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Selection of new chemicals to be used in conditioned aversion for non-lethal predation control

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ABSTRACT

Globally, native predators and scavengers are threatened through the incidence of illegal poisoning due to increasing human-wildlife conflicts. The use of conditioned taste aversion (CTA) may mitigate such conflicts. CTA is a robust learning paradigm that occurs when animals associate a food with a discomfort induced by a chemical, thereby avoiding that food in subsequent encounters. We reviewed the potential of 167 chemical compounds to be used in CTA, considering effects, margin of safety, accessibility, and detectability. After the review, 15 compounds fulfilled the required characteristics, but only five (thiabendazole, thiram, levamisole, fluconazole and fluralaner) were finally selected to be tested in CTA assays with dogs. Of the tested compounds, thiabendazole, thiram and levamisole caused target food rejection by dogs and reduced the time spent eating during post-conditioning. However, despite being microencapsulated, levamisole appeared to be detectable by dogs, whereas thiram and thiabendazole were not. Fluconazole and fluralaner did not produce any CTA effect. Thiabendazole, thiram and levamisole can therefore induce CTA, and thus are potential candidates as aversive compounds for wildlife management. Thiram is an undetectable, relatively safe and accessible compound that can induce CTA in canids, and opens new possibilities to develop methods of non-lethal predation control.

1. Introduction

Predation conflict between humans and predators has been occurring since the prehistoric age. This conflict is especially pronounced where medium-large wild canids such as red foxes (*Vulpes vulpes*), coyotes (*Canis latrans*) or grey wolves (*Canis lupus*) coexist with livestock or game species (Macdonald and Sillero-Zubiri, 2004). Humans avoid damage caused by wildlife to their crops, livestock and game species through the use of lethal predator control (Reynolds and Tapper, 1996; Bergstrom et al., 2014). Lethal predator control has negative effects on ecosystems and endangered species through food chain alterations or secondary poisoning (Gordon et al., 2017; Wallach et al., 2017; Margalida and Mateo, 2019). Also, its current social acceptability is low and there is a demand of non-lethal approaches for wildlife control (Cowan et al., 2000; Bergstrom, 2017).

Conditioned taste aversion (CTA) is a non-lethal predation control method that has been considered a potential tool to reduce predation of wildlife (Nicolaus et al., 1989a; Cowan et al., 2000), as to reduce predation of nests by foxes in nesting ground bird species. CTA is a natural mechanism in animals to prevent poisoning and intoxication (Gustavson et al., 1974). Thus, the referent food is avoided after an illness induced by the ingestion of a non-lethal dose of that food (Garcia et al., 1974). CTA can be induced experimentally by adding a chemical substance in a specific food to which it is intended to create an aversion. The correct selection of the aversive compound is very important to induce an effective CTA, which must satisfy with several characteristics: (1) to induce slight gastrointestinal adverse effects as vomit or diarrhoea; (2) to have a wide (or great) acute margin of safety (MOS), which means a high toxic dose together with a low effective dose (a wide MOS is also required to avoid intoxication in the event that an individual consumes numerous doses); (3) to have a short period of latency (30 min-two hours) to assure the correct learning of CTA (Garcia et al., 1974); and (4) to be odourless and tasteless to avoid detection in the referent food (Gentle et al., 2004; Nielsen et al., 2015).

CTA has been experimentally studied in rodents (Gill et al., 2000; Massei and Cowan, 2002), wild birds (Nicolaus et al., 1989b; Avery et al., 1995), wild mammals (Nicolaus et al., 1989c; Norbury et al., 2005) and reptiles (Price-Rees et al., 2013). In the case of wild canids, aversion has

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Table 1
List of chemicals selected as potential CTA-inducing compounds, doses used and effective dose on dogs and LD_{50} for rats. Oral doses in mg/kg of body weight.

Substance	Main use	Odour	Taste	CTA studies	Acute oral LD_{50}		Potential CTA dose in canids	Ref.
					Rat	Dog		
Niclosamide	Anthelmintic	Unknown	Unknown	No	> 1000–2500	> 6000	500	a
Epsiprantel	Anthelmintic	No	No	No	> 5000	> 200	25	b, c
Pyrantel (pamoate)	Anthelmintic	No	No	No	> 5000	> 690	30	d, e
Oxantel	Anthelmintic	Unknown	Unknown	No	980	170	10	f, g
Praziquantel	Anthelmintic	Weak	Yes (bitter)	No	2000–3000	> 200	200	h, i
Fenbendazole	Anthelmintic	Weak	No	No	> 10000	500	200	j, k
Levamisole	Anthelmintic	Yes*	Yes (bitter)*	Yes (canids, mustelids, rodents)	480	Unknown	50 (40–80)	l, d
Thiabendazole	Anthelmintic/Antifungal	No	No	Yes (canids, bears, rodents)	3100	Unknown	200	m, n
Fluconazole	Antifungal	No	No	No	1271	300	30–70	o
Clotrimazole	Fungicide	No	Weak (metal)	Yes (rodents)	708	> 2000	140	p
Thiram	Fungicide	No	No	No	3700–4000	Unknown	40	q, r
Afoxolaner	Insecticide	Unknown	Unknown	No	> 1000	Unknown	120–200	s
Fluralaner	Insecticide	Unknown	Unknown	No	> 2000	Unknown	200–300	t
Spinosad	Insecticide	Yes (rancid)	Yes (bitter)	No	> 3738	Unknown	90	u
Metaldehyde	Mollusquicide	Yes (mint)	No	No	690–927	500	100	v, w

