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Experimental tests of nonsurgical reproductive inhibitors to prevent coyote reproduction

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Abstract: Sterilization is an effective nonlethal tool to reduce livestock depredation by coyotes (*Canis latrans*) because adults without pups to provision are less likely to kill livestock. Surgical sterilization is costly and invasive, so identifying nonsurgical methods for canids that allow wide-scale application is important. We conducted a preliminary assessment of 2 types of reproductive inhibitors (gonadotropin releasing hormone [GnRH] vaccine and deslorelin, a GnRH agonist) on coyote reproductive capabilities. We treated captive coyotes with a GnRH vaccine (n = 6 males, n = 6 females) or deslorelin (n = 6 males), measured number of litters and pups born, and compared their behavior and hormone levels to captive coyote pairs in which the male was surgically vasectomized (n = 6). At least half of the pairs receiving treatment with either of the nonsurgical reproductive inhibitors produced pups, and litter size was larger than expected compared to historical records. Male coyotes treated with deslorelin showed decreased testosterone levels, whereas there was no difference in testosterone levels in males treated with GnRH vaccine compared to controls. Behavior did not differ between any groups. Despite the lack of efficacy of either reproductive inhibitor tested, our research suggests that hormonal alterations that disrupt reproduction of coyotes are unlikely to negatively affect behavior, and further investigation of nonsurgical reproductive inhibitors for wild canids is warranted.

Key words: *Canis latrans*, coyote, deslorelin, fertility control, GnRH vaccine, nonlethal control, nonsurgical sterilization

Social monogamy, the long-term behavioral association between a male and female (Reichard 2003), is prevalent in many wild canids (Kleiman and Eisenberg 1973, Kleiman 1977). Social monogamy can also extend to include genetic monogamy, which is defined as exclusivity in mating (Reichard 2003). Coyotes (*Canis latrans*) exhibit social and genetic monogamy (Hennessy et al. 2012), with pair bonds and territorial tenure being long-term (Gese 2001, Hennessy et al. 2012).

There are high energetic costs to pup rearing. Canids without pups to provision will remain territorial but live longer and kill significantly fewer livestock and native ungulates than neighboring packs with pups (Till and Knowlton 1983; Spence et al. 1999; Bromley and Gese 2001*a*, *b*; Seidler and Gese 2012). Indeed, territorial, mated pairs of coyotes with pups to support are often the primary predators of domestic livestock (Sacks et al. 1999, Blejwas et al. 2002) and large wild prey, such as mule deer (*Odocoileus hemionus*) and elk (*Cervus canadensis*; Gese and Grothe 1995). Lethal removal of coyote pups resulted in a similar reduction in livestock depredation by coyotes when compared to lethal removal of breeders and pups, suggesting the cost of pup provisioning was the cause of most depredation events (Till and Knowlton 1983). However, lethal removal of pups is controversial (Kellert 1985, Andelt 1987). An alternative method to reduce pup production, such as sterilization, could provide a viable nonlethal management tool to reduce conflict and ensure the long-term survival of canids by eliminating costs associated with pup provisioning.

Field studies in free-ranging canids have investigated sterilization in gray wolves (*Canis*

lupus; Mech et al. 1996, Spence et al. 1999), red fox (*Vulpes vulpes*; Bubela and Augee 1993, Saunders et al. 2002), and coyotes (Bromley and Gese 2001*a*, *b*; Seidler and Gese 2012; Gese and Terletzky 2015). Several studies have relied on tubal ligation and vasectomy because hormones remain intact and social structure, especially pair bonds, remains constant (e.g., Bromley and Gese 2001*a*, Saunders et al. 2002, Seidler and Gese 2012). While maintaining hormones may be beneficial, the process of sterilizing wild canids is arduous, and nonsurgical methods are necessary for sterilization to serve as a widespread, nonlethal management tool (Boitani et al. 2004, Massei and Cowan 2014). Nonsurgical methods for reproductive inhibition can affect hormones, and it is unclear if hormonal changes impact behavior and social structure (Asa et al. 2005). It has been suggested that alterations in sex steroid hormones to control reproduction in the species may have an effect on social systems (Asa and Valdespino 1998), particularly in territory fidelity and mating behavior (Seidler and Gese 2012). However, this hypothesis remains experimentally untested in wild canids.

Coyotes have territories that are defended year-round (Gese 2001). Male coyotes gradually produce increasing amounts of gonadal testosterone during the presumptive breeding season (November to March) and often reach peak levels in January, then experience testicular regression the remainder of the year (Minter and DeLiberto 2008). During this period of testicular atrophy, testosterone levels are basal, testicular volume is minimal, sperm production is zero, and accessory glands do not produce seminal fluid (Minter and DeLiberto 2008). The non-reproductive season also coincides with pup rearing and dispersal (Bekoff and Wells 1980). Data on the role that gonadal androgens play in behavior that may impact pair bonds are conflicting (i.e., Beach 1970, Bhasin et al. 1988, Hart and Eckstein 1997). Thus, it is unclear whether reduced gonadal androgens would alter behavior of wild canids and, if behavior is altered, whether this impacts pair bonds.

