Positive Reinforcement Conditioning as a Tool for Frequent Minimally-Invasive Blood and Vaginal Swab Sampling in African Lions (*Panthera leo*).

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Abstract

Information regarding the physiology of African lions is scarce, mainly due to challenges associated with essential routine research procedures. The aim of this experiment was to test the possibility of training six captive lionesses by positive reinforcement conditioning (PRC) to voluntarily allow the collection of vaginal swabs and blood samples. This was done with the final goal of avoiding frequent anesthesia, and potential stressful management during research. All lionesses mastered basic clicker and targeting principles within two weeks. Routine sampling was possible after 20 weeks of training, enabling collection of about 750 vaginal swabs and 650 blood samples over the course of the study (18 months). These samples served to describe in detail the African lioness' ovarian cycle by combination of behavioral observations, longitudinal steroid hormone monitoring, and vaginal cytology. They also helped establish the ideal time for ovulation induction and artificial insemination of lionesses presenting natural estrus. The animals remained calm and cooperative during all sessions, and demonstrated curiosity in the training. PRC training of captive lionesses proved to be a suitable, minimally invasive method for repeated collection of vaginal swabs and blood. Additionally, PRC may serve as behavioral enrichment for African lions in captive settings. Compared to chemical or physical restraining methods, this non-invasive management approach may reduce distress and physiological negative side effects, thus opening up new avenues for feline research.

Keywords: African lion, behavioral training, blood collection, operant conditioning, vaginal swab collection.

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Introduction

At present, the red list of the International Union for the Conservation of Nature lists 25 of the 38 known cat species, as "Vulnerable" or "Endangered" in at least part of their natural habitat (IUCN, 2018). Therefore, captive population management and breeding constitutes an extremely important tool for the preservation of feline species, as part of inclusive conservation programs. To be effective, these programs require a thorough understanding of feline ecology and behavior, but also of feline physiology. In order to gather additional physiological information, researchers must apply capture and handling methods in line with the codes of ethics and welfare guidelines published by professional societies and involved countries (Proulx, Cattet, & Powell, 2012).

Given the global decline of around 40% in the last two decades, and the continued decreasing population trend, less than 30 000 individual lions (*Panthera leo*) remain to date (Bauer, Packer, Funston, Henschel, & Nowell, 2016). However, since they breed exceptionally well in captivity (Bauer *et al,* 2016), African lions represent an accessible model species for studying the biology and reproductive physiology of large, non-domestic cats.

Information regarding physiological parameters, such as normal ranges for hematology and blood chemistry, or endocrine mechanisms is currently scarce for large felids such as the African lion. This is mainly due to the general intractability of these animals, since routine research, and diagnostic or therapeutic procedures may be challenging in both wild and captive individuals. Physical restraint, such as the use of squeeze cages, may pose risk of injury to both the non-domestic animal and personnel, while chemical restraint often requires the use of expensive drugs in large doses coupled with a lengthy latent period before taking effect (Pistey & Wright, 1961).

Additionally, these techniques may affect homeostasis, altering certain blood parameters such as cortisol levels, hematology or serum chemistry, increasing the variability of data, and the number of subjects needed to achieve statistically significant results in research (Brockway, Hassler, & Hicks, 1993).

Positive reinforcement conditioning (PRC) training, originally implemented in laboratory animals (Phillippi-Falkenstein & Clarke, 1992; Bloomsmith, Stone, & Laule, 1998; Laule, Bloomsmith, & Schapiro, 2003; Gillis, Janes, & Kaufman, 2012), is becoming an increasingly common practice in zoos, as part of the daily behavioral enrichment of the animals, and husbandry routines (Phillips, Grandin, Graffam, Irlbeck, & Cambre, 1998; Savastano, Hanson, & McCann, 2003; Broder, MacFadden, Cosens, Rosenstein, & Harrison, 2008). The PRC approach facilitates the frequent performance of non-invasive or minimally invasive techniques such as oral, intramuscular or subcutaneous drug administration and blood collection. Also, ultrasound scans become possible in non-sedated animals, reducing the stress associated with physical restraint, and the possible physiological effects of anesthesia (*e.g.* hypoventilation, hypotension)