[a]Hayes and Laws, 1991; [b]Corwin et al., 1989; [c]Lynn, 2009; [d]Lanusse et al., 2009; [e]Pitts and Migliardi, 1974; [f]Mackenzie, 2016; [g]Robinson et al., 1976; [h]Frohberg and Schulze, 1981; [i]Frohberg, 1984; [j,k]Scholz and Schultes, 1973a, b; [l]Symoens et al., 1979; [m]Robinson et al., 1965; [n]Robinson et al., 1978; [o]NIIRDN, 1990; [p]Tettenborn, 1972; [q]Lee et al., 1978; [r]Maita et al., 1991; [s]EMEA, 2015; [t]Walther et al., 2014; [u]US EPA, 1997; [v]Booze and Oehme, 1985; [w]Gupta, 2012; *Microencapsulation has been used to reduce the detectability of this compound (see Tobajas et al., 2019).

been successfully induced to red foxes (*Vulpes vulpes*) and grey foxes (*Pseudalopex griseus*) to reduce the bait consumption (Gentle et al., 2004; Nielsen et al., 2015), and also to reduce nest predation (Maguire et al., 2009). Although several chemical compounds have been shown effective to induce CTA, most of them do not meet all characteristics required for CTA (Gill et al., 2000). Issues of safety and detectability limit the number of compounds that may be used for practical applications of CTA in wildlife management. The application of the CTA with safety must be one of the main characteristics for its use in wildlife management. Up to now, few CTA experiments performed have taken into consideration this important issue, and some substances had to be discarded due to their high toxicity or detrimental effects on animal health (Nicolaus and Nellis, 1987; Conover, 1989; Dueser et al., 2018). The other major problem is that the detectability of the compounds by the animals causes rapid discrimination between foods with and without CTA compounds (Burns, 1980; Nicolaus and Nellis, 1987; Gentle et al., 2004; Nielsen et al., 2015). In this case, if the compound added to the referent food is detected, the animal associates the illness with the substance and avoids only treated referent food, acting as secondary repellent (Sayre and Clark, 2001; Cagnacci et al., 2005). A potential way to mask odour and taste of substances is microencapsulation, which isolates the chemical substance with an impermeable coating (Shukla et al., 2011). However, it requires experimental research to test their effectivity (Cotterill et al., 2006; Tobajas et al., 2019). Expensive chemical compounds that need to be fabricated, handled under special conditions, and cannot be preserved in the field for a long time are not suitable for their use as aversive for wildlife management. Hence, the search for new, safe, accessible and undetectable compounds is paramount for the development of CTA for wildlife management. For these reasons, we reviewed the compounds previously used in CTA and assessed potential new candidates that accomplish the desirable characteristics as aversive compounds for canids. We evaluated the potential of five selected compounds in a pilot study to induce CTA in penned dogs, to test them as candidates to be applied as aversive for wildlife management.

2. Material and methods

2.1. Review and selection of aversive compounds

We conducted a literature review using Web of Science, Scopus, Toxnet databases, Google Scholar with the terms “taste aversion”, “learned aversion”, “odour aversion”, “food aversion”; and also in databases of chemical substances registered in Spain (i.e. pesticides, biocides and pharmaceuticals) in order to identify: (1) substances that have been used in CTA in canids or other wildlife species, (2) substances that have been described as potential CTA inducers in rats, (3) other new substances of the same chemical family as the known aversive substances that could be candidates to be tested in CTA, (4) LD_{50} in rat and dog of the selected substances, and their difference as a precautionary principle, (5) odour and taste of the selected substances, (6) potential doses to produce CTA in canids, and (7) commercial availability of the product. Based on the LD_{50} values in rat and the potential doses to induce CTA we calculated the expected acute MOS of each substance. The acute MOS has been calculated as the ratio between LD_{50} in rat and the potential dose to induce CTA. The potential dose to induce aversion has been selected from the review analysis and is defined as the dose that induces adverse gastrointestinal effects related to the CTA mechanism, mainly nausea and vomits. The purpose of this literature review was to identify substances that have been successfully used to induce CTA and to identify new candidates that could be used in further experimental CTA tests with canids. Therefore, the selected substances should have an acute MOS above 10 (i.e. the lethal dose should be at least 10-fold higher than the potential CTA dose). Moreover, the selected compounds should be also odourless and tasteless, and available in the market with an approved use as a veterinary drug or other use.

A total of 167 substances were included in the literature review (see full list in supplementary material, Table S1). Based on the data obtained from all these substances, we first selected the 15 chemical compounds that have been or could be used to induce CTA (Table 1). These substances were chosen because among their adverse effects they include gastrointestinal symptoms related to the CTA mechanism (i.e. nausea, vomits). From these compounds we finally selected five compounds that could be good candidates to induce CTA in wild canids with a low risk of lethality as expected by the MOS > 10.

