The effects of O,P'-dicofol on two generations of American kestrels

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ABSTRACT

M.Sc. Kelly N. M. MacLellan Renewable Resources (Wildlife)

THE EFFECTS OF O,P'-DICOFOL ON TWO GENERATIONS OF AMERICAN KESTRELS

A two generation laboratory study was conducted on a captive population of American kestrels (*Falco sparverius*) to investigate the possible teratogenic effects of the pesticide Dicofol. Paired females were exposed to three levels of Dicofol. Integrity of the reproductive tract of the resulting embryos were examined. Viable eggs were hatched and theses birds were permited to breed the following year. Breeding performance for these birds was measured based on their ability to form pair bonds and exhibit normal behaviour in the presence of a mate. Clutch completion, fertility, hatchability and number of hatchlings reared to fledging were used as reproductive parameters. Females dosed with 20 ppm Dicofol laid eggs that were significantly ($p<0.05$) thinner than eggs of control birds. Male embryos from dosed females were significantly ($p<0.05$) different from control chicks. Feminization was confirmed by the presence of primordial germ cells. Second generation adults showed altered reproductive parameters related to their parental dose groups.

Rank-order trials were conducted on second generation males based on parental dose levels to determine the aggressiveness of these individuals when placed in a competitive arena. Primary perch sites and food items were obtained by control birds significantly ($p<0.05$) more often than exposed males.

RÉSUMÉ

LES EFFETS DU O,P'-DICOFOL SUR DEUX GÉNÉRATIONS DE CRÉCERELLES d'AMÉRIQUE

Une étude en laboratoire portant sur deux générations de crécerelles américaine (*Falco sparverius*) en captivité a été menée dans le but d'enquêter sur les effets tératogéniques éventuels du pesticide DICOFOL. Des femelles accouplées ont été exposées à trois concentrations de DICOFOL. On a examiné la totalité de l'appareil reproducteur des embryons. On a fait éclore les oeufs viables et permis à ces oiseaux devenus adultes de s'accoupler l'année suivante. On a mesuré la capacité de reproduction de ces oiseaux à partir de leur habileté à former une union monogame et à exhiber un comportement normal en présence du sexe opposé. D'autres paramètres en matière de reproduction ont été utilisés, à savoir, une couvée réussie, la fécondité, l'éclosivité et le nombre de petits élevés jusqu'à ce qu'ils aient toutes leurs plumes. Les oeufs des femelles à qui l'on avait injecté 20 ppm de DICOFOL étaient considérablement plus minces que ceux pondus par les femelles du groupe témoin. Les embryons mâles nés des femelles injectées étaient très différents des oisillons du groupe témoin. On a confirmé la féminisation par la présence de cellules germinales primordiales. Les oiseaux adultes de la deuxième génération ont exhibé des paramètres reproducteurs altérés apparentés à leur groupe parental infecté.

Des expériences d'ordre hiérarchique ont été faites sur des mâles de la deuxième génération en tenant compte des doses administrées à leurs parents, afin de déterminer le degré d'agressivité de ces individus lorsqu'ils étaient placés dans un milieu compétitif. Les oiseaux mâles du groupe témoin étaient beaucoup plus habiles à obtenir les meilleurs perchoirs et la nourriture de choix que les mâles affectés.

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PREFACE

Organochlorine compounds are both stable and lipophilic in nature (Noakes and Benfield 1965, Ecobichon and Saschembrecker 1968). Once released into the environment they are persistent (Dimond and Sherburne 1969) and are biologically magnified (Hickey and Anderson 1968).

The insecticidal properties of the organochlorine, Dicofol, (2,2,2-trichloro-1,1-di-(4-chlorophenyl)ethanol) were first described by Barker and Maughan (1956). It was introduced in 1955 by the Rohm and Haas Co. under the code number FW-293, trademark, Kelthane. Kelthane has been found to contain at least 17 compounds, including o,p'- and p,p'- isomers of DDT, DDD, DDE, and chloro-DDT (collectively termed DDT-r), as well as the respective o,p'- and p,p'- isomers of dicofol (Rothman 1980). Dicofol is a non-systemic acaricide recommended for the control of mites on a wide range of crops, e.g. alfalfa, apples, citrus, grapes, melons, pecans, tea, ornamentals, etc. (Spencer 1982). For a review of Dicofol (kelthane) as an environmental contaminant see Clark (1990).