(Gilroy & DeYoung, 1986; Lambeth, Hau, Perlman, Martino, & Schapiro, 2006). PRC training has been known to reduce stress, as evidenced by cortisol response and defensive reactions in rhesus macaque (*Macaca mulatta*), and by hematology and serum chemistry profiles in captive chimpanzees (*Pan troglodytes*) (Reinhardt, 2003; Lambeth *et al*, 2006). In addition to refining the research methodology through controlling the undesirable variable of stress, voluntary cooperation by the study subjects during sample collection proved to diminish the handler's risk of injury (Reinhardt, 2003). Despite the occasional application of PRC for blood collection and vaccination of tigers and lions at various zoological institutions, the published literature on this topic remains scarce at the time of writing. Overall, protocols and training guidelines for felid species are rarely accessible, if they exist at all. Only a few reports show the use of training by positive reinforcement for welfare applications in non-domestic feline species such as cheetah (*Acinonyx* jabatus; Bergman & Janssen, 2005), snow leopard (*Uncia uncia;* Broder *et al,* 2008), and Bengal tiger (*Panthera tigris* tigris; Lin & Wang, 2018). Therefore, we hypothesized PRC training would enable frequent blood sampling and vaginal swabbing in large felids. The final goal of this study was to confirm that PRC may be used as an easy, cost effective, minimally-invasive tool to advance physiological research and improve husbandry of captive African lions.

The objectives of this study were: a) to train six African lionesses by PRC to allow frequent, routine collection of vaginal swabs and blood from the lateral coccygeal vein, without any additional physical or chemical restraint, and b) to record all steps involved in order to create an accessible, practical protocol that may be used by others implicated in research and management of captive large felids.

This study was prerequisite for a research project focused on the reproductive physiology of African lions and the applicability of assisted reproduction techniques into large felids' conservation programs. The final results of the aforementioned project were not the purpose of this study, and are reported elsewhere (Callealta, Ganswindt, Gonçalves, Mathew, & Lueders, 2018; Callealta, Ganswindt, Malan, & Lueders, 2019; Callealta, Ganswindt, & Lueders, submitted; Callealta, Lueders, Callealta, & Ganswindt, submitted).

Materials and methods:

Study animals

Six, captive born female African lions held at a private conservation center near Brits (North West province, South Africa; -25° 30' 55.23", 27° 40' 24.55") were chosen for this study. Two females were juvenile at the start of the training (2.5 years), and the remaining four females were fully grown adults (7-9 years). Although all individuals were hand-raised at the same facility, they had not been in direct contact with humans after six months of age. Animals were healthy and in good body condition and had not had previous experience with operant conditioning training.

For research purposes, study animals were kept in three adjacent outdoor enclosures: enclosure A housed a group of three adult females; enclosure B housed one adult male and one adult female; and enclosure C housed two juvenile females. All animals remained within visual, auditory, and olfactory range of each other. Each mesh wire enclosure was between 800 and 1200 m^2. Enclosures contained trees, shelter and a separation compartment; substrate was natural (*i.e.* grass, soil). Animals were fed every 7-10 days and water was accessible *ad libitum*. Food items consisted mainly of full cow and horse carcasses, and were supplemented with game meat and farmed chicken. Animals remained at the private conservation center upon completion of the study.

This study was conducted with the permission of the Animal Ethics, Use and Care, and

Research Committees (V052-17) of the University of Pretoria, South Africa.