2.2. Laboratory experimentation of aversive compounds

Five selected substances were tested for the ability to induce CTA in penned dogs to reduce the intake of the referent food (preferred food for the dogs). Two of these compounds had been successfully used

before in CTA with canids (i.e. thiabendazole and levamisole). Thiabendazole was taken because of the available literature on this substance as CTA inducer. Levamisole has also been tested as CTA inducer, but important practical limitations may exist with this compound because of its potential detectability by taste and odour. The other three substances (thiram, fluconazole and fluralaner) fulfilled most of the requirements to be used as aversive but have not been tested before.

2.2.1. Animals

Five males and seven females of adult Beagle dogs (*Canis lupus familiaris*) were used in the experiment. Individual body weight ranged from 9.3 to 15.7 kg. The experiment was performed following the appropriate European regulations in the Laboratory Animals Section (Research Support Service, University of Murcia). The project has been evaluated by the Ethics Committee of the University of Murcia and approved subsequently by the Government of the Region of Murcia (Spain) with the permit Nº A13170703. Animals were fed every morning with their usual dry dog food (Gosbi® Premium Performance). During the study period a novel wet dog food (Gosbi® Fresko Chicken) was used as referent food to induce aversion. Dogs were fasted 24 h before each test. The dogs were housed individually in randomly assigned separate indoor pens (size: 1.6 × 4.3 × 3 m) were they were feed during the whole study period. We followed the Guidelines for accommodation and care of animals of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (European Commission, 2007), conforming to Directive 2010//63/EU: room temperature: 20–24 °C; relative humidity: 45–65%; air exchange: 10–15 times/h; 12 h light/darkness cycle. Tap water was provided *ad libitum* and the dogs were released for exercise and physical contact with their roommates for 30 min every day.

2.2.2. Tested aversive compounds

The selection of substances and doses were based on literature as described above (Table 1). Thiabendazole is a benzimidazole that has anthelmintic properties and has been previously used to generate CTA in several mammalian species (Gustavson et al., 1983; Conover, 1989; Ternent and Garshelis, 1999). Levamisole chlorhydrate (levamisole) is an imidazothiazole with anthelmintic properties that has been used in CTA studies with laboratory rats (Massei and Cowan, 2002), foxes (*Vulpes vulpes* and *Pseudalopex griseus*) (Massei et al., 2003a; Nielsen et al., 2015) and Eurasian badgers (*Meles meles*) (Cagnacci et al., 2005). Thiram is a dithiocarbamate fungicide that has been used as a repellent both in birds and mammals (Nolte and Barnett, 2000; Werner et al., 2010). Fluralaner, one of the new and structurally-unique isoxazolines, is an ectoparasiticide selected because it is considered a safe drug causing vomiting, decreased appetite and diarrhoea as the most common adverse reactions at the recommended therapeutic doses (25–56 mg/kg) in dogs (EMEA, 2015). Fluconazole is a triazole used as antifungal with a wide acute MOS. Nausea, vomiting and anorexia have been described at therapeutic doses of fluconazole in dogs (Mueller, 2007).

The effective doses of the aversive substances were obtained from previous toxicity studies based on their ability to cause digestive symptoms (vomiting, nausea and/or diarrhoea), but without causing severe adverse effects (Table 1). These single oral doses were 200 mg/kg for thiabendazole, 50 mg/kg for levamisole, 40 mg/kg for thiram, 200 mg/kg for fluralaner and 30 mg/kg for fluconazole (see references in Table 1). Because no vomits or food avoidance were found during conditioning with fluconazole and fluralaner, the dose during the reinforcement phase (see Section 2.3) was increased to 70 mg/kg for fluconazole (seven times the maximum therapeutic dose for dogs, Kukanich, 2008) and to 300 mg/kg for fluralaner (about six times the maximum therapeutic dose for dogs; Walther et al., 2014), but in both cases well below LD_{50} values (Table 1).

In order to reduce the levamisole bitter taste described in previous studies with canids (Massei et al., 2003a; Gentle et al., 2004), this was microencapsulated with Precirol® Ato 5 (glyceryl palmitostearate) as the hydrophobic binder using a melt-granulation technique (Hamdani et al., 2003; Mašić et al., 2012). The other chemicals were used in the pure composition, except for fluralaner that was used in the commercial form. Levamisole was microencapsulated by the Drug Development Service, Faculty of Pharmacy, University of Barcelona. Fluconazole and thiram were purchased from Sigma-Aldrich®, fluralaner (Bravecto™) from MSD Animal Health, and thiabendazole from Alfa Aesar®.

2.2.3. Experimental design of the aversive compounds assay

In order to evaluate the treatment effect of the compounds on the dogs feeding behaviour, we performed a Before-After Control-Impact (BACI) design (Underwood, 1994). We followed three phases during the CTA experiment: pre-conditioning (only food, four trials); conditioning (food + aversive agent, single trial); post-conditioning (only food, four trials); reinforcement (food + aversive agent, single trial). This assay was performed with a pair of dogs per each chemical tested and a pair of dogs for the control group, in order to test the potential of these substances to induce CTA on dogs. This decision was made following the current ethical and animal welfare standards to reduce the number of individuals used in the animal experimentation.

