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Pen trial of estrogen-induced conditioned food aversion to eggs in raccoons (*Procyon lotor*)

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ABSTRACT

Aversive conditioning is a promising but unproven non-lethal approach to reducing mammalian depredation on the eggs of ground-nesting birds, terrapins and sea turtles. This research tested the efficacy of oral estrogen concealed in a bland carrier as an aversive agent for wild-caught raccoons (*Procyon lotor*) under controlled conditions. Nine treatment group raccoons were given six estrogen-injected eggs every other day during a 14-day treatment phase, and then given a combination of two estrogen-injected eggs, two fresh eggs, and two carrier-only injected eggs every other day during a 14-day challenge phase. Nine control group animals received six carrier-only injected eggs every other day during the treatment phase, and then two fresh eggs and four carrier-only injected eggs every other day during the challenge phase. All treatment animals exhibited a conditioned food aversion (CFA) after 1–8 egg feedings (15–116 mg of estrogen per kilogram of body mass). All later sampled at least a few eggs, but they consumed fewer eggs than the control animals during both the treatment phase ($p < 0.001$) and challenge phase ($p < 0.001$). No raccoon could distinguish treated from untreated eggs during the challenge phase ($p = 0.740$); the treatment was undetectable by visual or olfactory cues. We observed no conspicuous changes in the feeding activity, behavior or demeanor of the treatment animals. Treatment and control animals ate ($p = 0.629$) and drank ($p > 0.05$) comparably. Treatment animals gained less mass than control animals ($p = 0.013$), but there was no apparent relationship between estrogen intake and mass change ($p = 0.912$). Testes of treatment males were similar in volume and mass ($p = 0.712$) to those of control males. Treatment animals experienced higher frequencies of abnormal feces ($p < 0.005$) and dermatitis ($p = 0.001$) than control animals. A treatment female died during the trial from an aborted late-term pregnancy, probably induced by the estrogen. Necropsies revealed no obvious tissue or organ damage from estrogen exposure. The conditions of this pen trial provide a conservative test of the potential for using an estrogen-induced CFA as a management tool for reducing egg consumption in the wild. Ingestion of 20–80 mg kg^{-1} of estrogen delivered over 1–4 days would be sufficient to bring about a reduction in egg predation using this method. A full-scale field trial of estrogen is likely to be productive under circumstances where all of the target population is subject to treatment.

1. Introduction

Aversive conditioning is a promising but unproven non-lethal approach to reduce mammalian depredation on the eggs of ground-nesting birds, terrapins and sea turtles (Nicolaus and Nellis, 1987; Conover and Lyons, 2003; Shivik et al., 2003; Macdonald and Baker, 2004). A potentially powerful technique is the use of conditioned food aversion (CFA; Conover, 2002) to "teach" mammalian nest predators, such as raccoons (*Procyon lotor*) and red foxes (*Vulpes vulpes*), to avoid the eggs of ground-nesting wildlife (Conover, 1989; Nicolaus et al., 1989b; Reynolds, 1999; Cowan et al., 2000; Macdonald and Baker, 2004).

An ideal aversive compound would (1) produce a severe short-term illness in the predator (Nicolaus et al., 1989b), (2) cause this illness only after a brief time delay (~2 h), allowing the predator to consume an effective dose of the compound (Conover, 1997), (3) have an effective (illness-producing) dose far below the lethal dose (Gill et al., 2000), (4) be undetectable to the predator when present at appropriate concentrations in a bait (Conover, 1984, Gill et al., 2000), (5) be physically stable in baits when distributed under field conditions (Nicolaus

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et al., 1992), (6) produce no chronic or long-lasting health effects (Gill et al., 2000), (7) work equally well for protection of both solitary and colonial nesters, and (8) be capable of deployment without the observer making a close approach to the actual nest or colony (Conover, 1990; Conover and Lyons, 2003). The expectation is that predators will develop an aversion to treated eggs (the mimic), will generalize this aversion to non-treated eggs (the model), and will cease depredating all eggs (Cowan et al., 2000).

A host of potential aversive compounds have been proposed and tested for this application with raccoons, including emetine dihydrochloride (Conover, 1989, 1990), oral estrogen (Nicolaus et al., 1989a), cinnamamide and thiabendazole (Gill et al., 2000), carbachol (Cox et al., 2004), and pulegone (Conover and Lyons, 2003). Most have proven ineffective, effective for only a short duration, difficult to deploy safely, laden with side effects, or toxic in the environment (Conover, 1990). Oral estrogen appears to be a particularly promising alternative, which has been reported to provide a non-toxic, but effective means of inducing CFA in raccoons (Nicolaus et al., 1989b). A variety of free-ranging small and medium-sized predators have been observed to significantly reduce their consumption of eggs after consuming surrogate eggs containing estrogen hidden in a bland carrier (Semel and Nicolaus, 1992; Nicolaus et al., 1989b). On the other hand, Ratnaswamy et al. (1997) used estrogen-treated chicken eggs to induce in raccoons an aversion to sea turtle eggs on a barrier beach in Florida, U.S.A. Consumption of treated eggs by some unknown number of raccoons, out of a very large raccoon population, failed to prevent depredation of turtle nests. Ratnaswamy et al. (1997) thus concluded that the adoption of this technology awaits further research. While there are a host of methodological and practical reasons why the results of Ratnaswamy et al. (1997) might have been negative, the reality is that there has been no subsequent widespread adoption of what was once viewed as a major breakthrough in wildlife damage management technology.

Previous studies have deployed treated eggs in field situations with little control over either the number and identity of predators involved (Nicolaus et al., 1989b; Ratnaswamy et al., 1997) or the actual exposure to the treatment (Semel and Nicolaus, 1992). Our objective was to further test the efficacy of oral estrogen as an aversive agent for raccoons under controlled conditions. Specifically, we wanted to learn: (1) Does ingestion of eggs treated with a mild dose of estrogen reliably induce aversion? (2) Do treated raccoons cease eating eggs or simply reduce egg consumption? (3) Can raccoons distinguish between estrogen-injected eggs and similar but non-injected eggs? (4) Does this treatment produce changes in behavior or appetite? The administration of exogenous estradiol is known to influence feeding behavior in animals, expressed primarily as a decrease in meal size (Geary, 2001). And (5) Does this treatment affect raccoon physical condition or health? High levels of estrogen prevent or terminate pregnancy (Asa, 2005), and under- and over-exposure to estrogen influences testicular development and function (Coveney et al., 2001, Sierens et al., 2005).