Training enclosure description

Training took place in the separation compartment attached to each enclosure. Fully enclosed compartments measured 5 x 5 m in size, were fenced off by 3 m-high, 50 mm reinforced square tubing, and surrounded by 5 x 5 cm fully galvanized diamond mesh wiring (Fig 1). Compartments were connected to the main enclosure by a reinforced square tubing sliding gate. To facilitate training development and sampling procedures, four features were implemented in each separation compartment: (a) a concrete platform of 55 x 160 cm along the fence, indicating the location where the lionesses should lie down during the training session (Fig 1); (b) five 60 cm-high wooden poles of 10 cm diameter located along the concrete platform, opposite the cage fence - these poles created a passage that allowed the lioness to walk into the platform, but prevented her from turning over, *i.e.* she had to walk forward to exit the platform (Fig 1); (c) a 10 x 10 cm access window located at the front of the concrete platform that the trainer used to introduce food rewards (henceforth *window a*; Fig 2); and (d) a mechanical safety lock opening of 13 x 55 cm located on the fence along the concrete platform which was used by the researcher to access animals and collect the required samples (henceforth *window b*; Fig 1 & 2). The passage created by the wooden poles, as well as the two openings, allowed the trainer to interact with the animals, and the researcher to access them with a minimum risk of injury. Before training implementation, individuals accessed the separation compartment only when fed a whole carcass or if veterinary intervention was necessary. Thus, they generally showed no interest in this area, or even tried to avoid it, unless they could see a carcass inside. To address this, lions were given free access to the compartment at night to facilitate acclimatization to the new training enclosure. In addition, care-takers left portions of meat in this compartment before the start of each session, until the lionesses started entering the enclosure voluntarily.

Conditioned training methodology

A combination of classical and operant conditioning was used to train these lions. The first phase was to introduce basic clicker training. For one week, the sound of a clicker (conditioned stimulus) was repeatedly paired with the taste of a food reward (unconditioned stimulus). Initially, the trainer activated a clicker (Fig 3) every 15-30 seconds in front of the lioness. Subsequently, using 35 cm-long barbeque tongs (Fig 3) for safety reasons, a 50-75 g red meat reward (positive reinforcement) was given immediately after the "click" sound. This exercise was repeated 10 times per session, twice a day, five days per week. After the 10th successful response, the session was finished by a voice reward ("good!"), and the trainer clapped his hands in front of the lioness, who could then exit the training enclosure. Once she reached the housing enclosure, the lioness received a chicken as a final reward for her good work.

Once animals were habituated to the "click" sound and understood it was followed by a reward, clicking was subsequently used as a continuous conditioned reinforcement (or bridging stimulus) to achieve the next desired behaviors. During the following training sessions, food rewards were offered using a variable interval to stay unpredictable and keep the lionesses' attention high, and after completion of every intended training step.

The second phase of training development involved the implementation of basic targeting. For this, a handmade target made from 35 cm-long barbeque tongs and a tennis ball wrapped in duct tape (Fig 3) was presented against the fence of the training enclosure. The trainer encouraged each animal to approach the target and touch it with the nose. Every time the lioness moved closer to the target or accidentally touched it, the clicker was activated (conditioned reinforcement) and a food reward was immediately offered (positive reinforcement). Once all animals learnt to voluntarily touch the stationary target and lie in front of it, the trainer started to present the target at different locations along the fence, encouraging the lioness to follow and touch it at each location. When a lioness displayed the desired behavior, the action was reinforced with a “click” and a food reward. When unwanted behaviors, such as trying to claw or bite the target instead of touching it with the nose were displayed, a session time-out of 30 seconds to 5 minutes was established. During a time out, the lioness was ignored and the target removed from the fence until she stopped these undesirable behaviors. If, however, after 5 minutes from the beginning of the training session (or session time out), the subject did not show any interest in the activity (*e.g.* not paying attention to the target or pacing along the training enclosure), the trainer allowed her to exit the enclosure, and considered the session terminated, offering the lioness no reward.

By following the moving target and increasing the duration of interaction with the target, individuals were trained to step onto the concrete platform, lie down, and wait for the 5-20 minutes it took the researcher to complete the required sampling procedures (Fig 4). During this time, the lioness was not required to continuously touch the target, but at least pay attention to it. The “click” sound was used as a bridging signal every 10-15 seconds, and a food reward was given every 2-3 clicks to keep the lioness’ attention high. The target training was divided into sessions of 10 food rewards. Each session was performed twice a day, five days per week, for about 10 weeks, until all lionesses mastered the basic intended behaviors, and were ready for the next, more practical, stage of the training.

