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ABSTRACT
Contraception may provide a useful nonlethal management tool when it is desirable to reduce populations of birds. We tested the efficacy of 20,25 diazacholesterol, and immunization with avian gonadotropin-releasing hormone (AGnRH-I) and chicken riboflavin carrier protein (cRCP) as contraceptives and investigated their modes of action in Coturnix quail (Coturnix coturnix japonica). Females that were paired with males treated with 20,25 diazacholesterol produced lower percentages of eggs that were fertile and hatched. Females treated with 20,25 diazacholesterol and paired with control males laid fewer eggs, and lower percentages of their eggs were fertile and hatched. Treatment with 20,25 diazacholesterol reduced testosterone levels in males and progesterone levels in females. Nonesterified cholesterol levels were reduced, whereas desmosterol levels increased in birds treated with 20,25 diazacholesterol. Treatment with AGnRH-I and cRCP immunocontraceptive vaccines did not decrease average egg production and hatchability or hormone levels, but this failure might have been due to the vaccination protocol. If registered, wildlife managers may be able to use 20,25 diazacholesterol when other methods, such as lethal control, are undesirable for reducing damage caused by specific breeding behaviors such as the building of nests.

(Key Words: avian gonadotropin-releasing hormone, chicken riboflavin carrier protein, 20,25 diazacholesterol, immunocontraception, reproductive inhibition)

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INTRODUCTION

Birds cause numerous conflicts including consumption of food crops (White et al., 1985; Dolbeer et al., 1986; Wilson et al., 1989), fruits and vegetables (Clark, 1986), livestock feed (Glahn, 1982; Glahn and Otis, 1986), and fish at aquaculture facilities (Parkhurst et al., 1987; Stickley et al., 1995; Andelt et al., 1997). Birds also cause public health concerns by transmitting disease (Gough and Beyer, 1982; Glahn and Stone, 1984) and safety concerns by striking aircraft (Dolbeer, 1989; Conover et al., 1995). Care must be taken in selecting and using techniques that resolve conflicts because the public has concern about lethal control techniques. Contraception is a possible nonlethal alternative that is more acceptable to the general public than lethal control measures (Arthur, 1981; Messmer et al., 1997; Stout et al., 1997).

A number of contraceptives have been tested in birds, but several, such as the synthetic estrogen BDH10131 (Kendle et al., 1973), mestranol (Wentworth, 1968; Wentworth et al., 1968; Sturtevant, 1970), α-chlorohydrin (Aire and Olusanya, 1980), triethylendiamine (TEM) (Davis, 1961; Vandenbergh and Davis, 1962; Messersmith, 1971; Bhat and Maiti, 1989), thiotepa (Potvin et al., 1982), and surgical sterilization (Converse and Kennelly, 1994), have produced unacceptable results. The desirable qualities of any wildlife contraceptive are that it last for one breeding season with a minimal number of days of treatment and that it be reversible so as not to permanently remove the animals from the gene pool. In addition, for practical reasons, the contraceptive should be oral to eliminate the need to capture animals and must be relatively inexpensive (Turner and Kirkpatrick, 1991; Garrott, 1995). Mestranol, α-chlorohydrin, and TEM might possibly permanently sterilize adults and young being fed by crop milk. Thiotepa and BDH10131 are associated with a limited duration of effect that requires an extended treatment

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Abbreviation Key: AGnRH-I = avian gonadotropin-releasing hormone; cRCP = chicken riboflavin carrier protein; FCA = Freund’s complete adjuvant; FIA = Freund’s incomplete adjuvant; FSH = follicle-stimulating hormone; GnRH = gonadotropin-releasing hormone; KLH = keyhole limpet hemocyanin; LH = luteinizing hormone; TEM = triethylendiamine.
period to cover the breeding season. Surgical sterilization is prohibitively expensive.

The cholesterol inhibitor 20,25 diazacholesterol is a promising contraceptive because, in contrast to immuncontraceptives that currently must be injected, it can be delivered orally. Research on the efficacy of 20,25 diazacholesterol produced conflicting results with red-winged blackbirds (Agelaius phoenicus) (Fringer and Granett, 1970; Lacombe et al., 1986, 1987, 1990), pigeons (Columbia livia) (Schortemeyer and Beckwith, 1970; Sturtevant and Wentworth, 1970), house sparrows (Passer domesticus) (Sanders and Elder, 1976; Mitchell et al., 1979), grackles (Quiscalus quiscula) (Fringer and Granett, 1970), Japanese quail (Coturnix coturnix japonica), and parakeets (probably Melopsittacus undulatus) (Powell, 1966). Conclusions from these studies were limited because little physiological work with hormone levels was conducted to determine the mode of action of the compound.