We conducted a preliminary assessment of 2 types of reproductive inhibitors, gonadotropin releasing hormone (GnRH) and a GnRH agonist, deslorelin, Suprelorin134® (Peptech Animal Health, Macquaria Park, NSW, Australia; hereafter referred to as deslorelin) to determine their effects on reproduction in coyotes. We also obtained baseline data on behavior and hormones as measures of potential explanatory factors related to the success or failure of the reproductive inhibitors.

We focused on suppressing GnRH because it is a key reproductive hormone that regulates the production of the sex steroids progesterone, estrogen, and testosterone. Both hormonal and immunological methods have been used to successfully suppress the function of GnRH and induce infertility in a number of species (Eymann et al. 2007, Boutelle and Bertschinger 2010, Miller et al. 2013). Alternatively, vaccination against GnRH can induce infertility in a number of species, including deer (Gionfriddo et al. 2011), elk (Killian et al. 2009), bison (*Bison bison*; Miller et al. 2004), pigs (*Sus scrofa*; Massei et al. 2012), horses (*Equus caballus*; Killian et al. 2009, Gray et al. 2010), prairie dogs (*Cynomys* spp.; Yoder and Miller 2010), and cats (*Felis catus*; Levy et al. 2011). A reproductive inhibitor that could last 6 years would cover the reproductive lifespan of most wild coyotes (Kilgo et al. 2017). In a preliminary trial, DeLiberto et al. (1998) showed that vaccination against GnRH could suppress circulating levels of progesterone and testosterone in female and male coyotes, respectively, and therefore had the potential to disrupt fertility.

Deslorelin was been developed for short-term suppression of the reproductive axis in male dogs. Captive male coyotes given a high dose of deslorelin formulated into 12-month slow-releasing implants exhibited full downregulation of the reproductive axis for 25 months, as supported by the complete absence of sperm, and no physiological side effects were detected (MacGregor et al. 2013, 2016). These data suggested deslorelin was capable of inhibiting fertility in male coyotes with no ill effects to health.

Study area

All methodology was approved by the Institute for Animal Care and Use Committee at the U.S. Department of Agriculture (USDA) National Wildlife Research Center (QA-2137). Captive coyotes maintained at the USDA-Wildlife Services-National Wildlife Research Center's Predator Research Facility in Millville,

Utah, USA (66.4 ha) were used for this study. The facility manages and cares for coyotes using methods to maintain wild behavior (Shivik et al. 2009). About 100 adults are housed at the facility as male–female pairs in outdoor enclosures (0.1–1.0 ha in size) with natural earthen floors. Twenty-four pairs of adult coyotes were used, selected from those no longer needed for breeding purposes. All pairs were housed in 0.1-ha outdoor enclosures during the study. The enclosures are surrounded by chain-link fencing and contain a manmade den box (a second box is added in pens with pregnant females), 2 shade tables, and an *ad libitum* source of water.

Methods

We randomly assigned 6 pairs of coyotes to 1 of 3 groups: female GnRH vaccine, male GnRH vaccine, or male deslorelin. In addition, 6 coyote pairs where a male had already received a vasectomy were selected at random for the experimental control group. Vasectomies are commonly used at the facility to manage the colony and have been used effectively on wild coyotes (Bromley and Gese 2001*a*, *b*).

Coyotes in the 2 GnRH vaccine groups received a single injection of vaccine on October 22, 2013. The vaccine was a mineral oil-based vaccine made into a water-in-oil emulsion. Each 0.5-ml dose contained approximately 500 µg of GnRH conjugated to a carrier protein (blue protein, Biosonda), and killed *Mycobacterium avium* was added as an immunostimulant (Perry et al. 2008). Coyotes were lightly sedated with Dexdomitor (0.33 ml/kg), and the vaccine was administered intramuscularly in the back of the left hind leg. Males in the deslorelin group were anesthetized with a mixture of 100-mg ketamine and 20-mg xylazine (Kreeger and Arnemo 2007) and then treated with 47 mg deslorelin in the form of 10 x 4.7-mg controlled release Suprelorin® implants. The dosage was 10 times that recommended for domestic dogs of similar size but was previously shown to be effective at suppressing sperm production in captive male coyotes housed alone (MacGregor et al. 2017).

Testosterone measurement

To obtain testosterone levels from treated and control males, blood was collected from the cephalic vein into heparinized tubes, centrifuged, and plasma was stored at -20°C. Coyotes were either manually restrained for blood collection or, if needed for human safety, anesthetized or sedated as detailed above. Blood was collected on days 0, 57, 121, 245, and 442 from the time of treatment (October 2013 to January 2015). Total testosterone was estimated by radioimmunoassay (RIA; TKTT2; Siemans Healthcare Diagnostics, Inc., Los Angeles, California, USA) with assay sensitivity of 0.04 ng/ml, and intra- and inter-assay coefficients of variation were 1.68% and 4.15%, respectively (MacGregor et al. 2017).