All red meat and chicken rewards used during the training sessions were consistently weighed and formed part of the animals’ weekly nutritional intake.

Touch desensitization and sampling

Once the lionesses voluntarily waited on the concrete platform near the target for five minutes, touch desensitization was implemented in the training sessions. During the five minutes, while the trainer provided conditioned reinforcement every 10-15 seconds, and offered positive reinforcement every 30 seconds, the researcher used a 50 cm-long snake handling hook (Fig 3) to approach the lioness' tail through window *b*. Once the hook came into contact with the individuals tail, the stimulus bridge was activated, and a food reward was given. This routine was repeated until the individual stopped moving the tail away after noticing the touch of the hook. The next step was to place the hook on the tail, providing positive reinforcement only if the lioness tolerated the contact for at least three seconds. Once the subjects were habituated to the touch of the hook to the tail, attempts were made to hold, palpate, and handle the tail manually. As before, the trainer would provide positive reinforcement only when the subject showed no resistance to the researcher's handling. In this way, all lionesses learnt to tolerate palpation of the hind-quarters, perineum, vulvar lips, and entrance to the vagina.

After six weeks of touch desensitization, collection of the first samples was possible. Insertion of a cotton swab to about 2 cm-deep into the vagina and gentle rotation against the mucosal wall enabled the researcher to obtain vaginal swabs (Fig 5). Conditioned and positive reinforcement rewards were given to the subject as soon as the cotton swab was introduced in the vagina, and right after collection of the sample. The collection of approximately 5 ml whole blood samples from the dorsal or lateral coccygeal veins was performed using a 21G butterfly needle attached to a 10 ml syringe (Fig 5). Conditioned and positive reinforcement rewards were provided as soon as the needle was inserted through the skin, and right after the blood sample was collected. The trainer had to ensure that the animal was completely focused on the target before the researcher could attempt any sample collection. This active communication routine decreased the risk of accidents during the handling and sampling procedures. Sample collection by positive reinforced clicker training took place 1-3 times per week, depending on the stage of the reproductive cycle the female presented.

By the end of the study, each lioness received (per session) one food reward when she: a) touched the target for the first time once in the training enclosure, b) laid on the concrete platform, c) allowed vaginal swab, and d) allowed blood collection. The remaining six rewards were given following an unpredictable routine to keep the lioness' interest in the activity high.

Data collection

At the beginning of each training session, meteorology data such as temperature, humidity, wind speed, and rain probability were recorded. For each individual session, the researcher captured starting and ending times in a data-sheet, and gave every attempted training step a score of either "1" (successful) or "0" (unsuccessful). Table 1 shows the complete training routine the lionesses followed along this study. The lionesses were considered to correctly perform one intended behavior after three or more days scoring "1" for that specific training step (Table 2). All relevant behaviors observed during training (*e.g.* "does not approach training enclosure", "tries to claw/bite target", "presents hind-quarters after palpation of perineum") were also noted down.

Results

Entering the training enclosure, and basic clicker training

Despite previous avoidance, all females (n=6) became confident around the separation compartment within the first week of training. After a few days, they started to voluntarily approach this compartment as soon as the trainer and researcher began to prepare the training setup. The lionesses needed 2 ± 0.68 (mean ± SE) sessions (range: 1-5) to voluntarily enter the enclosure. All females showed some kind of aversion to the clicker sound the first time they heard it. In general, they would get a mild fright and suddenly jump back. To overcome this problem, the trainer made the "click" sound softer by hiding the clicker either inside a pocket or behind his back. The females rapidly got habituated, and there was no need to hide the clicker anymore from that first session forth.