Immuncontraception acts on the animal’s immune system to control reproduction. Gonadotropin-releasing hormone (GnRH) is a promising antigen for use as an immuncontraceptive vaccine. Antibodies generated against avian GnRH (AGnRH-I) or GnRH were effective in inhibiting reproduction in cowbirds (Molothrus ater; Thompson et al., 1994), starlings (L. A. Miller, 2000, USDA/APHIS/WS/NWRC, personal communication), and several species of mammals (Ladd et al., 1988; Awoniyi et al., 1993; Meloen et al., 1994). The amino acid sequence of AGnRH-I differs from mammalian GnRH by one amino acid, which may confer some avian specificity. Mammalian GnRH vaccines were effective for ≥1 yr in rats (Rattus norvegicus) (Miller et al., 1997) and ≥2 yr in white-tailed deer (Odocoileus virginianus) (Miller et al., 2000), which may make AGnRH-I suitable for use as an immuncontraceptive lasting at least one breeding season in birds.

Another promising antigen for use in an immuncontraceptive vaccine is chicken riboflavin carrier protein (cRCP). Antibodies to cRCP inhibited reproduction in lab mice (inbred Swiss strain; Natraj et al., 1994) and common marmosets (Callithrix jacchus; Natraj, 1991). Immunological homologues to cRCP were demonstrated in lab mice and rats (Muniyappa and Adiga, 1980; Natraj et al., 1987), primates (Macaca radiata; Visweswariah and Adiga, 1987a; Callithrix jacchus; Natraj and Khoklute, 1989), and humans (Natraj et al., 1987; Visweswariah and Adiga, 1987b). A cRCP vaccine may be preferable to other contraceptive agents because it should not interfere with reproductive and territorial behaviors. No research has previously been conducted to test the effect of antibodies to cRCP on reproduction of birds.

Our objectives were to assess the efficacy of 20,25 diazacholesterol and immunization with AGnRH-I-keyhole limpet hemocyanin (KLH) and cRCP-KLH in Coturnix quail by measuring numbers of eggs produced, fertility, and hatchability of eggs. Our objectives also were to document the effects of all 3 contraceptives on testosterone and progesterone levels; the effect of 20,25 diazacholesterol on cholesterol, desmosterol, and corticosterone levels; the effect of AGnRH-I on corticosterone levels; and the ability of AGnRH-I and cRCP to induce an antibody response.

We proposed that 20,25 diazacholesterol would reduce fertility by preventing side chain cleavage of cholesterol or reduce cholesterol levels directly by preventing the conversion of desmosterol to cholesterol (Dietert and Scallen, 1969; Counsell et al., 1971; Emmons et al., 1982). Cholesterol is the parent compound for steroid hormones, which include the reproductive hormones testosterone and progesterone. We hypothesized that the block of desmosterol to cholesterol would result in increased desmosterol and decreased cholesterol and, therefore, decreased testosterone, progesterone, and corticosterone, thus reducing egg production and fertility.

We proposed that vaccination with AGnRH-I vaccine would create antibodies to native AGnRH-I. In the male, treatment with AGnRH-I should indirectly lower testosterone levels through its effect on follicle-stimulating hormone (FSH) and lutetizing hormone (LH), thus lowering sperm production and reducing fertility and hatchability. In females, treatment with AGnRH-I should lower FSH and LH levels, reducing follicular development, thus indirectly reducing progesterone levels and egg production. Because the AGnRH-I vaccine may affect copulatory behavior, we predicted that treated females might lay a lower percentage of fertile eggs. In birds, corticosterone is formed from progesterone, thus we predicted decreased progesterone could also decrease corticosterone. We did not expect a reduction in testosterone to have any effect on corticosterone levels.

We proposed that vaccination with cRCP vaccine would create antibodies to native cRCP, thus blocking riboflavin binding to the riboflavin transport protein and preventing storage of riboflavin. We proposed this block would cause embryos of treated birds to consistently die at an early stage because riboflavin is essential for embryonic development, and so a lower percentage of eggs would hatch in the treated group than in the control group. We predicted that treatment would have no effect on progesterone levels and therefore would not affect the number or fertility of eggs laid by treated females.

Coturnix quail were used in this study because, in contrast to many wild species of birds, Coturnix quail reproduce readily in captivity, and their reproductive cycles are easily manipulated by changing the photoperiod (Padgett and Ivey, 1959; Wilson et al., 1962; Schafer et al., 1977). Testicular regression can be induced using a 6L:18D light cycle, and testicular development can be induced using a 16L:8D light cycle (Schafer et al., 1977). Egg production can be reduced by decreasing the number of hours of light exposure (Wilson et al., 1962). Coturnix quail are sexually mature at 42 d, and mature females can lay ≤300 eggs per year in the laboratory (National Academy of Sciences, 1969). Coturnix quail exhibit reproductive seasonality in natural settings (National Academy of Sciences, 1969; Wada, 1979). Once the male is removed from the female, sperm can be stored and is viable in the female reproductive tract for 6 to 9 d (Sittman and Abplanalp, 1965; Woodard and Abplanalp, 1967).
MATERIALS AND METHODS