We report results based on a pen trial with captive raccoons that was conducted in preparation for a field trial with free-ranging animals. We recognize that captive behavior may differ from free-range behavior for a variety of reasons (Gustavson and Gustavson, 1985), and that captive-study results are likely to provide an inherently conservative assessment of the potential for CFA to reduce egg predation in field applications (Nicolaus and Nellis, 1987). That is, with restricted opportunity for avoidance of the mimic food at a distance, limited alternative foods and exposure to potentially averse-resistant individuals in neighboring pens, captive animals may exhibit a reduced susceptibility to aversion simply because of their circumstances (Conover, 1989). A captive study, if successful, confirms the formation of a CFA under conditions where such confirmation is least likely. Nevertheless, a captive study has the potential to provide insights that are unattainable with free-ranging animals, particularly with respect to effective dosages, behavioral responses and physical effects.

2. Materials and methods

2.1. Animals

We live-trapped 32 adult (ages 1–7 years according to tooth aging) raccoons from the Skidmore Island and mainland sections of Eastern Shore of Virginia National Wildlife Refuge in Northampton County, Virginia, U.S.A. (Martin, 2007). Each individual was sedated lightly with an intramuscular injection of Ace-Ketamine administered at a dosage of $0.2\,ml\,kg^{-1}$ body mass (ketamine concentration $100\,mg\,ml^{-1}$; acepromazine concentration $10\,mg\,ml^{-1}$; Dueser et al., 2013, Kreeger et al., 2002). Each was then examined by a veterinarian for external signs of injury or illness; animals that appeared listless or unhealthy were excluded from the pen trial. Obviously pregnant females also were excluded. All trapping and handling conformed to American Society of Mammalogists guidelines (Sikes et al., 2011) as well as Utah State University Animal Care and Use Committee policies under Protocol 952. We live-trapped 32 adult raccoons, but only 18 were included in the pen trial. Ten were released back to Skidmore Island, 2 were released back on the mainland, and 2 were retained as replacement animals for the pen trial (but not used).

Eighteen of the healthy individuals were selected at random for inclusion in the pen trial. Each was assigned randomly to a cage and to either the treatment group (4 females, 5 males) or control group (5 females, 4 males), and then caged within sight of five other raccoons, both control and treatment. Multiple randomizations using a coin toss were carried out to balance the assignments between genders and source populations. All of the animals were approximately the same size (~3.8 kg) at the outset, increasing the likelihood that they would be similarly susceptible to the effects of the treatment. We weighed the animals at the beginning and at the end of the trial. We used the average of these two values to estimate egg, food, water, and estrogen consumption per kilogram of body mass.

2.2. Animal care

The raccoons were housed in an 18-cage pen facility in a rural, forested setting ~15 km from the capture site (37.390618°N, 75.924661°W; Martin, 2007). There were three pens, each consisting of six cages made of pressure-treated lumber and wire. Each cage was a cube 1.2 m on each side (floors were ½-inch hardware cloth, and the walls and ceiling were 2-inch mesh kennel wire). Each was outfitted with a 38 l plastic den box, 1 l water bottle, set of food bowls attached to a wooden platform, and a "pacifier" (a 20 cm length of 5 cm diameter PVC pipe smeared on the inside with peanut butter) designed to provide a diversion from chewing on the wooden framework. The pen facility was designed to minimize stress on the animals (*sensu* Morgan and Tromborg, 2007). The den box provided retreat space, and the platform and pacifier provided environmental enrichment. The entire facility was located beneath a deciduous forest canopy, providing exposure to a normal diurnal light cycle and natural background sights, smells and sounds. A sloped roof of 6 mil black plastic sheeting provided additional protection from sun and rain. We minimized unnecessary activity and noise.

Each individual received a daily ration of 140 g of dry dog food and water *ad libitum*. Each was treated over the first 3 days with three doses of the drug fenbendazole (Panacur®, $50\,mg\,kg^{-1}$) mixed with the dog food in an effort to reduce the health effects of potentially heavy endoparasite loads. With crude protein content of 18.0%, crude fat content of 6.5% and energy density of $\sim 13.3\,kJ\,g^{-1}$ (or $\sim 3.17\,kcal\,g^{-1}$, calculated *as per* Dzanis (1998)), this food provided a diet on which the raccoons should have been able to maintain or gain weight. We recorded daily food and water consumption for each animal to ensure that they were adequately provisioned, and we made frequent observations of how each interacted with the dog food and eggs. We also recorded stool characteristics during the treatment and challenge

Fig. 1. Study design for test of estrogen-induced conditioned food aversion to eggs in raccoons (*Procyon lotor*).

phases, classified as either normal (i.e., firm) or abnormal (i.e., soft, runny or diarrhea). Finally, we made casual observations of any signs of stress (e.g., fear, stereotypic pacing, failure to feed and reduced activity; Broom, (1991), Morgan and Tromborg, (2007)).

During feeding events, the food bowls and water bottles were removed from each cage, cleaned and refilled, and feces were scooped from the cage. Food bowls containing new treated or fresh eggs or dog food were returned to the cages in as short a time as possible, always within 2.0 h. Cages were pressure washed only every second or third day to minimize disturbance. We covered feces, spilled food and egg drippings under the pens with hydrated lime after every washing. All animals were monitored daily for general appearance and wellbeing. All of the animals bore at least a few ticks, but each appeared to be healthy and vigorous at the outset.

2.3. Egg preparation

We employed 17 α-ethinyl estradiol, a powdered form of estrogen (Spectrum Chemical Mfg. Corp.), as the aversive agent (Martin, 2007). To prepare the powdered estrogen for injection, we made a gel carrier by mixing 18 g of arrowroot powder with 500 ml cold water and heating on a stove while constantly stirring. Once the solution cleared and gelled, we allowed it to cool and blended 500 ml of the gel with 5.0 g of estrogen powder. The carrier was used to facilitate injection of the estrogen into the egg, keep the estrogen suspended in the yolk, and prevent the estrogen from losing potency by becoming bound with albumen (Nicolaus et al., 1989a; Nicolaus et al., 1992).

We added six drops of blue food coloring to the arrowroot-estrogen mixture to provide a color contrast with the egg contents, allowing us to detect whether or not an estrogen plug had been consumed. Medium white chicken eggs (average size ~50 ml; energy density ~7.50 kJ ml^{-1} or ~375 kJ per egg; Carey et al., 1980) were prepared by using a 30 ml plastic syringe with a 16-gauge needle to pierce the shell at the tapered end and suck out 2 ml of the contents, both yolk and albumen. We then injected a 1 ml plug of the estrogen-arrow root gel mixture (10 mg/ml) using a 3 ml syringe with a 16-gauge needle thrust into the yolk. The resulting needle hole was then sealed using a glass rod dipped in melted paraffin. Nicolaus et al. (1989b) and Semel and Nicolaus (1992) reported that a 10 mg dose of estrogen per egg was more effective in inducing a CFA than either a higher or lower dose. Following their recommendation, we injected each egg with a 10 mg dose of estrogen. All eggs were stored at 3 °C; treated eggs were stored at 3 °C for 1–2 days before use.

