

Using the CLOD to Deliver Pentachlorobenzene to Coyotes (*Canis latrans*)

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ABSTRACT: The Coyote Lure Operative Device (CLOD) is a bait delivery device designed to deliver chemicals such as vaccines, sterilizing agents, predacides, and physiological markers to coyotes. Coyotes have activated the device in the field when it is filled with placebo baits, but measured delivery of an active ingredient has never been attempted. We developed a dose-response relationship for pentachlorobenzene (PeCB) residues in coyote serum and used the CLOD to deliver PeCB to captive coyotes. Twenty-two days post dosing, PeCB residues were detected in serum samples collected from every coyote that activated the CLOD. No residues were found in controls. We conclude that PeCB can be used as a physiological marker for identification of coyotes that activate CLODs. Using PeCB to quantify CLOD consumption met with moderate success and recommendations for future research are provided.

KEY WORDS: *Canis latrans*, CLOD, coyote, Coyote Lure Operative Device, pentachlorobenzene, physiological marker

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INTRODUCTION

The Coyote Lure Operative Device (CLOD) was developed by Marsh *et al.* (1982) to deliver baits to free ranging coyotes (*Canis latrans*). Potential deliverables include oral vaccines, predacides (Johnston 2005), physiological markers (to verify bait consumption, for example), and contraceptive agents (Ebbert and Fagre 1987, Buseck 2004). The CLODs used in this study consisted of a 30-ml plastic vial with a rigid nylon core that was attached to a steel stake anchored in the ground (Figure 1). Because coyotes readily consume sugar-containing substances (Marsh *et al.* 1982, Mason and McConnell 1997), the plastic vial contains a corn syrup carrier solution. The sweet contents act as a "reward" and encourage repeat activation (Barnum *et al.* 1982, Fagre and Ebbert 1988, Berentsen *et al.* 2006), which is important when attempting to deliver multiple doses. As a pre-baiting technique, CLODs containing non-toxic placebo baits are deployed in the field to establish bait acceptance, prior to adding an active ingredient. Once regular activation occurs, the active ingredient can be added to the carrier solution, and coyotes ingest the active ingredient along with the palatable carrier solution. Free-ranging coyotes have activated CLODs when filled with placebo bait (Ebbert 1988, Hein and Andelt 1994), and captive coyotes have activated CLODs more readily with repeated exposure (Berentsen 2004, Berentsen *et al.* 2006). Studies involving delivery of predacides (Johnston 2005) and contraceptive agents (Buseck 2004) to captive coyotes through the CLOD have produced promising preliminary results. Stolzenberg and Howard (1989) added Rhodamine B to early versions of the CLOD to simulate poison baits and mark bait spillage, but the amount of carrier solution ingested by recipients is

unknown. Quantifying the amount of carrier contents ingested by recipients will allow end-users of the device to determine the number of doses of active ingredient to be added to CLODs prior to deployment.

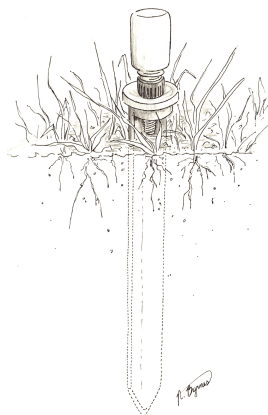


Figure 1. CLOD as it appears when driven into the ground.
Adapted from Berentsen *et al.* (2004).

Physiological markers have been used in behavioral studies (Crabtree *et al.* 1989) and for analyzing patterns of predation (Windberg *et al.* 1997). They also have been used to determine the amount of fluid ingested from livestock protection collars (Knowlton and Ebbert 2002) and to identify mammals feeding on prey items (Knowlton and Olmstead 2001). Furthermore, various markers have been used to monitor bait consumption (Follmann *et al.* 1987, Eason and Batcheler 1991, Southey *et al.* 2002), to identify coyotes responding to control devices (Burns *et al.* 1990), and to assess