Measurement of antibodies to GnRH

We collected blood serum on days 0, 31, 58, 91, 121, and 142 from coyotes treated with GnRH vaccine to determine antibody responses to GnRH using enzyme-linked immunosorbent assay (ELISA). Briefly, wells of microtiter plates (Immulon 2HB flat bottom; Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with antigen by adding 50 µl of GnRH-BSA conjugate (80 µg/ml) in carbonate bicarbonate buffer and incubated overnight at 4°C in a sealed plastic bag. The plates were then washed 2x with 200 µl/well PBST (phosphate buffered saline plus 0.05% [v/v] Tween 20, pH 7.4) at room temperature. Blocking buffer (200 µl; 20% [v/v] SeaBlock [Thermo Fisher Scientific] plus 0.05% [v/v] Tween 20 in 0.01 M PBS) was added to each well and incubated for 1 hour at 25°C, followed by another 2x washes with PBST. Serial dilutions of sera obtained from immunized coyotes were added to the wells and incubated 1 hour at 25°C, followed by 2x washes with PBST. Bound anti-GnRH antibody was detected using 50 µl horseradish peroxidase conjugated rabbit anti-dog IgG (Sigma; diluted 1:6,000) incubated 1 hour at 25°C followed by 2x washes. Enzyme substrate (50 µl of 3,3′,5,5′- tetramehtylbenzidine [TMB] dihydrochloride in phosphate citrate buffer; Sigma) was added to each well, and the reaction was terminated after 3–5 minutes by the addition of 50 µl of 2 M sulfuric acid. The absorbance of each well was measured at 450 nm. Endpoint titers were determined based on cut-off values, which were calculated for each dilution as the mean plus 3 standard deviations using the pre-vaccination samples from all animals. Titers are reported as the reciprocal of the highest dilution of serum that gave a value

Table 1. Definitions of behavioral classifications used during focal sampling on 24 mated pairs of adult coyotes (*Canis latrans*) at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA. Sampling occurred at least 3 times per month (September 2013 to February 2014).

Category	Term	Behavioral state or event
Rest	Lying down	Mid-section of body in contact with ground
	Sit	back part of body in contact with ground
	Stand	Stationary, upright position
Active	Self-groom	Lick own body
	Scratch	Scratch own body
	Dig	Scratch soil/dirt
	Mark urine dig	Dig-like behavior, typically with back legs after urinating
	Walk	Locomotion without in-air phase
	Trot	Locomotion with in-air phase
	Run	Locomotion with in-air phase where hind legs extend to Meet or pass front legs
	Pace	Walking back and forth over the same, small area
Biological functions	Raised leg urinate	Urinate with hind leg lifted
	Squat urinate	Urinate in squatting posture, hind leg may be slightly lifted
	Overmark urinate	Urinate in same spot where other coyote urinated <5 min
	Defecate	Defecate
	Eat	Consume solid food
	Drink	Consume water
Social interactions	Sniff site	Investigate soil/dirt/plant/etc.
	Sniff mate	Investigate other coyote
	Play invitation	Stamp or bow forelegs or use forelegs to paw mate
	Play chase	Chase mate, non-aggressive
	Present	Female orients to male for mounting
	Attempt mount	Male attempts to mount female
	Mount	Male mounts female
	Tie	Mount is successful
Antagonistic interactions	Charge/lunge	Advance toward mate, ears typically back
	Growl	Growl at mate
	Gape	Open mouth, oriented toward mate
	Agnostic chase	Chase mate, aggressive
	Submissive crouch	Crouch or semicrouch body position
	Submissive whining	Long and high-pitched, may accompany crouch
	Bite	Snapping jaws shut
	Bark	Short, loud vocalization often linked to aggression

above the respective cut-off value. Pooled serum from animals with known high titers was used as a positive control in each plate.

Pregnancy and litter counts

We tested all treated females for pregnancy using WITNESS® 244 Relaxin Canine Pregnancy Test (Zoetis, New Jersey, USA). Dates of testing varied based on observed copulation dates but were in conjunction with blood draws for hormone tests when possible. Those found to be pregnant received additional daily food rations to support the pregnancy. We calculated expected whelp dates as 63 days after observed copulations and monitored females more closely within approximately 10 days of the estimated whelping date to identify whelping date and obtain a 2-day litter count.

We attempted to obtain 2-day litter counts of all pups born to treated pairs per standard captive care protocol for the facility (Standard Operating Procedure: ACUT005.02); however, 2-day counts were not possible if the female gave birth in an earthen den instead of 1 of 2 manmade den boxes placed within each pen. Two-day counts were used to compare treated litter sizes to litter sizes counted at the facility over a 10-year period (2010–2015) and to litter sizes for treated animals prior to or after this study. We used a 10-year window for comparison to ensure only pregnant females maintained under the same animal care protocol were considered; the same animal care staff have been on site, and current standard operating procedures related to daily care and colony management have been in place since the 2005 breeding and pup-rearing seasons. This ensured nutrition and density factors were similar.

Behavior sampling

We recorded behavior of all treatment and control coyotes at randomly selected times from all daylight hours for 15-minute sampling periods. Each coyote was observed for behavioral samples at least 3 times each month. Behavior data included information on pair interactions obtained via focal sampling of individuals to determine the type and duration of behavior observed by pairs under the different treatments. We classified behavior into 4 major categories (Table 1). We also noted any copulatory activity observed during behavioral observations or opportunistically by animal care staff.