At the outset of this study, we had planned to use a flour-water mixture as the estrogen carrier as per Semel and Nicolaus (1992). The use of the flour-based carrier quickly proved to have several drawbacks. We could smell the flour-estrogen mixture, so we assume raccoons could as well. Furthermore, this mixture began to coagulate and clog the hypodermic needle after about an hour, when the gluten became stringy. The mixture had to be used immediately and could not be stored. Furthermore, outside of refrigeration, the dough began to ferment in less than 24 h and either blew off the wax plug or cracked the egg from the pressure.

We therefore tested a variety of other possible carriers before beginning the actual trial, including wheat flour, potato starch, tapioca starch, guar gum, rice starch, arrowroot starch, cornstarch, gum Arabic, gelatin, and pectin (Martin, 2007). Each of these food thickeners was mixed with water, cooked and then tasted and smelled by a panel of four human judges. Only the arrowroot starch was undetectable by taste or smell, had a smooth consistency, and remained injectable after being refrigerated overnight. Furthermore, a sample left outside in humid 35 °C heat for several days showed no signs of spoilage. The results showed arrowroot starch to be a good choice because the raccoons proved unable to distinguish between injected and non-injected eggs.

2.4. Study design

The study design consisted of five phases (Fig. 1):

2.4.1. Setup phase (June 11–28)

Raccoons were captured, sedated, examined by a veterinarian, caged and treated with fenbendazole as captured.

2.4.2. Acclimation phase (June 28–July 7)

The 18 caged raccoons were acclimated to captive conditions and normal feeding and cage maintenance procedures on a standard schedule. They received only dog food and water during acclimation.

2.4.3. Treatment phase (July 8–20; egg-feeding days 1–7)

The treatment phase was designed to assess the rate of onset of a CFA following exposure to estrogen-injected eggs. The animals assigned to the treatment group received 6 estrogen-injected eggs without dog food on egg-feeding day 1. They received dog food every day thereafter, along with 6 estrogen-injected eggs every other day for the next 12 days (7 egg feedings). The animals assigned to the control group received 6 carrier-injected eggs with no estrogen on the same schedule. We tallied the number of eggs consumed (i.e., eaten or broken) by each animal per feeding.

All eggs were presented at the normal feeding time between 1700

and 1800 h. At 0900 h the next day, we recorded egg condition as "intact" or "consumed," and we recorded food and water consumption for each animal. We converted consumption values to approximate caloric values using the caloric densities of ~375 kJ per egg and ~13.3 kJ g^{-1} for dry dog food. Because there was some spillage of both egg contents and dog food, these consumption values are maximal values; actual intake might have been somewhat less in many cases.

We maintained two additional raccoons in kennels out of sight of the caged animals during the treatment phase. We gave these animals large numbers of fresh eggs (12–18) to determine how many they would consume at one feeding.

2.4.4. Challenge phase (July 21-August 3; egg-feeding days 8–14)

The challenge phase was designed to test the willingness of the treatment group raccoons to "sample" eggs and their ability to discriminate among fresh eggs, estrogen-injected eggs, and carrier-only injected eggs. Each individual in the treatment group received dog food every day, along with 2 eggs of each type (which were marked with a pencil for identification) every other day for an additional 7 egg-feeding days. Each individual in the control group received 2 fresh eggs and 4 carrier-only injected eggs on the same schedule. For each egg-feeding day, we tallied the number of each type left undamaged.

2.4.5. Conclusion (August 5–7)

At the conclusion of the study, we euthanized each animal with Beuthanasia D and followed a systematic tissue collection protocol during necropsy to obtain tissue sets to examine for general condition, the presence of lesions, and endoparasite infections (Appendix A). We extracted a premolar to section for age. We visually compared the appearance of tissues and organs between treatment and control animals, measured the volume and mass of both testes for each male, and submitted tissues to The Utah Veterinary Diagnostic Laboratory for histopathology diagnosis. Tissues were cut into blocks with maximum dimensions of 1 × 1 × 0.5 cm and preserved by freezing and/or fixing in 10% buffered formalin, except for bone marrow, which was taken by splitting a 2-cm section of femur and dropping it into formalin.

2.5. Statistical analyses

Each animal in the treatment group was housed within view of one to three (average 1.9) other treatment animals and one to three (average 2.3) control animals. The responses of individual animals may, therefore, not have been strictly independent. This raises the possibility that the establishment and persistence of an aversion could have been delayed or impeded by exposure of an averse-prone animal to a nearby averse-resistant or control animal, rendering the test for an aversion inherently conservative. The basic data consisted of repeated observations on sets of control and treatment animals, but inequality of sample sizes during the challenge phase rendered repeated-measures analysis infeasible. We thus resorted to "per individual" analyses based on average values over time for each animal ($n_{control}$ = 9 individuals and $n_{treatment}$ = 9 or 7 individuals, depending on the dependent variable). For comparative purposes, we report sample descriptions as means and standard errors ($\bar{x}$ ± 1 se). Nevertheless, all comparisons of sample groups were analyzed in XLSTAT (Addinsoft, 2017) using non-parametric tests (nominal $\alpha = 0.05$) with a correction for continuity and a Bonferroni correction for multiple comparisons (Mann-Whitney U, Kruskal-Wallis H with X^2 approximation, and X^2 test of association; Zar, 1999). Because we were testing the hypothesis that the estrogen treatment would result in reduced egg consumption, we used one-tailed tests for control-treatment comparisons of egg consumption. We used two-tailed tests for all other comparisons.

3. Results

3.1. General behavior and feeding behavior

The general behavior of the caged raccoons was highly variable. Most of the animals were social, non-aggressive and curious; two males occasionally growled in the presence of caretakers. Every individual spent much of the day lounging or sleeping on top of the nest box, making no effort at concealment. Some individuals showed immediate interest in their food at each feeding, while others exhibited disinterest for a time. Some chewed the wooden framework of their cages while others did not, and some habitually stole their neighbors' pacifiers through the wire. We saw little evidence of typical behavioral indicators of stress such as fearfulness, reduced exploratory behavior, increased vigilance, aggression and tendency to startle (Morgan and Tromborg, 2007). Most individuals learned to drink from a water bottle on the first day of acclimation, but some took 2–3 days to catch on. We observed no consistent difference in behavior or sociality between treatment and control animals.