ingestion of oral rabies vaccines by non-target species (Olson *et al.* 2000). Pentachlorobenzene (PeCB) has been proposed as an effective long-term marker for use in coyotes (Kimball *et al.* 1996, Johnston *et al.* 1997, 1998) and can be reliably detected in serum, adipose tissue, muscle, and feces for at least 168 days post-ingestion (Kimball *et al.* 1996, Johnston *et al.* 1998). It is a white, crystalline, solid material at room temperature, previously used in production of the fungicide pentachloronitrobenzene (McDonald 1991). Our experiment was conducted in two phases. We conducted a dose-response experiment (hereafter “dose-response phase”) to establish the amount of PeCB residue in the serum of coyotes 22 days after ingesting known quantities of the marker. In the second phase (“consumption phase”), we used the CLOD to deliver a known quantity of PeCB to coyotes, verified consumption of CLODs by analyzing PeCB residues extracted from sera of coyotes, and estimated the amount of marker and thus carrier solution consumed by study subjects.

METHODS

Study Area

Studies were conducted at the USDA APHIS WS National Wildlife Research Center (NWRC) Predator Research Facility near Millville, Utah, in July and September 2003. The dose-response phase was conducted in 1.2 × 3.6 × 1.8-m outdoor kennels, while the CLOD consumption phase took place in 0.10-ha outdoor pens. These pens consist of three 0.10-ha enclosures converging on a central observation building (Heffernan 2001). The amount of PeCB in blood serum samples was quantified at the NWRC in Fort Collins, CO, in August and October 2003.

Analysis by Gas Chromatography

PeCB residues were quantified by injecting a 1.0- μ l sample of extracts into a Hewlett Packard 5890 gas chromatograph (GC) with an electron capture detector, as described in Johnston *et al.* (1998). The analysis process was conducted in two phases. The dose-response phase involved developing standards and establishing a dose-response relationship between known quantities of PeCB and the chromatograph peak area derived by the GC. The consumption phase involved developing a second set of standards and exposing coyotes to a known amount of PeCB in CLODs, then using the equations established during the first phase to calculate the amount of PeCB and thus carrier solution consumed by coyotes.

Development of Standards

For the dose-response phase, standard solutions (1 ppb to 1,000 ppb) were prepared and used to create a standard curve of known concentration of PeCB and the chromatographic peak area provided by the GC. Standard extracts were removed from the refrigerator and placed in random order on the autosampler rack where they were allowed to reach room temperature before analysis. The following linear relationship was generated using the REG procedure (SAS Institute 2002) (Figure 2):

$$\text{Concentration (ppb)} = (\text{Area Count} - 19979) / 1397 \quad [1]$$

Equation 1 was used to calculate the concentration (ppb) of PeCB residue in the 1.0- μ l sample analyzed by the GC during the dose-response phase of the experiment.

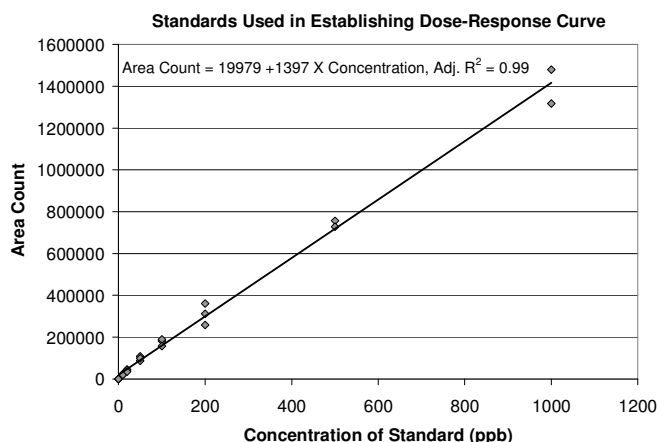


Figure 2. Relationship of known standard concentrations and the chromatographic peak area count for PeCB generated during the dose-response phase of the experiment.

Because several weeks elapsed between the dose-response phase and the consumption phase, a second set of standards (1-2,000 ppb) was analyzed during the consumption phase, generating the following equation (Figure 3):

$$\text{Extract Concentration (ppb)} = (\text{Area Count} + 2042) / 4902 \quad [2]$$

Equation 2 was used to calculate the concentration (ppb) of PeCB residue in the 1.0- μ l sample analyzed by the GC during the consumption phase of the experiment.

