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

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Abstract

The use of conditioned food aversion (CFA) can reduce the predation conflict and therefore the incidence of illegal poisoning, which is one of the most important conservation threats for predators and scavengers around the world. CFA 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 CFA, considering effects, margin of safety, accessibility, and detectability. After the review, 15 compounds fulfilled the required characteristics, but only five were finally selected to be tested in CFA 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 CFA effect. Thiabendazole, thiram and levamisole can therefore induce CFA, and thus are potential candidates as aversive compounds for wildlife management. Thiram is a new undetectable, safe and accessible compound that can induce CFA in canids, and opens new possibilities to develop methods of non-lethal predation control.

Keywords: Conditioned taste aversion; learned aversion; predation conflict; non-lethal predator control; wildlife management

1. Introduction

Predation conflict between humans and predators has been occurring since the prehistoric age. This conflict is especially pronounced almost everywhere where the 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 try to avoid damages caused by wildlife to their crops, livestock and game species, by using mainly lethal predator control (Reynolds & Tapper 1996; Bergstrom et al. 2014). Lethal predator control has negative effects on the ecosystems (Gordon et al. 2017; Wallach et al. 2017) and endangered species (Margalida and Mateo 2019), and nowadays, its social acceptability is low and the public demands non-lethal approaches for wildlife control (Cowan et al. 2000; Bergstrom 2017).

Conditioned food aversion (CFA) is a non-lethal predation control method that has been rarely used but is considered a potential tool to reduce predation of wildlife (Nicolaus et al. 1989a; Cowan et al. 2000). CFA is a natural mechanism in animals to prevent poisoning and intoxications (Gustavson et al. 1974). Thus, a toxic food is avoided after an illness induced by the ingestion of a non-lethal dose of that food (Garcia et al. 1974). CFA 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 CFA, which must comply 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, that is also required to avoid intoxications in case that a single individual monopolizes and consumes numerous doses; (3) to have a short period of latency (30 min-two hours) to

64 assure the correct learning of CFA (Garcia et al. 1974); and (4) to be odourless and
65 tasteless to avoid its detection by the predators (Gentle et al. 2004; Nielsen et al.
66 2015).

67 CFA has been experimentally studied in rodents (Gill et al. 2000; Massei and
68 Cowan 2002), wild birds (Nicolaus et al. 1989b; Avery et al. 1995), wild mammals
69 (Nicolaus et al. 1989c; Norbury et al. 2005) and reptiles (Price-Rees et al. 2013). Several
70 chemical compounds have been shown effective to induce CFA, but most of them do
71 not accomplished with all characteristics required for CFA, and more effective CFA
72 compounds need to be found (Gill et al. 2000). Issues of safety and detectability
73 severely limit the number of compounds that may be used for practical applications of
74 CFA in wildlife management. The application of the CFA with safety must be one of the
75 main characteristics of the potential candidates for its use in wildlife management. Up
76 to now, the CFA experiments performed have not taken into consideration this
77 important issue, and some had to be discarded due to their high toxicity or detrimental
78 effects on animal health (Conover 1989; Dueser et al. 2018). The other major problem
79 is that the detectability of the compounds by the animals causes rapid discrimination
80 between foods with and without CFA compounds (Burns 1980; Nicolaus and Nellis
81 1987; Gentle et al. 2004; Nielsen et al. 2015). In this case, if the compound added to
82 the bait is detected, the animals associate illness with the substance and avoids only
83 treated baits, acting as secondary repellent (Sayre and Clark 2001; Cagnacci et al.
84 2005). Microencapsulation is a potential way of masking the odour or taste, but it
85 requires experimental research to test their effectivity (Cotterill et al. 2006; Shukla et
86 al. 2011). Expensive chemical compounds that need to be fabricated and/or handled
87 under special conditions, and/or 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 CFA for wildlife management. For these reasons, we reviewed the compounds previously used in CFA and assessed potential new candidates that accomplish the desirable characteristics as aversive compounds for canids. Finally, we evaluated the potential of five selected compounds in a pilot study to induce CFA in penned dogs, to test them as potential 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 and databases of chemical substances registered in Spain (i.e. pesticides, biocides and pharmaceuticals) in order to identify: (1) substances that have been used in CFA in canids or other wildlife species, (2) substances that have been described as potential CFA 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 CFA, (4) LD_{50} in rat and dog of the selected substances, (5) odour and taste of the selected substances, (6) potential doses to produce CFA in canids, and (7) commercial availability of the product. Based on the LD_{50} values in rat and the potential doses to induce CFA 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 CFA. The purpose of this literature review was to identify substances that have been successfully used to induce CFA and to identify new