Targeting

Three out of six lionesses showed signs of mild aggression towards the target the first time they saw it (*e.g.* they hissed at it, and/or tried to claw or bite it). However, these behaviors disappeared quickly, and all six females learnt to voluntarily search and touch the stationary target within 1.33 ± 0.42 sessions (range: 1-3). It took them an average of 1.83 ± 0.48 sessions (range: 1-4) to learn to lie down in front of the target and touch it to get the reward. Exercises with a moving target along the fence were repeated for 10 weeks until all new features were implemented in the separation compartment, and the training enclosures were ready. All subjects learnt to approach the concrete platform, lie down, and touch the target in 4.0 ± 1.15 sessions (range: 1-8) on average.

Touch desensitization

All females (n=6) needed on average 13.0 ± 4.10 sessions (range: 3-27) to allow the researcher to firmly hold their tails, externally locate the coccygeal vein, and to pour a small volume of alcohol on the targeted injection site, as well as to access the entrance of the vagina. Handling of the tail was the most challenging step of the training. In general, during the first sessions this step was attempted, all females refused contact moving the tail away, despite remaining alert to the target on the platform. Only one lioness (Lioness 5), that had suffered a tail fracture as a cub, abandoned the platform and show mild aggression towards the researcher (*e.g.* hissing or growling) during these first sessions.

Vaginal swab and blood sampling

Once the subjects were successfully touch-desensitized, the lionesses needed on average 3.17 ± 1.05 (range: 0-7) and 1.83 ± 0.60 (range: 0-4) sessions to allow collection of vaginal and blood samples, respectively. During estrus, some females appeared more receptive at the time of vaginal swab sampling than during pro-estrus, when females were usually more restless and numerous attempts were needed for successful sampling.

Exceptions

One of the females (Lioness 5) needed special training to meet the targeted goals in time. This particular female required reinforcement more frequently than the other lionesses: a “click” sound (bridging stimulus) every five seconds, and food reward (positive reinforcement) every 10-15 seconds while lying on the platform, waiting near the target. This lioness responded better and was more attentive to the target when the food rewards consisted of chicken instead of red meat. To prevent this individual from quickly losing interest in the target and leaving the concrete platform, the trainer allowed her to lick the food reward while the sampling procedures were taking place.

Overall summary

Routine sampling from all six lionesses was possible after 20 weeks of training (1-2 sessions per day, five days per week), which facilitated collection of about 750 vaginal swabs and 650 blood samples over the total course of the underlying research project (18 months). These samples served to describe in detail the African lioness’ ovarian cycle by combination of behavioral observations, longitudinal steroid hormone monitoring, and vaginal cytology. They also helped establish the ideal time for ovulation induction and artificial insemination of lionesses presenting natural estrus. These results have been reported elsewhere (Callealta, Ganswindt, Gonçalves, Mathew,

& Lueders, 2018; Callealta, Ganswindt, Malan, & Lueders, 2019; Callealta, Ganswindt, & Lueders, submitted; Callealta, Lueders, Callealta, & Ganswindt, submitted).

Discussion

In this study, we conditioned six African lionesses by positive reinforcement training to allow collection of vaginal swabs and blood samples. This is the first time, to our knowledge, that the training in parallel of such a number of large felids has been documented in the scientific literature, compared to common previous reports of single cases (*e.g.* Bergman & Janssen, 2005; Broder *et al,* 2008; Lin & Wang, 2018). Our results support the benefit of positive reinforcement training on captive African lion welfare, handling, and research methodology, and we suggest this practice may be beneficial for other captive felid species. Routine sample collection was possible after 20 weeks; however, all trained animals were hand-reared. Even though hand-rearing might still be common in some breeding facilities, this practice is currently in decline. As training of only hand-reared lions may be a practical limitation of this study, further research would be needed in the future to investigate the differences in learning speed and training performance of hand-reared and parent-reared animals under the same conditions.

