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Evaluation of the Efficacy of a Rat Agreement Based Reinforcement Procedure

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EVALUATION OF THE EFFICACY OF A RAT AGREEMENT BASED REINFORCEMENT PROCEDURE

by

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Since 2007, giant African pouched rats (Cricetomys gambianus) have been used successfully for detecting Tuberculosis (TB) positive patients. The rats are trained to detect TB-positive sputum samples through the use of operant conditioning techniques, in which an indicator response is rewarded with food. If the rats are to be used for first line screening of patients reinforcement could not be provided because the true status of the sample would be unknown. The present study evaluated the effects of a reinforcement-for-agreement procedure that could be used to reinforce indication responses when the true status of the sample is unknown. Four rats evaluated 100 sputum samples per session under two phases of the study: baseline and the reinforcement-for-agreement phase. During the reinforcement for agreement phase two rats evaluated samples under extinction and the remaining two rats evaluated the same samples and were rewarded only if they indicated on samples that both the first two rats made an indication response. Sensitivity and specificity rates did not greatly differ between the two conditions. These findings suggest that the reinforcement-for-agreement procedure may be a tenable option to use during first line screening or in areas in which other diagnostic methods are unavailable.

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Katherine B. La Londe

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CHAPTER I

INTRODUCTION

Developing countries are faced with many problems, including diagnosing and treating infectious diseases. Tuberculosis (TB) is an airborne infectious disease caused by bacteria *(Mycobacterium tuberculosis)* and is spread from person to person through microscopic droplets released into the air when someone coughs, speaks or sneezes. TB is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent. Over 95% of TB deaths occur in low- and middle-income countries and it is among the top three causes of death in women aged 15 to 44. Sub-Saharan Africa has the greatest proportion of new cases, with over 260 new cases per 100,000 people in 2011.

In order for individuals to be treated for TB, the disease must first be diagnosed, which in developing countries can be difficult and expensive. The United Nations (UN), The World Health Organization (WHO), and hundreds of Non-governmental Organizations (NGO) around the world are working together to develop effective and efficient procedures to diagnosis and treat TB in developing countries.

Diagnostic Assessment and Treatment

The Stop TB Partnership (WHO, 2010) launched a global plan to halt and reverse the TB epidemic and reduce prevalence and death by half by 2015. In Africa alone this project will cost $18.3 billion dollars. A majority of this money will be spent on increasing case detection in Africa by 80% by 2015 (with the goal of identifying 6.9 million new cases). Sophisticated methods (e.g., chest x-rays, polymerase chain reaction analysis) are currently used in developed countries but are not widely available in African countries. In these countries, diagnosing TB is mainly done by microscopic examination of sputum smears stained by the Ziehl Nelsen (ZN) method, which makes the acid-fast bacilli (AFB) that cause the disease visible as purple rods. Other methods sometimes used include fluorescent microscopy (FM) and culturing, the "gold standard" diagnostic, which requires laboratory facilities that are not readily available in resource-limited areas. In Tanzania, there are only three TB culture laboratories serving nearly 40 million people (WHO, 2010). Therefore, the ZN method is a vital tool for diagnosing TB in developing countries. Microscopy requires trained technicians to view slides that contain stained sputum samples and the procedure is both expensive and time consuming. On average, technicians can evaluate 20 specimens in an 8-hour working day (Weetjens, Mgode, Davis, Cox, & Beyene, 2009). Microscopy has been reported to have greater than 80% sensitivity for detecting cases of pulmonary tuberculosis in some settings (Van Deun, 2004) yet other reports have shown low and variable sensitivity rates (ranging from 20% to

60%) (Mendelson, 2007). Additionally smear-negative tuberculosis is disproportionately higher in HIV-positive than HIV-negative individuals and has been correlated with poor treatment outcomes, including death, in areas with high HIV prevalence rates (Mendelson, 2007). Finally, there are multiple variables that lead to false-positive and false-negative sputum smears (e.g., food particles in the sample and improper sputum collection) (Van Deun, 2004). Clearly, effective and efficient alternatives to microscopy are sorely needed. Among the alternatives being evaluated is the using of trained pouched rats to detect the smell of *M. tuberculosis* in human sputum samples.

TB Detection Rats: The Anti-Persoonsmijnen Ontmijnende Product

Anti-Persoonsmijnen Ontmijnende Product (APOPO) is a Belgian NGO specializing in the application of rat scent detection technology to solve humanitarian problems. APOPO personnel use operant conditioning techniques to train African pouched rats (*Cricetomys gambianus*) to detect the odor of 2,4,6-trinitrotluene (TNT), the primary explosive in most landmines and the microorganism *(Mycobacterium tuberculosis)* that causes TB in human sputum samples. Appropriately trained rats are used operationally for both landmine and TB detection (Mahoney, Weetjens, Cox, Beyene, et al., 2001; Poling, Weetjens, Cox, Beyene, Sully, 2010b), allowing previously hazardous land to be returned to locals and patients with active TB to be detected and subsequently treated. APOPO also is currently conducting pilot studies

to evaluate the feasibility of using the rats to detect *salmonella* and living humans buried in rubble (e.g., collapsed buildings).

Signal Detection Theory

The rats are trained using operant conditionings procedures (Skinner, 1957) using a signal detection task (SDT; Green and Swets, 1966). A standard SDT involves two stimulus classes: 1) signal superimposed on noise and 2) noise alone; and two response classes 1) the organism responds "yes" (i.e., stimulus is present) and 2) the organism responds "no" (i.e., stimulus is absent). This arrangement produces four stimulus-response events; hits, correct rejections, false alarms, and misses. The table below illustrates the four possible outcomes in a SDT using the rat's behavior and the presence and absence of TB bacteria.

