Analysis

[4] Vieira de Castro, AC, Fuchs D, Munhoz Morello G, Pastur S, de Sousa L, Olsson IAS. Does training method matter? Evidence for the negative impact of aversive-based methods on companion dog welfare. PloS one 2020;15(12): e0225023.

Is the study Correlational Research or Causal Research?

This study was identified by the authors as "quasi-experimental."

From the Introduction: *"We used a quasi-experimental approach in which dog-owner dyads were recruited to participate through the training school at which they were enrolled."*

From the Discussion Section, the authors state that causality cannot be inferred: *"Firstly, because this was a quasi-experimental rather than an experimental study, we cannot infer a true causal relationship between training methods and dog welfare."*

Which sub-type of study best describes the research?

Not applicable for quasi-experimental.

Are there any potential problems with the study?

Yes. The cortisol testing performed may lack the scientific rigor to be considered valid.

From the study: *"These samples were sent to the Faculty of Sport Sciences and Physical Education of the University of Coimbra, Coimbra, Portugal, where they were assayed for cortisol concentration using standard ELISA kits (Salimetrics®)."*

From the Salimetrics web site FAQ page (https://salimetrics.com/how-do-i-test-saliva/) - ***Emphasis added.***

Can Salimetrics kits be used to measure analytes in specimens from other species / animals?

***Yes, in theory**, since the molecular structure of small molecule hormones is the same across species, it is possible to detect it in multiple species, **however Salimetrics kits have only been validated for human saliva and we are unable to guarantee the performance of the kit when used with specimens that have not been validated**. In addition, the levels of salivary analytes can be very different between species, and some species may not have certain analytes in their saliva. **We recommend careful pilot testing be conducted to verify assay performance in saliva samples from non-human species**. Salimetrics assay kits, developed for the measurement of proteins such as IL-6, sIgA, and CRP, **should not be used for measurements in non-human species since the homology across species is fairly low and the likelihood of the assay working is minimal**. The Salimetrics cortisol assay has been shown to work in horses, dogs, porcine, deer, antelope, fish, sea lions based on published literature.*

Another study recommends multiple physiological markers be used when evaluating stress, not saliva sampling for cortisol alone. (only cortisol tesing was used in the study under evaluation, *"2.4.1. Phase 1 –Evaluating welfare within the training context - To obtain physiological data on stress during training, six saliva samples were collected per dog to allow assay of salivary cortisol [9 ,22]."*)

Physiological stress reactivity and recovery related to behavioral traits in dogs (*Canis familiaris*) Rian C. M. M. Lensen1, Christel P. H. Moons2, Claire DiederichID1*
1 Department of Veterinary Medicine (IVRU), University of Namur, Namur, Belgium, 2 Department of Nutrition, Genetics and Ethology, Ghent University, Merelbeke, Belgium

"Saliva sampling is a relatively easy, non-invasive method that has often been used in dogs to monitor short-term physiological changes [16]. Cortisol is a well-known stress marker in dogs and reflects the activity of the hypothalamic-pituitary-adrenal (HPA) axis [17]. Saliva concentrations of this marker increase in response to sudden non-social stressors [18], fear-inducing events [19], and novel environments [12]. However, physiological markers are regulated through complex pathways [20,21]; and their expression could be influenced by factors like circadian fluctuations [22], physical health [23], activity [24], as well as stimulus intensity [18] and experience [25]. Hence, the measurement of multiple physiological parameters is recommended for a more accurate estimation of stress levels [17,26]."

Additionally, there is potential variability in the testing process, which includes:

1) Materials
2) People
3) Equipment
4) Method
5) Environment

Contributors to variability include but are not limited to:

1) Sampling method variation within operator (consistency of the same person to sample accurately over multiple repetitions).
2) Sampling method variation between operators (consistency of different people to accurately sample over multiple repetitions for each person).
3) Testing method variation within operator (consistency of the same person to sample accurately over multiple repetitions).
4) Testing method variation between operators (consistency of different people to accurately sample over multiple repetitions for each person).
5) Differences in testing protocols between Sport Sciences and Physical Education of the University of Coimbra and the Salimetrics laboratory.
6) Calibration of equipment and precision/accuracy of measurement system.
7) Differences in sampling environments.
8) Differences in testing environments.
9) Lack of proper test method validation.

The variability factors would be considered in the validation of the method for testing on canine cortisol, but there is no mention of this by the researchers.

Also, Salimetrics provides information on validating the procedure:
What Kit Validation test procedures should I run in my laboratory?
Salimetrics kits have been extensively validated during assay development. If you wish to revalidate these kits, we recommend you use the criteria as outlined in the FDA document "Bioanalytical Method Development and Validation":
https://www.fda.gov/downloads/drugs/guidances/ucm368107.pdf.

