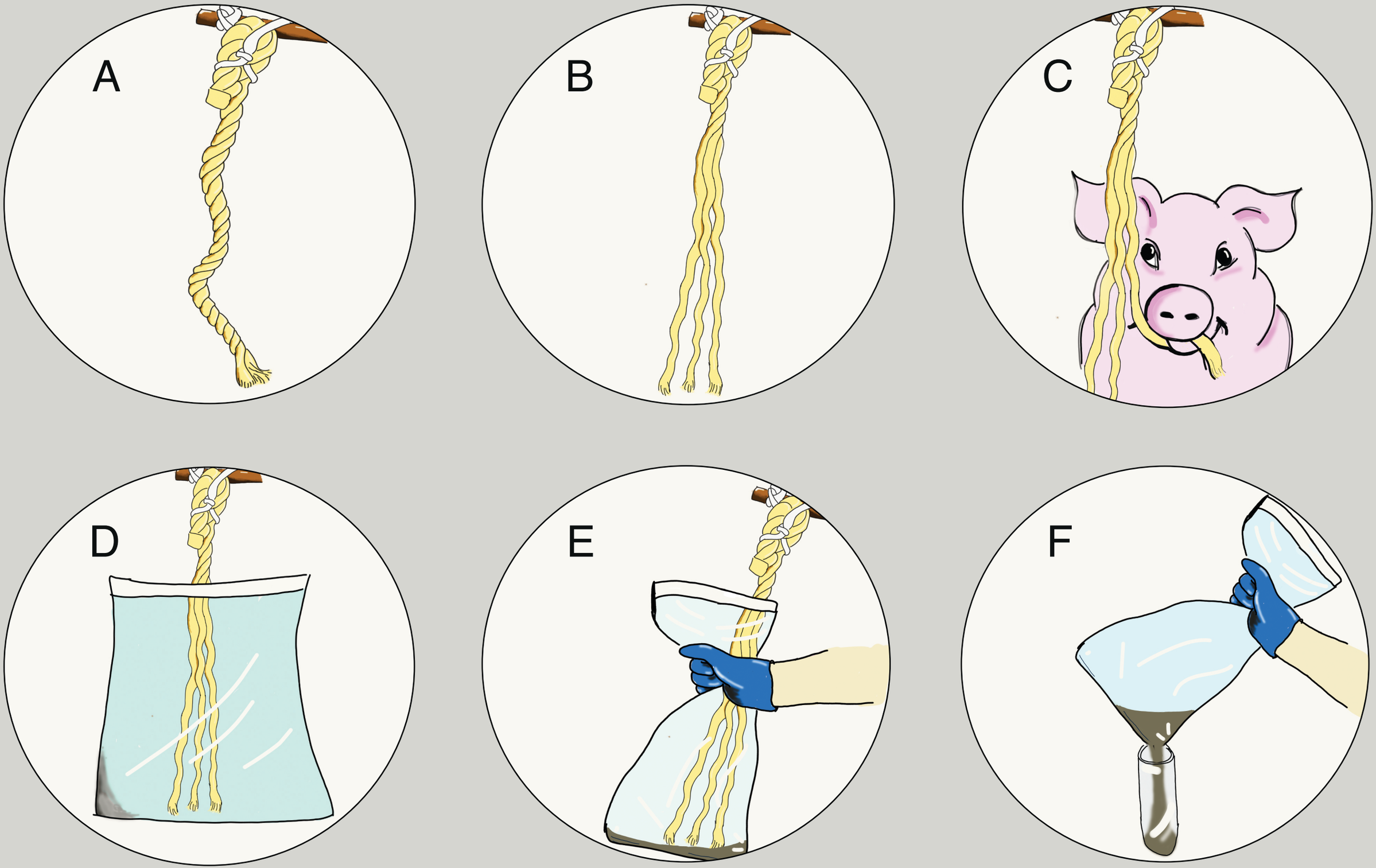