Both groups of raccoons averaged 4.2 years of age (range 1–7), so it is possible that all had prior experience with eggs of some type. They quickly learned to manipulate and consume eggs. They used a variety of methods, but all attempted to consume the entire contents of the egg. They usually bit off one end and licked out the contents, and then sometimes ate the shell. Some individuals simply crunched up and swallowed the entire egg, while others spit out the chewed shell. There was no apparent discrimination between yolk and albumen, and no obvious attempt on the part of the treatment group animals to avoid ingesting the estrogen plug. Perhaps because only a few eggs were presented at each meal, the raccoons tended to eat rather than simply damage the eggs. Unlike Semel and Nicolaus (1992), we observed very few occasions when eggs were opened, but not consumed.

The two raccoons that were given 12–18 fresh eggs at each feeding continued to break eggs even as they became satiated. They tended to eat yolk in preference to albumen and to spill large quantities of egg contents. Spillage of egg contents was much more common than with the treatment and control animals.

3.2. Number of eggs consumed

All of the raccoons ate every egg presented on egg-feeding day one. Eight of the nine control group animals ate every egg provided subsequently throughout the treatment and challenge phases (Fig. 2A). One male (animal #1) rejected two eggs on egg-feeding day two, but ate every egg presented thereafter. The control animals were eager consumers of eggs, eating 754 of the 756 eggs presented (99.7%). They consumed 5.97 ± 0.032 eggs per day during the treatment phase and 6.0 ± 0.000 during the challenge phase ($p = 0.084$).

Treatment group animals exhibited much greater variability, but some degree of aversion became evident for all nine (Fig. 2B). Eight rejected some or all eggs subsequent to egg-feeding days 1–4 ($\bar{x}$ = 2.1 days; 8–24 eggs consumed before rejection). All rejected some eggs on a minimum of four feedings (out of 7). Every individual subsequently "sampled" eggs on one or more occasions. The ninth animal (#17) did not reject an egg until egg-feeding day nine, after consuming 48 eggs. We watched this female closely, but found no indication that estrogen plugs were being rejected. The treatment resulted in a significant reduction in egg consumption compared with the control animals (Kruskal-Wallis $X^2_3 = 26.075$, $p = 0.0001$). Treatment group animals consumed 430 of the 684 eggs presented (63%). They consumed only 53% of the eggs available during the challenge phase. Treatment group animals consumed many fewer eggs per day than the control group animals during both the treatment (4.4 ± 0.325; $p < 0.001$) and challenge phases (3.1 ± 0.674; $p < 0.001$). Six treatment group animals ate fewer eggs per day during the challenge phase than during the treatment phase, even though only two of the six challenge eggs

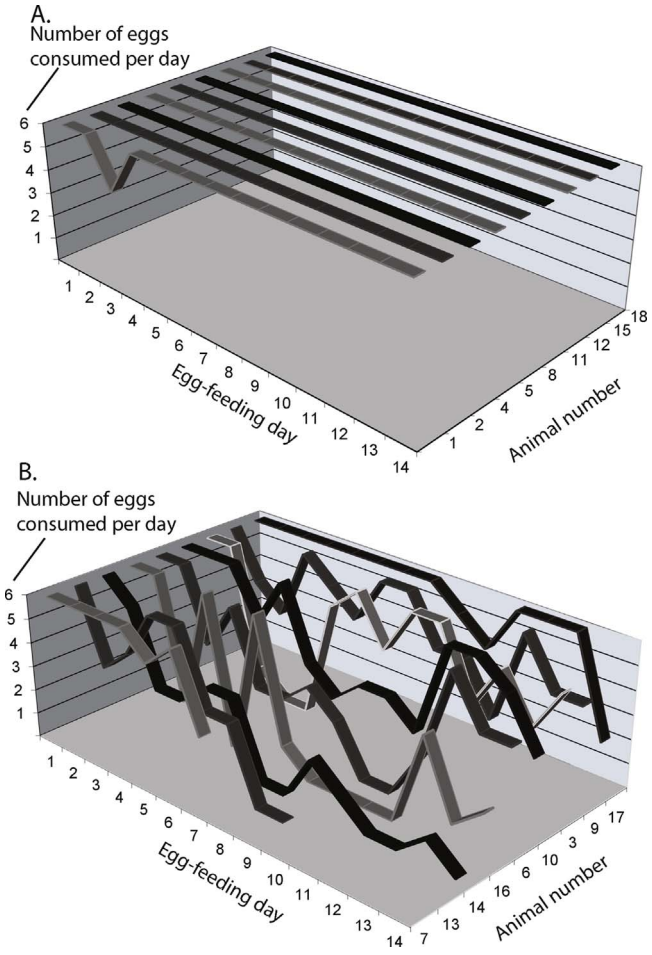

Fig. 2. Number of eggs consumed per individual per egg-feeding day (n = 14) for nine control group and nine treatment group raccoons (*Procyon lotor*). The raccoons were presented with eggs every other day during the trial. Egg-feeding days 1–7 constituted the treatment phase and days 8–14 were the challenge phase. (A) *Control group animals* – During the treatment phase, each individual received six eggs injected with the estrogen carrier (arrowroot-starch gel), but no estrogen, on each egg-feeding day. During the challenge phase, each received a combination of two fresh and four carrier-injected eggs per egg-feeding day. (B) *Treatment group animals* – During the treatment phase, each individual received six estrogen-injected eggs on each egg-feeding day. During the challenge phase, each received a combination of two fresh, two estrogen-injected, and two carrier-injected eggs per egg-feeding day. Animals #7 and #13 died during the challenge phase of the trial.

available contained estrogen. There was no difference between males and females either in the tendency to exhibit an aversion or in the percentage of eggs eaten after aversion (Mann-Whitney $U_{(2)4,5} = 17$, $p = 0.111$). Two treatment group animals, a male and a female, died early in the challenge phase (see Section 3.9 below).

All seven of the treatment group animals remaining through the challenge phase rejected some eggs on 3–7 feeding days ($\bar{x} = 5.6$ days; Fig. 3). None resumed eating all of the eggs available. As a result, the treatment group animals consumed fewer eggs per day during the challenge phase (3.1 ± 0.674) than during the treatment phase (4.4 ± 0.325), but the difference was statistically non-significant (Mann-Whitney $U_{(2)7,9} = 43$, $p = 0.244$). On the other hand, the treatment group animals consumed significantly fewer eggs per day than the control group animals during the challenge phase (6.0 ± 0.000; Mann-Whitney $U_{(2)7,9} = 0$, $p = 0.001$; Fig. 3). Exposure to estrogen-treated eggs significantly reduced egg consumption even when a mixture of fresh and treated eggs was available.