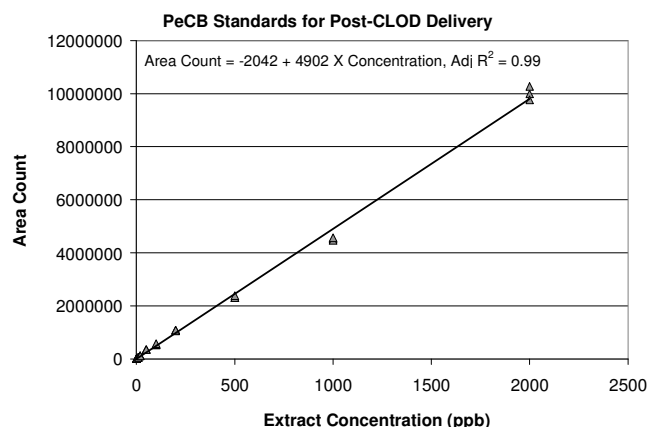


Figure 3. Relationship of known standard concentrations and the chromatographic peak area count for PeCB generated during the CLOD consumption phase of the experiment.

Dose-Response Relationship

Eighteen adult coyotes, 12 males and 6 females >1 year old, were fed 250 g of wet coyote ration (Fur Breeders Agricultural Cooperative, Sandy, UT) containing known amounts of PeCB dissolved in 1.0 ml mineral oil. Three replicates, 2 males and 1 female, were conducted for each dose category of 5, 10, 20, 40, 80, and 160 mg. Three control subjects were fed only the 250 g

food ration. Experienced handlers observed the subjects to ensure all food was eaten. Subjects that did not eat the entire meal were replaced with naïve subjects or censored from analysis.

Twenty-two days post-dosing, a 5.0-10.0 ml blood sample was drawn from each subject. Blood samples were allowed to clot and were centrifuged (Kimball *et al.* 1996). Serum samples were extracted by pipette, placed in labeled plastic collection tubes and frozen at -24°C until analyzed. Serum was thawed at room temperature. Two 0.50-ml aliquots were removed from each serum sample and placed in centrifuge tubes. PeCB was extracted by adding 1.0 ml toluene, mixing for 10 seconds on a vortex mixer, and then centrifuging for 45 min on high speed. The toluene layer (serum extract) was removed using a glass pipette, transferred to a labeled autosampler vial with 0.50-ml inserts, and refrigerated at 5°C until analyzed. A single sample from each serum extract was analyzed. After injecting serum extracts, chromatographic response area counts were recorded for each extract and the marker residue concentration was calculated based on the chromatographic peak area vs. concentration regression equation derived from the standard curve (Equation 1). This provided the concentration of PeCB within the 1.0- μ l sample analyzed by the GC. The concentration of PeCB in the 0.5-ml serum sample was corrected for dilution of extract with the equation:

$$\text{Serum ppb} = \text{Extract ppb} \times (1.0 \text{ ml toluene} / 0.5 \text{ ml serum}) \quad [3]$$

The serum concentration (ppb) was then plotted against the dose to generate the dose-response curve.

Marker Delivery Using the CLOD

Sixteen adult coyotes, 8 males and 8 females >1 year old, were selected from the colony. All were naïve to the CLOD and had not been used in establishing the dose-response relationship. Seven subjects were tested on Day 1 and 9 subjects on Day 2. On each study day, a carrier solution was prepared using a 19:1 ratio of light corn syrup and powdered sugar (Ebbert 1988). Next, 640 mg PeCB was added to 16.0 ml of emulsifier (Tween 80:Span 80; 1:1) (Sigma/Aldrich, Milwaukee, WI) and heated gently to dissolve the PeCB. This provided a stock marker solution of 40 mg PeCB per ml emulsifier mixture. The emulsifiers were chosen as a result of mineral oil's poor solubility in the corn syrup/powdered sugar mixture. The dose of 40 mg was chosen because during the dose-response phase, all study subjects offered a food ration containing the 40 mg dose consumed the entire meal, resulting in a slightly higher number of replicates for this dose category. A 1.0-ml aliquot of stock marker solution was put in each CLOD unit head and 24 ml of heated carrier solution was added for a total volume of 25.0 ml and concentration of 1.6 mg PeCB per ml carrier solution. Loaded CLODs were placed in the center of each experimental arena, and 0.2 ml of Fatty Acid Scent (FAS) (Pocatello Supply Depot, Pocatello, ID) was applied to elicit biting and chewing responses. Study subjects were exposed to the device for 2 hr in the morning, prior to feeding. Shade shelters and den boxes were present and water was available *ad libitum*.