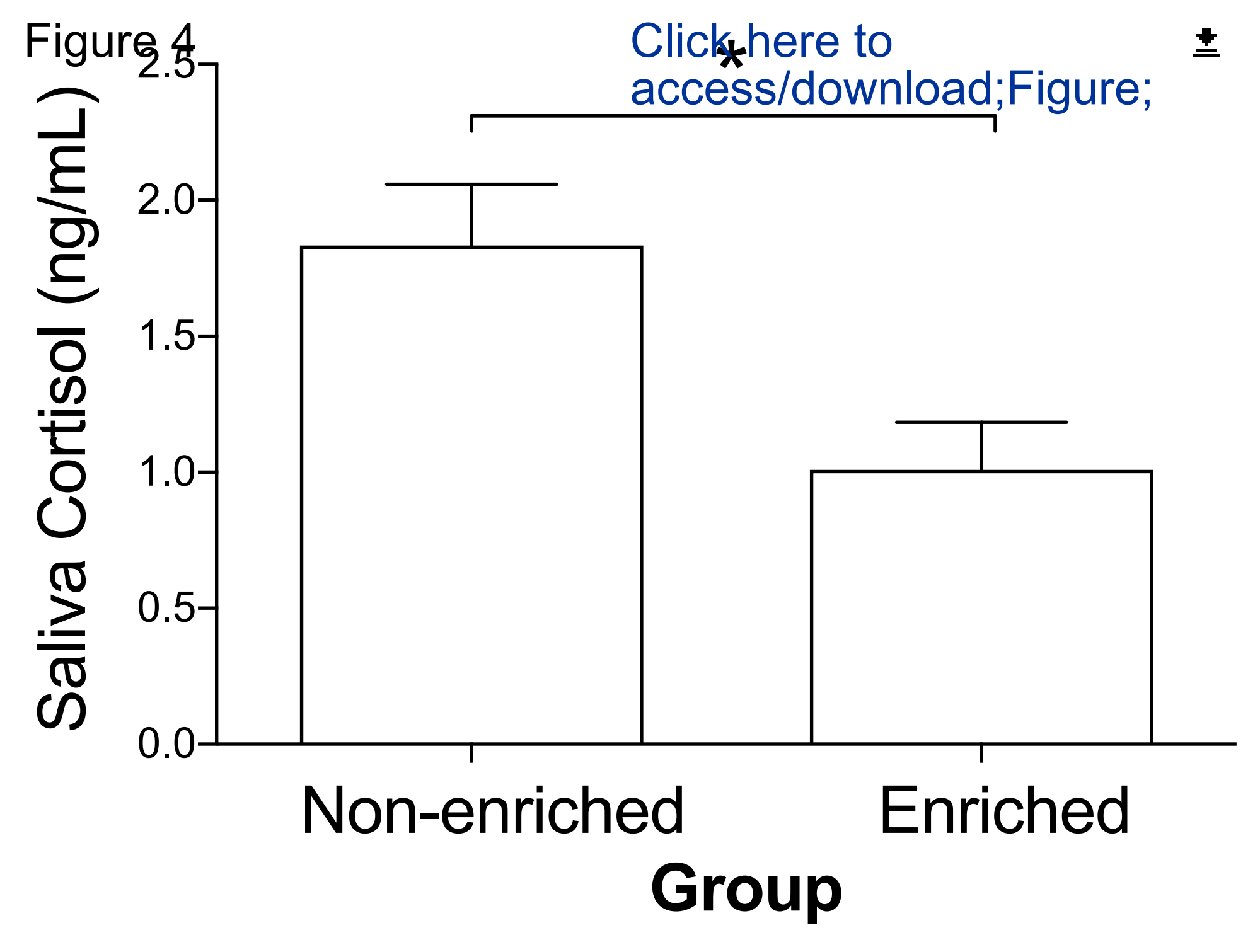