112 candidates that could be used in further experimental CFA tests with canids.
113 Therefore, the selected substances should have an acute MOS above 10 (i.e. the lethal
114 dose should be at least 10-fold higher than the potential CFA dose). Moreover, the
115 selected compounds should be also odourless and tasteless, and available in the
116 market with an approved use as a veterinary drug or other use.

117 A total of 167 substances were included in the literature review (see full list in
118 supplementary material, Table S1). Based on the data obtained from all these
119 substances, we first selected the 15 chemical compounds that have been or could be
120 used to induce CFA (Table 1). These substances were chosen because among their
121 adverse effects they include gastrointestinal symptoms related to the CFA mechanism
122 (i.e. nausea, vomits). From these compounds we finally selected five compounds that
123 could be good candidates to induce CFA in wild canids with a low risk of lethality as
124 expected by the MOS > 10. In a second phase of the study, the five selected substances
125 were tested in a pilot experiment to induce CFA in penned dogs. Two of these
126 compounds had been successfully used before in CFA with canids (i.e. thiabendazole
127 and levamisole). Thiabendazole was taken because of the available literature on this
128 substance as CFA inducer. Levamisole has also been tested as CFA inducer, but
129 important practical limitations may exist with this compound because of its potential
130 detectability by taste and odour. The other three substances (thiram, fluconazole and
131 furalaner) fulfilled most of the requirements to be used as aversive but have not been
132 previously tested.

133

134 *2.2. Animals*

Five males and seven females of adult Beagle dogs (*Canis lupus familiaris*) were used in the experiment. Dog's 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 dry (Gosbi® Premium Performance) and/or wet (Gosbi® Fresko Chicken) dog food and they were fasted during 24 hours before each test. The dogs were housed individually in separate indoor pens (size: 1.6 × 4.3 × 3 m), following 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 minutes every day.

2.3. Tested aversive compounds

The selection of substances and doses were based on literature as described above (Table 1). Thiabendazole and levamisole have been tested in the past for their CFA effects. To our knowledge, thiram, fluconazole and fluralaner have never been used as CFA agents. Thiabendazole is a benzimidazole with anthelmintic properties that has been previously used to generate CFA in several mammalian species (Gustavson et al. 1983; Conover 1989; Ternent and Garshelis 1999). Levamisole

159 chlorhydrate (levamisole hereafter) is an imidazothiazole with anthelmintic properties
160 that has been used in CFA studies with laboratory rats (Massei and Cowan 2002), foxes
161 (*Vulpes vulpes* and *Pseudalopex griseus*) (Massei et al. 2003a; Nielsen et al. 2015) and
162 Eurasian badgers (*Meles meles*) (Cagnacci et al. 2005). Thiram is a dithiocarbamate
163 fungicide that has been used as a repellent both in birds and mammals (Nolte and
164 Barnett, 2000; Werner et al. 2010). Fluralaner, one of the new and structurally-unique
165 isoxazolines, is an ectoparasiticide selected because it is considered a safe drug causing
166 vomiting, decreased appetite and diarrhoea as the most common adverse reactions at
167 the recommended therapeutic doses (25–56 mg/kg) in dogs (EMA, 2014). Fluconazole
168 is a triazole used as antifungal with a wide acute MOS. Nausea, vomiting and anorexia
169 have been described at therapeutic doses of this in dogs (Mueller, 2007).