Because species at the top of the food chain are particularly sensitive to organochlorines (Lincer 1972), many birds of prey have been highly contaminated leading to reproductive failure and eggshell thinning by the pesticide DDT (Burlington and Lindeman 1950, Albert 1962, Keith 1966, Ratcliffe 1967, 1970, Hickey and Anderson 1968).

Today, many organochlorines have been replaced by the less environmentally persistent organophosphate compounds. However, the use of products containing Dicofol has continued.

Dicofol has come under the scrutiny of the United States Environmental Protection Agency because of environmental contamination of its technical product with DDT and related compounds (DDT-r) (Hunt *et al.* 1986, Riseborough *et al.* 1986). Kelthane products marketed after May 1986 were required to contain less than 2.5 % DDT and related compounds and those marketed after May 1988 were required to contain less than 0.1% DDT (Moore 1986).

Besides the DDT contamination, some eggshell thinning and abnormal reproductive behaviour exhibited with Kelthane compounds may actually be caused by the active ingredients themselves, i.e. p,p'- dicofol (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethanol) and o,p'-dicofol. For example, chronic exposure of Ring Doves (*Streptopelia risoria*) to Dicofol at 34 mg/kg in the feed caused an average of 7.2 % eggshell thinning, while DDE fed at 37 mg/kg caused an average of 5.6 % thinning over the 41 day test period (Schwarzbach 1991). Mallards (*Anas platyrhynchos*) fed various levels of Dicofol laid eggs with reduced shell thickness, strength and weight (Bennett *et al.* 1990). Similar findings with Kelthane have been reported in Screech owls (*Otus asio*) (Wiemeyer *et al.* 1989) and in American kestrels (*Falco sparverius*) (Clark *et al.* 1990). No studies have addressed the effects of pure Dicofol.

The purpose of this study was to investigate the impact of pure Dicofol on captive American kestrels, in both the first dosed generation and their subsequent non-dosed progeny with respect to eggshell thinning and reproductive performance (Chapter 1).

Chapter 2 addresses the pair bonding behaviour of the second generation non-dosed birds when placed in the presence of a normal unaffected mate. Males of this generation were also tested for aggressive tendencies related to rank order in a captive situation.

This study is expected to increase understanding of the action of organochlorine pesticides and their disruption of avian reproductive systems. Valuable information will also be generated on the possible teratogenic effects of Dicofol to kestrel embryos (including feminization of male chicks), as well as the quantification of the reproductive performance of birds hatched from contaminated females. This study includes a collection of data on reproductive performance of two generations of kestrels, eggshell thickness measures, gross and histological information on the integrity of the reproductive tracts of Dicofol exposed birds, pair bonding behaviour and aggressive behaviour in a captive situation.

As permitted by McGill University guidelines concerning thesis preparation, the author has chosen the option of submitting the thesis as a set of papers. The first chapter of this thesis entitled **"Reproductive and Morphological Effects of O,P'-Dicofol on Two Generations of Captive American Kestrels"** will be submitted to the *Archives of Environmental Contamination and Toxicology*.

Chapter 2 entitled **"Behaviour of Captive American Kestrels Hatched from O,P'-Dicofol Exposed Females"** will be submitted to *Animal Behaviour*. Both chapters have been written in the style required by the respective journals. David M. Bird of the Avian Science and Conservation Centre of McGill University and D. Michael Fry of the University of California at Davis will appear as co-authors on both manuscripts. Laird J. Shutt of the Canadian Wildlife Service will also appear as an author on the second paper. These authors are acknowledged for their contribution towards the initial conception of this research and their guidance throughout. Data collection, analysis, and manuscript preparation were completed independently by the candidate.

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REGULATION ON THESIS PRESENTATION

The following is included in accordance with the regulations of the McGill University Faculty of Graduate Studies:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must still conform to all other requirements explained in this document, and additional material (e.g. experimental data, details of equipment and experimental design) may need to be provided. In any case, abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstract, introduction and conclusion are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted.

While the inclusion of manuscripts co-authored by the Candidate and others is not prohibited for a test period, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should also be noted that the task of the External Examiner is much more difficult in such cases."