Figure 4



Name of Material/ Equipment	Company	Catalog Number
Bite-Rite	Ikadan System USA Inc.	
ELISA; Saliva Cortisol Kit	DRG International Inc. NJ, USA	Slv2930
HALM 60/80 CM	W. Domino A/S, DK	49084/ 85
TEGO Swine Oral Fluids Kit	ITL BioMedical, USA	A100930

Comments/Description
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Including everything needed for the saliva sampling protcol

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CORRESPONDING AUTHOR

Name:	Tal Raz
Department:	Koret School of Veterinary Medicine
Institution:	The Hebrew University of Jerusalem
Title:	PI
Signature:	
Date:	March 31, 2019

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Revision note for Manuscript JoVE60070_R1

Dear Editor and Reviewers,

We want to express our appreciation for the thorough review of our manuscript and valuable comments. Accordingly, the manuscript was extensively revised. We hope that the following responses and the corresponding revision of the manuscript fulfill the Editor's and Reviewers' requirements for considering this manuscript for publication in JoVE.

Please note: we included in our R1 submission two copies of the manuscript; one is a "clean copy", and the other is a copy with "Track changes" (using Word). The Track changes copy is located at the end of the submitted PDF file. Lines numbers referred to in this Revision Note are related to the revised, clean-copy manuscript.

Responses to comments made by the editor

Comments:
•Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
• Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Answer:
Corrected as required.

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Comments:
• Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) Will animals be available for filming?
2) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.

Answer:
The relevant text was highlighted as instructed (see the revised manuscript- “clean copy”. Animals will be available for the film.

--- --- --- --- --- --- --- --- ---

Comment:
Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Answer:
The discussion was extensively revised according to the Editor and Reviewers comments.

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Comment:
Figure/Table Legends: Define the error bars in fig 4.

Answer:
The error bar is Standard Error Mean (SEM). It was defined in the figure legend.

--- --- --- --- --- --- --- --- ---

Comment:
References: Please spell out journal names.

Answer:
All references are according to the Journal’s style.

--- --- --- --- --- --- --- --- ---

<u>Responses to comments made by Reviewer #1:</u>

Comment:
The paper has two objectives: to provide methodological information on how to provide environmental enrichment for pigs and to demonstrate how to non-invasively collect saliva sample. The problem of environmental enrichment is particularly important and has been recently evaluated by other authors (see Giuliotti, et al., Effect of Different Environment Enrichments on Behavior and Social Interactions in Growing Pigs. Animals 2019, 9, 101. doi: 10.3390 / ani9030101) with interesting results that deserve to be mentioned.

Answer:
Thanks to the reviewer's suggestion, the recommended paper is cited in the manuscript.
--- --- --- --- --- --- --- --- ---

Comment:
In the form in which it was written, the paper is halfway between a review and a research article and presents a very detailed protocol but few experimental data.
in conclusion, in my opinion, the paper must be reworked, giving a greater space to the analysis of cortisol.

Answer:
We appreciate the reviewer's comment and suggestions. Accordingly, the manuscript was extensively revised, and clarifications were made, also about the cortisol analysis (Lines 80-93; 211-272, 327-333; 344-356). As the JoVE journal focuses on methods, the protocols provided in our manuscript in details and the results are representative outcomes following the use of these protocols, in accordance with the journal guidelines.

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Comment:
The evaluation of the behavior of the pigs is completely absent: in the section "Representative results" only superficial indications of the behavior of the animals are reported; instead it would have been important that it was assessed more accurately. I therefore suggest, in the absence of precise behavioral data, to avoid references to animal behavior and to focus attention only on salivary cortisol.

Answer:
We appreciate and agree with the reviewer's comment, which was also noted by Reviewer #2. The manuscript was revised accordingly, and the behavioral descriptions were removed from the manuscript.

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Comment:
As regards salivary cortisol, it is not clear if the reported data refer to the total average concentration or if they are the sum of the concentrations detected at the different times analyzed. Furthermore, the statistical test used is not reported. I advise you to report the cortisol concentrations obtained at the different sampling times.

Answer:
Thank you for this comment. The relevant information was added to the figure legend (Lines 327-333).

--- --- --- --- --- --- --- --- ---

Responses to comments made by Reviewer #2:

Comment:
Manuscript Summary:
The manuscript provides a protocol for enriching the pigs's environment and for sampling pig saliva to assess cortisol levels as an indicator of acute stress. Although the topic is not new, the manuscript summarizes some interesting aspects to evaluate when providing environmental enrichments. I attached my comments in a pdf file and indicated below my main concerns. The manuscript will need to undergo a linguistic revision and some of its core aspects will need to be thoroughly reconsiedered and discussed before it can be accepted for publication. Please note that I attached a pdf file with my comments since I had to make many observations on the text.

Answer:
We are sincerely thankful for the reviewer's vital comment and suggestions. We found the PDF extremely helpful and have revised the manuscript accordingly.