The experimental protocol was reviewed by the Colorado State University and National Wildlife Research Center’s Animal Care and Use Committees and complied with the Animal Welfare Act. The experiment consisted of 1) 20 female Coturnix quail treated with 20.25 diazacholesterol and paired with 20 untreated males, 2) 20 males treated with 20.25 diazacholesterol and paired with 20 untreated females, 3) 20 females vaccinated with AGnRH-I-KLH and paired with 20 untreated males, 4) 20 males vaccinated with AGnRH-I-KLH and paired with 20 untreated females, 5) 20 females vaccinated with cRCP-KLH and paired with 20 untreated males, and 6) a control group of 20 untreated males paired with 20 untreated females. The untreated members of each group were used to provide fertility and hatching data. Birds were weighed and separated by sex. Six of the heaviest of 120 females were randomly assigned to each of 6 treatment groups, then the next 6 heaviest females were randomly assigned to groups, and so on until each group contained 20 females. The same procedure was used for the males. Ten birds of each sex from each treatment group were randomly assigned to cage racks and to cages within racks. All birds were 8 wk of age at the beginning of the study (at pretreatment wk 1). Birds were maintained on a 16L:8D light cycle, which we determined in a pilot study to be the optimal light cycle for peak sex hormone levels (Yoder, 2000). Quail were maintained on a layer diet that consisted of 3.6 to 4.6% calcium, ≥ 0.5% phosphorus, ≥ 16% crude protein, and 2.88 kcal/g metabolizable energy.

We drew 500 μL of blood from the jugular vein of each bird treated with 20.25 diazacholesterol and from all control birds once each week for 3 wk pretreatment (at 8, 9, and 10 wk of age), once per week for 2 wk of treatment (at 11 and 12 wk of age) and 8 wk posttreatment (at 13 to 21 wk of age), and then once every 2 wk for 11 wk. We drew 500 μL of blood from the jugular vein of AGnRH-I- and cRCP-treated birds once each week for 3 wk pretreatment (at 8, 9, and 10 wk of age); once each in wk 7 (at 14 wk of age), wk 11 (at 18 wk of age), and wk 13 (at 20 wk of age); and then once every 2 wk for 11 wk. Birds in the AGnRH-I and cRCP groups were weighed once a week during the treatment period. This procedure resulted in 10 males and 10 females fed treated bait for 14 d, 5 males and 5 females fed treated bait for 13 d, and 5 males and 5 females fed treated bait for 12 d. Data were combined regardless of the length of treatment for analysis. Control birds were fed the same product without the 20.25 diazacholesterol. Uneaten bait was weighed each day. We calculated the average dose of 20.25 diazacholesterol fed to each bird each day post hoc as described in Yoder (2000). Quail were weighed once a week during the treatment period and once every 2 wk thereafter to ascertain if weight loss occurred.

Primary and booster AGnRH-I and cRCP vaccines were prepared according to the method described by the manufacturer for maleimide-activated keyhole limpet hemocyanin (KLH). In wk 3, birds in the AGnRH-I treatment group were given a 0.3-mL primary vaccination of 100 μg of AGnRH-I-KLH conjugate in Freund’s complete adjuvant (FCA), and cRCP birds were given a 0.3-mL primary vaccination of 100 μg of cRCP-KLH conjugate in FCA. Two 0.3-mL booster vaccinations consisting of 100 μg of AGnRH-I-KLH conjugate in Freund’s incomplete adjuvant (FIA) or 100 μg of cRCP-KLH conjugate in FIA were given to the respective groups during wk 7 and 11. Control birds were sham vaccinated with 0.15 mL of saline in 0.15 mL of FCA or FIA. Vaccinations were given...
weighed once every 2 wk to ascertain if weight loss occurred.

Because sperm can remain viable in the reproductive tract of female quail for ≤9 d (Sittman and Abplanalp, 1965; Woodard and Abplanalp, 1967), we did not begin collecting eggs to determine fertility and hatchability for the 20,25 diazacholesterol groups until 1 wk after treatment ceased due to the necessary rearrangement of pairs for treatment purposes. We did not expect any effects on fertility and hatchability from the AGnRH-I and cRCP vaccines until fertility and hatchability from the AGnRH-I and cRCP vaccines until 2 wk after the last booster vaccine was given, thus we began collecting eggs 3 wk after the last vaccination. Eggs were collected and incubated to determine viability of the embryos and chicks. Unhatched eggs were opened after the expected hatching date to determine viability. The stage of development of the embryo at death was determined for the cRCP and control groups only according to Padgett and Ivey (1960). Fertility was defined as the presence of an embryo or a blastodisc. Eggs were considered to be infertile if neither an embryo nor a blastodisc could be located or the egg had degraded to the point that such a determination was not possible. We determined plasma progesterone, testosterone, and corticosterone levels for all treatment and control groups using a solid-phase \(^{125}\) IRIA technique with antibody-coated tubes\(^7\) and a Genesys 5000 series multiwell gamma-counter.\(^8\) Desmosterol and nonesterified cholesterol levels were determined for the 20,25 diazacholesterol groups using HPLC following the procedure of Johnston et al. (2001). Antibody titer levels were determined for the AGnRH-I and cRCP groups using an ELISA technique developed at the National Wildlife Research Center (Yoder, 2000).

**Statistical Analyses**

We divided the study into pretreatment (1 to 3 wk) and treatment and posttreatment (4 to 23 wk) phases for comparisons among diazacholesterol and control groups. Data were combined for the diazacholesterol groups regardless of the length of treatment. We also divided the study into pretreatment (1 to 3 wk), priming (4 to 7 wk), first boost (8 to 11 wk), and second boost (12 to 24 wk) phases for comparisons among AGnRH-I, cRCP, and control groups. We used a one-way ANOVA (PROC GLM; SAS Institute Inc., 1988), and \(P < 0.05\) for all analyses.