Two observers collected all behavioral data; they first trained together by observing and recording behavior of the same coyotes to ensure inter-observer reliability. We randomly selected which individual coyote of each pair was to be observed in a given sampling period but collected data on all treated coyotes throughout the breeding season (December 15, 2013 to February 15, 2014) and their mates during the peak of pair bonding (November and December; Carlson and Gese 2008). Although breeding may begin in mid-December, most female estrus occurs in early February at the facility (J. Young, personal observation).

Statistical analysis

For all statistical tests, $P < 0.05$ was considered statistically significant. Testosterone data were analyzed by repeated measures ANOVA and Tukey's post hoc test (GraphPad Software, Inc., La Jolla, California, USA). Testosterone values were log transformed before analysis and presented as mean ± SEM.

We used Fisher's exact test/odds ratio to test whether coyotes or their mates with lesions became pregnant. We used a chi-square test to compare litter sizes between treated coyotes and coyotes used for regular colony maintenance. Litter size was grouped as <5, 5, 6, 7, and >7 to ensure most categories had >5 data points for analysis and presented as mean ± SEM.

We performed a linear mixed effects analysis of the relationship between the proportions of time spent in selected behavioral categories and treatments using the *lme4* package (Bates et al. 2012) in R (R Core Team 2012). Visual inspection of residual plots revealed little deviation from homoscedasticity and normality for the proportion of time spent in the behavioral categories of interest. Using data from the entire breeding season, we first evaluated only those coyotes that received treatments or previously received a vasectomy and served as controls ($n = 24$). The main effect was treatment and the fixed effect was sex. Since behavioral observations were repeated within and across months, coyote identity and month were used as random effects. We next compared behavior throughout the breeding season of coyotes and

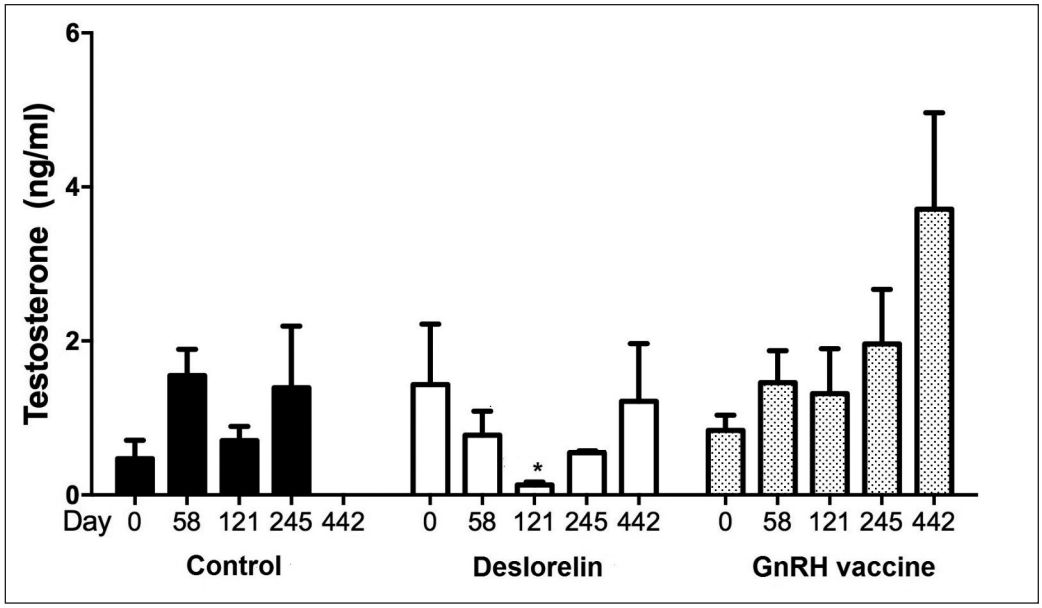

Figure 1. Plasma concentrations of testosterone from captive male coyotes (*Canis latrans*) at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA, treated with deslorelin (n = 6) or gonadotropin releasing hormone (GnRH) vaccine (n = 6) compared to vasectomized controls (n = 6). Deslorelin treated coyotes were implanted with 47 mg of deslorelin in October 2013 (day 0) and monitored for 2 breeding seasons with 58, 121, 245, and 442 days post treatment corresponding to December 2013, Februrary 2014, June 2014, and January 2015, respectively. The GnRH vaccine treated coyotes were injected day 0 and monitored as above. Data are represesnted as mean ± SEM: * significantly different from vasectomized control males at day 121.

their mates that had offspring to those that did not. Sex remained as a fixed effect, but we removed month as a random effect and added pregnancy. Finally, we compared behavior of treated coyotes and their mates during the peak of pair bonding behavior (November and December; n = 48). Sex remained as a fixed effect, but we added breeding pair identity as a third random effect. We evaluated statistical significance for fixed effects using likelihood ratio tests of the full models against the models lacking the factor in question. Time spent within each behavior category are presented as mean ± SEM.