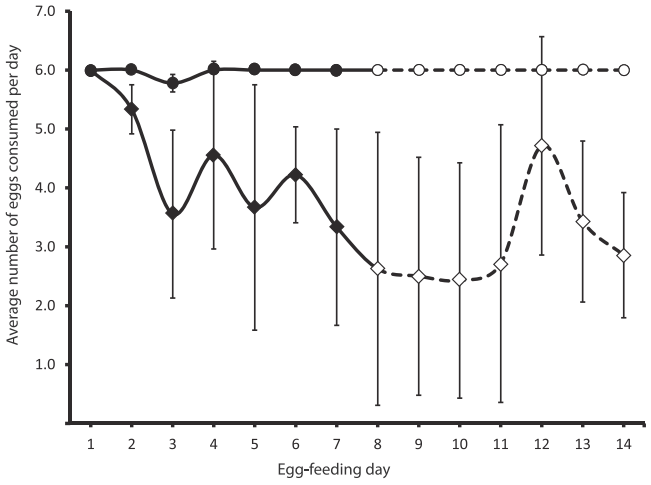

Fig. 3. Average number of eggs ($\bar{x}$ ± 1 se) consumed per individual per egg-feeding day (n = 14) for nine control group and nine treatment group raccoons (*Procyon lotor*). The raccoons were presented with eggs every other day during the trial. Egg-feeding days 1–7 constituted the treatment phase and days 8–14 were the challenge phase. Closed circles represent control group animals during the treatment phase; open circles represent control group animals during the challenge phase. During the treatment phase, each control group animal received six eggs injected with the estrogen carrier (arrowroot-starch gel), but no estrogen, on each egg-feeding day. During the challenge phase, each received a combination of two fresh and four carrier-injected eggs per egg-feeding day. Closed diamonds represent treatment group animals during the treatment phase; open diamonds represent treatment group animals during the challenge phase. During the treatment phase, each treatment group animal received six estrogen-injected eggs on each egg-feeding day. During the challenge phase, each received a combination of two fresh, two estrogen-injected, and two carrier-injected eggs per egg-feeding day.

3.3. Amount of estrogen consumed

Treatment animals apparently varied in their sensitivity to the estrogen. The average raccoon consumed 16 eggs (range 6–48) or 160 mg of estrogen (range 60–480 mg) before it began to reject eggs. This amounted to 41.3 mg kg^{-1} of estrogen (± 10.938; range 14.8–116.4 mg kg^{-1}). Animal #17 (a female) ate all but seven of the 84 eggs presented (92%), including 53 of the 56 (95%) treated eggs. In contrast, animal #14 (also a female) ate only 33 of the 84 eggs presented (39%), and only 26 of 56 (46%) treated eggs. There was no significant relationship between the age of the individual and the amount of estrogen ingested before the onset of an aversion ($r_{s,8} = 0.271$, $p = 0.536$). There was no difference between males and females in their tendency to eat eggs during either the treatment phase (71% vs 76% of eggs eaten) or the challenge phase (52% vs 49% of eggs eaten). The total estrogen consumed per individual ranged from 260 to 530 mg ($\bar{x}$ = 373 mg). Although females (average 380 mg) consumed more estrogen than males (average 354 mg), the difference was non-significant (Mann-Whitney $U_{(2)4,5} = 11.00$, $p = 0.903$). On a body-mass basis, females (93.9 mg kg^{-1} ± 13.501) and males (93.6 mg kg^{-1} ± 4.644) consumed comparable amounts of estrogen (Mann-Whitney $U_{(2)4,5} = 8.00$, $p = 0.713$). Similarly, females (47.7 mg kg^{-1} ± 23.857) and males (36.1 mg kg^{-1} ± 8.650) consumed comparable amounts of estrogen prior to first rejecting eggs (Mann-Whitney $U_{(2)4,5} = 9.00$, $p = 0.903$).

3.4. Types of eggs consumed during challenge phase

Treatment group animals did not distinguish among fresh eggs, carrier-only eggs, and estrogen-injected eggs during the challenge phase (Fig. 4). They consumed only 162 of the 306 eggs presented (53%). Means for the total daily consumption of the three types of eggs were not different. The animals consumed comparable numbers of fresh, carrier and treated eggs (Kruskal-Wallis $X^2_2 = 0.602$, $p = 0.740$). The estrogen treatment satisfied the requirement that it be undetectable

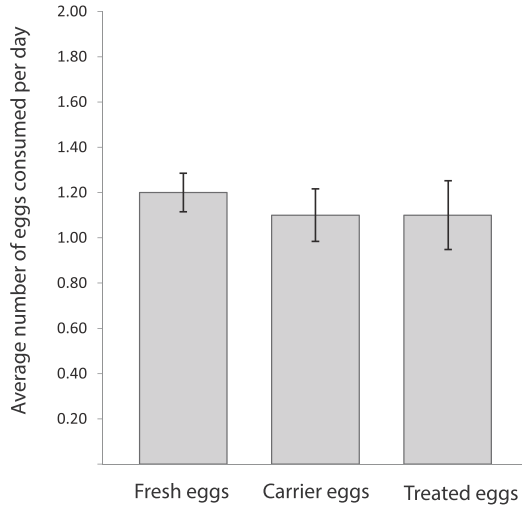

Fig. 4. Average number of eggs of each type ($\bar{x} \pm 1$ se) consumed per egg-feeding day (n = 7) during the challenge phase for nine treatment group raccoons (*Procyon lotor*). Each animal received a combination of two fresh, two estrogen-injected, and two carrier-injected eggs per egg-feeding day during the challenge phase.

from visual and olfactory cues.

3.5. Amounts of food and water consumed

Treatment group and control group animals ate comparable amounts of dog food per day during the acclimation (483 vs 459 kJ kg^{-1}), treatment (457 vs 438 kJ kg^{-1}) and challenge phases (465 vs 424.9 kJ kg^{-1}, Kruskal-Wallis $X^2_5 = 3.464$, $p = 0.629$). There were no treatment or phase differences in the amount of dog food consumed per kilogram of body mass per day. The consumption of estrogen-treated eggs had no effect on the willingness of the raccoons to consume normal rations of non-egg foods. During the challenge phase, four of the nine control raccoons ate dog food before eggs, three ate eggs first, and two alternated which they ate first. In contrast, all of the treatment animals ate dog food before eggs after exposure to estrogen, and dog food became the preferred food type.

Treatment group and control group animals drank comparable amounts of water during the acclimation phase (73.7 vs 76.4 ml kg^{-1}). Treatment group animals drank more during the treatment phase (95.3 vs 78.2 ml kg^{-1}), and control group animals drank more during the challenge phase (90.8 vs 60.7 ml kg^{-1}). The overall comparison was significant (Kruskal-Wallis $X^2_5 = 13.342$, $p = 0.020$), but after correction for the number of contrasts (Boneferroni corrected significance level = 0.0033), none of the pair-wise comparisons were significant. Treatment group animals exhibited a pronounced tendency to drink more water than the control group animals during the treatment phase, but less during the challenge phase. Consumption of treated eggs may have had a modest effect on water consumption, but this tendency disappeared after the treatment phase.