Dogs were enrolled in the experiment on day 1 (start of pre-conditioning) (Fig. S1). During pre-conditioning period (15 days), we performed four two-choice tests between the dry food and the novel referent wet food, and dogs were fed with the usual diet of dry food the rest of the days. The amount of consumed food was calculated daily. Wet food was usually preferred over dry food and then this wet food was chosen as the target food against which we wanted to induce aversion. Although it is known that prior exposure of a food before conditioning reduces the aversion acquisition (Kalat and Rozin, 1973), we decided to perform the pre-conditioning phase with the target food to achieve a conservative experiment. On day 15 (conditioning trial), the dogs were randomly assigned to each substance or control group. A male and a female of Beagle dogs were conditioned with each substance, except for thiram for which 2 females were used (as no more males were available for the test). Prescribed amounts of aversive compounds were mixed homogenously with the wet food and offered for 30 min to each dog. A control pair (a male and a female) received the same amount of wet food, but without any substance added, and were handled in the same conditions. The dogs were checked by a veterinarian every 2 h for signs of illness such as nausea, vomiting and diarrhoea for 8 h after exposure, and 24 h later for confirm no consumption changes of their normal diet.

During the post-conditioning, two-choice tests were performed on days 19, 26, 33 and 45 to compare consumption of the previously conditioned food (wet food, but without the aversive substance) versus the non-conditioned dry food. The rest of the days the dogs were fed with their normal diet based on dry food. Reinforcement of aversive conditioning with wet food containing the chemical was performed on day 22. In each trial, dry and wet food was weighed (± 1 g) with a precision balance (Mettler® PJ15, Mettler Instrumente®, Greifensee-Zurich, Switzerland) in stainless-steel dog bowls and was offered to each dog for 30 min. Bowls were then removed from the dog pens and weighted to calculate the amount of food eaten and the proportion of food rejected (PFR). Dog behaviour was recorded with a video camera (Spartan, HCO Outdoor Products, Norcross, GA, USA) to observe the signs of adverse effects of conditioning and to estimate the latency time (LT; time from food offer to start eating) and the time spent eating all food (TSE). LT and TSE (in min) were used as CTA indicators (Massei et al., 2002; Webb et al., 2008). If at the end of the 30-min presentation dogs had not started or eaten all the food, LT and TSE, respectively, were recorded as 30 min. Feeding behaviour was also video recorded during the pre-conditioning phase.

2.2.4. Haematology and serum biochemistry analysis

Haematology and serum biochemistry were studied to evaluate the possibility of detrimental effects on health. Blood samples were obtained from all the dogs, including controls, one day before and one day after the conditioning and reinforcement with the aversive substances. Blood samples (4–5 mL) were obtained by puncturing the brachial vein, using a 5 mL syringe and a 21 G needle. All the analyses were made at the Interdisciplinary Laboratory of Clinical Pathology, Interlab-UMU, Campus of Excellence Mare Nostrum, University of Murcia, Spain.

2.2.5. Data analysis

The effect of treatment (fixed factor) on PFR, LT and TSE were analysed by a generalized linear mixed model (GLMM) to investigate differences between treatments in the pre-conditioning, and in the post-conditioning phase. The relationships between pre-conditioning and post-conditioning phases of PFR, LT and TSE were modelled by GLMM using the interaction "treatment × phase" as a fixed effect. BACI analysis approaches include generalized linear mixed models (McDonald et al., 2000), where a significant interaction between treatments (each substance and control group) × time (pre-conditioning and post-conditioning) indicates that the experimental treatment had an effect on dogs feeding behaviour. Individual was fitted as a random effect in all models. Where differences between treatments or significant effects of "treatment × phase" were found, pair-wise comparisons for each group were performed. Paired t-tests were used to compare haematology and serum biochemical parameters before and after treatment with each aversive substance. Normality of residuals was checked, and non-normal data were logit transformed for the PFR and log transformed for LT and TSE. Significance of statistical tests was considered at $p \leq 0.05$. All statistical analyses were carried out with the SPSS statistical package 24.0 software (IBM Inc., Chicago, USA).

3. Results

3.1. Potential aversive compounds

From 167 substances evaluated during the review of the available literature (Table S1), 15 were considered as potential candidates to be used in CTA studies with canids. These included anthelmintics, fungicides, insecticides and mollusquicides of 12 chemical groups: salicylanilides (i.e. niclosamide), pyrazinobenzazepines (i.e. epsiprantel), pyrazinoquinolines (i.e. praziquantel), tetrahydropyrimidines (i.e. pyrantel and oxantel), imidazoles (i.e. clotrimazole), benzimidazoles (i.e. fenbendazole and thiabendazole), imidazothiazoles (i.e. levamisole), triazoles (i.e. fluconazole), dithiocarbamates (i.e. thiram), isoxazolines (i.e. fluralaner and afoxolaner), spinosyns (i.e. spinosad) and aldehydes (i.e. metaldehyde). Five of these compounds were odourless and tasteless and this information was not available for four other substances. The remaining six compounds had some odour and/or taste that may affect the conditioning process if the animals associate the adverse effect with these physical properties of the substances. (Table 1). Only three of these substances had been previously used in CTA assays (i.e. thiabendazole, levamisole and clotrimazole). The oral acute LD_{50} in rat was available for all the considered compounds, ranging from 480 mg/kg of levamisole to > 10,000 mg/kg of fenbendazole. The oral acute LD_{50} in dog was only available for nine of the considered compounds (Table 1). The acute MOS was < 10 in four substances, between 10 and 100 in nine substances and > 100 in two substances (Fig. 1).