LITERATURE REVIEW

CHLORINATED HYDROCARBONS IN THE ENVIRONMENT

The 1948 Nobel Prize in medicine was awarded to Dr. Paul Mueller for his discovery that DDT (1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane) acted as a contact poison on flies and other insects (Costa *et al.* 1986). Since that time the effectiveness of DDT and other chlorinated hydrocarbons has been widely documented throughout the first half of this century. DDT was easy to produce, inexpensive, incredibly effective, and long lasting which led to its widespread agricultural use (Pagel and Jarman 1991). However, the advancement of research showed that many of these compounds had toxicological effects on various organisms other than insects which ultimately led to the ban of DDT and other chlorinated hydrocarbons in the 1970's. The discovery of the ramifications of DDT and its metabolite DDE (1,1-bis(4-chlorophenyl)-2,2,-dichloroethylene) precipitated much of the baseline wildlife toxicology research seen today.

DDT AS A CONTAMINANT

Eggshell thinning was brought to the forefront of environmental science in 1967 when Ratcliffe first linked organochlorine pollutants with the decrease in raptor eggshell thickness. Ratcliffe noted increased incidence of egg destruction in peregrine falcons (*Falco peregrinus*), sparrowhawks (*Accipter nisus*) and golden eagles (*Aquila chrysaetos*). Examination of museum egg

collections of all three species found a significant decrease in thickness from 1945-1950. As a standard he devised the Ratcliffe index which is still used today:

$$\frac{\text{weight of shell (mg)}}{\text{length of shell (mm) x breadth (mm)}}$$

Much attention has since been given to the peregrine (Hickey & Anderson 1968, Berger *et al.* 1970, Cade *et al.* 1971) because of its endangered status. Reports of unusual reproductive problems by peregrine falcons including egg breakage and eating, as well as other adult behaviourial anomalies quickly followed Ratcliffe's work (Hickey and Anderson 1968). Eggshell thinning of 19% or greater attributed to organochlorines was also reported by Hickey and Anderson (1968) for various species of North American raptors. Cooke (1973) reviewed the literature on a number of avian species which have exhibited eggshell thinning from environmental pollutants since world war II. In North America and Great Britain the thickness declines have been due largely to DDT and its metabolites. Bald eagles (*Haliaeetus leucocephalus*) studied by Wiemeyer *et al.* (1984) from 1969 to 1979 showed reproductive failure of 100% in North American regions with more than 15 ppm DDE. Regions with 5 ppm DDE were associated with 10% eggshell thinning and areas with less than 3 ppm DDE (wet weight) appeared normal.

Organochlorine residues (primarily DDT) found in the eggs of Richardson's merlin (*F. columbarius richardsonii*) showed reductions of 13% in shell weight and 20% in shell thickness when compared to

pre-1946 egg specimens (Becker & Hull Sieg 1987).

Peregrine falcons are at the top of the food chain and each successive increase in trophic level results in a 10-fold increase in contamination due to the bioaccumulation and lipophilicity of DDT. Thus organochlorines are systematically concentrated in the upper trophic layers of animal pyramids (Hickey and Anderson 1968). The correlations noted between the presence of DDT-r (collective term for other isomers and analogs of DDT related compounds) in the eggs and decreased eggshell thickness have not been exclusive to raptorial species (Peakall 1975).

Poor reproductive success related to DDD and DDT contaminants was reported in brown pelicans (*Pelecanus occidentalis*) and mallard ducks (*Anas platyrhynchos*) in the United States (Lamont *et al.* 1970), as well as in various gull species (Keith 1966, Hunt and Hunt 1973, Gilman *et al.* 1977, Mineau *et al.* 1984).

LABORATORY STUDIES ON DDT

Laboratory studies were conducted on white leghorns (*Gallus domesticus*) to determine the effects of dietary DDT on egg contents and pesticide accumulation in the body fat of laying hens (Cecil *et al.* 1972, 1973). In hens fed 5 to 50 ppm o,p'-DDT, p,p'-DDT or DDE 10% of o,p'-DDT dietary levels were present in the eggs, and 13% and 87%, respectively, for p,p'-DDT and p,p'-DDE. There was no evidence for conversion of o,p'-DDT to p,p'-DDT in the birds fed o,p'-DDT (Cecil *et al.* 1972). Pesticide levels in the hen's fat were approximately 13 times greater than those found in their eggs.