Despite minor inter-individual differences in their performances, all lionesses mastered basic clicker training and targeting principles in two weeks, matching previous results described by Gillis *et al* (2012) for squirrel monkeys (*Saimiri boliviensis*). The final training goal that enabled routine vaginal and blood sample collection was achieved in approximately five months. This was slightly longer than the time-frame reported by Broder *et al* (2008), who needed about four months to perform a transabdominal ultrasound on one unanesthetized snow leopard (*Uncia uncia*). Nevertheless, one must be careful to compare results from different studies, due to the great variety of possible training conditions (*e.g.* rewarding technique, staff experience, training facilities), and individual experiences both within and between species. In this case, the lionesses had to perform basic targeting exercises for 10 weeks before starting with touch desensitization. The original architecture of the training enclosures impeded direct contact with the lionesses and, as a result, building modifications to and around the separation compartments were necessary before new training routines could be implemented. If the training enclosures had been ready before the start of the study, this time could have been shorter.

It may be interesting to note that, even though the complete training and sampling routine was implemented and mastered by all six females, specific situations seemed to affect the performance of some of the lionesses. For example, most lionesses did not show interest in the training the day they were given their normal food portion, nor the day right after a regular meal. On the other hand, probably due to increased hunger as their normal feeding day approached, all individuals demonstrated behavioral signs of boredom or impatience, such us pacing along the fence of the training enclosure, digging in front of the food reward's bucket, clawing at the fence where food rewards were given, or leaving the concrete platform before the end of the session. Nevertheless, these behaviors as well as those observed during estrus and pro-estrus, or those related to meat preference, are only initial observations noted by the trainer and researcher during the training development. Thus, further investigation to support these observations and improve the training efficiency would be needed, and is recommended for future studies.

The lionesses demonstrated increased curiosity and interest in shaping new tasks in exchange of food rewards. Further, the time required to allow partially invasive procedures such as vaginal swabs or blood sample collection once touch-desensitized, was minimal, basically entailing no extra challenge for them. These results lead us to conclude that it could be relatively simple to implement new training routines that are typically required in basic animal care and handling maneuvers. Should this approach be chosen, drug injection or oral administration, superficial wound treatment, antiparasitic medication spraying, or pregnancy monitoring via ultrasound scanning would be possible after an initial period of training.

Prior to the start of this study, the lionesses avoided entering the separation compartment unless a carcass was present. At the end of this study, all females would approach the separation compartment as soon as the trainer and researcher began to prepare the setup for the new session. Once the lionesses understood the association between the training enclosure, the bridging stimulus, and the food rewards, they seemed stimulated by the training sessions. On occasion, the subjects would wait on the concrete platform, or in front of the training tools and food rewards for the trainer to start the session. Since PRC training implementation, previous tedious, time-consuming tasks such as moving the animals to the separation compartment to clean the main enclosure, became easy, rapid, and non-stressful for both animals and personnel. "Working for food" appeared to be a positive activity for these animals, as seen before in primates (Reinhardt, 2003), livestock (Hemsworth, 2003), cats (Broder *et al,* 2008), and even reptiles (Hellmuth, Augustine, Watkins, & Hope, 2012). The ultimate goal of this study was to empirically demonstrate that PRC training may serve to improve research, vet care, and husbandry of captive lions, and not to test whether this approach was enriching. Nevertheless, the observed positive responses suggest that this activity is in fact stimulating for large felids, where environmental enrichment is especially challenging (AZA, 2012). This study therefore lends support to the idea that a wellorchestrated training program may be utilized as another tool for environmental enrichment for this species, as long as it gives the animals control over their environment, allows them to choose, and teaches them to deal with new challenges, as previously supported by Mellen & Shepherdson (1997), Melfi (2013), and Westlund (2014).

Conclusions

In summary, the PRC training of captive African lionesses resulted in a minimally-invasive, suitable, repeatable and cost effective method for the collection of vaginal swabs and blood samples. Based on the positive responses displayed by six out of six trained lionesses, our results indicate that this kind of training is feasible for both physiological studies and veterinary treatment. Our sampling methodology by PRC training may reduce the psychological stress component associated with traditional physical and chemical restraint techniques, and avoids physiological effects associated with anesthesia. The use of PRC would be beneficial to handlers and management as routines and behaviors previously warranting negative reinforcement or anesthesia, can now be progressively shaped. In addition to opening new avenues of physiological research and veterinary treatment options, this approach appeared to be stimulating for these animals, and thus could be potentially considered a form of behavioral enrichment for African lions in captive settings.