Table 1. Signal Detection Task

	TRUE STATE OF AFFAIRS IN THE SPUTUM SAMPLE	
Rat Indication	Mycobacterium tuberculosis **present**	Mycobacterium tuberculosis **absent**
Indication Response (TB present)	Hit	False Alarm
No indication response (TB absent)	Miss	Correct rejection

By dividing the total hits by the total hits plus total misses and multiplying by 100% a quantitative measure of the sensitivity of the rats is provided. Sensitivity summarizes how good the rat is at identifying which samples have *Mycobacterium*

tuberculosis bacteria present. In contrast, specificity refers to the probability that a test indicates that a sample is disease free when in fact it is disease free. In the case of the rats' task, this is how well the rats withhold a response (i.e., correct rejection) when that sample is in fact negative. By dividing total correct rejections by total correct rejections plus total false alarms and multiplying by 100% a quantitative measure of specificity is determined.

Operant Conditioning Training Procedures

Operant conditioning procedures are used to establish indication responses in the presence of a stimulus (a hit). That is, rats are trained through the differential reinforcement of hits to make indication responses (e.g., pausing or scratching) only in the presence of the TB bacteria. TB-positive and TB-negative human sputum samples are obtained from Direct Observation of Treatment – Short Course (DOTS) centers located in Dar es Salaam, Tanzania. DOTS centers provide free TB screening via microscopy. Because microscopy has high specificity (i.e., a low false positive rate), almost all of the samples deemed TB-negative at the DOTS centers are indeed free of *M. tuberculosis*. Because the sensitivity of microscopy is variable, but typically low, some samples deemed TB-positive at the DOTS centers actually do not contain *M. tuberculosis*. This does not pose a serious issue in training the rats, but it can falsely reduce their specificity, because all rat identifications of DOTS-negative samples that actually contain *M. tuberculosis* are considered as false alarms, which is incorrect relative to the true state of affairs.

Training rats to identify TB begins early in the rat's life and requires progressive stages, which are described in detail in past publications (e.g., Poling et al., 2010; Verhagen et al., 2003).

First, the rats are socialized to human contact, smells, and noises. When rats do not engage in escape behaviors when they are picked up or hear novel sounds they begin clicker training (Pryor, 2002). In this phase they are placed in an experimental chamber with three holes located in a line along the chamber's center. A click sound is established as a conditioned reinforcer by delivering food immediately after each click. The click also becomes a discriminative stimulus to approach the food hole located on the side of the chamber because food is only presented through this hole, and only following clicks. Once the rat reliably walks to the food hole upon the sound of the click, it is trained to detect the target scent. A TB-positive sample is placed under one hole in a floor of the cage and rats are trained to place their nose in that hole; the other holes are closed during this training. The rat's behavior is conditioned by progressively delaying the click when its nose is in the hole in order to reach the terminal target. The terminal target is to have the rat hold its nose in the hole for approximately 5 s.

The final training stage is discrimination training in which two additional holes are opened which contain samples absent of TB and the location of the TB sample is randomly distributed among the three holes. Pausing above TB-positive samples for at least 5-s is reinforced with food. All other responses have no

programmed consequences. Finally, rats graduate to working in a 10-hole experimental chamber. Rats are exposed to 50 to 100 samples per day of which 5-20% are known TB positives. When rats display high sensitivity and specificity under these conditions, they are used operationally in second-line screening.

Operational Use of Giant African Pouched Rats *(Cricetomys Gambianus)*

When APOPO started breeding and socializing Giant African Pouched rats *(Cricetomys gambianus)* there was research to suggest rats could be trained for use in explosive detection (Nolan, Weinstein, & Weinstein, 1978). This particular species was evaluated for demining in Africa for several reasons. First, the olfactory system of *Cricetomys gumbianus* is highly sensitive resulting in superior smelling capacity. Breeding and training occur quickly and the rats can live up to eight years in captivity, allowing a long working life. The rats are resistant to sub-Saharan African diseases, which reduces the likelihood of illness. Thus, training *cricetomys gumbianus* to detect explosives seemed both efficient and potentially effective given years of stimulus discrimination research using laboratory rats (e.g., Iverson & Lattal, 1991 a, b). After some initial success with the landmine detection rats, APOPO started investigating the use of *cricetomys gumbianus* to detect the presence of TB.

APOPO started second-line screening in 2007 after successful completion of a proof of principle study (Weetjens, Mgode, Machangu'u et al., 2009). In this study, two rats were trained using the procedures described above. The rats evaluated positive and negative samples determined through culturing (gold standard). Over

seven days the rats evaluated 817 sputum samples, of which 67 were TB positive. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each rat. The PPV is defined as the number of true positives divided by the number of true positives plus false positives. NPV is defined as the number of true negatives divided by the number of the true negatives plus false negatives. Both rats had sensitivities of 73%, which is well above the usual sensitivity of microscopy (Steingart et al., 2006), as well as high specificities (93% and 93.8%), good PPVs (48 and 51), and high NPVs (both 97.5).

Based on these findings, the Tanzanian Ministry of Health allowed the rats to be used during second-line screening of sputum samples provided by DOTS centers' patients. Second-line screening means that sputum samples are first screened at DOTS centers, then they are screened by APOPO's rats. As noted, APOPO collects both positive and negative samples from the DOTS centers located in Dar es Salaam. In Dar es Salaam, the incidence of TB is 418 per 100,000 people (APOPO, 2012). Once the samples arrive at APOPO's laboratory they are inactivated using heat treatment in an autoclave, which ensures the sputum is not contagious, as is essential for the trainers' and rats' protection. Samples identified as positive at the DOTS centers are used as reinforcement samples. That is, if a rat makes an indication response above a DOTS-positive sample, a click is provided followed by the food reinforcer. If two or more rats make the indication response on a DOTS-negative sample the sample is later checked by FM in APOPO's laboratory. If FM confirms

the presence of *M. tuberculosis* in the sample, APOPO personnel report the additional case findings to the DOTS centers, whose staff tracks the patient and initiate treatment. After the proof of principle study, Weetjens et al., (2009) reported initial results of APOPO's second-line screening. Sputum smears were first analyzed by DOTS centers microscopy then by the rats. Positive indications by the rats on samples evaluated as negative by the microscopies were confirmed by a second microscopy. Samples from 15,041 patients were evaluated. The DOTS centers detected TB in 1,838 of the patients, whereas the rats detected the disease in 2,415 patients. The cases detected by rats but missed by DOT centers increased TB detection by 31.4%. Since then, APOPO has continued to train and use rats in second-line screening.