Results

Testosterone

Deslorelin suppressed plasma testosterone compared to vasectomized control males for 245 days after implantation, although this was only significant on day 121 (Figure 1). Testosterone returned to pre-treatment levels 442 days after implantation. Males that impregnated their mates (0.11 ± 0.02 ng/ml, n = 3) and those that did not (0.17 ± 0.10 ng/ml, n = 3) had significantly reduced testosterone on day 121 (during breeding season; Supplemental Table 1) when compared to vasectomized control males (0.63 ± 0.58 ng/ml, n = 6; Supplemental Table 2). In contrast, GnRH vaccine treatment did not reduce testosterone levels at any time point compared to vasectomized controls (Figure 1). However, the 2 males that did not sire offspring had very low testosterone levels at days 58 and 121 (Supplemental Table 3).

Antibodies to GnRH

There was an overall poor response to the GnRH vaccine, as shown by antibody titers in GnRH vaccine treated animals (Figure 2). However, the animals that did not produce offspring were those that had the best responses to the vaccine (i.e., highest titers). Three females and 3 males receiving the vaccine had lesions at the injection sites. There was no correlation between whether coyotes had lesions at injection sites and if they or their mate became pregnant (Fisher's exact test, odds ratio = 0.36; P = 0.56).

Pregnancy and litter counts

Three of 6 females paired with males that received deslorelin (6.7 ± 0.3 pups/litter, n = 3),

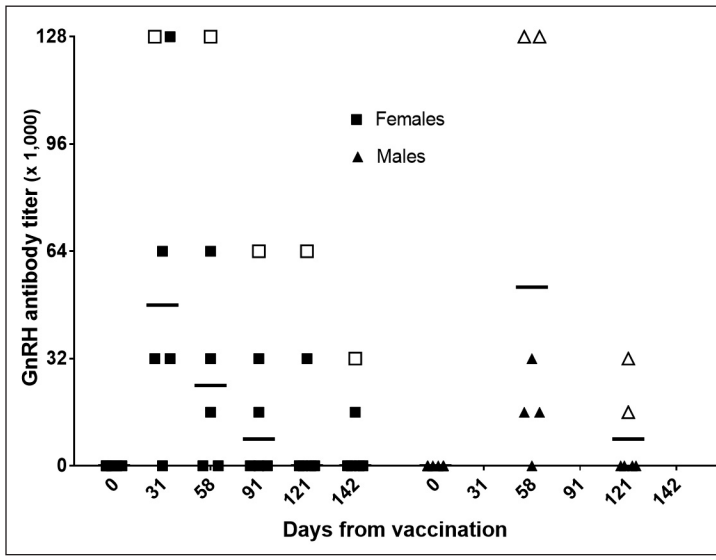

Figure 2. Antibody titers in coyotes (*Canis latrans*) treated with gonadotropin releasing hormone (GnRH) vaccine at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA, September 2013 to February 2014. Open symbols are from animals that did not produce litters. Horizontal lines represent median titers.

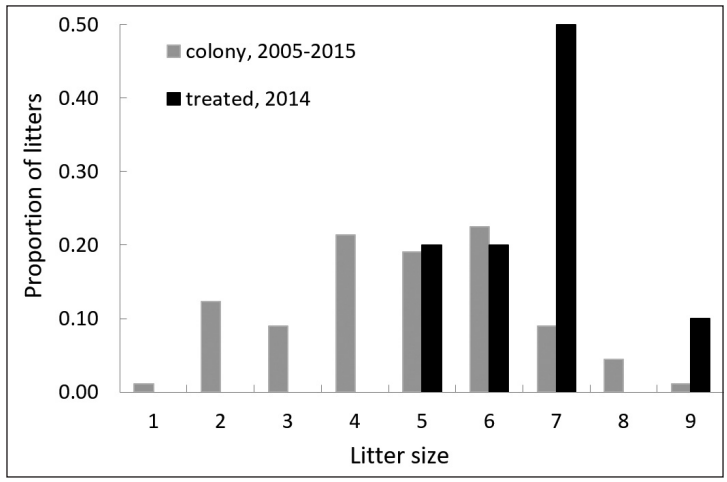

Figure 3. Proportion of litter sizes containing 1–9 pups based on pup counts at 2-day old counts for all litters born at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA, 2005–2015 (n = 89) and pups born in 2014 to coyotes (*Canis latrans*) treated with 1 of 2 nonsurgical reproductive inhibitors in 2013 (n = 10). Although 12 litters were born in spring 2014 to coyotes treated with nonsurgical reproductive inhibitors in fall 2013, we were unable to obtain 2-day counts on 2 litters.

4 of 6 females paired with males that received GnRH vaccine (6.3 ± 1.0 pups/litter, n = 4), and 5 of 6 females to receive GnRH vaccine (7.0 ± 0.0 pups/litter, n = 4) became pregnant and gave birth to live pups. We were unable to get a 2-day count for 2 GnRH vaccine treatment litters, but on May 14 we were able to count 6 pups from a litter born on April 6. The other litter was born on April 13 and attended to by the female for a couple of weeks before they disappeared after a severe storm, when it is likely she killed them. There was no significant difference in the number of pups per litter across treatment type ($F_{2,8}$ = 0.184, P = 0.835). Treated coyotes gave birth to more pups per litter than expected based on litter sizes of captive coyotes over 10 years (χ^2_{291} = 16.21, df = 4, P < 0.001; Figure 3). They also had more pups/litter relative to litter size of the same coyotes during other years prior to or after this experiment (treatment year = 6.6 ± 0.4 pups/litter, n = 9 litters; other years = 5.2 ± 0.3 pups/litter, n = 26 litters; t = -2.57, df = 16.83, P = 0.02). None of the females with a vasectomized male became pregnant or had pups.