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Disclosure of Interest:

The authors report no conflict of interest or economic interest.

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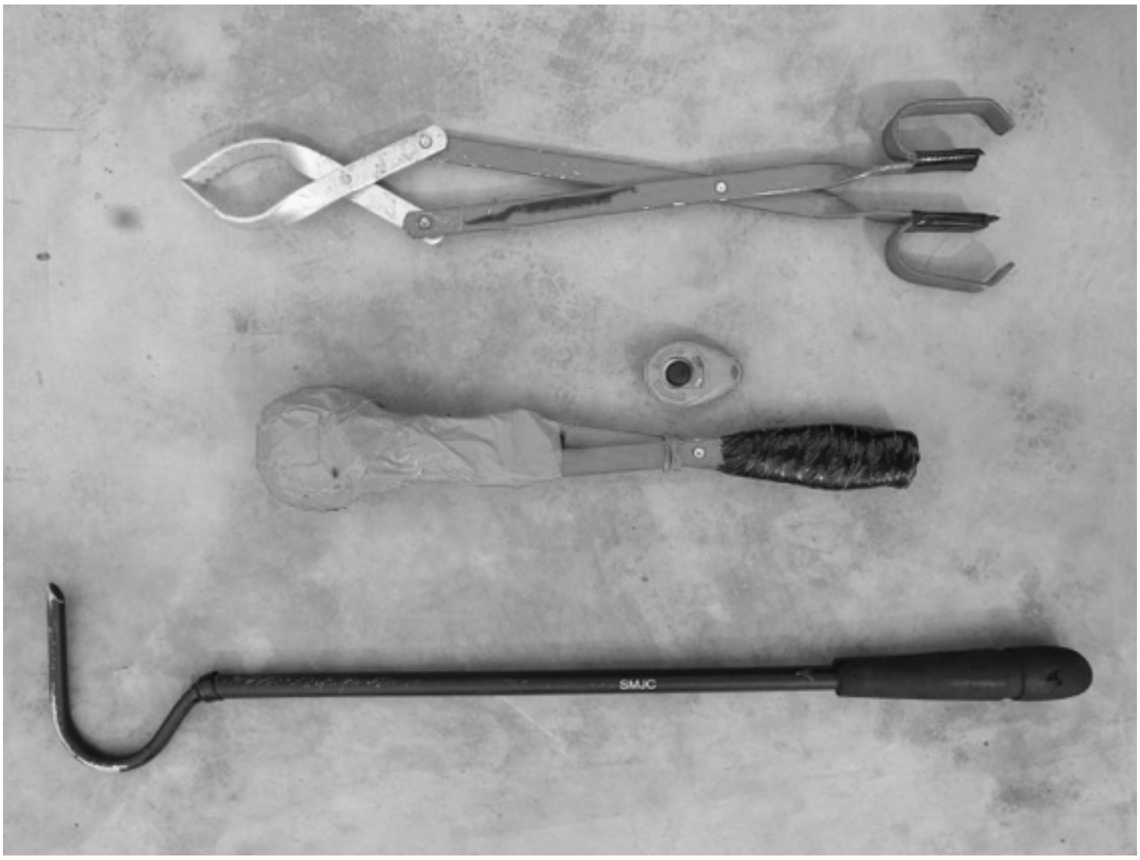
Figures

1. Training enclosure: fencing and mesh wiring may be noticed. Concrete platform is indicated by black arrows; wooden poles are indicated by stars. *Window b* can also be observed, closed, on the left.

2. *Window a* (left) was used by the trainer to safely introduce the food rewards in the cage. *Window b* (right) was used by the researcher to safely access the animal and collect the samples needed. Both windows are indicated by white arrows.