--- --- --- --- --- --- --- --- ---

Comment:
Please note that this paper (and cortisol assay in saliva) entirely focuses on acute stress, whereas the lack of enrichment has to be considered as a chronic, more than an acute stressor, therefore more appropriate assays (for example in hair) should be used. The Authors should explain and discuss the reasons for their choice

Answer:
We agree with the reviewer regarding this excellent comment. Indeed, hair cortisol is a valuable parameter for animal welfare, as it might reflect chronic stress. Protocol for the analysis of hair cortisol has been detailed in a recent publication in JoVE (Meyer et al., 2014; doi:10.3791/50882); it is definitely a good option, and this was clarified in the revised manuscript (see introduction and discussion; Lines 85-93; 193-194; 316-327). Obviously, researchers and producers should be aware of several options, as each has its advantages and disadvantages. Hair cortisol, as mentioned, is an excellent choice, but such analysis also has some disadvantages, as it requires animal handling, the number of repeated sampling is limited by the hair growth rate, and the analysis is more cumbersome as it requires long extraction process. Therefore, in complementary to the previous publication of Meyer et al., we included a protocol for saliva collection for cortisol analysis, as an additional option for researchers and producers. The sampling and analyses were done in several pens, every two weeks, and we found that mean saliva cortisol concentration was significantly lower in the enriched group as compared to the non-enriched group.

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Comment:
The position of the rope is another critical point. It the rope touches the ground it will get soiled (and therefore less interesting for the animals), and expose the pigs to a sanitary risk. Also, if it gets soiled it might collect cortisol from other sources (faeces/urine) which may affect the cortisol assay. Have these aspects been considered by the Authors?

Answer:
Thanks for this important comment. In the revised manuscript we clarified this point specifically, in each section of the protocols. Our first intention was that in some circumstances, for the training of young piglets, ropes may touch the floor. However, we understand that this was not properly explained in the original version of the manuscript, and to avoid any confusion the text was revised accordingly.

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Comment:
How often does the material need to be replaced approximately? This is another important critical point. If too much manpower or material has to be used, the enrichment cannot be considered as practical or viable...

Answer:
Thanks to the reviewer's comment, the information was clarified in each section. The overall maintenance of the enrichment devices did not require intensive manpower. Typically, every 4-5 days, one of the workers walked around the pens and added/changed ropes and jutes, if it was needed (approximately 15-20 min all together).
Regarding filling of straw in racks, this can be done every 7-10 days. However, the farm workers in this study preferred to add a small amount of fresh straw every 1-3 days, as it took only a few minutes altogether. As far as we are concerned, the effort is not so tremendous as compared to the benefits.

--- --- --- --- --- --- --- --- ---

Comment:
The description of the experimental trial will need to include much more details on number of piglets per pen, pen characteristic, type of enrichment provided, statistical analysis, behavioral observations, etc.

Answer:
Thanks to the reviewer's suggestion, the relevant details were elaborated along the revised manuscript.

--- --- --- --- --- --- --- --- ---

Comment:
The results presented on saliva cortisol disagree with those indicated on the graph. Please double-check. Also, are cortisol values comparable to those observed by other Authors?

Answer:
Thank you for noticing this technical mistake. It was corrected. Please see Figure 4.
The range of saliva cortisol concentrations in this study were comparable to those observed previously (Schonreiter et al., 1999; Cook et al., 2013; Morgan et al., 2018). This was clarified in the manuscript (see Lines 344-345).

--- --- --- --- --- --- --- --- ---

Comment:
Please be careful when using "nesting behavior" or "rooting behavior" since none of these behaviors can be carried out in the described rearing conditions.

Answer:
We appreciate and agree with the reviewer's comment, which was also noted by Reviewer #1. Accordingly, the manuscript was revised, and the behavioral descriptions were vastly removed, or rephrased (in one place we used the term "nesting-like behavior", Line 114).

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Comment:
Several parts of the discussion belong more to the Introduction section. I suggest removing them.

Answer:
The discussion was revised extensively, according to this comment as well as the comments made by the Editor and Reviewer #1.

--- --- --- --- --- --- --- --- ---

Comment:
Be sure to double-check the figure legends.

Answer:
All figure legends have been thoroughly checked and revised where needed.

--- --- --- --- --- --- --- --- ---

We hope the responses and the corresponding revision of the manuscript fulfill the Editor's and Reviewers' requirements for considering this manuscript for publication in JoVE.

We are looking forward to hearing from you.

Sincerely,

Tal Raz
The corresponding author