Only those substances with an acute MOS > 10 were considered to be included as CTA inducers. Thus afoxolaner, clotrimazole, metaldehyde, niclosamide and praziquantel were firstly excluded. According to the MOS calculated, pyrazinobenzazepines and tetrahydropyrimidines would be the best candidates to be tested as CTA inducers, but these anthelmintics also show important differences in LD_{50} values between rat and dog (i.e. oxantel, Table 1). In addition, pyrantel and epsiprantel are expensive substances and difficult to obtain in pure form, and commercial formulations may have other ingredients that could introduce some flavour or increase their toxicity (i.e. pyrantel tartrate). Hence, these substances were not finally selected following a precautionary principle. For the same reason, fenbendazole was also excluded. Spinosad was discarded because it has a known taste and odour and could be detected. We selected 5 substances to be tested experimentally with penned dogs: thiabendazole, microencapsulated levamisole, thiram, fluconazole and fluralaner (Fig. 1). Thiabendazole was included in the experimental tests because it is a confirmed CTA inducer in canids. We also included levamisole because it is a CTA inducer in several mammal species, but here we have employed a microencapsulation to reduce its detectability. Finally, three other substances with MOS > 10 that can produce gastrointestinal symptoms related to the CTA mechanism (nausea, vomits) were selected for the experimental tests with dogs. Tiram and fluconazole were selected because they are odourless and tasteless and the presumable amount of the chemical compound needed to induce CTA is small. The organoleptic characteristics of the fluralaner are unknown, but it was included because we wanted to diversify types of compounds or modes of action. Fluralaner is also a novel chemical that apart from gastrointestinal problems has not shown any other adverse effect (Walther et al., 2014).

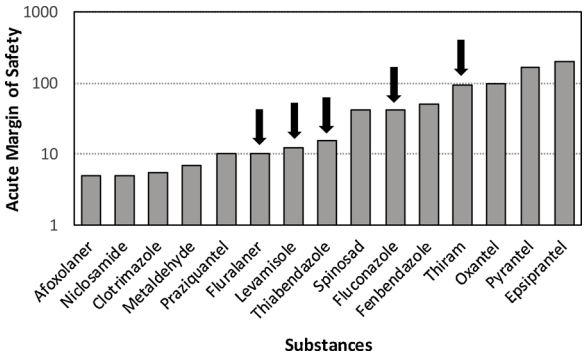

Fig. 1. Acute margin of safety (MOS) in a logarithm scale of the substances that can be used to induce conditioned taste aversion in canids. MOS was calculated as the ratio between LD_{50} in rat and the potential dose to induce CTA in animals (canids if this information was available). Substances with a MOS above 10 were considered good candidates as CTA inducers if other requirements were also fulfilled. The substances finally used in the experimental tests with penned dogs are marked with arrows.

3.2. Conditioned taste aversion

During the first two-choice test (day 19) after conditioning, one dog from the levamisole group, and another one from the thiram group rejected the target food (wet food). Moreover, both dogs of thiabendazole, thiram and levamisole treatments increased LT and TSE compared to the control group and also respect to the pre-conditioning phase. The dogs of these treatment groups lasted more time to start tasting the food and ate the food by sticking small nibbles, stopping to eat and recede from food several times. In contrast, the other dogs from the fluconazole, fluralaner and control groups ate the food without stopping in a shorter time. During the reinforcement on day 22, all the unconditioned dogs (one from thiram and thiabendazole group and both from fluconazole and fluralaner group) ate all the wet food except the dog exposed to levamisole, which rejected the food. After reinforcement, previously conditioned dogs with levamisole and thiram continued showing aversion in the two-choice tests performed at days 26 and 33, and one additional dog from the thiabendazole group showed food aversion at day 26. As during the first two-choice tests, after reinforcement the dogs from the thiabendazole, thiram and levamisole treatment groups showed a misgiving behaviour and they increased the LT and TSE. No signs of food aversion in the fluralaner and fluconazole groups were found during the experiment.

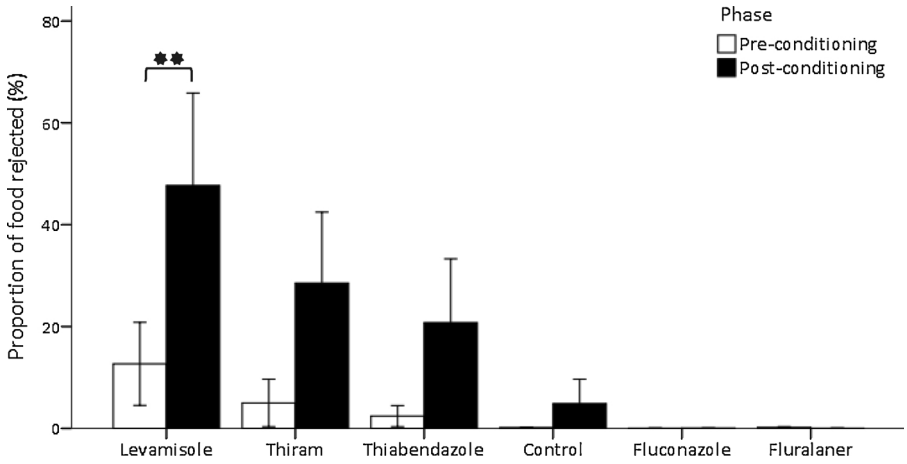

Fig. 2. Proportion of wet food rejected (untransformed data) by treatment and control dogs across four 30-min two-choice pre-conditioning and four 30-min two-choice post-conditioning taste aversion tests. Error bars represent standard errors of the mean; ** indicate differences with $p < 0.01$.