3. Training tools. From top to bottom: feeding tongs, clicker, handmade target, and tail handling hook.



4. Training set-up: the picture shows a lioness laying on the concrete platform, holding by the target. On the left, the researcher and assistant handle the tail through *window b*. On the right, the trainer holds the target on his right hand and the clicker on his left hand, while the feeder holds a bucket with the food rewards and tongs.

5. Blood sample (left) and vaginal swab (right) collection through *window b*.

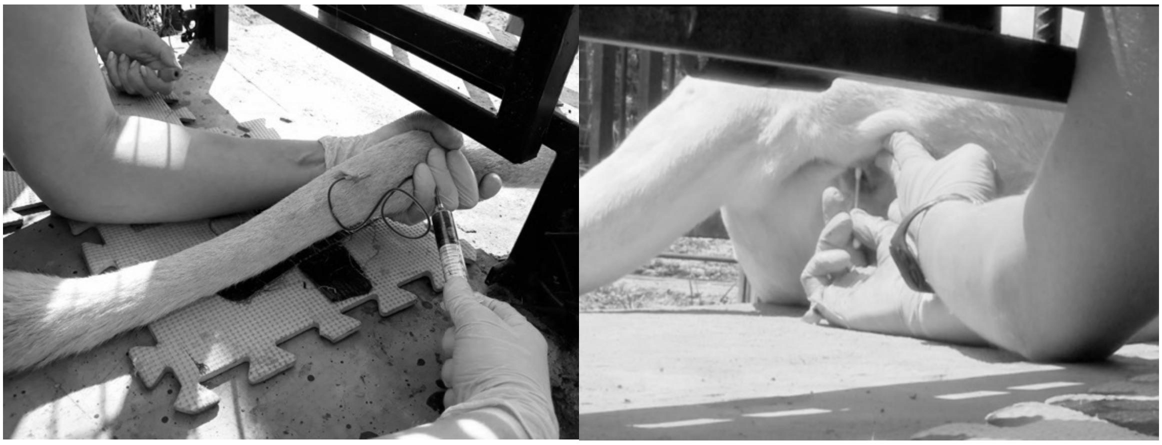


Table 1. Positive Reinforcement Conditioning Training steps:

a. Classic Conditioning:
    i. Pairing conditioned stimulus with unconditioned stimulus
b. Basic Clicker Training
    i. Establishing conditioned-primary reinforcer relationship
c. Target Training
    i. Touching stationary target
    ii. Lying down by stationary target
    iii. Following moving target
    iv. Lying down on concrete platform
    v. Increasing holding time
d. Touch Desensitization and sample collection
    i. Allowing tail contact with handling hook
    ii. Allowing tail rubbing with handling hook
    iii. Allowing tail pressure with handling hook
    iv. Allowing tail contact with hands
    v. Allowing tail rubbing with hands
    vi. Allowing tail holding with hands
    vii. Allowing palpation of hind-quarters with hands
    viii. Allowing pressure of hind-quarters with fingers
    ix. Allowing palpation of perineum with hands
    x. Allowing palpation of vulvar lips
    xi. Allowing vaginal swap collection
    xii. Allowing location of coccygeal vein by palpation
    xiii. Allowing alcohol pouring on the targeted injection site

xiv. Allowing pressure of the targeted injection site with capped butterfly needle

xv. Allowing blood sample collection

Table 2. Individual training effort. This table shows the number of training sessions each lioness needed to correctly perform the main intended behaviors.

	Training effort					
Training steps	Lioness 1	Lioness 2	Lioness 3	Lioness 4	Lioness 5	Lioness 6
Classic conditioning	1	1	1	1	1	1
Entering training enclosure	3	5	1	1	1	1
Touching stationary target	2	0	1	1	3	1
Lying in front of target	1	2	1	4	2	1
Following moving target to concrete platform	7	8	2	3	3	1
Tail touch desensitization	5	21	27	3	17	5
Allowing vaginal swabbing	5	3	7	3	0	1
Allowing blood collection	4	2	1	3	1	0