In 2009, samples were collected from seven DOTS centers in Dar es Salaam. Rats identified an additional 620 patients, not found by the centers. This increased new case detection rate by 44% (Poling et al., 2009)

In 2010 APOPO's second-line rats investigated samples from 12,329 patients. Each patient supplied two or three samples and between eight and ten rats inspected each sample. Samples the DOT centers determined to be TB-negative but were indicated on by at least two rats were re-evaluated by a technician at APOPO's laboratory using ZN or FM methods. Overall, there were 22,858 DOTS-negative samples. Using a cut-off of having at least two rats indicate on a sample the overall sample-wise sensitivity relative to the combined results of DOTS and APOPO microscopy was 89% and specificity was 76.3%. The overall patient-wise sensitivity

was 95.6% and specificity was 73.6% This resulted in a total of 716 new cases of TB found by APOPO rats that were not detect by the DOTS center (Mahoney et al., 2011).

APOPO's research suggests that pouched rats can substantially increase new-case detections when used for second-line screening of sputum samples initially evaluated by ZN microscopy. Unfortunately, in resource-poor areas many people do not have access to microscopy, and there is widespread recognition that for effective worldwide TB detection a cheap, fast and accurate first-line screen is needed (WHO, 2010). It is possible that trained pouched rats could meet some of this need, but using the rats for first-line screening poses one substantial challenge: How can differential reinforcement be arranged for pouched rats under conditions where the only available TB diagnostic is the rats? As previously described, the status of samples received from DOTS centers (i.e., TB-negative or TB-positive) is used to determine whether identification responses to those samples are reinforced. In first-line screening, samples would come directly from patients to APOPO and an alternative strategy for arranging reinforcement would be required. If such an arrangement were not tenable, than it would not be possible to arrange differential reinforcement and the rats would be forced to work in extinction, which would adversely affect their performance. Although many variables affect performance in extinction (e.g., Cooper, Heron, & Heward, 2007), and its effects change over time (Grow, Kelley, Roane, &

Shillingsburg, 2008; Morgan & Lee, 1996), responding inevitably weakens and falls to near zero levels when extinction is prolonged.

For example, Mahoney et al. (2012) systematically evaluated how long landmine-detection rats would work under extinction before performance decreased. Rats searched land under reinforcement and extinction procedures. Under the reinforcement conditions the rats' correct indications (i.e., scratching within 1 m of a landmine) were reinforced. This condition continued until rats had 100% accuracy for 4 consecutive days at which time an extinction condition was implemented. Extinction was identical to the reinforcement phase except correct indications had no planned consequences (i.e., food was not delivered). This phase continued until performance was at 0% for two consecutive days. Next, these phases were repeated. Four of the five rats' performance fell to 0% within 3 days. When the reinforcement condition was reinstated the rats' responded differently. One rat (Toyota) had variable responding for 6 days until returning to baseline accuracy. Mar remained at 0% accuracy for 8 days until returning to baseline accuracy. Two rats retuned to 100% accuracy within 2 days (Nijad and Bila) and one rat within 3 days (Enda). When the final extinction condition was implemented, responding for all the rats decreased to 0% between two and four days (3 days on average). These results suggest that rats can work on average for 3 days under extinction procedures before accuracy decreases. When reinforcement procedures are reinstated, responding is variable among rats and it may take up to 8 days to return to reinforcement levels. Therefore,

reinforcement is needed to maintain high levels of accuracy and to avoid variability in responding.

Reinforcement for Agreement: The Purpose of the Present Study

In 1966 Thom Verhave built and tested an apparatus in which pigeons inspected batches of pharmaceutical capsules for "skags" (i.e., capsules that are off-color, have gelatin sticking out, or dented). Pigeons were quickly trained on the visual discrimination task and displayed high hit rates and low false alarm rates. During training, when the pigeons correctly identified a "skag" by pecking a key, food was delivered. Food was withheld for incorrectly identifying a "skag" and there was a brief black-out for false alarms. Although the apparatus and pigeons were never used operationally (due to concern about the public's perception of using birds to inspect medication) Verhave acknowledged that his procedure for identifying "skags" could not be used in first line screening. He proposed two reinforcement procedures that could be used to reinforce hits during first-line screening. One involved planting known "skags" into batches of capsules allowing programmed intermittent reinforcement, which is similar to APOPO's current second-line screening procedures for TB. It is also similar to a procedure used with operational landmine-detection rats, who work in areas where the locations of landmines are unknown. The procedure used involved placing bags containing TNT in contact with the ground prior to operational search by the rats, removing the bags, and reinforcing indication responses within 1 m of the location where a bag was placed. With this procedure, all

rats reliably detected landmines, although indications over landmines had no planned consequences (Mahoney et al., 2013, in press).

Verhave' s second proposed reinforcement procedure was an agreement procedure in which a minimum of two pigeons would be used to simultaneously inspect each capsule. After initial training, birds would begin on-line inspection in which the pigeons would only receive reinforcement if they both agreed on whether a capsule was a "skag." Verhave maintained that if pigeons were adequately trained the agreement-contingency procedure would be sufficient to maintain the desired behavior with the small probability that the birds would agree to treat a "skag" as an acceptable object (i.e., a miss). If such a problem did exist, he suggested adding more animals into the agreement-contingency circuit. Because Verhave' s apparatus was never used operationally, he never tested the two procedures. The purpose of the present study was to evaluate a procedure similar to Verhave' s agreement-contingency procedure. In this study, an ABA design was implemented to compare performance under a procedure like that used in second-line TB screening and under a reinforcement-for-agreement procedure. If performance did not deteriorate substantially under the reinforcement-for-agreement procedure, than the procedure may be a tenable strategy for maintaining accurate TB detection in first-line screening applications.

CHAPTER II

METHOD

Subjects, Materials, and Setting

Four adult *cricetomys gambianus* served as subjects. All were experienced rats with at least 2 years of experience. All worked operationally as second-line screening rats. During the two weeks prior to the study, each rat's daily hit rate was consistently above 80%, its false alarm rate below 5%, and it completed evaluation of 100 samples within 30 minutes. Because the research took place in Tanzania, Institutional Animal Care and Use Committee IACUC approval from Western Michigan University was not required, although the committee was informed of the project and provided with a written description of it. APOPO's Institutional Animal Care and Use committee reviewed and approved the research protocol (Appendix A).