The mean percentage of fertile eggs was calculated by combining the number of eggs that hatched and the number of eggs that were fertile (either by the presence of an embryo or blastodisc) post expected hatching date, dividing this number by the total number of eggs set, and multiplying the result by 100. The mean number of eggs that hatched was calculated by dividing the number of eggs that hatched by the number of eggs set and multiplying the result by 100. We divided the incubation period into 6 stages of embryonic development, incubation 1 to 3 d, 3 to 6 d, 6 to 9 d, 9 to 12 d, 12 to 15 d, and 15 to 17 d, and assigned each embryo to a stage of development at death. We compared average stage of development at death among the cRCP and control groups using a one-tailed ANOVA. Only weeks that eggs were incubated to term were used for this analysis.

Because pretreatment levels of testosterone and corticosterone varied among birds, we averaged the 3 pretreatment values for each bird, subtracted this value from the response mean of 4 to 23 wk by bird for the diazacholesterol and control group comparisons and from the mean of each of the subsequent phases for AGnRH-I, cRCP, and control group comparisons. We compared the testosterone differences among treatment and control groups, and the corticosterone differences among the control and 20,25 diazacholesterol groups and among the control and female AGnRH-I groups using a one-tailed or two-tailed ANOVA.

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\(^7\)Product numbers TKPG1, TKTT1, and TKRC1, respectively, Diagnostic Products Corp., Los Angeles, CA.

\(^8\)Laboratory Technologies Inc., Schaumburg, IL.
Results

20,25 Diazacholesterol Males

The percentage of eggs that were fertile and hatched were reduced by 20,25 diazacholesterol in treatment compared with control groups (Figure 1, Table 1). Testosterone levels decreased more in treated males than in control males. Differences in corticosterone levels did not vary between treatment and control males \((P = 0.376)\). Nonesterified cholesterol levels were lower in treated males than in control males. Desmosterol levels were greater in treated males than in control males. Control males gained more weight than treated males during wk 4 to 23. The average dose of 20,25 diazacholesterol per male per day was 59.42 mg/kg (range = 0.01 to 101.40 mg/kg). Dose was negatively correlated with testosterone levels and the percentage of eggs that were fertile \((P = 0.0002)\), nonesterified cholesterol levels \((P = <0.0001)\), and percentage of eggs that hatched \((P = 0.0015)\). Dose was positively correlated with desmosterol levels \((P = 0.0002)\).

20,25 Diazacholesterol Females

The number of eggs laid per female per week and the percentages of eggs that were fertile and hatched were reduced by 20,25 diazacholesterol in treatment compared with control groups (Figure 2, Table 2). Progesterone and nonesterified cholesterol levels were lower in treated females than in control females. Differences in corticosterone levels did not vary between treatment and control females \((P = 0.895)\). Desmosterol levels were higher in treated females than in control females. Control females gained more weight than treated females during wk 4 to 23. The average dose of 20,25 diazacholesterol per female per day was 61.03 mg/kg (range = 1.47 to 103.37 mg/kg). Dose was negatively correlated with progesterone levels \((P = 0.05),\) nonesterified cholesterol and the number of eggs laid per female per week \((P = <0.0001)\), the percentage of eggs that were fertile \((P = 0.0458)\), and the percentage of eggs that hatched \((P = 0.0001)\). Dose was positively correlated with desmosterol \((P = <0.0001)\).

AGnRH-I Males

The percent of eggs that were fertile did not vary between treated and control males (Table 3). The percentage of eggs that hatched was marginally lower in the treatment compared with the control group. Changes in testosterone levels from pretreatment values did not differ between the treated and control males during the priming and first boost phases, but testosterone decreased more
in treated males than in control males during the second boost phase. Changes in corticosterone did not differ between treated and control males from pretreatment values during the priming phase (P = 0.952), first boost phase (P = 0.194), and second boost phase (P = 0.936). Changes in body weight from pretreatment values during the 3 phases did not differ between treated and control males. Treated males had antibody titers of 3,711 during the priming phase, 7,789 during the first boost phase, and 9,061 during the second boost phase. Control males had no antibody titers during all 3 phases.

**AGnRH-I Females**

The number of eggs laid per female per week during all 3 phases and the percentage of eggs that were fertile and hatched during the second boost phase did not differ between treated and control females (Table 4). Progesterone levels did not differ between treated and control females in the priming and first boost phases, but progesterone levels were lower in treated females than in control females during the second boost phase. Changes in corticosterone from pretreatment levels did not differ between treated and control females during the priming phase (P = 0.750), first boost phase (P = 0.257), or second boost phase (P = 0.974). Treated females gained more weight than control females during the priming, first boost, and second boost phases. Treated females had antibody titers of 2,025 during the priming phase, 8,625 during the first boost phase, and 7,275 during the second boost phase. Control females had no antibody titers during all 3 phases.