Comparing PFR between pre-conditioning and post-conditioning phases (Fig. 2), we found a significant effect of the "treatment x phase" interaction ($F_{11, 84} = 2.756$, $p = 0.004$). Although thiabendazole, thiram and levamisole groups increased the PFR after the conditioning (Fig. 2), difference between pre- and post-conditioning phases was only significant for the levamisole groups ($p = 0.002$). The effect of the interaction "treatment x phase" was also significant for LT ($F_{11, 84} = 106.55$, $p < 0.001$; Fig. 3), and TSE ($F_{11, 84} = 3.903$, $p < 0.001$; Fig. 4). In the case of LT, the difference between the pre- and post-conditioning phase in each treatment group was significant for levamisole ($p < 0.001$), thiram ($p = 0.001$) and thiabendazole ($p < 0.001$; Fig. 3). In the case of TSE (Fig. 4), differences were significant for levamisole ($p = 0.035$), thiram ($p = 0.029$) and thiabendazole ($p = 0.002$). No significant differences were found in PFR, LT and TSE for the fluconazole and fluralaner treatment groups.

3.3. Clinical signs related with aversive ingestion

During conditioning, although all the dogs ingested the total dose of aversive substances by eating 100% of the target food, vomiting and/or diarrhoea were only found in dogs exposed to levamisole, thiram and thiabendazole (Supplementary material, Table S2). Dogs exposed to fluralaner and fluconazole did not show any symptoms. First vomiting after exposure to each substance occurred at different times: between 30–40 min after exposure to thiram; between 1.5–2.5 h after exposure to thiabendazole; and 2 h 20 min – 3 h after exposure to levamisole (Table S2). Diarrhoea was found in one dog 5 h 15 min after exposure to thiram and in another dog 3 h after exposure to levamisole (Table S2). For the reinforcement, as mentioned above, doses of fluralaner and fluconazole were increased due to the lack of conditioning related symptoms. Despite this, no conditioning related symptoms were found in dogs after reinforcement with these substances. Levamisole exposed dogs in the reinforcement barely ate the food (about 4% food ingested) as did a thiram-exposed dog (17% food ingested). The other thiram exposed dog ingested all food and vomited 20 min later.

3.4. Haematology and serum biochemistry

Exposure to these substances did not seem to cause physiological adverse effects in the dogs, as both haematological and serum biochemical parameters did not significantly differ between before and after treatment (Supplementary material, Table S3). Only levamisole caused a significant increase of neutrophils in the dogs after conditioning (female: 5.3–10.55 × 10^3 cells/μL; male: 4.2–10.02 × 10^3 cells/μL, $p = 0.03$). Despite this increase, the values were within the reference values established in the laboratory for dogs and decreased back after the reinforcement.

4. Discussion

The selection of substances with the literature review and the subsequent experimental tests have yielded three substances than could be

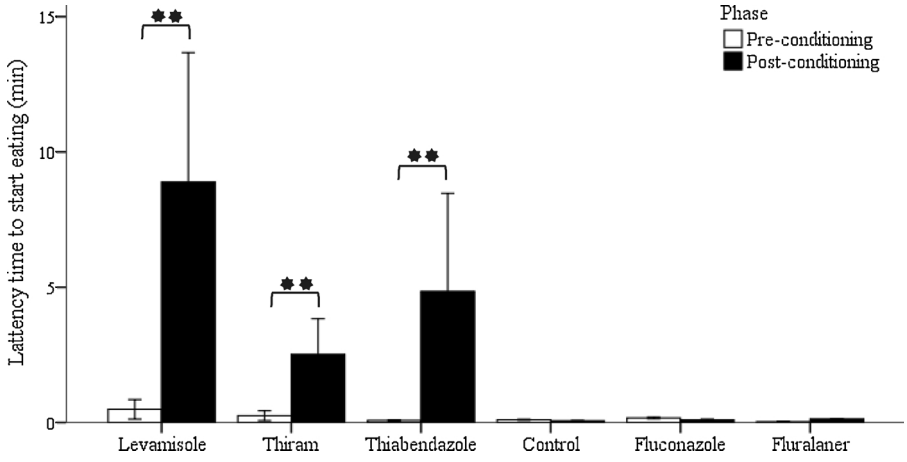

Fig. 3. Latency time to start eating the wet food (seconds) by treatment and control dogs across four 30-min two-choice pre-conditioning and four 30-min two-choice post-conditioning taste aversion tests. Error bars represent standard errors of the mean; ** indicate differences with $p < 0.01$.