Rats were individually housed in a colony at APOPO's TB facilities. The rats were not feed outside of experimental sessions to ensure they were slightly food deprived during experimental sessions.

APOPO trainers implemented the experimental sessions under the supervision of a training supervisor and the author. All trainers were certified by APOPO and were selected because they demonstrated good adherence to APOPO's standard operating procedures. The trainers had previously worked on several research projects in the past and were competent in data collection and following research protocols.

Materials included clickers to signal availability of food, food for reinforcers, timers, datasheets, positive and negative TB samples collected from DOT centers (explained above) and experimental chambers. Rats received mashed banana through a 20 ml syringe presented by trainers during correct responses. Datasheets were used to record all rat behavior. Training supervisors were trained on data-collection procedures by the author. During baseline condition a datasheet used during normal training was used. It was a graphic display that contained highlighted cells indicating TB-positive samples determined by the DOT centers. If the rat made the indication response, a checkmark was made in the corresponding cell on the datasheet. If food was delivered after the indication response a circle was placed next to the checkmark to indicate food delivery. During the reinforcement for agreement procedure a different datasheet was used. These datasheets were a graphic display of the bars (i.e., a metal bar containing holes to insert the samples) evaluated. Each row contained 10 cells that corresponded to the 10 samples that were evaluated in each bar. During the reinforcement for agreement procedure, both trainers and data collector were blind to the location of positive samples, but the same data collection procedures were used.

The experimental chamber was a 10-hole cage (205 cm long x 55 cm wide x 55 cm high). Hollow metal bars containing 10 pots, into which sputum samples were placed, could be fit into holders that placed each sample immediately below one of

the holes. Each session, 10 bars were placed below the holes allowing the animals to evaluate 100 samples per session, which took between 15 and 25 minutes. Samples of sputum were obtained from DOTS centers in Dar es Salaam.

Experimental Design

An ABA design was used to compare the rat's performance under baseline or normal training conditions and the reinforcement-for-agreement procedure. Rats evaluated samples under baseline conditions until visual inspection indicated that responding was stable. Then the reinforcement for agreement procedure was implemented. This continued until responding was again stable. Finally, the rats again evaluated samples under baseline conditions. Because the purpose of this study was to determine if the reinforcement for agreement procedure would be a viable option for first-line screening the ideal outcome of the study would be little or no difference in sensitivity and specificity levels between baseline and reinforcement for agreement procedures.

Baseline. Before each session, the experimental chamber was cleaned with acetone to eliminate any scents from the pervious animal. A rat was then placed in the experimental chamber and a timer was started. The trainers placed the first bar (i.e., bar A), which contained 10 sputum samples, below the holes in the chamber's floor. The chamber contained movable plates that covered the holes allowing the trainer to make only one sample (hole) available for rat evaluation at a given time. Holes were

opened in sequence from left to right and the rat was given a single opportunity to sniff each hole. If a rat sniffed a hole and paused above it for 5 s, the trainer would call out the name of the hole to the data collector. The data collector would indicate a positive indication by saying "reward" or an unknown sample by saying, "suspect." If the rat made a positive indication the trainer produced an audible click using a handheld clicker. If the rat orientated and approached the food hole at the end of the cage within 3 s of the click a small amount of mashed banana was delivered via a syringe. If the rat made a correct indication and received a food reinforcer, this was indicated on the data sheet with a checkmark and circle. If the rat did not orientate or approach the food hole within 3 s, food was withheld. When the rat finished evaluating the first bar, the bar was removed and a next bar was placed under the chamber for inspection. This continued until the rat evaluated 10 bars, totaling 100 samples per session. The time taken to evaluate 10 bars was recorded. If the rat did not make an indication response on a known positive sample an "M" was indicated on the data sheet to indicate a miss. There were no planned consequences for misses and correct rejections. If two or more rats indicated on DOTS negative samples, it was later checked by FM in APOPO's laboratory. At the end of each session a sensitivity and specificity score were determined for each rat. For example, if a rat indicated 8 out of the known positive samples, it was given the sensitivity score of 80%. If it

correctly rejected 79 of the 90 remaining (i.e., DOTS-negative) samples (i.e., had 11 false alarms), its specificity score would be 87.8%

Reinforcement for Agreement Procedure. This phase was the same as training except now the first two rats evaluated the 100 samples under extinction and trainers and data collectors were blind to the location of the positive samples. For the first two rats there were no planned consequences for indication responses. If these rats both made an indication response on a given sample it was highlighted on the datasheet and then used as a reinforcement sample for the following rats. That is, if the third rat made a positive indication response over a sample that both rats 1 and 2 made an indication response on, a click and food were delivered. If the third rat made an indication response that none or only one of the previous rats had made an indication over, there was no planned consequence, but the indication was recorded on the datasheet. This continued for the remaining rats. The order of the rats was randomly predetermined, with no single rat serving in the first or second position for more than 3 consecutive days.

Interobserver Agreement

The author and the head of behavioral research and training collected interobserver agreement data during 52% of sessions on both rat and trainer behavior. This was done to ensure reliable data collection and adherence to procedures. If both the training supervisor and secondary data collector indicated a response on the same

sample it was recorded as an agreement. If either data collector indicated a response and the other did not it was recorded as a disagreement. The second observer agreed with the primary data collector on 99% (range across rats: 97% to 100%) of rat indications. The two data collectors agreed on 97% of indications made and 99% of samples in which the rat did not make an indication response.