### TABLE 2. Fertility, hormone levels, cholesterol and desmosterol levels, and weight for females treated with 20,25 diazacholesterol and control Coturnix quail at the National Wildlife Research Center, Fort Collins, Colorado, January to July 1997

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AGnRH-I</th>
<th>Control</th>
<th>p&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>20,25 diazacholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of eggs laid per female per week&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.65 ± 0.17</td>
<td>4.50 ± 0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Percentage of fertile eggs&lt;sup&gt;3&lt;/sup&gt;</td>
<td>21.0 ± 11.0</td>
<td>70.0 ± 5.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Percentage of eggs hatched&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.0 ± 0.0</td>
<td>36.0 ± 4.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td>20 ± 0.45 ± 0.06</td>
<td>19 ± 0.77 ± 0.08</td>
<td>0.002</td>
</tr>
<tr>
<td>Nonesterified cholesterol (µg/mL)</td>
<td>18 ± 224.4 ± 26.50</td>
<td>19 ± 461.7 ± 44.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Desmosterol (µg/mL)</td>
<td>18 ± 283.67 ± 29.60</td>
<td>19 ± 32.36 ± 3.36</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change in body weight (g)&lt;sup&gt;5&lt;/sup&gt;</td>
<td>-0.70 ± 4.97</td>
<td>19 ± 20.90 ± 3.56</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<sup>1</sup>All P-values are 1-tailed except the P-value for weight, which is 2-tailed.

<sup>2</sup>Values are for the combined treatment and posttreatment period (4 to 23 wk).

<sup>3</sup>Average weight was 204.86 g (SE = 5.27) for the control group and 184.13 (SE = 4.75) for the 20,25 diazacholesterol group for the combined treatment and posttreatment period (4 to 23 wk).

### TABLE 3. Fertility, hormone levels, and weights for males treated with AGnRH-I<sup>1</sup> and control Coturnix quail at the National Wildlife Research Center, Fort Collins, Colorado, January to July 1997

<table>
<thead>
<tr>
<th>Parameter and phase</th>
<th>AGnRH-I</th>
<th>Control</th>
<th>p&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of fertile eggs&lt;sup&gt;3&lt;/sup&gt;</td>
<td>18 ± 56.0 ± 7.0</td>
<td>20 ± 62.0 ± 7.0</td>
<td>0.250</td>
</tr>
<tr>
<td>Percentage of eggs hatched&lt;sup&gt;4&lt;/sup&gt;</td>
<td>18 ± 14.0 ± 3.0</td>
<td>20 ± 24.0 ± 5.0</td>
<td>0.066</td>
</tr>
<tr>
<td>Change in testosterone (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime&lt;sup&gt;5&lt;/sup&gt;</td>
<td>19 ± -0.05 ± 0.27</td>
<td>20 ± 0.09 ± 0.17</td>
<td>0.336</td>
</tr>
<tr>
<td>First boost&lt;sup&gt;6&lt;/sup&gt;</td>
<td>19 ± -0.53 ± 0.35</td>
<td>20 ± -0.06 ± 0.19</td>
<td>0.118</td>
</tr>
<tr>
<td>Second boost&lt;sup&gt;6&lt;/sup&gt;</td>
<td>19 ± -0.38 ± 0.31</td>
<td>20 ± 0.03 ± 0.20</td>
<td>0.038</td>
</tr>
<tr>
<td>Change in body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime&lt;sup&gt;5&lt;/sup&gt;</td>
<td>19 ± 18.55 ± 2.67</td>
<td>20 ± 13.00 ± 1.94</td>
<td>0.184</td>
</tr>
<tr>
<td>First boost&lt;sup&gt;6&lt;/sup&gt;</td>
<td>19 ± 28.12 ± 3.01</td>
<td>20 ± 23.40 ± 2.81</td>
<td>0.258</td>
</tr>
<tr>
<td>Second boost&lt;sup&gt;6&lt;/sup&gt;</td>
<td>19 ± 30.29 ± 3.38</td>
<td>20 ± 29.04 ± 2.86</td>
<td>0.779</td>
</tr>
</tbody>
</table>

<sup>1</sup>Avian gonadotropin releasing hormone-I.

<sup>2</sup>All P-values are 1-tailed except P-values for weight and corticosterone, which are 2-tailed.

<sup>3</sup>Values are for 14 to 24 wk and were obtained from females paired with treated males.

<sup>4</sup>Values were obtained by subtracting the pretreatment means from each of the phase means (priming, first boost, and second boost phases).

<sup>5</sup>Average testosterone levels for the priming, first boost, and second boost phases were 3.37 ng/mL (SE = 0.19), 3.22 ng/mL (SE = 0.19), and 3.38 ng/mL (SE = 0.27), respectively, for the control group and 2.69 ng/mL (SE = 0.20), 2.21 ng/mL (SE = 0.39), and 2.36 ng/mL (SE = 0.32), respectively, for the AGnRH-I group.