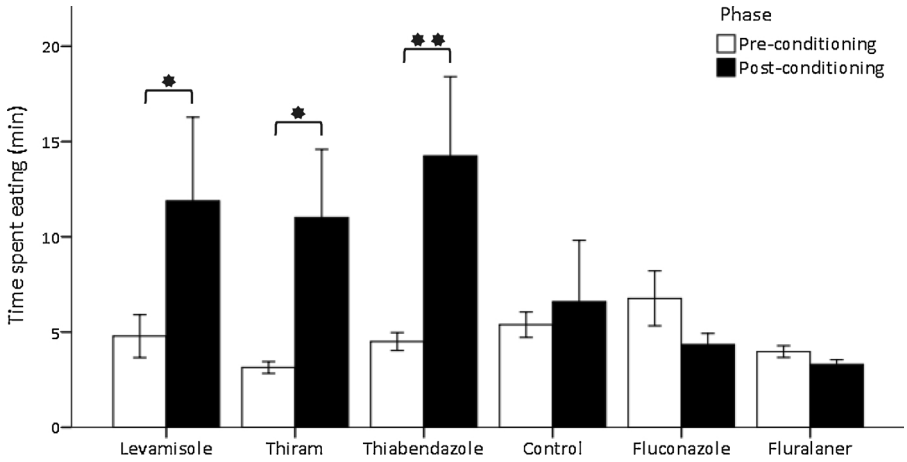

Fig. 4. Time spent eating all wet food (minutes) by treatment and control dogs across four 30-min two-choice pre-conditioning and four 30-min two choice post-conditioning taste aversion tests. Error bars represent standard errors of the mean; * and ** indicate differences with $p < 0.05$ and $p < 0.01$, respectively.

used as CTA agents to reduce the conflicts with wild predators. Thiabendazole and levamisole were already known as CTA inducers in canids, and thiram is a new candidate with a very high acute MOS (aprox. 100), which should be confirmed as CTA inducer in further experimental studies with more animals. Moreover, thiram seemed to be not detected by dogs during the conditioning process, which is an important aspect to be considered in case thiram was used with wild canids to reduce the predatory conflict.

4.1. Review and selection of substances

The list of substances reviewed in the present study reveals many potential candidates to be CTA inducers in canids. However, the adverse effects produced by some of the substances reviewed and used in CTA may have negative consequences in the health of the animals. Some of the aversive compounds used, such as amphetamine, amitriptyline, bupropion (Miller and Miller, 1983; Bryant et al., 1993), and more recently, fluoxetine hydrochloride (Massei and Cowan, 2002), affect the central neural system. Although some of these compounds can induce CTA in rats, all of them modify their natural behaviour exciting or depressing the central neural system, which could lead vulnerability and risk situations for the conditioned individuals in the wild. Also, many repellents, such as anthraquinone, d-pulegone, cinnamic aldehyde, cinnamamide and capsaicin, have been used (Avery et al., 1998; Gill et al., 2000; Conover and Lyons, 2003), but these act differently to CTA agents because they only prevent predation in the presence of the chemical. Oestrogens, like 17α-ethinyloestradiol, have also been used as aversive compounds with good results in rats (Gill et al., 2000) and carnivore species (Nicolaus et al., 1989c; Semel and Nicolaus, 1992; Dueser et al., 2018). However, their ability to induce abortion or even death in pregnant individuals (Yasuda et al., 1981; Dueser et al., 2018) limit its use for CTA during the breeding periods. Finally, other compounds such as insecticides and fungicides have been tested for CTA, mainly causing an cholinergic agonist effect and gastrointestinal irritation (Dimmick and Nicolaus, 1990; Massei and Cowan, 2002; Cox et al., 2004; Maguire et al., 2009). These groups of substances, especially those with an cholinergic agonist effect, (e.g. levamisole, thiabendazole, trimethacarb and carbachol) have shown good results in CTA studies (Gustavson et al., 1983; Nicolaus and Nellis, 1987; Dimmick and Nicolaus, 1990; Massei et al., 2003a). Due to the high toxicity of trimethacarb and carbachol, these are not recommended for being used in the field as CTA inducers (Schafer, 1972; Conover, 1990).

Taste and odour of most compounds used in CTA studies modify the original food and taste of foods (Burns, 1980; Nicolaus and Nellis, 1987; Gentle et al., 2004; Nielsen et al., 2015). Only 17α-ethinyloestradiol and thiabendazole appear to be undetectable (Gustavson et al., 1983; Nicolaus et al., 1989a; Gentle et al., 2006; Dueser et al., 2018). To solve detection problems, a masking odour or taste has been used, although with limited success in baits (Cotterill et al., 2006; Nielsen et al., 2015). Other possibility suggested is the microencapsulation technique (Burns, 1983; Mašić et al., 2012), but it requires an increase of cost and specialized machinery. However, the new formulations and manufacturing methods could enable its use in an effective and economical way.