3.6. Net change in body mass as a function of food consumption

Body-mass dynamics differed between treatment and control animals. Control animals weighed an average of 3.8 kg ± 0.230 at the beginning of the trial. They gained an average of 0.72 kg ± 0.124 in body mass (18.4%) by the end of the trial. Treatment animals weighed 3.9 kg ± 0.164 at the beginning. Five (2 males and 3 females) gained an average of 0.45 kg ± 0.121 (12%) and four (3 males and 1 female) lost an average of 0.44 kg ± 0.138 (10%). There was no overall difference in body mass based on either treatment group or study phase (Kruskal-Wallis $X^2_3 = 4.185$, $p = 0.242$). Nevertheless, treatment animals gained less mass on average than control animals (Mann-Whitney

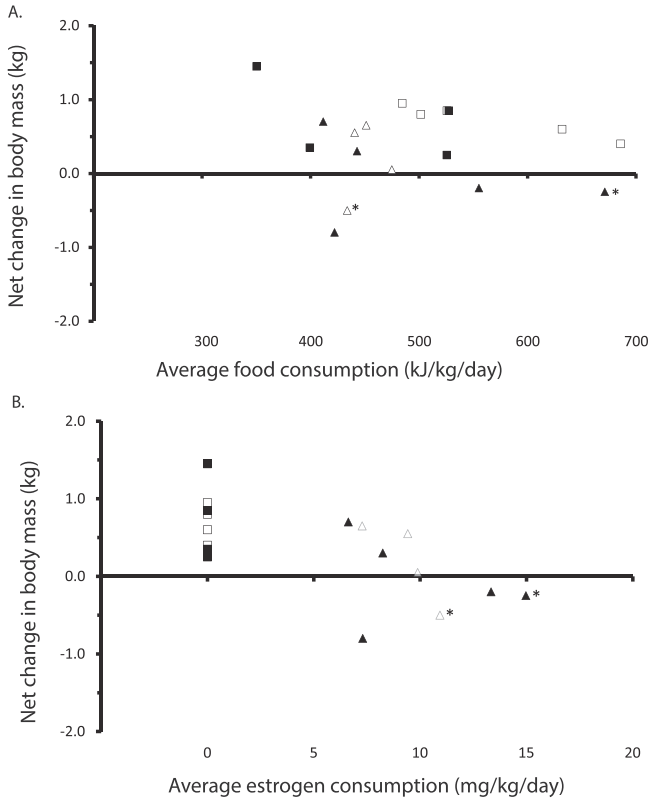

Fig. 5. Net change in body mass (kg) of 18 captive raccoons (*Procyon lotor*) between the beginning and end of the pen trial (A) as a function of average daily food consumption (kJ kg^{-1}/day) and (B) as a function of average daily estrogen consumption (mg/kg/day). Closed squares represent control group males; open squares are control group females. Closed triangles represent treatment group males; open triangles are treatment group females. Two treatment group animals that died before the end of the trial are marked with asterisks.

$U_{(2)9,9} = 12$, $p = 0.013$), and four treatment animals lost mass while no control animals did. There was no overall difference in percentage mass change between sexes (Mann-Whitney $U_{(2)9,9} = 30$, $p = 0.354$). Although three of five treatment males lost mass, and one of four females lost mass, there was no difference in the frequency of mass gain/loss between sexes for the treatment animals ($X^2_{C,1} = 1.103$, $p = 0.294$).

There was no apparent relationship between daily caloric intake and either body-mass dynamics or survival (Fig. 5A). The average control group animal consumed 515 kJ kg^{-1} per day (± 9.580) during the treatment and challenge phases, while the treatment group animals consumed 448 kJ kg^{-1} per day (± 18.888). There was no overall difference in daily consumption (Mann-Whitney $U_{(2),9,9} = 60$, $p = 0.456$). The correlation between average daily caloric intake and change in body mass was positive but non-significant (Spearman $r_{s,16} = 0.186$, $p = 0.082$). Even with exposure to estrogen, animals were able to gain mass on as little as 419 kJ kg^{-1} per day. Both of the treatment animals that died during the trial consumed at or above the average daily caloric intake.

3.7. Net change in body mass as a function of estrogen consumption

There also was no apparent relationship between estrogen intake and body-mass dynamics. The average estrogen dose was 9.8 mg kg^{-1} per day (± 0.956; Fig. 5B). The cumulative estrogen dose ranged from 68.9 to 128.5 mg kg^{-1} (93.7 mg kg^{-1} ± 6.030). The dose received by the seven surviving treatment animals ranged from 76.5 mg kg^{-1} to 128.5 mg kg^{-1} (41.3 ± 10.938) over a 14 egg-feeding day exposure period. The dose received before eggs were rejected ranged from 14.8 mg kg^{-1} to 116.4 mg kg^{-1}. The average cumulative estrogen dose

was 365.6 mg ± 29.208. Some of the treatment animals gained mass, while others lost comparable amounts over the same period. The correlation between cumulative estrogen dose ingested and change in body mass over the trial was essentially zero (Spearman $r_{s,7} = -0.050$, $p = 0.912$).

3.8. Overall health, volume of testes, and general histopathology

Somewhat surprisingly, treatment raccoons exhibited few, if any, outward signs of illness in the hours after eating estrogen-injected eggs. Although raccoons are capable of vomiting, there were none of the usual signs of distress following ingestion of an aversive agent, such as head shaking, retching or emesis (Gustavson, 1977). Both control and treatment animals exhibited bouts of abnormal feces (i.e., soft, runny or diarrhea), perhaps related to the stress of confinement and the basic diet of dog food. In fact, the treatment animals experienced a higher average frequency of abnormal feces (57% of 31 observation days) than the control animals (38%; Mann-Whitney $U_{(2)9,9} = 73$, $p < 0.005$).

Although our study animals were already adults, we examined testis gross morphology to determine whether short-term exposure to oral estrogen resulted in reduced testis volume. The testes of the treatment males (2845 mm^3 ± 1,005.266) were similar in volume to those of the control males (2475 mm^3 ± 981.108; Mann-Whitney $U_{(2)4,5} = 12$, $p = 0.712$). The testes of the treatment males (4880 mg ± 1,437.150) weighed less than those of the control males (7667 mg ± 4,212.811), but small sample size and high variability rendered the difference non-significant (Mann-Whitney $U_{(2)3,5} = 6$, $p = 0.766$). Identical results were obtained when testes mass and volume were standardized for body mass. At least over the time period of the pen trial, estrogen exposure appeared not to influence testis size.