Procedural Integrity

Procedural integrity data were also collected on 50% of sessions. During training (baseline) sessions, the following behaviors were scored: 1) whether the trainer called out the indication location, 2) whether the note taker affirmed a "hit" on a positive sample, 3) whether the trainer delivered the click contingent upon a correct rat indication, and 4) whether the reinforcer was delivered dependent upon a correct rat indication and approach of the food-hole within 3 s. During the reinforcement for agreement procedures, the following behaviors were scored: 1) whether the trainer called out the location of an indication response, 2) whether the note taker affirmed that they heard the "hit" by saying "OK" and the data collector recorded a hit in the correct corresponding cell, 3) whether cells in which the first two rats made an indication response were highlighted after the session, 4) whether the trainer delivered the click dependent upon a correct rat indication and approached of the food-hole within 3 s (only for rats who followed the first two extinction rats), and 5)

whether extinction rats never received reinforcement during the session. Overall, procedural integrity was 99%.

CHAPTER III

RESULTS

Figure 1 shows the sensitivity data for the four rats across baseline, reinforcement for agreement and the return to baseline. Data were averaged in blocks of three days. During baseline responding was fairly consistent across the four rats. Casey's average sensitivity during baseline was 73.8% , which means on average she was correctly indicating approximately 7 samples (out of ten) per session. During the reinforcement for agreement phase her responding was 67.3% , which means she was correctly indicating between 6 and 7 positive samples. This suggests that, on average, under the reinforcement for agreement procedure she missed roughly one positive sample compared to the initial baseline condition. During the return to baseline her average sensitivity was 78.9%, which was slightly above her initial baseline average. Baseline sensitivity for Kim was 70% , during reinforcement for agreement it was 62.4% , and during the return to baseline it was 77.8% . Laila's baseline sensitivity was 73.3%, during reinforcement for agreement it was 63.1%, and during the return to baseline it was 77.4%. Peter's average baseline sensitivity was 72.8%, during the reinforcement for agreement it was 65%, and during the return to baseline was 78.9%

Figure 1. Individual Sensitivity Percentage

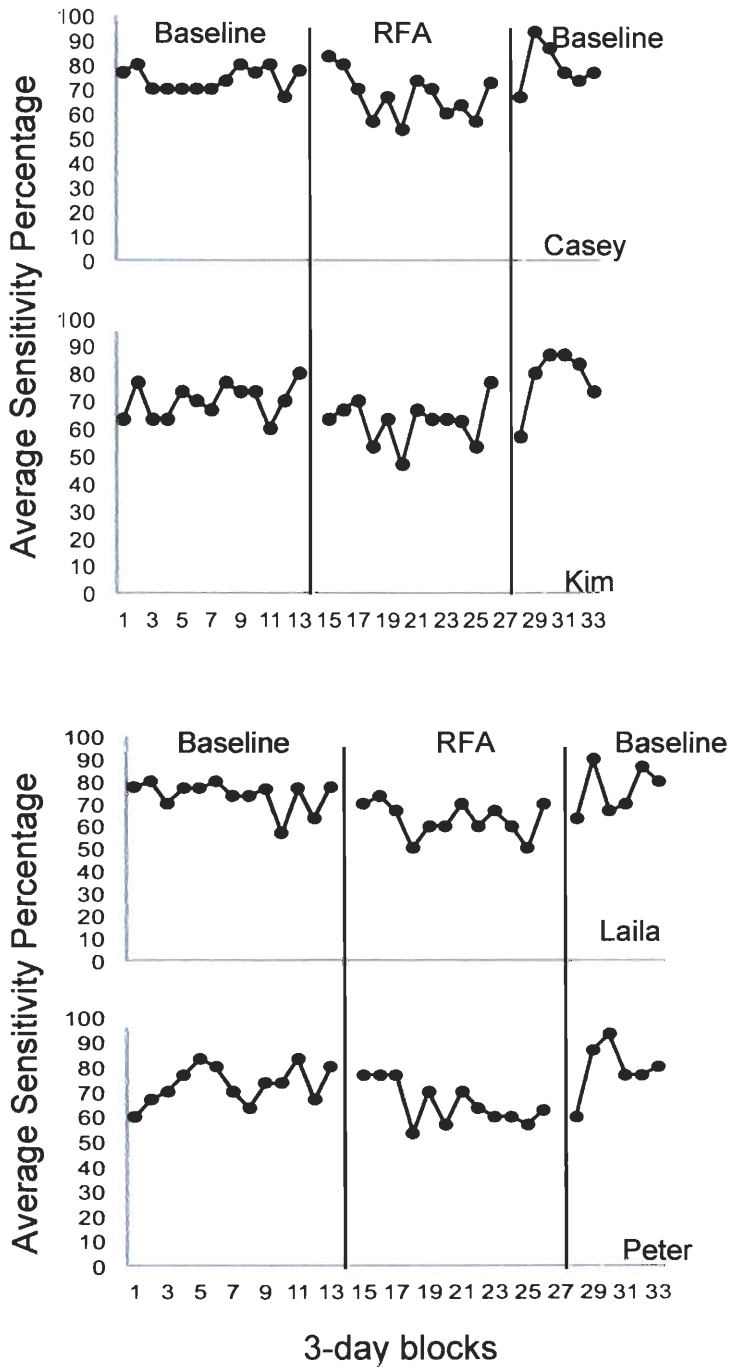
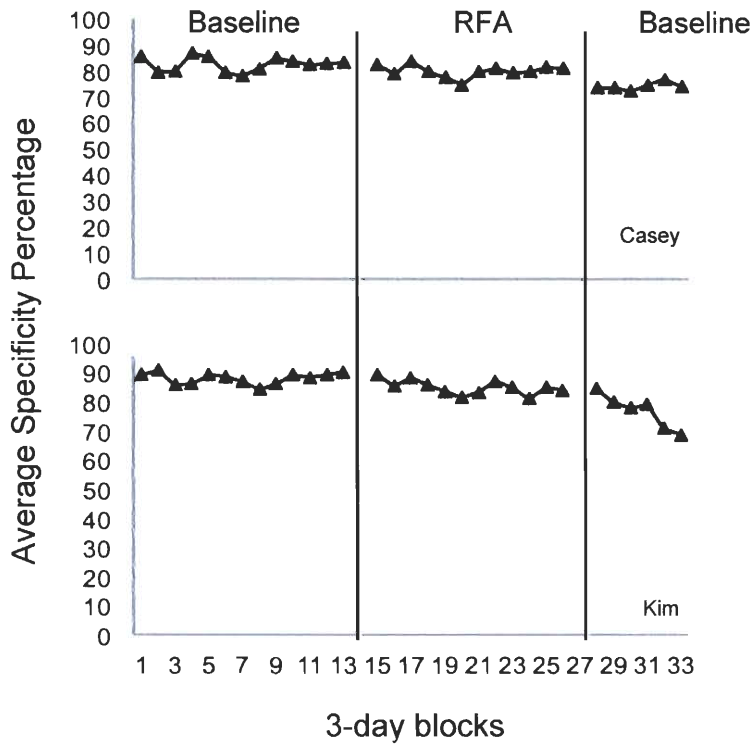