Twenty-two days post-dosing, blood samples were drawn as described above.

CLOD Post-Consumption Analysis

Extraction of PeCB from sera was performed as previously described. Samples were analyzed by gas chromatography, with modifications to separate chromatographic responses in the control samples that were eluting at the same retention time as PeCB (Berentsen 2004). Area counts obtained from analyzing blood serum samples of coyotes offered CLODs containing 40.0 mg PeCB were converted to extract concentrations (ppb) by using Equation 2. The resulting extract concentration was converted to serum concentration using Equation 3. To calculate the actual dose ingested, the serum concentration was entered into the dose-response curve and solved for *dose*. Dose (mg) was converted to ml consumed by dividing the dose by the concentration of PeCB in the CLODs (1.6 mg/ml).

RESULTS

The dose-response curve for PeCB concentrations in coyote serum created a linear relationship (Figure 4): $\text{Serum concentration} = 3.8209 + (6.6893 \times \text{dose})$ (Adj. $R^2 = 0.83$), although individual variation within dose categories was higher than anticipated with considerable overlap.

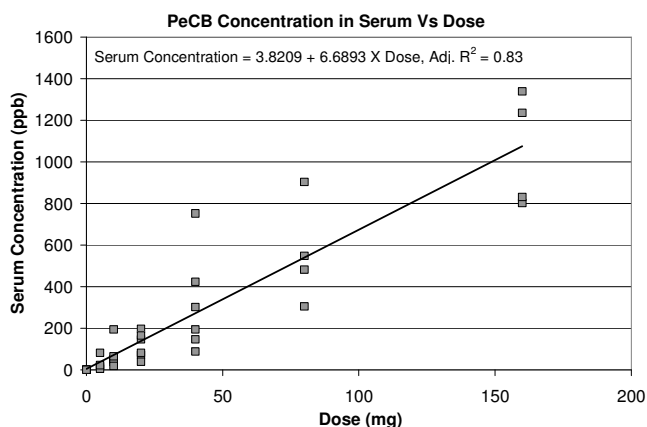


Figure 4. Dose-response curve of serum residue concentrations vs. dose from coyotes ingesting known quantities of PeCB in Millville, Utah, 2003.

Eight (4 males and 4 females) of 16 subjects exposed to the CLOD activated and ingested the contents. No liquid remained in unit heads after activation. Subjects that did not activate CLODs served as control subjects during analysis with no PeCB residue detected. The PeCB concentration in sera collected from coyotes offered 40.0 mg of PeCB in CLODs ranged from 0.87 mg to 6.11 mg, and CLOD carrier solution ingested ranged from 0.54 ml to 3.82 ml (Table 1).

DISCUSSION

This study demonstrated that the CLOD could be used to deliver an active ingredient to coyotes. Serum PeCB residues were detectable for at least 22 days post-dosing. It also showed that residues from 40-mg doses of PeCB

Table 1. Serum residues and estimated consumption concentrations based on dose-response curve from coyotes dosed with 40.0 mg pentachlorobenzene in CLODs in Millville, Utah, 2003.

Animal ID	Estimated PeCB consumption (mg)	Estimated CLOD carrier solution consumption (ml)
M 5294	2.13	1.13
F 5328	2.79	1.74
M 5368	1.53	0.96
F 5413	1.18	0.74
F 5622	3.20	2.00
M 5623	0.87	0.54
F 5626	6.11	3.82
M 5714	2.54	1.59