An email was sent to Ana Catarina Vieira de Castro on May 27, 2024 for clarification regarding the cortisol testing. This paper will be updated when a response to the email (below) is received.

2020 Research - Question Regarding Cortisol Testing

BB **Blue Bridge Dog Training <jeff@bluebridgedogtraining.com>** Today at 5:43 AM

To: ana.castro@ibmc.up.pt

Hello.

In your research (*Does training method matter? Evidence for the negative impact of aversive-based methods on companion dog welfare*), cortisol testing was used as a measure of physiological stress. Was the test method validated by Faculty of Sport Sciences and Physical Education of the University of Coimbra, Coimbra, Portugal, per the recommendation of Salimetrics® (using *the FDA document "Bioanalytical Method Development and Validation")?*

The Salimetrics® FAQ page has the following question and answer:

What Kit Validation test procedures should I run in my laboratory?
Salimetrics kits have been extensively validated during assay development. If you wish to revalidate these kits, we recommend you use the criteria as outlined in the FDA document "Bioanalytical Method Development and Validation"

Additionally, they make the following statement on the FAQ page:

Can Salimetrics kits be used to measure analytes in specimens from other species / animals?
Yes, in theory, since the molecular structure of small molecule hormones is the same across species, it is possible to detect it in multiple species, however Salimetrics kits have only been validated for human saliva and we are unable to guarantee the performance of the kit when used with specimens that have not been validated. In addition, the levels of salivary analytes can be very different between species, and some species may not have certain analytes in their saliva. We recommend careful pilot testing be conducted to verify assay performance in saliva samples from non-human species. Salimetrics assay kits, developed for the measurement of proteins such as IL-6, sIgA, and CRP, should not be used for measurements in non-human species since the homology across species is fairly low and the likelihood of the assay working is minimal. The Salimetrics cortisol assay has been shown to work in horses, dogs, porcine, deer, antelope, fish, sea lions based on published literature.

Any clarification on the cortisol testing related to Salimetrics® recommendations would be appreciated. I did not see any reference to the limitations of the test and validation of the procedure in your study.

Thank you.

Jeff Leavitt, IACP-CDT
Blue Bridge Dog Training
6155 Old Dayton Pike
Hixson, TN 37343
(423) 991-8655

Breaking through Barriers
Transforming Lives

https://www.bluebridgedogtraining.com

Additional Comments

1) In the Discussion section, using a quasi-experimental rather than an experimental approach was justified with the following statement: *"However, conducting an experimental study where dogs are designedly subjected to aversive-based methods would raise ethical concerns,…"* If ethical concerns are raised by using aversive methods in an experimental study, why are there no ethical concerns to study the effects of aversive methods when used by someone else? Using aversive methods should carry the same ethical weight whether done by the researcher or another party, for ethical consistency.
2) 2.4. Data collection
   2.4.1. Phase 1 –Evaluating welfare within the training context.
   "Owners were instructed on how to properly collect samples of their dog's saliva during the first training session, when the first sample (PT1) was collected by the first author of the study."

   The effects of sampling variability can greatly influence test results. The final number of subjects was as follows:
   "Consequently, our final sample comprised 92 subjects, 28 from Group Aversive (Schools A and D: n = 14), 22 from Group Mixed (School C: n = 8, School F: n = 14), and 42 from Group Reward (School B and G: 15 dogs, School E: 12 dogs)."

   However:
   "2.6.1.2. Cortisol analysis. Two dogs (one from School B and one from School E, both from Group Reward) did not cooperate with the saliva collection procedure and, as such, no saliva samples were extracted from them. For the remaining 90 dogs, ***only 23 dog owners (seven from Group Aversive, five from Group Mixed and 11 from Group Reward) were able to appropriately collect six saliva samples.*** *The samples from these subjects were selected for analysis. An additional 40 dog owners (14 from Group Aversive, 11 from Group Mixed and 15 from Group Reward) were able to properly collect* ***at least four saliva samples****. From these 40 dogs,* ***eight were randomly selected*** *to have their samples analyzed (one from Group Aversive, three from Group Mixed and four from Group Reward).* ***In total, 8 dogs from Group Aversive, 8 dogs from Group Mixed and 15 dogs from Group Reward had their samples selected for analysis*** *(Schools A, C, D, E and F: n = 4; School B: n = 5; School G: n = 6). These samples were sent to the Faculty of Sport Sciences and Physical Education of the University of Coimbra, Coimbra, Portugal, where they were assayed for cortisol concentration using standard ELISA kits (Salimetrics®)."*
   Given 31 (8+8+15) subjects (dogs) with a corresponding 31 owners, or 31 "sample technicians," there is no indication that (a) sampling variability between owners or within owner was checked, or (b) sampling technique was consistent over the 3-month period for Phase 1.