Behavioral sampling

We recorded 106 hours of behavioral observations on treated coyotes (n = 24) and 36 hours of behavioral observations on their mates (n = 24). Coyotes spent most of their observed time resting and the least time engaged in antagonistic behavior (Figure 4). When analyzing only treated coyotes, a linear mixed model showed that treatment affected social interactions (χ^2 (2) = 6.52, P = 0.04). Estimated effects from the model indicated that compared to male coyotes treated with deslorelin (4.2 ± 0.7 % overall time budget), coyotes treated with GnRH vaccine spent more time engaged in social interactions (males: 4.4 ± 0.6 %; females: 3.7 ± 0.4 % overall time budget), and coyotes treated with vasectomy spent relatively less

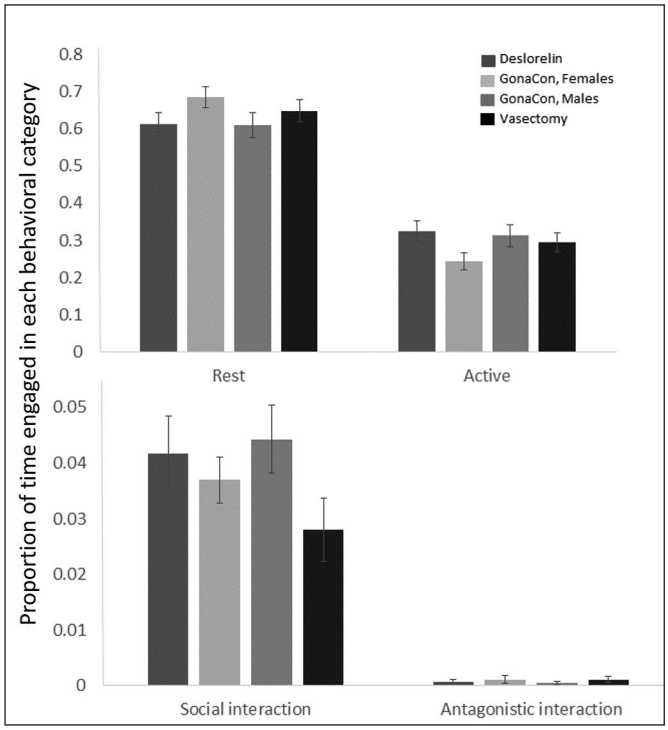

Figure 4. Average proportion of time (± SE) captive coyotes (*Canis latrans*) treated with deslorelin implants (gonadotropin releasing hormone [GnRH]; *n* = 6) or GnRH vaccine (Gonacon; *n* = 6 males, *n* = 6 females) were engaged in rest, active, social, or antagonistic behavior during the breeding season (December 15, 2013 to February 15, 2014), at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA. Males that previously received a vasectomy were also observed as controls (*n* = 6).

time engaged in social interactions (2.9 ± 0.6% overall time budget). However, there were no significant differences on the proportion of time spent engaged in resting (χ^2 (2) = 0.74, P = 0.69), antagonistic behavior (χ^2 (2) = 0.71, P = 0.70), or active (χ^2 (2) = 0.37, P = 0.83; Figure 4). When using a linear mixed model to evaluate all coyotes in treated pairs, we found the models that included treatment did not perform better than those without treatment on the proportion of time coyotes spent engaged in social interactions (χ^2 (2) = 3.35, P = 0.19), antagonistic interactions (χ^2 (2) = 1.48, P = 0.48), resting (χ^2 (2) = 1.91, P = 0.39), or active (χ^2 (2) = 2.37, P = 0.31). Similarly, the models that included whether a pair had offspring did not improve fit on the proportion of time coyotes spent engaged in antagonistic interactions (χ^2 (2) = 1.44, P = 0.49), resting (χ^2 (2) = 2.07 P = 0.35), social interactions (χ^2 (2) = 0.61, P = 0.74), or active (χ^2 (2) = 2.58, P = 0.28).

Discussion

Despite testing chemical reproductive inhibitors known to prevent reproduction in other wildlife, in this study at least half of each group of coyotes treated with a reproductive inhibitor successfully produced pups, and average litter size was larger than normal. Even so, not all coyotes reproduced, and suppression of fertility was highly variable for the 2 methods we tested that target GnRH. Inconsistent results have also been shown in female African wild dogs (*Lycaon pictus*) and wolves, with some pregnancies occurring and reproductive function not suppressed in male bush dogs (*Speothos venaticus*) treated with deslorelin (Bertschinger et al. 2001, 2002).