can be used to demonstrate consumption of CLODs by coyotes. However, estimation of the amount of CLOD contents consumed was lower than expected and did not match direct observation, which suggested most if not all contents were consumed. Studies by Johnston *et al.* (1998) demonstrated a single oral dose of 65 mg PeCB could be detected in serum samples for 168 days. During the dose-response phase of our experiment, the concentration of PeCB residue in serum samples for subjects dosed with 40 mg PeCB was comparable to that described by Johnston *et al.* (1998) for a 65-mg dose at approximately the same time period. However, after offering coyotes 40 mg PeCB in the CLOD, the serum concentrations were considerably lower than those obtained during the dose-response phase and estimates provided by Johnston *et al.* (1998). Johnston *et al.* (1998) dissolved PeCB in sesame oil and fed it to coyotes by oral gavage or in gelatin capsules while subjects recovered from anesthesia. Similarly, during the dose-response phase, we dissolved the PeCB in mineral oil before feeding it to study subjects in their food ration. However, we did not use a fatty or lipophilic substance as a carrier solution to deliver PeCB during the CLOD consumption phase. Because fatty/lipophilic substances are absorbed more readily when delivered in a lipophilic carrier, PeCB absorption may have been greater during the dose response phase of the study as compared to the CLOD consumption phase. This may have contributed to a lower PeCB absorption during the CLOD delivery than during the dose vs. response experiments.

The presence of PeCB in coyote serum after ingesting CLODs containing 40 mg of the marker was an indicator of CLOD content ingestion. However, the quantity of CLOD contents consumed by each animal was lower than observations suggest. Direct observation of study subjects chewing on CLODs indicated that most, if not all, CLOD contents were consumed. The variability within dose categories could be the result of variation in physiology of the study subjects. Future research involving a higher number of replicates for each dose category, using animals of approximately equal weight and body fat, may help reduce this variability. Another consideration for future studies would be to administer the PeCB doses by oral gavage to ensure that 100% of the marker is ingested. Also, the addition of a surrogate standard such as tetra- or hexachlorobenzene to the analytical method would reduce the variability associated with the chemical

analyses. Finally, utilizing an experimental design in which identical carrier solutions are used for both the dose vs. response and CLOD PeCB delivery field studies would likely permit a more accurate assessment of the portion of CLOD contents ingested under field conditions.

MANAGEMENT IMPLICATIONS

The results of this study demonstrate that PeCB can be delivered to coyotes using the CLOD. This marker could be useful in determining which animals respond to CLODs in the field. Exposing coyotes to CLODs with PeCB prior to coyote removal can allow managers to look for marker residue in blood or tissue samples. This can provide vital information on sex and age of coyotes activating CLODs and help determine selectivity. Residues of PeCB can also be found in feces (Kimball *et al.* 1996; Johnston *et al.* 1997, 1998), which can allow researchers to collect fecal samples in areas where coyotes have activated CLODs containing the marker. Because DNA analysis allows researchers to identify animals by extracting DNA from feces (Paxinos *et al.* 1997, Ernest *et al.* 2000) and saliva (Williams *et al.* 2003), it is feasible that multiple techniques could be used to identify animals responding to CLODs as well as the fraction of coyotes in an area that activate CLODs. Capture and radio collaring of coyotes could provide the opportunity to collect tissue samples for DNA analysis and establish a library of genotypes of known individuals. However, this relies on most of the coyotes within a territory being captured, which can be difficult. Finally, by analyzing feces containing the biomarker and recovering saliva samples from chewed CLODs, genotypes could be compared with the known library and establish the identity of responding coyotes. For short-term marking, different colored metal flakes (Nass and Knowlton 2004) could be substituted for long-acting biomarkers to determine whether individual animals are responding to multiple CLOD locations.

Observations suggest coyotes ingest most or all of the CLOD contents. The presence of PeCB in coyote serum supports the observation that coyotes ingest CLOD contents but, without further research, cannot be used to quantify the amount consumed. For effective delivery of single-dose toxicants, pharmaceuticals, or vaccines, the CLOD should contain the desired single animal dose for the material of interest. Adding PeCB to the CLOD contents and analyzing feces for PeCB residues could be used to verify consumption of an additional ingredient, such as a vaccine. Similar techniques have been employed using various markers to monitor consumption of oral rabies vaccines (Olson *et al.* 2000) and tallow baits (Nass and Knowlton 2004). To increase the chance of successful delivery of any active ingredient, the carrier solution used should be compatible with the delivery agent and be palatable to coyotes. Despite the fact that the CLOD originated in the early 1980s, it has not yet been developed for operational use. Further research is necessary to establish a variety of potentially palatable carrier solutions and active ingredients that could be used in the CLOD as well as information regarding the social status of coyotes that activate CLODs.

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Experiments were approved by Institutional Animal Care and Use Committees at the NWRC (QA-970) and at Utah State University (IACUC #1073).

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