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

181 In order to reduce the levamisole bitter taste described in previous studies with
182 canids (Massei et al. 2003a; Gentle et al. 2004), it was microencapsulated with

183 Precirol® Ato 5 (glyceryl palmitostearate) as the hydrophobic binder using a melt-
184 granulation technique (Hamdani et al. 2003; Mašić et al. 2012). The other chemicals
185 were used in the pure composition, except for fluralaner that was used in the
186 commercial form. Levamisole was microencapsulated by the Drug Development
187 Service, Faculty of Pharmacy, University of Barcelona. Fluconazole and thiram were
188 purchased from Sigma-Aldrich®, fluralaner (Bravecto™) from MSD Animal Health, and
189 thiabendazole from Alfa Aesar®.
190
191 *2.4. Experimental design of the aversive compounds assay*
192 In order to evaluate the treatment effect of the compounds on the dogs
193 feeding behaviour, we performed a Before-After Control-Impact (BACI) design
194 (Underwood 1994). BACI analysis approaches include generalized linear mixed models
195 (McDonald et al. 2000), where a significant interaction between treatments (each
196 substance and control group) × time (pre-conditioning and post-conditioning) indicates
197 that the experimental treatment had an effect on dogs feeding behaviour. We
198 followed the typical phases in CFA experiments: pre-conditioning (only food, four
199 trials); conditioning (food + aversive agent, single trial); post-conditioning (only food,
200 four trials); reinforcement (food + aversive agent, single trial). This initial assay was
201 performed only with a pair of dogs per each chemical tested, in order to test the
202 potential of these substances to induce CFA on dogs. This decision was made following
203 the current ethical and animal welfare standards to reduce the number of individuals
204 used in the animal experimentation. Those substances that yielded best CFA results in
205 this study will be tested with a larger number of animals in further studies (see Tobajas
206 et al. in press).

Dogs were enrolled in the experiment on day 1 (start of pre-conditioning). During pre-conditioning period, they were fed with two types of food, dry and wet, and 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 minutes 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 hours 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. 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 minutes. Bowls were then removed

231 from the dog pens and weighted to calculate the amount of food eaten and the
232 proportion of food rejected (PFR). Dog behaviour was recorded with a video camera
233 (Spartan, HCO Outdoor Products, Norcross, GA, USA) to observe the signs of adverse
234 effects of conditioning and to estimate the latency time (LT; time from food offer to
235 start eating) and the time spent eating all food (TSE). LT and TSE (in min) were used as
236 CFA indicators (Massei et al. 2002; Webb et al. 2008). If at the end of the 30-min
237 presentation dogs had not started or eaten all the food, LT and TSE, respectively, were
238 recorded as 30 min. Feeding behaviour was also video recorded during the pre-
239 conditioning phase.
240
241 *2.5. Haematology and serum biochemistry analysis*
242 Haematology and serum biochemistry were studied to evaluate the possibility
243 of detrimental effects on health. Blood samples were obtained from all the dogs,
244 including controls, one day before and one day after the conditioning and
245 reinforcement with the aversive substances. Blood samples (4–5 mL) were obtained by
246 puncturing the brachial vein, using a 5 mL syringe and a 21 G needle. All the analyses
247 were made at the Interdisciplinary Laboratory of Clinical Pathology, Interlab-UMU,
248 Campus of Excellence Mare Nostrum, University of Murcia, Spain.
249
250 *2.6. Data analysis*
251 The effect of treatment (fixed factor) on PFR, LT and TSE were analysed by a
252 generalized linear mixed model (GLMM) to investigate differences between
253 treatments in the pre-conditioning, and in the post-conditioning phase. The
254 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. Individual dogs were 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 CFA 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, for other four this information was not available and the other six have some odour and taste that may affect the conditioning process if the animals

279 associate the adverse effect with these physical properties of the substances (Table 1).
280 Only three of these substances had been previously used in CFA assays (i.e.
281 thiabendazole, levamisole and clotrimazole). The oral acute LD_{50} in rat was available
282 for all the considered compounds, ranging from 480 mg/kg of levamisole to >10,000
283 mg/kg of fenbendazole. The oral acute LD_{50} in dog was only available for nine of the
284 considered compounds (Table 1). The acute MOS was <10 in four substances, between
285 10 and 100 in nine substances and >100 in two substances (Fig. 1).