Figure 2 shows the specificity data for the four rats across baseline, reinforcement for agreement and the return to baseline. Again data were summarized in blocks of 3 days. During baseline Casey's average specificity was 82.6%, during reinforcement for agreement it was 80%, and during the return to baseline it was 74.3%. Kim's average specificity during baseline was 88.1%, during reinforcement for agreement it was 84.6%, and during the return to baseline it was 77% Laila's average specificity during baseline was 87.9%, during reinforcement for agreement it was 82.2%, and during the return to baseline it was 76.4% Lastly, Peter's average specificity during baseline was 83%, during reinforcement for agreement it was 80%, and during the return to baseline it was 71.7%

Figure 2. Individual Specificity Percentages



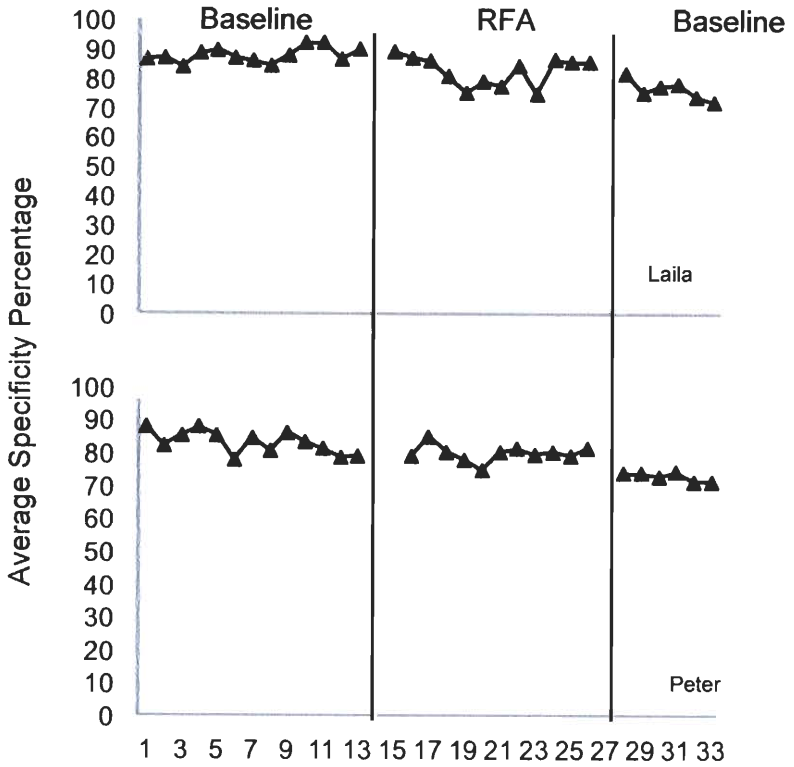


Table 2. Sensitivity and Specificity Summary

	Sensitivity			Specificity		
Rat	**Baseline**	**R.F.A**	**Baseline**	**Baseline**	**R.F.A**	**Baseline**
Casey	73.8	67.3	78.9	82.6	80	74.3
Kim	70.3	62.4	77.8	88.1	84.7	77
Laila	73.3	63.1	77.4	87.9	82.2	76.4
Peter	72.75	65	78.9	83	80	71.1
Average	72.5	64.5	78.3	85.4	81.7	74.7

Figures 4 and 5 show sensitivity and specificity averages across baseline, the reinforcement for agreement procedure, and the return to baseline for each rat using bar graphs. The sensitivity results were consistent across the four rats. During reinforcement for agreement the sensitivity drop was approximately 10%, which means on average rats were missing one positive sample per session. Once the baseline condition was reinstated average responding surpassed initial baseline sensitivity averages for all rats. Specificity averages were also consistent across the four rats. Specificity rates increased during the reinforcement for agreement procedure compared to the initial baseline, and then decreased once baseline was reinstated.