<sup>6</sup>Average weights for the priming, first boost, and second boost phases were 172.37 g (SE = 2.96), 182.78 g (SE = 4.04), and 188.41 g (SE = 5.99), respectively, for the control group and 176.68 g (SE = 3.20), 187.35 g (SE = 3.41), and 190.99 g (SE = 3.94) respectively for the AGnRH-I group.
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Table 4. Fertility, hormone levels, and weights for females treated with AGnRH-I and control Coturnix quail at the National Wildlife Research Center, Fort Collins, Colorado, January to July 1997

<table>
<thead>
<tr>
<th>Parameter and phase</th>
<th>AGnRH-I</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>x</td>
</tr>
<tr>
<td>Number of eggs laid per female per week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime</td>
<td>20</td>
<td>5.23</td>
</tr>
<tr>
<td>First boost</td>
<td>20</td>
<td>4.68</td>
</tr>
<tr>
<td>Second boost</td>
<td>20</td>
<td>4.39</td>
</tr>
<tr>
<td>Percentage of fertile eggs</td>
<td>20</td>
<td>57.0</td>
</tr>
<tr>
<td>Percentage of eggs hatched</td>
<td>20</td>
<td>20.0</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime</td>
<td>20</td>
<td>0.75</td>
</tr>
<tr>
<td>First boost</td>
<td>20</td>
<td>0.71</td>
</tr>
<tr>
<td>Second boost</td>
<td>20</td>
<td>0.55</td>
</tr>
<tr>
<td>Change in body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime1,5</td>
<td>20</td>
<td>32.87</td>
</tr>
<tr>
<td>First boost1,5</td>
<td>20</td>
<td>44.76</td>
</tr>
<tr>
<td>Second boost1,5</td>
<td>20</td>
<td>43.62</td>
</tr>
</tbody>
</table>

1Avian gonadotropin-releasing hormone-I.
2All P-values are 1-tailed except P-values for weight, which are 2-tailed.
3Values are for 14 to 24 wk.
4Values were obtained by subtracting the pretreatment means from each of the phase means (priming, first boost, and second boost phases).
5Average weights for the priming, first boost, and second boost phases were 198.36 g (SE = 5.08), 210.85 g (SE = 6.01), and 211.75 g (SE = 6.13), respectively, for the control group and 213.93 g (SE = 4.46), 225.82 g (SE = 5.56), and 224.68 g (SE = 6.18), respectively, for the AGnRH-I group.

CRCP Females

The proportion of eggs that were fertile and hatched and the average stage of embryonic development at death did not vary between treated and control birds during the second boost phase (Table 5). The number of eggs laid per female per week and progesterone levels did not differ between treated and control birds during the 3 phases. Changes in body weight from pretreatment values did not vary between the treatment and control females during the 3 phases. Treated birds had higher antibody titers of 6,658 during the priming phase, 5,059 during the first boost phase, and 8,808 during the second boost phase. Control birds had no antibody titers during any of the 3 phases.

DISCUSSION

20,25 Diazacholesterol

The decrease of testosterone in treated males and the decrease in percentage of fertile eggs laid by their mates suggests 20,25 diazacholesterol indirectly caused a reduction in spermatogenesis through lowered testosterone levels, as reported by Lacombe et al. (1986, 1987) in red-winged blackbirds. Powell (1966) also observed a lower percentage of fertile eggs laid by female Coturnix quail paired with treated males. Wofford and Elder (1967) observed complete inhibition of reproduction for 5 to 7 mo in pigeons fed 0.1% 20,25 diazacholesterol.

Lower levels of progesterone in 20,25 diazacholesterol treated females likely contributed to the reduction in egg laying (Figure 2) because progesterone is necessary for ovulation (Johnson, 2000). Powell (1966) and Wofford and Elder (1967) noted reductions in the rate of laying by 20,25 diazacholesterol-treated female Coturnix quail and female pigeons, respectively. One explanation may be that preovulatory follicles in females treated with 20,25 diazacholesterol may not produce enough progesterone and thus become atretic. Powell (1966) and Sturtevant and Wentworth (1970) found an increase in the number of atretic follicles in treated female Coturnix quail and female pigeons, respectively. Sanders and Elder (1976) observed that treated female house sparrows did not have mature follicles in the ovary.

We expected 20,25 diazacholesterol to reduce corticosterone levels because it is a steroid hormone. We recognize that corticosterone is difficult to accurately measure in the plasma because the stress of handling increases the levels. The rise in corticosterone due to handling was not of concern here but rather a marked decrease in corticosterone levels, which would indicate that 20,25 diazacholesterol impaired corticosterone production. However, because corticosterone levels were similar in treatment and control groups, it is possible that a larger dose of 20,25 diazacholesterol is needed to affect this hormone. Corticosterone occurs at higher levels than reproductive hormones and thus may not be as sensitive to treatment. Another possibility is that 20,25 diazacholesterol may be preferentially deposited in reproductive tissues rather than in adrenal tissue. Based on these results, it seems unlikely that treatment at suggested levels will negatively affect corticosterone, which is a stress hormone involved in the fight-or-flight response.
TABLE 5. Fertility, stage of embryonic development at death, hormone levels, and weights for females treated with cRCP\(^5\) and control Coturnix quail at the National Wildlife Research Center, Fort Collins, Colorado, January to July 1997