286 Only those substances with an acute MOS > 10 were considered to be included
287 as CFA inducers. Thus afoxolaner, clotrimazole, metaldehyde, niclosamide and
288 praziquantel were firstly excluded. According to the MOS calculated,
289 pyrazinobenzazepines and tetrahydropyrimidines would be the best candidates to be
290 tested as CFA inducers, but these anthelmintics also show important differences in
291 LD_{50} values between rat and dog (i.e. oxantel, Table 1). Hence these substances were
292 not finally selected following a precautionary principle. For the same reason,
293 fenbendazole was also excluded. We finally selected 5 substances to be tested
294 experimentally with penned dogs: thiabendazole, microencapsulated levamisole,
295 thiram, fluconazole and fluralaner. Thiabendazole was included in the experimental
296 tests because it is a confirmed CFA inducer in canids. We also included levamisole
297 because it is a CFA inducer in several mammal species, but here we have employed a
298 microencapsulation to reduce its detectability by canids. Finally, three other
299 substances with MOS > 10 that can produce gastrointestinal symptoms related to the
300 CFA mechanism (nausea, vomits) were selected for the experimental tests with dogs,
301 two odourless and tasteless (i.e. thiram, fluconazole) and another one with unknown
302 organoleptic characteristics (i.e. fluralaner) (Fig. 1).

303

304 *3.2. Conditioned food aversion*

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

323 Comparing PFR between pre-conditioning and post-conditioning phases (Fig. 2),
324 we found a significant effect of the "treatment x phase" interaction ($F_{11, 84}$ = 2.756, p =
325 0.004). Differences between pre- and post-conditioning phases for PFR were significant
326 for the levamisole (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 minutes 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 to 10.55 $x10^3$ cells/µL; male: 4.2 to 10.02 $x10^3$ 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 used as CFA agents to reduce the conflicts with wild predators. Thiabendazole and levamisole were already known as CFA inducers in canids, and thiram is a new candidate with a very high acute MOS (aprox. 100), which should be confirmed as CFA inducer in further experimental studies with more animals. Moreover, thiram was 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 CFA inducers in canids. However, the adverse effects produced by

some of the substances reviewed and used in CFA 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 CFA 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 CFA 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) makes them inappropriate candidates for CFA. Finally, other compounds such as insecticides and fungicides have been tested for CFA, mainly causing an agonist cholinergic 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 agonist cholinergic effect, (e.g. levamisole, thiabendazole, trimethacarb and carbachol) have shown good results in CFA 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 CFA inducers (Schafer 1972; Conover 1990).

399 Taste and odour of most compounds used in CFA studies modify the original
400 food and taste of foods (Burns 1980; Nicolaus and Nellis 1987; Gentle et al. 2004;
401 Nielsen et al. 2015). Only 17α-ethinyloestradiol and thiabendazole appear to be
402 undetectable (Gustavson et al. 1983; Nicolaus et al., 1989a; Gentle et al. 2006; Dueser
403 et al. 2018). To solve detection problems, a masking odour or taste has been used,
404 although with limited success on baits (Cotterill et al. 2006; Nielsen et al. 2015). Other
405 possibility suggested with few positive results is the microencapsulation technique
406 (Burns 1983; Mašić et al. 2012), but it requires an increase of cost and specialized
407 machinery. However, the new formulations and manufacturing methods could enable
408 its use in an effective and economical way.
409
410 *4.2 Assays with penned dogs*
411 The assay results with dogs showed that levamisole, thiabendazole and thiram
412 produced CFA in dogs. In contrast, fluralaner and fluconazole at 6 and 7 times the
413 therapeutic dose, respectively, did not produce CFA. Contrary to our expectations,
414 these two substances apparently did not cause any adverse effect in the dogs, neither
415 vomits nor diarrhoea. In the case of fluconazole, the lack of adverse signs may be due
416 to the low doses used here, but it seems to be undetectable by dogs and may have a
417 potential to produce CFA at higher doses. Fluralaner, in its commercial form, has a
418 strong smell so it is susceptible to modify importantly the organoleptic characteristics
419 of the food. The dose of fluralaner was increased until 300 mg/kg, this means a large
420 amount of commercial product that modified the characteristics of the amount of the
421 target food used. Therefore, the use of fluralaner as an aversive agent can be ruled
422 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 seeds 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 CFA agent. One dog acquired CFA 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 CFA. 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 CFA 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 CFA agent in several studies with controversial results. It has induced long-lasting CFA 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 mask 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 CFA behaviour, increasing PFR, LT and TSE. The different results between both dogs may be due to individual aversion behavioural differences, and a new experiment with a larger sample size is necessary to confirm its potential as aversive.