Coyotes in this study did not respond well to the GnRH vaccine. Half of the vaccine-treated animals developed lesions related to the injections, but the appearance of lesions was not correlated with strength of antibody response. Lesions were also reported in domestic dogs given a similar, mineral oil-based GnRH vaccine (Griffin et al. 2005). Lesions like the ones observed in this study could lead to severe infections and preclude its use as a management tool, even if it would have been more effective at preventing pregnancy. The 2 males with the best antibody responses to the vaccine had suppressed testosterone and did not sire offspring. Similarly, the female with the best response to the vaccine did not produce pups. Even so, 75% of the vaccine-treated coyote pairs produced pups. Thus, while there was some evidence that GnRH vaccination could inhibit reproduction, a much improved formulation would be necessary that is more effective and does not cause lesions to justify further testing.

We were also surprised with the lack of efficacy of deslorelin because it has been successfully used to suppress reproductive function in male coyotes housed alone (MacGregor et al. 2017) and in other captive carnivores housed with mates (Bertschinger et al. 2001, 2002). Further,

it has been used successfully in domestic dogs (Trigg et al. 2001, Goericke-Pesch et al. 2009, Junaidi et al. 2009). Based on results from this study, it is possible that the poor efficacy after treating males with deslorelin related to timing of administration. Implant placement too near the breeding season may have resulted in 3 of the 6 pairs treated with deslorelin having offspring. Time to downregulation in canids is variable between individuals and species, especially in males. In domestic dogs, testosterone production reaches a nadir by 6 weeks (Goericke-Pesch et al. 2009, Junaidi et al. 2009), whereas in African wild dogs (Newell-Fugate 2009) and gray wolves (Bertschinger et al. 2001), it may take >4 months for both testosterone reduction and azoospermia occur. Thus, in the current study, administration of deslorelin may have been too close to the onset of the breeding season to stop sperm production before their female counterparts entered estrus. Indeed, 2-thirds of the coyotes receiving the implants showed castration-level testosterone suppression at the start of the subsequent breeding season (day 442). This further suggests deslorelin may have been implanted too close to the immediate breeding season to work successfully in all coyotes.

Implants appeared to continue to have suppressive capabilities long after the 6-month minimum length of efficacy of the implant formulation. Although the *in-vitro* release rates for the implants are ~1 μg/day for 1 year (Trigg et al. 2001), experimental studies suggest either the implants may release for longer than 6 months or the reproductive axis may be slow to return to pre-treatment functionality. Recent *in-vivo* release rates in captive male coyotes found the 6-month implants released deslorelin for 12–18 months (MacGregor et al. 2017). For broad-scale colony management reasons at the facility, males treated with deslorelin were separated from their mates during the subsequent breeding season. Thus, it is unknown if the suppression observed in 4 of the males would have also prevented production of offspring. Declines in basal follicle-stimulating hormone and testosterone concentrations did not result in fewer sired offspring in male common brushtail opossums (*Trichosurus vulpecula*) treated with deslorelin (Eymann et al. 2007). This may be similar to recent studies in which male coyotes treated with deslorelin produced sperm even though pituitary hormones and testosterone were suppressed (MacGregor et al. 2017).

Even though coyotes showed some evidence of altered hormones, we found almost no differences in captive coyote behavior, suggesting reproductive inhibitors that target GnRH may not have significant impacts on pair bonds that could result in their dissolution. The differences observed among treatments in the amount of time spent in social interactions is unlikely to equate to biological differences since relatively little time was observed to be spent within this category already. While promising, we interpret these results with caution because it is unclear if results would be similar had the reproductive inhibition methods been more successful or if hormones had been suppressed in all treated individuals. Further, dissolution of the pair bond may be more complex or even suppressed in captive animals that are unable to disperse or abandon their territory. Additionally, we collected only minimal behavioral data and during daylight hours due to logistical issues (i.e., minimize stress to pregnant coyotes related to human presence) and may have missed behavioral shifts during active bouts overnight or at crepuscular hours. Because we relied on live observations from long distances to avoid human disturbance, we were unable to use night-vision equipment that would have enabled overnight observations. Even so, our observations are likely representative of captive coyote behavior because coyotes at the facility are more active during daylight hours, similar to wild populations without human persecution (Kitchen et al. 2000). The fact that male coyotes treated with deslorelin and showing suppression of testosterone for several months to a year were successfully maintained with their mates (i.e., no fighting, which would result in manual splitting) implies behavior and pair bonding may not be influenced by changes in hormones caused by nonsurgical reproductive inhibition. Similar results have recently been reported in red wolf (*C. rufus*), where pairs remained territorial whether surgery involved altering hormones (i.e., spay and neuter) or not (i.e., vasectomy and tubal litigation; Gese and Terletzky 2015).

In addition to the unexpected number of pairs

that produced offspring from our 3 treatment groups, the large litter sizes that treated pairs produced was also unexpected. Although within the range of litter sizes observed from captive coyotes, pairs within which 1 animal was treated with a reproductive inhibitor produced above-average litter sizes compared to a 10-year average at the facility and relative to their own reproductive output in other years. It is very unlikely that treatment of males, which made up the bulk of treated animals, would indirectly influence the physiology of females and litter size. Alterations in hormone levels of females treated with GnRH vaccine could possibly have had some effect on litter size, but hormones were not measured in those females. Moreover, a previous study using GnRH-treated females showed of those observed to tie and become pregnant, litter sizes were equal to that of the colony in the same year (Carlson and Gese 2009). In our study, there were no differences in litter size between treatment groups. Litter size can vary according to density, prey availability, nutrition, and breeding experience (Knowlton 1972, Todd et al. 1981, Windberg 1995, Gese et al. 2016).