White leghorn hens fed p,p'-DDT or a combination of the pure isomers (o,p'-DDT 20% and p,p'-DDT 80%) at levels of 10 and 50 ppm showed increasing egg residues which reached a plateau after 4 months on the experimental diets (Cecil *et al.* 1973). After 40 weeks, abdominal fat levels were 15 to 19 times the dietary levels. Birds fed lower calcium diets were found to retain more DDT and DDE and hence, showed higher residues in the fat. Data from hens showed that residues accumulated in the egg in direct relationship to dietary amounts, whereas residues in hen's fat were 13 times those reported in eggs. Although the hen's eggshell-forming mechanism appears quite resistant to chronic administration of DDT, mobilization of body residues caused by fasting and stress during reproduction in wild birds could cause the more drastic eggshell thinning observed in their populations.

Species differences also exist. For example, captive American kestrels (*F. sparverius*) fed 10 ppm p,p'-DDE on a dry weight basis laid shells 10% thinner rollowing DDE administration compared to those in their previous reproductive year (Wiemeyer and Porter 1970).

MECHANICS OF EGGSHELL THINNING

The mechanisms which actually cause the thinning are much disputed. Carbonic anhydrase is believed necessary to supply carbonate ions required in the deposition of calcium carbonate in the eggshell. Bitman *et al.* (1970) studied carbonic anhydrase

levels in Japanese quail (*Coturnix coturnix*) treated with DDT and DDE. Carbonic anhydrase levels decreased by 16 to 19% when birds were given 100 ppm p,p'-DDT or p,p'-DDE, resulting in eggshells 10 to 15% thinner than controls. Peakall (1970) found that circulating estradiol levels were reduced in ring doves (*Streptopelia risoria*) fed 10 ppm p,p'-DDT, which caused reduction of eggshell weight and inhibition of carbonic anhydrase in the oviduct.

An enzymatic basis of DDE-induced thinning was reported by Miller *et al.* (1976) for Pekin ducks and ring doves. This supports the proposal that thinning results from a disruption of shell gland (uterus) function rather than a reduction in the circulating blood calcium supply to the organ. More recent information on the hatchability of budgerigars (*Melopsittacus undulaatus*) in relation to the eggshell thickness showed most embryonic mortality to be related more to abnormal porosity rather than shell thickness (Baker and Baker 1992). DDE is known to disrupt embryo respiration by affecting the porosity of the eggshell (Pagel and Jarman 1991), suggesting that embryos in other species could have died from abnormal eggshell porosity.

ENVIRONMENTAL LEVELS OF DDT RELATED TO EGGSHELL THINNING

Audet *et al.* (1992) studied organochlorine and mercury levels in osprey (*Pandion haliaetus*) eggs in the United States and concluded that median DDE levels did not significantly decline in eggs

collected from 1973 to 1986. Many eggs surpassed the 10% eggshell thinning value associated with egg breakage.

Speich *et al.* (1992) concluded that total DDT in the eggs of waterbirds near Washington, DC did not account for the amount of thinning observed. They also discovered the presence of right oviducts in females exposed to DDT and they even suggested the possibility of Kelthane contamination (known to contain small amounts of DDT - see later).

Lincer (1972) used laboratory and wild American kestrels to show that small decreases in shell thinning could be associated with predator species feeding primarily at low trophic levels and/or resident northern prey. More drastic decreases in shell thinning were associated with predators feeding at high trophic levels, especially those associated with aquatic habitats. According to Lincer (1972), not one raptor population exhibiting 18% or more eggshell thinning has been able to maintain a stable, self-perpetuating population.

Environmental concentrations of DDT can be significantly different between regions. Newton and Wyllie (1992) reported continued declines in DDE residues from eastern Britain and a concomitant improvement in eggshell thickness and breeding success in the local sparrowhawk population. Long term trends in organochlorines in predatory birds from Britain have shown population recoveries and increased shell thickness with an associated decrease in environmental levels of organochlorines. Species differences were related partly to diet, habitat and

migratory patterns (Newton *et al.* 1993).