Histopathology reports for the treatment and control animals were very similar. There was no condition shared by the treatment animals that was not also common among the control animals (Skirpstunas, 2006). The raccoons were laden with endoparasites and long-standing, chronic mild-to-moderate organ damage. Sarcocytosis (*Sarcocystis* sp.) was evident in the heart and skeletal muscle of several animals, but was not considered a pathologic condition. Lesions possibly attributable to at least three protozoan organisms were widespread. Intestinal parasite loads were considered low and of no clinical significance. Seven of the raccoons (6 treatment animals and 1 control) exhibited dermatitis and patchy hair by the end of the trial, including two (1 treatment male and 1 treatment female) that were diagnosed with dermatophytosis (ringworm infection). The frequency of dermatitis was higher in treatment group ($p = 0.001$). We were unable to detect any tissue or organ conditions that might be directly attributable to the effects of the treatment.

3.9. Deaths of two treatment animals

Animal #7 (male, age 5) was captured on June 12. He ate 38 treated eggs (380 mg estrogen) between July 8 and July 20 (7 treatment days). He died on egg-feeding day 1 of the challenge phase due to a prolapsed rectum. He had consumed a slightly larger cumulative dose of estrogen (104.7 mg kg^{-1}) than most of the treatment animals (93.7 mg kg^{-1}). He had produced unusual feces on 53% of the days, only slightly above the overall median of 50%.

Animal #13 (female, age 7) was captured on June 24. Her pregnancy went undetected during the initial physical examination. She ate 31 treated eggs (310 mg estrogen) between July 8 and July 20 (7 treatment days). She failed to eat eggs on egg-feeding days 1 and 2 of the challenge phase (July 22–24), before she died on July 25. She received a substantially smaller cumulative dose of estrogen (76.5 mg kg^{-1}) than most of the treatment animals. Given a 63-day gestation period (Llewellyn, 1953) and the sizes of the 4 fetuses she carried at the time of her death (130 mm total length), animal #13 must have been near-term when she died. Calculating back from day 63 (July 25), she must have been at day 41 of pregnancy when captured and day 54 when she began eating treated eggs. Necropsy indicated that she died of sepsis from an aborted late-term pregnancy.

4. Discussion

Our objective was to test the efficacy and safety of oral estrogen as an aversive agent for raccoons under controlled conditions. Every treatment raccoon became averted to eating eggs after 1–8 feedings of six eggs injected with 10 mg of 17 α-ethinyl estradiol. The average amount of estrogen required to induce a CFA was 41.3 mg kg^{-1}, far below the oral LD_{50} for laboratory rats (1200 mg kg^{-1}, Gill et al., 2000). There was no gender difference in either the tendency to exhibit an aversion or the percentage of eggs eaten after aversion. The aversion was neither absolute (1 female ate 92% of the 84 eggs presented) nor persistent (all of the animals "sampled" eggs at some later time) under the conditions of the pen trial. Importantly, the raccoons were unable to detect the presence of estrogen or to distinguish between treated and fresh eggs. They consumed estrogen-injected, carrier-injected and fresh eggs equally during the challenge phase.

Evidence for CFA included a decline in the number of eggs consumed and a preference for eating dog food before eggs. Treatment group raccoons consumed 63% of the eggs available during the treatment phase but only 53% of those available during the challenge phase, even though four of the six challenge eggs available at each challenge feeding contained no estrogen. A 10 mg dose of estrogen per egg was sufficient to inhibit, but not stop, egg consumption. The results of the challenge phase confirmed that the raccoons were averting to the taste and appearance of egg rather than the smell or taste of the carrier or the taste or smell of estrogen. Therefore, estrogen and arrowroot gel provided an effective and undetectable aversive dose.

The minimum cumulative estrogen dose required to induce a CFA was somewhere between 15 and 116 mg kg^{-1} given in daily doses of ~15 mg kg^{-1} body mass. Semel and Nicolaus (1992) reported that aversion was induced in free-ranging raccoons by an average dose of 23.5 mg kg^{-1} (range 4.6–61.1 mg kg^{-1}). Given that they had less control over their subjects, Semel and Nicolaus (1992) may have failed to detect some cases of estrogen ingestion, so that their estimates may be conservative. On the other hand, because our raccoons were constrained in movement, they may have ingested higher cumulative doses than would have been the case with more freedom of choice in movement and food selection (Conover, 1989). It is therefore reasonable to conclude that a cumulative dose 20–80 mg kg^{-1} is likely to provide an effective dose for raccoons in the range of 4–5 kg body mass. Such a dose could be delivered with only 8–32 treated eggs delivered over 1–4 feeding bouts.

A somewhat surprising result was that the CFA formed without any outward signs of illness or distress. The lack of visible symptoms makes it impossible to surmise what effects the animals experienced from ingesting even relatively large cumulative doses of estrogen, in the 300–400 mg range. They continued to eat, drink, and engage in normal behaviors in spite of whatever distress they encountered. Semel and Nicolaus (1992) reported a similar absence of illness in the treated raccoons they observed. Gustavson (1977) reviewed several cases of acquired aversion in the absence of a reliable indicator of illness (e.g., emesis). In reality, CFAs often are induced without obvious signs of illness (Bernstein, 1999). In humans, the most frequent symptom of oral estrogen is nausea, which, while unpleasant, rarely interferes with eating and does not cause weight loss (Murad and Haynes, 1980). This general pattern appears also to apply to raccoons. The apparent absence of suffering and ill effects recommends in favor of estrogen-induced CFA as a humane aversive treatment.

The estrogen treatment had little effect on food and water consumption, body-mass dynamics or general physical condition. Although the administration of exogenous estrogen can influence feeding behavior (e.g., reduced meal size; Geary, 2001), our animals exhibited no

such effect. Treatment and control animals had comparable daily caloric intake throughout the trial. Treatment group animals exhibited a pronounced tendency to drink more water than the control group animals during the treatment phase, but actually drank less during the challenge phase. There was no apparent relationship between daily caloric intake and either body-mass dynamics or survival; treatment animals gained less on average than control animals, but were still able to gain mass on intake of less than 500 kJ kg^{-1} per day. There also was no apparent relationship between estrogen intake and body-mass dynamics; some treatment animals gained mass while others lost, so that the correlation between estrogen intake and change in body mass was essentially zero. Although under- and over-exposure to estrogen can influence testis development and function (Coveney et al., 2001, Sierens et al., 2005), we observed no effect of estrogen on testes mass. Finally, histopathology examination detected no obvious effect of estrogen on tissue or organ condition (Skirpstunas, 2006).