Of these factors, only breeding experience could significantly vary among captive coyotes maintained at the facility over the 10-year period from which data were extracted. It is possible that litter size was higher than average because we used older, experienced breeder coyotes for this study and differences in litter size were unrelated to the actual treatments. The lack of variation in litter size among treatments supports this hypothesis. However, the larger litter sizes from the same individuals in other years, both before and more importantly after the treatment year, suggest further investigations into how nonsurgical reproductive inhibitors affect coyotes is warranted.

Management implications

Although neither compound tested can currently be recommended for inhibiting coyote reproduction, we did find evidence that targeting GnRH shows promise. We found no adverse behavioral or health effects from treatment of male coyotes with deslorelin. While more research into the mechanism by which deslorelin suppresses the male reproductive axis may be warranted, testing in females should also be considered, and the development of an implant capable of a consistent duration of drug release is necessary. Coyotes are monoestrus and seasonal breeders, which should facilitate the use of reproductive control methods. Successful fertility control in coyotes will necessitate the reproductive tool be consistent in efficacy, not produce unintended consequences to individual and group fitness, and be easy to administer.

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Supplemental Table 1. Pre- and post-treatment data for captive male coyotes (*Canis latrans*) treated with deslorelin at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA. An asterisk (*) denotes an insufficient sample. Data include plasma testosterone, pregnancy of mate, and observed copulatory ties. Sampling occurred over study duration (October 2013 to January 2015) on day 0, 58, 121, 245, and 432 post implantation. Day 121 occured within February 2014, the timeframe most females are in estrus at the colony.

Coyote ID	Days post treatment	Testosterone (ng/ml)	Pregnant (mate)	Copulatory tie observed
6133	0	0.76	Yes	No
	58	0.62		
	121	0.07		
	245	0.55		
	442	3.05		
1031	0	1.73	Yes	No
	58	2.24		
	121	0.14		
	245	*		
	442	0.04		
1111	0	0.27	No	No
	58	0.28		
	121	0.07		
	245	0.55		
	442	0.04		
8071	0	0.55	Yes	Yes
	58	0.86		
	121	0.12		
	245	0.55		
	442	4.04		
1151	0	0.10	No	Yes
	58	0.52		
	121	0.26		
	245	0.55		
	442	0.06		
941	0	5.19	No	No
	58	0.13		
	121	*		
	245	0.55		
	442	0.04		

Supplemental Table 2. Pre- and post-treatment data for captive male coyotes (*Canis latrans*) that had a surgical vasectomy and served as controls at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA. An asterisk (*) denotes an insufficient sample. Data include plasma testosterone, pregnancy of mate, and observed copulatory ties. Sampling occurred over study duration (October 2013 to January 2015) on day 0, 58, 121, and 245. Day 121 occured within February 2014, the timeframe most females are in estrus at the colony.

Coyote ID	Days post treatment	Testosterone (ng/ml)	Pregnant (mate)	Copulatory tie observed
1073	0	0.20	No	No
	58	1.02		
	121	1.36		
	245	0.55		
6135	0	0.23	No	No
	58	1.55		
	121	0.23		
	245	0.55		
701	0	0.53	No	No
	58	0.66		
	121	0.51		
	245	0.55		
113	0	1.36	No	No
	58	1.79		
	121	0.70		
	245	0.71		
1181	0	0.04	No	No
	58	1.66		
	121	0.26		
	245	4.61		
1041	0	*	No	No
	58	2.63		
	121	0.72		
	245	0.26		

Supplemental Table 3. Pre- and post-treatment data for captive male coyotes (*Canis latrans*) treated with gonadotropin releasing hormone (GnRH) vaccine at the U.S. Department of Agriculture, National Wildlife Research Center – Predator Research Facility, Logan, Utah, USA. An asterisk (*) denotes an insufficient sample. Data include plasma testosterone, pregnancy of mate, and observed copulatory ties. Sampling occurred over study duration (October 2013 to January 2015) on day 0, 58, 121, 245, and 442 post implantation. Day 121 occured within February 2014, the timeframe most females are in estrus at the colony.

Coyote ID	Days post treatment	Testosterone (ng/ml)	Pregnant (mate)	Copulatory tie observed
1021	0	0.55	Yes	No
	58	0.67		
	121	2.34		
	245	0.72		
	442	2.39		
6071	0	1.75	No	No
	58	0.55		
	121	0.05		
	245	1.98		
	442	*		
1011	0	0.55	No	No
	58	0.54		
	121	0.04		
	245	1.02		
	442	6.23		
931	0	0.55	Yes	
	58	2.42		
	121	1.99		
	245	2.29		
	442	5.35		
921	0	1.06	Yes	
	58	1.71		
	121	0.13		
	245	0.55		
	442	*		
8113	0	0.56	Yes	Yes
	58	2.85		
	121	3.32		
	245	5.21		
	442	0.88		

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