Chlorinated hydrocarbons are heavily regulated in North America, but their levels in wild bird populations still persist. There is ongoing use of these compounds in Latin American countries (Elliott and Shutt 1993) where many raptors overwinter. DDE and polychlorinated biphenyls (PCB's) reported in blood samples taken from bald eagle nestlings indicate early exposure to these compounds increases with age to cause moderate eggshell thinning in some populations (Wiemeyer *et al.* 1984, Anthony *et al.* 1993). Reproductive failures and chick deformities in double-crested cormorants (*Phalacrorax auritus*) associated with organochlorine use were recently reported in the upper Great Lakes, indicating persistent contaminant burdens in aquatic ecosystems (Somers *et al.* 1993, Yamashita *et al.* 1993).

SEX-REVERSAL EFFECTS OF DDT ON AVIAN SPECIES

Early laboratory studies on poultry (Burlington and Lindeman 1950) were conducted to better understand the toxicity and metabolic effects of DDT compounds on non-target species. DDT markedly retarded the growth of cockerel testes and inhibited the development of secondary sex characteristics such as wattles and combs. These findings were among the earliest reports of the estrogenic-like effects of DDT on certain species of birds. This was further supported by data suggesting that DDT can decrease sperm production in domestic fowl (Albert 1962). The ability of

DDT to act as an estrogen inducer was reviewed by Stancel *et al.* (1980) and Robison and Stancel (1982).

Sex reversal in birds is not a new topic, in fact histological studies were carried out on avian gonads as early as 1923 (Fell 1923). Modification of the sex development of chick embryos with male and female hormones was studied on white leghorns (Willier *et al.* 1937). Experimental modification of the accessory sexual apparatus in the hen resulted in transformation of the left gonad into an ovary or ovotestis in the genetically pre-determined male embryo (Greenwood and Blyth 1938). Embryotoxic and teratogenic effects on unhatched eggs have also been seen in hens fed polychlorinated biphenyls (PCBs) (Cecil *et al.* 1974), stilbestrol (Boss and Witschi 1947), estradiol (Narbaitz and Teitelman 1965), and mestranol (Wentworth et al. 1968).

Chickens and Japanese quail hatched from eggs injected during incubation with 0.2 to 5 ppm estrogen showed repressed crowing, strutting and copulatory behaviour (Adkins 1975, Whisett *et al.* 1977). Developmental abnormalities in male embryos exposed to estrogens caused marked changes in the reproductive behaviour of these birds at maturity.

Teratology of the avian uro-genital tract after exposure to DDT in chicken and quail embryos showed a strong disturbance in the organogenesis of the genital apparatus of the birds (Lutz-Ostertag and David 1973).

David (1975a & b) examined application of DDT to embryos at specific stages in incubation and the amount of teratology present

in the resulting urogenital tract. Results indicate that when pesticides occur at certain stages of incubation, they can have a significantly greater affect than at other stages in embryonic development. To exert its sterilizing affect on the germ cells, DDT must be administered before the onset of incubation. Administration of the compound after the gonocytes have already colonized the gonads results in a significant deficit in the germ cell population.

DDT-exposed eggs were opened and the gonads grafted onto non-treated embryos (David and Lutz-Ostertag 1975). Treated testicular grafts onto male host embryos had a feminizing affect in approximately 82% of embryos tested. Testicular grafts on female hosts showed varied results, e.g. no effect, some masculinization, and some feminization.

David (1976) compared the effects of p,p'-DDT to those of commercial DDT on the embryonic gonads of avian species. Results were contraindicative for the quail and chicken species, but there was a decrease in the number of primary gonadocytes present due to treatment.

DDT transferred from hens into corresponding eggs showed compound levels comparable to those in the laying hens (David 1977). The commercial DDT used in the experiment showed elevated levels of o,p'-DDT and conversion of p,p'-DDT to p,p'-DDE in the egg and its contents, as well as the corresponding tissues. A large variation in the amount of DDT passed from hen to egg, due in part to whole egg analysis, did not account for variations in size

and weight of the egg. In spite of the variability there were many diverse anomalies related to reproduction and development.