On the other hand, the treatment animals experienced more frequent bouts of abnormal feces. This suggests that the estrogen may have affected the digestive system, even if we were unable to detect an effect with observations of behavior. Similarly, the fact that six (66%) of the treatment animals, and only one (11%) of the control animals, exhibited patchy hair loss suggests that the estrogen might have been involved in some way. Furthermore, two of the affected treatment animals exhibited symptoms of dermatophytosis, a readily communicable disease in social species and in animals that are stressed or immunocompromised (Mishra et al., 1994; Ellis and Mori, 2001; Ramsay, 2011). Although confinement and forced proximity over an extended period of time can suppress immune function (Blecha, 2000), this relatively low incidence of dermatophytosis suggests that our animals were not particularly susceptible. Again, however, even this low level of incidence suggests some involvement of the estrogen.

Gill et al. (2000) compared the aversive effectiveness of oral estrogen with two other compounds, cinnamamide and thiabendazole, which they considered to pose less health risk to the target species. The compounds were administered to laboratory rats (*Rattus norvegicus*) by oral intubation at rates of 4 mg kg^{-1}, 160 mg kg^{-1} and 100 mg kg^{-1}, respectively. All three compounds induced an aversion to a novel food with a single dose. Estrogen induced the most persistent CFA, lasting for > 11 post-treatment tests (6 months). Even though the effective dose of estrogen was far below the oral LD_{50} for rats, Gill et al. (2000) expressed concern about the relative safety of estrogen because it has the potential to disrupt reproductive processes and fetal development (Badawy and Abdul-Karim, 1978; Yasuda et al., 1981, Matsuura et al., 2004).

We suspect the death of the female from sepsis was a result of the cumulative dose she received. We estimated that she consumed 310 mg of estrogen or 76.5 mg kg^{-1} before dying from an aborted pregnancy. Based on previous reports of the effects of high levels of estrogen on pregnancy (Asa, 2005) and fetal development in mammals (e.g., Badawy and Abdul-Karim, 1978; Yasuda et al., 1981; Matsuura et al., 2004), this death confirms a potential risk associated with high cumulative doses of estrogen. Confinement also may have been a contributing cause (Morgan and Tromborg, 2007), since no similar instances have been reported for free-ranging raccoons. Nevertheless, field application should be planned to both minimize overlap with the breeding season of the target species and to minimize exposure of protected or endangered non-target species (Gill et al., 2000).

The death of the male from rectal prolapse was not an obvious consequence of estrogen ingestion, but this condition is sometimes associated with immune deficiency (Miller et al., 2014). It is possible that the immune system of this animal may have been suppressed by high doses of exogenous estrogen (Gilmore et al., 1997; Whitacre, 2001).

Overall, the high survival rate of treatment and control animals, even with the variety of parasites and health problems identified in the necropsies and the complications of pen stress, was encouraging. Semel and Nicolaus (1992) observed similarly high survival rates for tagged raccoons in their study. Many of their raccoons survived long enough to participate in feeding trials that occurred a year apart. Consumption of estrogen at the dosages reported here is unlikely to influence survival, except perhaps for any pregnant females that might feed heavily on treated eggs.

The CFA was neither absolute nor persistent under the conditions of our pen trial. CFA formation may be influenced by social and environmental factors (Gustavson and Gustavson, 1985), the specific methods employed (Baker and Macdonald, 1999), and variation between the sexes and between individuals (Semel and Nicolaus, 1992). An aversion might fail to be absolute or to persist for several reasons that might pertain to this pen trial: (1) pre-exposure or learned safety of wild-caught animals (Kalat and Rozin, 1973), (2) social learning in the visual presence of other animals (Semel and Nicolaus, 1992), (3) restricted feeding times and alternative foods (Conover, 1997), (4) forced close proximity to the referent food, and (5) normal behavioral variation among individuals (Gustavson and Gustavson, 1985). Despite these circumstances, all of the treatment raccoons (1) developed an aversion to egg consumption after pairing estrogen-treated egg flavor with estrogen-induced illness, (2) developed this aversion typically after only a few egg feedings, (3) were unable to distinguish treated from untreated eggs, (4) consumed fewer eggs than control animals even when fresh eggs were available, and (5) learned to prefer an alternative food (i.e., dog food) over eggs. It is thus highly likely that free-ranging raccoons will exhibit a CFA when feeding choices are diverse, feeding is *ad libitum* and avoidance-at-a-distance is possible. Given that avoidance-at-a-distance is the ultimate objective of any CFA-based management strategy, these results should encourage further development of deception-based food aversion (Conover, 1997) as a management tool for the protection of the eggs of ground-nesting wildlife, with estrogen as a strong candidate as an aversive agent.

5. Conclusions

Oral estrogen is an effective aversive agent when combined with a bland carrier and injected into eggs. Estrogen clearly produced a reduced tendency of raccoons to eat eggs after only a few (1–4) feeding sessions. The estrogen was undetectable to the raccoons, and the estrogen-arrowroot combination was stable under field conditions. The treatment was equally effective for males and females, did not affect appetite or thirst, and appeared not to affect behavior or demeanor. The testes of the treatment males appeared not to be affected by exposure to estrogen. The treatment may have caused a higher incidence of dermatitis, but it produced no detectable chronic or long-lasting health effects at an effective dose rate. We conclude that ingestion of 20–80 mg kg^{-1} of estrogen would deliver an aversive dose for raccoons in the 4–5 kg range. Such a dose could be delivered in 1–4 days, suggesting that 1–2 weeks of treatment should be sufficient to bring about a reduction in egg predation using this method. The total number of treated eggs required to deliver such a treatment would depend on the number of raccoons in the vicinity. Other types of eggs (e.g., bantam chicken and Japanese quail) might provide effective surrogate eggs for delivering the treatment in the field. Our results say little about persistence, but other studies indicate that the treatment should be effective over a period of time sufficient to protect eggs over an avian breeding season. As with any CFA-based management strategy, effectiveness in a field application will depend critically on the timing and spatial extent of the deployment and on the percentage of target animals treated. Any field application should be planned to both minimize overlap with the breeding season of the target species and to minimize exposure of protected or endangered non-target species.

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Appendix A. Tissue preservation protocol in preparation for histopathological analyses

Tissue preservation protocol in preparation for histopathological analyses

Samples of the following tissues were preserved by freezing at −20 °C and by immersion in 10% buffered formalin: skeletal muscle, lung, heart, liver, spleen, kidney, brain, urinary bladder, large intestine, small intestine, and stomach. Samples of thyroid, adrenals, pituitary, and bone marrow were only preserved in10% buffered formalin; eyeball was only frozen.

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