<table>
<thead>
<tr>
<th>Parameter and phase</th>
<th>cRCP</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(\bar{x})</td>
</tr>
<tr>
<td>Percentage of fertile eggs(^3)</td>
<td>17</td>
<td>70.0</td>
</tr>
<tr>
<td>Percentage of eggs hatched(^3)</td>
<td>17</td>
<td>14.0</td>
</tr>
<tr>
<td>Number of eggs laid per female per week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime</td>
<td>20</td>
<td>5.57</td>
</tr>
<tr>
<td>First boost</td>
<td>19</td>
<td>4.92</td>
</tr>
<tr>
<td>Second boost</td>
<td>18</td>
<td>4.50</td>
</tr>
<tr>
<td>Average stage of embryonic development at death(^6)</td>
<td>17</td>
<td>1.22</td>
</tr>
<tr>
<td>Progesterone (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime</td>
<td>20</td>
<td>0.80</td>
</tr>
<tr>
<td>First boost</td>
<td>18</td>
<td>0.67</td>
</tr>
<tr>
<td>Second boost</td>
<td>18</td>
<td>0.79</td>
</tr>
<tr>
<td>Change in body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prime(^5)</td>
<td>20</td>
<td>24.44</td>
</tr>
<tr>
<td>First boost(^5)</td>
<td>19</td>
<td>32.98</td>
</tr>
<tr>
<td>Second boost(^5)</td>
<td>18</td>
<td>33.80</td>
</tr>
</tbody>
</table>

\(^1\)Chicken riboflavin carrier protein.

\(^2\)The \(P\)-values for weight, progesterone, number of eggs laid, and percentage of fertile eggs are 2-tailed. The \(P\)-values for the percentage of eggs hatched and average stage of embryonic development are 1-tailed.

\(^3\)Values are for 14 to 24 wk.

\(^4\)Values are obtained by subtracting the pretreatment means from each of the phase means (priming, first boost, and second boost phases).

\(^5\)Average weights for the priming, first boost, and second boost phases were 198.36 g (SE = 5.08), 210.85 g (SE = 6.01), and 217.75 g (SE = 6.13), respectively, for the control group and 207.28 g (SE = 3.59), 215.77 g (SE = 4.33), and 217.35 g (SE = 4.59), respectively, for the cRCP group.

The increase in desmosterol while nonesterified cholesterol decreased in treated birds indicated that 20,25 diazacholesterol may act as an antagonist to the \(\Delta^{25}\)-reductase enzyme, which converts desmosterol to cholesterol, as suggested by Dietert and Scallen (1969) and Emmons et al. (1982). However, 20,25 diazacholesterol also may act as an antagonist to the cytochrome P450 enzyme, causing an inhibition of the side chain cleavage. Counsell et al. (1971) suggested that inhibition of side chain cleavage might be caused by the substitution of a nitrogen for C-25.

We observed signs of toxicity, such as listlessness, weight loss, difficulty breathing, and loss of muscle control in some of the 20,25 diazacholesterol-treated birds. Eight of 20 treated females and 4 of 20 treated males died by wk 9, and the cause of death was suspected to be toxic effects of 20,25 diazacholesterol. Only 4 of 20 control females and 1 of 20 control males died by wk 9. Necropsies of treated birds indicated liver damage such as fatty livers and friable livers. Elder (1964), Powell (1966), Woulfe (1967), and Sturtevant and Wentworth (1970) also observed mortality and toxic effects on quail and pigeons treated with 20,25 diazacholesterol. Because cholesterol is an integral component of cell wall structure, the reduced cholesterol may cause toxicity. Ashraf et al. (1984) suggested that the increased desmosterol levels may contribute to an increase in membrane fluidity. Reddy et al. (1982) found that 20,25 diazacholesterol has a direct effect on muscle fibers, changing them morphologically and biochemically, and causes myotonia. We speculate that 20,25 diazacholesterol may cause cytotoxicity to hepatocytes when it is stored in the liver. The level of mortality observed in this study is a concern and highlights the need to perform dose-response studies. Other studies on red-winged blackbirds (Lacombe et al., 1986) and house sparrows (Mitchell et al., 1979) did not show such mortality. Feeding for a shorter period of time or lowering the dose may give satisfactory results without causing mortality.

Further analysis showed that birds treated with 20,25 diazacholesterol lost weight during the treatment period. Treated birds consumed less feed during the treatment period because the bait apparently was unpalatable, which contributed to weight loss and less weight gain overall. Upon removal of the treated bait, birds in the treatment group gained weight, and the differences in weight were not significant between female groups after wk 10 and between male groups after wk 11. Reduced consumption of 20,25 diazacholesterol treated bait and subsequent weight loss also was noted by Powell (1966) and Lacombe et al. (1987). Lacombe et al. (1987) suggested that 20,25 diazacholesterol may affect the metabolism of birds, contributing in part to weight loss. Although we cannot exclude the possibility that weight loss caused ovarian regression, we felt the weight loss was not the cause of the reduction in egg laying and fertility because they continued to be suppressed for 15 wk after birds gained weight. Weight loss only constituted a 10% loss in the treatment groups.