Figure 3. Average Sensitivity per condition

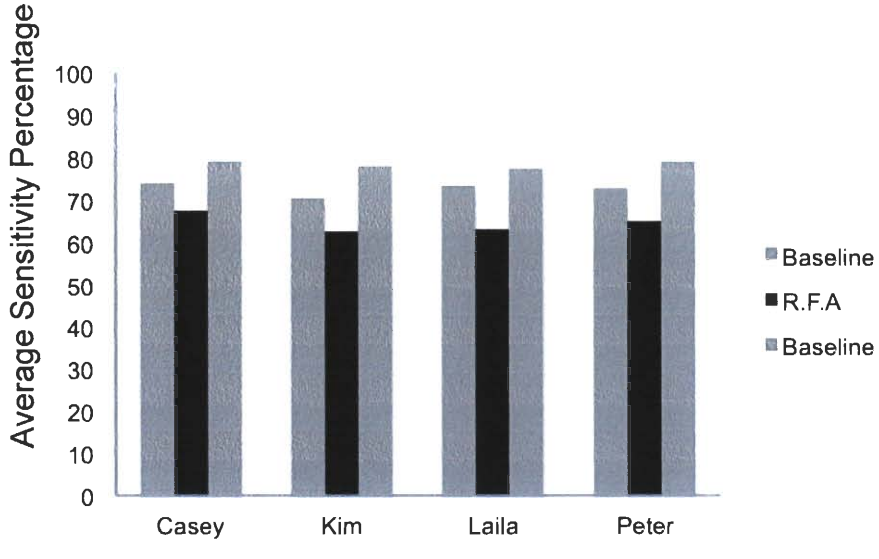
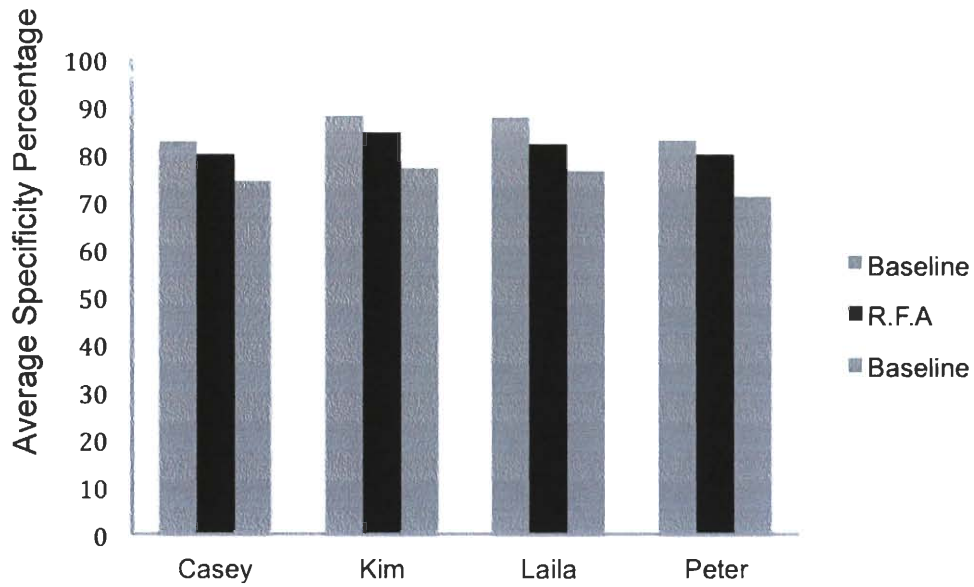


Figure 4. Average Specificity per condition



Figures 5 and 6 show each rat's sensitivity and specificity during only the reinforcement for agreement phase, when rats randomly rotating between working under extinction and agreement-contingency. Days in which a given rat evaluated samples under extinction (rats 1 or 2) were averaged and compared to days in which that same rat evaluated samples under the agreement-contingency (rats 3 or 4). Whether a given rat had higher sensitivity rates during extinction or the agreement-contingency differed across the four rats. One rat (Casey) showed no difference. Kim and Laila had higher sensitivity during the agreement-contingency days compared to days they evaluated samples under extinction and Peter on average had a higher sensitivity rate during extinction days compared to the reinforcement-for-agreement days

Three of the four rats had higher specificity averages during extinction (Casey, Kim, and Laila) than days in which they were evaluating samples under the agreement-contingency. Peter's average sensitivity was 1% higher under the agreement-contingency compared to extinction. Taken together these data demonstrate there was little difference between sensitivity and specificity when the rats were randomly rotating between evaluating samples under extinction and the reinforcement-for-agreement. Each rat evaluated samples under extinction 15 days total and 22 days in the agreement-contingency position.

Figure 5. Average Sensitivity during Extinction and Agreement-Contingency

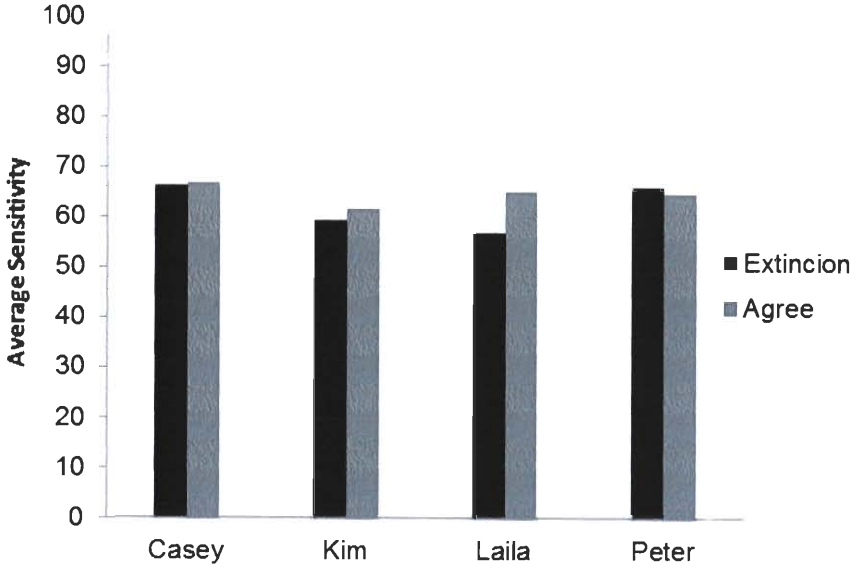


Figure 6. Average Specificity during Extinction and Agreement-Contingency

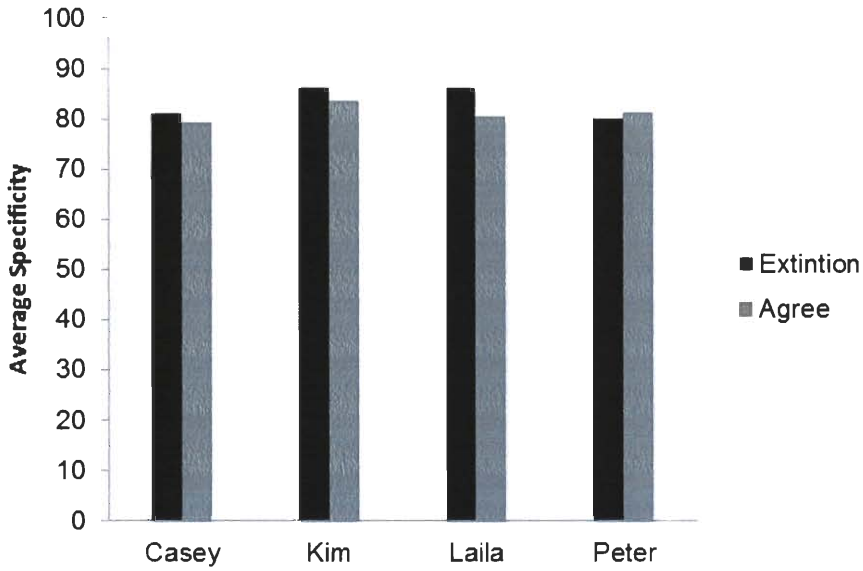


Table 3. Extinction and Agreement-Contingency Summary

	Sensitivity		Specificity	
Rat	EXT	RFA	EXT	RFA
Casey	66.2	66.8	81	79
Kim	59.3	61.7	86	84
Laila	56.9	65.0	86	81
Peter	65.9	64.8	80	81
Average	62.1	64.6	83.4	81.2