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 CFA generation in the wild, we can expect that, unlike penned dogs, wild animals could suffer a disruptive effect at early phases of predation, and this could favour prey escape.

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 CFA (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. Another consideration to keep in mind is that 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 foods 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 canids and that they are good candidates for use as aversive compounds in the wild. This pilot study identified thiram as a safe, accessible, cheap and undetectable substance that can induce CFA on canids. Further research with larger number of individuals, probably with revised doses, will be performed to confirm these preliminary results.

Conflict of interest

494 We declare that none of the authors of this manuscript has any actual or potential
495 conflict of interest including any financial, personal or other relationships with other
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497
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505
506 **References**
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728

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 et al., 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] Scholz and Schultes, 1973a; [k] Scholz and Schultes 1973b; [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.

Figure 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 LD50 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.

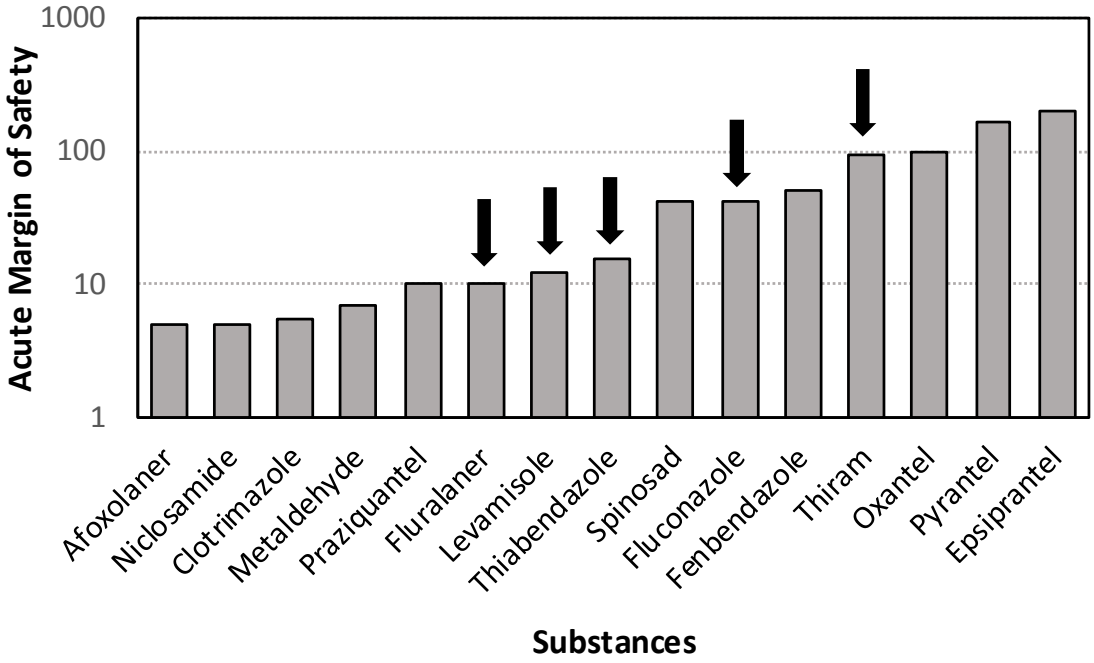

Figure 2. Proportion of wet food rejected (untransformed data) by treatment and control dogs across four 30-min two-choice pre-conditioning and four 30-min two-choice post-conditioning taste aversion tests. Error bars represent standard errors of the mean. ** indicate differences with $p < 0.01$.