Research on chick gonads *in situ* (Narbaitz and Adler 1966, McCarrey and Abbott 1978) and *in vitro* (Erickson 1974, Haffen 1975) have attempted to further our knowledge of sexual differentiation in avian gonads, which is speculative in certain stages of development. Biochemical data on plasma testosterone levels in chicks (Woods *et al.* 1975), radioimmunoassay of the steroids produced by embryonic gonads (Guichard *et al.* 1977), as well as research on the hypothalamus and quantification of hormones produced by the reproducing female (Opel 1979, Rehder *et al.* 1984) and developing chick embryos (Woods *et al.* 1981) have all endeavoured to unlock the mechanistic changes which lead to abnormal offspring when assaulted with xenobiotic substances. For an interpretation of the avian endocrine responses to environmental pollutants see Rattner *et al.* (1984). These endocrine studies on DDT and related compounds are not exclusive to avian species, as much work has also been done on mammalian species (Gobbetti 1980). For a comprehensive look at the estrogenic activity of pesticides and other xenobiotics on the uterus and male reproductive tract, see Bulger and Kupfer (1985).

The most distinctive evidence of the estrogenic properties of DDT was presented by Fry and Toone (1981). They injected 2 ppm o,p'-DDT into male gull eggs causing feminization of the resulting embryos. Estrogenic effects of DDT resulted in male gonads with a left oviduct, shell gland and short right oviduct, or an enlarged

left gonad flattened into an ovotestis, i.e. organ containing male and female reproductive cells, although the animal is genetically male. The most sensitive indicator of feminization of male embryos was the localization of primordial germ cells in a thickened ovary-like cortex present in the left testes (Fry and Toone 1981).

Some studies have examined the impact of feminization on birds in the wild. Organochlorine exposure causing feminization of male gulls with germ cells present in the cortex of the gonad, as well as females which developed both right and left oviducts was noted in Puget Sound, WA by Fry *et al.* (1987). Right oviducts in females persisted to sexual maturity and females laid thin-shelled eggs. Birds also exhibited an abnormal behaviour as a result of estrogenic pollutants. A sex skew in the study population was noted, and as a result female-female pairings and super numerary egg clutches were observed. Developmental feminization of males was associated with an inability to breed as adults and may serve to explain high sex skews on breeding grounds with reduced numbers of male gulls available to mate (Fry and Toone 1981).

Boss noted in 1943 that estrogenic hormones may not be entirely without importance in the natural course of avian behavioural development. Rattner *et al.* (1984) showed that hormonal changes in gull populations contaminated with organochlorines could ultimately induce behaviour anomalies in courtship, nest construction, breeding synchrony, and decreased incubation and parental care input. Quail embryos exposed to estradiol during incubation showed deficiencies in copulatory behaviour as adults, no matter how much

testosterone was available (Adkins, 1975). Similarly, Bryan *et al.* (1989) injected Japanese quail eggs with o,p'-DDT (1-10 mg) and noted long-term estrogen-like effects on bird behaviour.

Organochlorines have also been noted to cause abnormal aggressive behaviour toward mates and young chicks in Bengalese finches (*Lonchura striata*) fed diets with 32 ppm DDT and 38 ppm DDE (Jefferies 1967). Heinz (1976) showed that DDE-fed ducklings had a reduced flight response to danger and were hyper-responsive to maternal calls. Group avoidance behaviour was significantly suppressed in quail chicks fed Chlordane, Dieldrin, Endrin, and Aroclor 1254 (Kreitzer and Heinz 1974).

DICOFOL AS A CONTAMINANT

Hunt *et al.* (1986) studied migratory birds and their prey species and found that levels of DDT present in the United states could not be explained by illegal use of DDT or the transport of DDT by migratory birds from Latin America. Instead Hunt *et al.* (1986) attributed the continued environmental levels of DDE to the use of Kelthane and other biocides containing technical Dicofol (p,p'-dicofol; 1,1-bis(p-chlorophenyl)-2,2,2-trichloroethanol) as the principal active ingredient. DDE is metabolically derived from one or more of the components of Kelthane (active ingredient is Dicofol). The annual 1986 use of Dicofol in the United States was in the order of 1.4 million kg.