CHAPTER IV

DISCUSSION

The results of this study suggest the reinforcement-for-agreement procedure may be a tenable option to maintain responding similar to that obtained under normal second-line screening conditions. On average, there was little difference between baseline and reinforcement-for-agreement sensitivity rates across the rats. There was even less difference seen in specificity rates. These data suggest that the procedure did not teach the rats to indicate on negative samples. There are at least two reasons why the reinforcement-for-agreement procedure maintained sensitivity and specificity percentages similar to normal training conditions. First, all the rats in this study were used in second-line screening prior to the start of the study, which means they had an extensive history of intermittent reinforcement, because their identification responses to DOTS-negative samples are never reinforced, although some of those samples do in fact contain *M. tuberculosis*. During second-line screening any sample that at least two rats indicate over is later evaluated by APOPO's FM technicians. Sometimes it is the case that these samples are determined to be positive and APOPO contacts the DOT centers so these patients can begin treatment. These samples were not scheduled for reinforcement during evaluation so if the rat made an indication response it went unreinforced. Intermittent reinforcement makes responding more resistant to extinction (Keller and Schoenfeld, 1950). It may be the case that this intermittent

reinforcement history made the rat's responding resistant to the effects of extinction on days they evaluated first or second in the present study.

The second reason the reinforcement-for-agreement procedure may maintain responding roughly comparable to training is that the probability of reinforcement on a negative sample was low. In order for a rat to be reinforced on a negative sample, the first two rats would have had to indicate on that specific sample. When Verhave proposed a similar procedure he said the probability of two pigeons agreeing that an acceptable capsule was a "skag" was low and that more pigeons could be added to the reinforcement-contingency (e.g., three pigeons could evaluate the capsule) to negate this possibility. During reinforcement-for-agreement sessions the first rat may have had many false alarms but the second rat may have had very few. So even though the procedure can create a situation in which negative samples are scheduled for reinforcement, this is not likely to occur. Specificity rates were consistent throughout both conditions of the study, which suggests the rats were not trained to indicate on negative samples.

To maximize effectiveness and efficiency of the reinforcement-for-agreement procedure, specifics parameters of the procedure will have to be optimized. First, the number of times a rat can serve in position A or B within a given amount of time before degradation of responding is observed would needs to be evaluated. Second, the optimal number of rats in the agreement arrangement needs to be determined.

Given the slight decrease in sensitivity during the reinforcement for agreement condition, it may be beneficial to add a third rat to the reinforcement for agreement procedure. That is, three rats would evaluate samples under extinction and would all have to indicate on a given sample for it to be scheduled for reinforcement for the following rats. This would, in theory, decrease the probability of negative samples being identified as positive and the occurrence of rats being reinforced on negative samples.

Given these preliminary results it is possible the reinforcement for agreement procedure could be used operationally to identify individuals with TB. This would be especially useful in areas (e.g., rural communities, prisons) in which FM or other methods are unavailable or too expensive. If at least two or three rats indicated a sample as positive it could be presumed that the individual was TB positive and treatment could start, or these samples could be further analyzed with FM or other methods. A cost-benefit analysis could determine if this was an effective system in identifying patients and providing treatment without the use of established diagnostic methods.

One limitation of the present study is the variability in sensitivity rates across sessions. The literature on discrimination learning clearly indicates that the intensity of the stimuli that animals are initially trained to identify affects their subsequent performance (e.g., Catania, 2006). Animals trained to emit an indication response to

low intensity stimuli subsequently emit the response to low-intensity stimuli that are ignored by animals given similar training but to higher intensity stimuli. TB-positive samples are assigned by microscopists to one of four categories, (AFB; a few bacilli, +1, +2, and +3), depending on the number of bacilli present in the sample. It is easiest for microscopists to identify samples as positive when a large number of bacilli are present. The majority of positive sputum samples that APOPO receives from the DOTS centers are rated as +2 or +3. These samples are used as reinforcement samples during APOPO's training. Because most of the training samples contain a relatively high concentration of *M. tuberculosis* the rats are trained primary on high-intensity stimuli. This may limit their ability to indicate on low-intensity stimuli (i.e., samples containing low levels of *M. Tuberculosis*). The variability in responding within conditions and across the study may reflect the rat's training history with high intensity samples. Given that scheduled reinforcement samples are usually +2 or +3 the rats may less reliably make an indication response on AFB and +1 samples. The missed samples within the study may reflect this. APOPO is currently training rats on low concentrations samples to improve sensitivity and specificity rates. However, the rats used in the current study had an extensive history identifying high-intensity samples before this change was made and it may be reflected in these results.

APPENDIX
APOPO's Institutional Animal Care and Use

VAPOUR DETECTION TECHNOLOGY
apopo

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25th January 2013

RE: IACUC APPROVAL Evaluation of the Efficacy of a Rat Agreement Based Reinforcement Procedure

This letter affirms that Kate LaLonde's thesis entitled 'Evaluation of the Efficacy of a Rat Agreement Based Reinforcement Procedure project has been approved by the APOPO's Institutional Animal Care and Use Committee. The Institutional Animal Use Protocol Number is 2013-02. Specifically, the IACUC has reviewed the relevant Animal Research Protocol for technical, scientific, ethical, and legal merit and recommends the project for commencement.

The care and use of animals, specifically 6 giant African pouched Rats (*Cricetomys gambianus*) will be conducted in accordance with the US National Research Council's 1996 *Guide for the Care and Use of Laboratory Animals*.

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