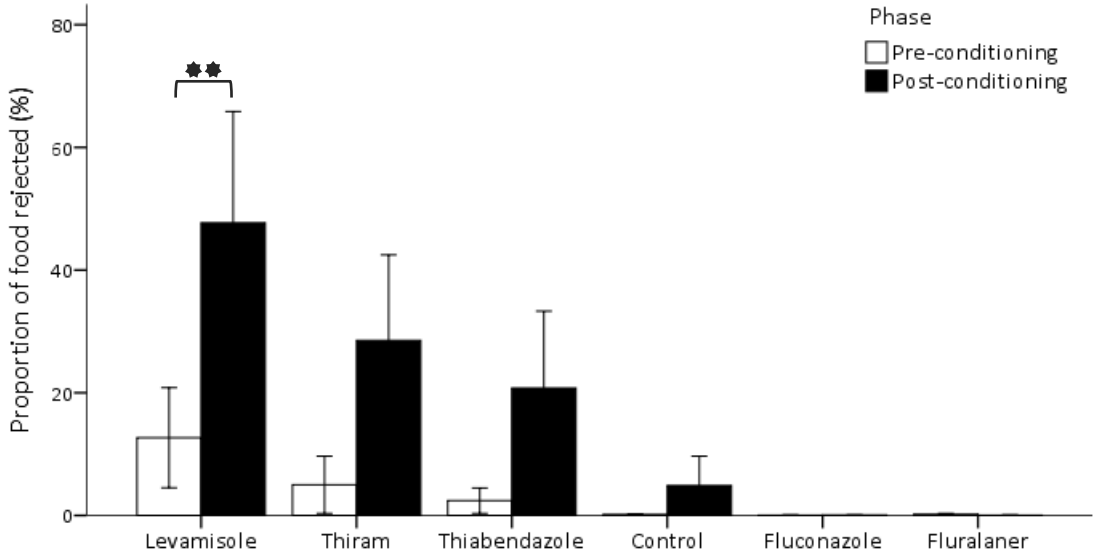

Figure 3. Latency time to start eating the wet food (seconds) by treatment and control dogs across four 30-min two-choice pre-conditioning and four 30-min two-choice post-conditioning taste aversion tests. Error bars represent standard errors of the mean. ** indicate differences with $p < 0.01$.

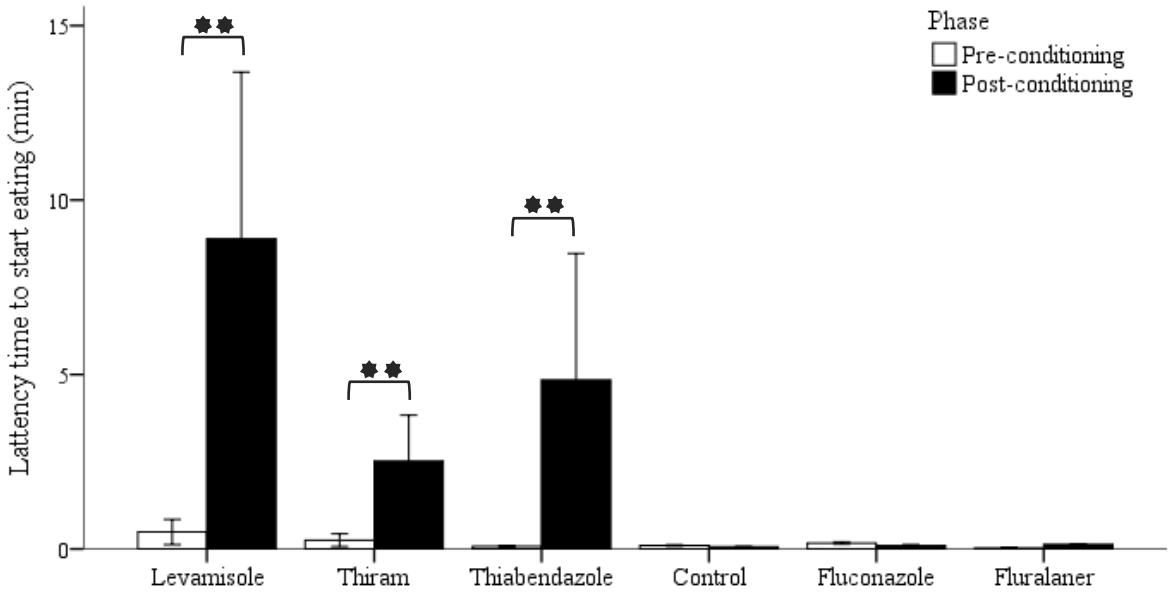

Figure 4. Time spent eating all wet food (minutes) by treatment and control dogs across four 30-min two-choice pre-conditioning and four 30-min two choice post-conditioning taste aversion tests. Error bars represent standard errors of the mean. * and ** indicate differences with $p < 0.05$ and $p < 0.01$,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