Dose-response work should be conducted on wild species of interest before 20,25 diazacholesterol is tested in the
field to determine an effective and nontoxic dose for each species. These lab tests should include a gavage study in which precise doses are known. Desmosterol and nonesterified cholesterol can be used as markers of efficacy in the absence of breeding by wild species in a laboratory setting. Dose and plasma values of desmosterol and nonesterified cholesterol can then be correlated, and this information can be extrapolated to determine dose when 20,25-diazacholesterol is fed ad libitum. Because we could not measure 20,25-diazacholesterol in the plasma of treated birds, even though it can be measured in spiked control plasma samples, a specific assay to measure plasma levels of 20,25-diazacholesterol metabolites is also needed. Because a daily dosing regimen is required, development of a more practical method of delivery that allows for a prolonged release of 20,25-diazacholesterol in a single dose would be advantageous. Nontarget and secondary hazards will also need to be assessed prior to wide scale use.

Immunocontraceptives

The reduced testosterone levels of AGnRH-I-treated males during the second boost phase likely was not biologically significant because fertility of treated males was not affected, and hatchability was only marginally decreased. Several investigators obtained lowered testosterone levels, reduced fertility, and reduced libido after vaccinating against GnRH in male cattle (Robertson et al., 1982), rats (Ladd et al., 1989), male piglets (Meloen et al., 1994), and brown-headed cowbirds (Thompson et al., 1994). On average, the AGnRH-I antibody titers produced in this study likely were not sufficient to reduce testosterone levels enough to reduce reproduction. However, 3 of the 20 birds responded to the AGnRH-I vaccine by producing antibody titers during the second boost phase ranging from 1:32,000 to 1:128,000, which reduced testosterone to 0 to 3.6 ng/mL and reduced fertility for 5 to 12 wk. The failure of the vaccine to produce high antibody titers in the majority of the birds may be due to the vaccine being metabolized too quickly. The vaccine depot must be present for several weeks for good antibody production. A route of administration other than subcutaneous vaccination may prevent the depot from being metabolized too quickly. The overall health of the animal may also affect the immune response. However, poor health would also tend to decrease fertility and egg production, which was not observed in this study and therefore was not a likely explanation for the vaccine failure.

Lower progesterone levels in AGnRH-I-treated females during the second boost phase likely were not biologically significant because egg laying was not reduced. On average, the AGnRH-I antibody titers produced in this study likely were not high enough to reduce progesterone levels due to the reasons mentioned above. Esbenshade and Brit (1985) found that progesterone and estradiol levels decreased to basal levels, and acyclicity occurred in gilts vaccinated against GnRH. In our experiment, 2 females had higher-than-average titers of 1:24,000 and 1:12,000 during the second boost phase. These birds did not lay eggs for 5 and 2 wk, respectively, and had progesterone levels ranging from 0 to 0.41 ng/mL. Control birds did not have antibody titers, and egg production was normal.

Although the exact cause is unknown, the higher weight gain of the AGnRH-I-treated females during the study may be due to a reduction in estradiol levels. We did not measure estradiol in our study; therefore, it is unclear if this was the case. Because GnRH affects FSH and LH, it is reasonable to expect vaccination against GnRH to lower estradiol levels. Because estradiol is the hormone responsible for sexual behavior in birds (Bell and Freeman, 1971), AGnRH-I-treated females might not have exhibited sufficient behavioral cues to elicit sexual behavior from the male. As a result, females might have been chased less by males, and more time might have been spent feeding, resulting in increased weight gain.

Similarly, low antibody titers and a lack of reduced hatchability with the cRCP vaccination indicate that the antibody titers generated were not high enough to have an effect. Vaccination against cRCP likely will not affect breeding behavior because it does not affect the reproductive hormones. Seshagiri and Adiga (1987) found that vaccination against cRCP in bonnet monkeys (Macaca radiata) did not alter estrogen and progesterone levels.

Management Implications

Of the 3 contraceptives investigated, 20,25-diazacholesterol gave the most promising results. It was efficacious for both sexes and reduced egg production and hatchability. Because 20,25-diazacholesterol is effective for 2 mo in Coturnix quail and is reversible, it is a promising contraceptive for field use. However, additional dose-response work will need to be conducted on 20,25-diazacholesterol to minimize the risk of birds receiving a toxic dose. Because the level of 20,25-diazacholesterol that causes toxicity will vary by species, lab tests will need to be conducted to establish appropriate dosages. It may be necessary to use desmosterol and nonesterified cholesterol as markers because many of the species of interest will not breed in a laboratory setting.

An advantage of the AGnRH-I vaccine is that it can potentially contracept both sexes. The potential length of efficacy of the AGnRH-I vaccine, and its reversibility, make it another promising field contraceptive. Although the overall results did not show an effect, it is clear from the few responders in the male and female treated groups that this vaccine has potential to work. Further research should be done on finding the best vaccination protocol to maximize antibody titers. This vaccination protocol should also be tested with cRCP to determine if the failure of the vaccine was due to the vaccine or the vaccination protocol. In order to be practical for field use, an oral vaccine or other method of delivery that does not require capture of birds (such as intradermal) will need to be developed.

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