DDT and related compounds were officially banned in 1972, but research on their effects is still being reported due to their long

half-life and persistence in the environment, which prolonged the reproductive failure in populations of peregrine falcons (Springer *et al.* 1984, Hunt *et al.* 1986). Reports of DDT's widespread distribution began again in 1984 because it was an unavoidable impurity in legal pesticides and as such, did not have to be listed on Dicofol and Kelthane product labels (Graham 1984). The chemical is produced in Italy by the addition of an extra oxygen atom to the DDT molecule and marketed in the United States by Rohm and Haas Company of Philadelphia and distributed widely under the trade name, Kelthane. A 3:1 mixture of p,p'-DDT (2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethane) and o,p'-DDT (2,2,2-trichloro-1-(4-chlorophenyl)-1-(2-chlorophenyl)ethane) is reported to be the starting material for manufacturing Dicofol (Rothman 1980, DiMuccio *et al.* 1988). Dicofol is structurally considered as the alpha-hydroxy analog of DDT (Brown *et al.* 1986). Dicofol is the active ingredient in the technical product marketed as Kelthane. In 1983, approximately 2 to 3 million pounds of Kelthane were used in the United States (USEPA 1984).

Dicofol is a non-systemic organochlorine acaricide analogous in composition to DDT. It is also marketed under the trade names, Acarin, Carbax, Decofol, Kelthane A, p,p' Kelthane, Mibol and Mitigan, as well as numerous others (USEPA 1988). Mode of action is upon contact with motile stages of all species of spider mites (*Tetranychus spp.*) and their eggs. Dicofol may be applied to crops such as alfalfa, apples, apricots, beans, citrus, clover, cucumbers, grapes, melons, peaches, pears, peppers, plums, squash,

strawberries, tomatoes, watermelons, turf, and ornamentals, to name but a few of its uses (Agri. Chem. 1985). The compound has been identified in soils 4 years after application (Agri. Chem. 1985), but it may persist longer.

Dicofol was the topic of much debate in 1982, when it was discovered that technical Kelthane contained as much as 15% DDT and DDT-r (Anon. 1984a & b). High performance liquid chromatographic research yielded 18 impurities in the acaricide marketed by Rohm and Haas (Rothman 1980). Among the components of Kelthane, chloro-DDT (1,1,12-tetrachloro-2,2-bis(p-chlorophenyl) ethane) is converted to DDE under certain laboratory conditions. While it was thought to explain the DDE levels metabolically formed in captive mallards (Risebrough *et al.* 1986), the study was not able to answer whether DDE could be derived from pure Dicofol. However, Dicofol administered to mice was metabolized into DCD (dechlorodicofol), DCBP (dichlorobenzophenone) and DCBH (dichlorobenzhydrol), but not DDE. However, the alpha-chloro-DDT impurity was metabolically dechlorinated and is thus a likely contributor to tissue levels of DDE (Brown and Casida 1987). Since humans are exposed to Dicofol in the diet, its impurities could contribute to the biological burden of DDE in humans. This prompted the Environmental Protection Agency to order a halt to the sale and production of Dicofol products containing more than 0.1% DDT-r by January 1989.

LABORATORY RESEARCH ON DICOFOL AND RELATED COMPOUNDS

A great deal of research had been compiled on Dicofol prior to the 1989 restriction ban. LD50 studies on the compound were completed long ago (Blackwell Smith *et al.* 1959). Mammalian data, i.e. from mongrel dogs receiving the compound, showed adrenocortical atrophy and reduced steroidogenesis (Cobey *et al.* 1958). Rats dosed with the compound showed increased liver and thyroid weights with decreased body growth rates (Verschuuren *et al.* 1973). Mammalian carcinogenicity was studied in rats and mice by Cueto (1980), who found a positive tumour response only in one strain and sex of rodent. Shirasu *et al.* (1976) studied mutation induction capacity in microbial systems with technical Kelthane and found it capable of inducing revertants in various species of bacteria. Chromosomal research using Kelthane on *Drosophilia melanogaster* showed point mutations in chromosomes but no evidence of sex-linked recessive lethals (Goplan and Njagi 1981).

There is a broad spectrum of experiments concerning the biological significance of residues on citrus foliage and fruits (Gunther 1969, Jeppson and Gunther 1970), as well as the metabolism of Dicofol in soils, water and by microorganisms (Williams 1977, Lyman and Anderson 1979, Walsh and Hites 1979).