4.2. Assays with penned dogs

The assay results with dogs showed that levamisole, thiabendazole and thiram produced CTA in dogs. In contrast, fluralaner and fluconazole at 6 and 7 times the therapeutic dose, respectively, did not produce CTA. Contrary to our expectations, these two substances apparently did not cause any adverse effect in the dogs, neither vomits nor diarrhoea. In the case of fluconazole, the lack of adverse signs may be due to the low doses used here, but it seems to be undetectable by dogs and may have a potential to produce CTA at higher doses. Fluralaner, in its commercial form, has a strong smell so it is susceptible to modify importantly the organoleptic characteristics of the food. The dose of fluralaner was increased up to 300 mg/kg, which means a large amount of commercial product that can modify the characteristics of the target food used. Therefore, the use of fluralaner as an aversive agent can be ruled out.

Thiabendazole showed an unexpected low aversive effect on dogs, contrary to previous studies on canids (Ziegler et al., 1982; Gustavson et al., 1983; Massei et al., 2003a) and other species (Massei and Cowan, 2002; Norbury et al., 2005; Gentle et al., 2006; O'Donnell et al., 2010). The dose used was the same or higher than in other studies with canids (Ziegler et al., 1982; Massei et al., 2003a), so the reduced effect may be due to the individual variability and differences in behaviour between domestic and wild canids. Massei et al. (2003a) found individual variability in the response of aversion to thiabendazole by red foxes, as other authors found with other species (Conover, 1989; Ternent and Garshelis, 1999). They suggested that the lack of effect in some individuals could be due to the detection of thiabendazole and subsequent aversion to this agent rather than to the test food. In our case, the dogs from the thiabendazole group did not detect the chemical. However, our results correspond to a pilot study with a small sample, so we should take them with caution.

Thiram is used in agriculture as a fungicide, but it also protects sown seeds from birds and mammals due to its repellent action (Nolte and Barnett, 2000; Lopez-Antia et al., 2014), but it has never been used as CTA agent. One dog acquired CTA to the target wet food, which was rejected during the post-conditioning, while the other dog ate twice the

treated food and had vomits in both cases without acquiring CTA. Therefore, thiram was apparently undetectable by dogs. This fact makes thiram a potential candidate as an aversive substance in predation control, since detectability is one of the main handicaps in the CTA applicability (Burns, 1980; Nicolaus and Nellis, 1987; Gentle et al., 2004). Another advantage of thiram is its low toxicity (Table 1). Accordingly, no negative effects on blood parameters were observed after two ingestions (Table S3).

Levamisole has been used as CTA agent in several studies with controversial results. It has induced long-lasting CTA in rats (Massei and Cowan, 2002) and grey foxes (*Pseudalopex griseus*) (Nielsen et al., 2015), but it failed in ferrets (*Mustela furo*) (Massei et al., 2003b; Norbury et al., 2005) and badgers (Cagnacci et al., 2005), and produced contrasting results in red foxes (Massei et al., 2003a; Gentle et al., 2004). The failures in the generation of aversion happened because animals detected the levamisole and only avoided consuming the food when the levamisole was present. The differences between the studies may be due to the ability of certain strong flavoured foods to mask the taste and smell of levamisole. On the other hand, the ratio between the amount of levamisole and the food may be relevant for masking the flavour of levamisole to a greater or lesser extent (Nielsen et al., 2015). In our study, one of the dogs possibly detected the levamisole despite it was microencapsulated, because it only rejected the target wet food when levamisole was present in the reinforcement. However, the other dog exposed to levamisole developed CTA behaviour, increasing PFR, LT and TSE. The different results between both dogs may be due to individual aversion behavioural differences, but additional experiments with a larger sample size have confirmed its potential as aversive (Tobajas et al., 2019).

LT and TSE increased significantly after conditioning with thiabendazole, thiram and levamisole, indicating that the dogs had an internal conflict between the awareness of the consequences of eating and the food palatability (Forbes, 1998). If these results should be applied to CTA generation in the wild, we can expect that, unlike penned dogs, wild canids could suffer a disruptive effect at early phases of predation, and this could favour prey escape. In this sense, the utilization of the CTA approach to protect ground nest predation from wild canids is the most reliable use of the CTA (Nicolaus and Nellis, 1987; Maguire et al., 2009).

The conclusions of the present study are limited by the reduced number of dogs used for each compound, and by the conservative design of the experiment. In this sense, the pre-exposure (pre-conditioning phase) to the target food reduces the strength of the aversion (Revusky and Bedarf, 1967; Mikulka and Klein, 1977). The dogs were used to the offered target food, which is then assumed as "learned safe" food, thus reducing the CTA (Kalat and Rozin, 1973). In the same way, Mikulka and Klein (1977) observed that leaving the food available for long periods of time in the aversion tests can mask a weak aversion, based on similar studies carried out by Fenwick et al. (1975) with short test intervals. Also, Carroll et al. (1975) observed that neophobia can be found with short test intervals but is not apparent in long test periods. Moreover, our experimental subjects were domestic animals fed by humans during all their life, thus they associate the food coming from humans as safe. In the case of wild animals, the processes of neophobia associated with illness after consumption of food would surely cause an increase in aversion in comparison with domestic animals (Mitchell, 1976).

In summary, the results provided here and in previous studies show that thiabendazole, thiram and levamisole can cause aversion in dogs and that they are good candidates for use as aversive compounds to reduce the predation by wild canids. This pilot study identified thiram as a safe, accessible, cheap and undetectable substance that can induce CTA on canids. Further research with larger number of individuals, with revised doses, will need to be performed to confirm these preliminary results.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2019.103905.

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