Another important research venue concerns phototoxicity to mites (Gough and Qayyum 1987), feeding inhibition (Walgenbach and Wyman 1987), mite resistance to Dicofol (Dennhey *et al.* 1987) and

interference with chitin deposition in the larvae (Grosscurt *et al.* 1988).

There is also a large research effort devoted to chemical experimentation of the pesticide itself, such as improved chromatographic detection and analysis for Dicofol residues (Kvalvag *et al.* 1979, Rothman 1980, Barbera *et al.* 1986).

EFFECTS OF DICOFOL ON AVIAN SPECIES

Concern over Dicofol in the environment continues, since evidence of the effects of the active ingredient (Dicofol) on wildlife still persists. For a complete review of Dicofol (Kelthane) as an environmental contaminant, see Clark (1990).

Mallard ducks, which are moderately sensitive to DDE eggshell thinning toxicity, were fed diets ranging from 0 to 100 ug/g Dicofol for 42 days (Bennett *et al.* 1990). No change in egg production occurred, but there was a significant increase in the number of cracked eggs from birds on the 100 ug/g Dicofol diet. Eggshell strength, thickness and weight were negatively related to the concentration of Dicofol in the diet administered. A positive control group fed DDE experienced relatively similar reductions in eggshell quality as those in the dicofol group.

Schwarzbach *et al.* (1988) exposed ring doves to p,p'-dicofol at 33.4 ppm or p,p'-DDE at 37 ppm. Birds fed the latter diet laid 5.6% thinner eggs than the controls, and the Dicofol birds laid eggs with eggshells 7.2% thinner. Both doses showed decreased egg

production when compared to controls. The greatest number of broken or cracked eggs was found in the Dicofol group with 16.9% of all eggs laid, compared to 7.9% from the DDE group and 5.7% of controls. Only Dicofol egg residues were positively correlated with eggshell thinning.

Dicofol metabolites were examined in ring doves fed 10 or 32 mg/kg p,p' dicofol. Dicofol was metabolized into 1,1-bis(4-chlorophenyl)2,2 dichloroethanol (DCD) by reduction and by oxidation into dichlorobenzophenone (DCBP). The major metabolite was DCD which did not produce eggshell thinning at the above doses. However, the Dicofol metabolites DCD, DCBP and DCBH (dichlorobenzhydrol) appeared to be less responsible for eggshell thinning than pure Dicofol (Schwarzbach 1991).

American kestrels and ring doves exposed to oral gavage of 3.0 and 0.3 mg Dicofol/kg body weight per day for 39 days showed that kestrels were more sensitive to Dicofol than doves. The major hepatic metabolite in the kestrel was DCD (mono-dechlorinated Dicofol), but most organochlorine was stored in tissues as Dicofol. DDE was not a major metabolite in kestrels. Compared with doves, kestrels had a reduced capacity to transform Dicofol to DCBP and DCBH had a steeper dose-response curve for Dicofol induced eggshell thinning (Schwarzbach *et al.* 1991). According to Schwarzbach *et al.* (1991), the metabolic breakdown of Dicofol in a bird's body is deactivating in terms of the compound's ability to cause eggshell thinning. Thus, the metabolic capacity of a species to break down Dicofol into DCBP and DCBH may be significant in determining the

sensitivity of avian species with respect to eggshell thinning from Dicofol.

Wiemeyer *et al.* (1989) compared old Kelthane (3.4 % DDT-r) with new Kelthane (no detectable DDT-r) when fed to reproducing eastern screech owls (*Otus asio*), a species sensitive to a variety of environmental contaminants. Eggshell weights and thickness were significantly reduced in the treated owls. Eggs laid by old-Kelthane birds were significantly higher in p,p'-DDE concentrations compared with those receiving new Kelthane.

BEHAVIOURIAL EFFECTS OF DICOFOL ON REPRODUCTION

Reproductive parameters were measured through two breeding seasons of American kestrels fed Kelthane (containing no DDT-related compounds) (Clark *et al.* 1990). Dietary concentrations of $\geq$3 ug/g thinned eggshells and lowered the thickness index. Eggshell thickness and weight were reduced at dietary levels of >10 ug/g in a dose-related fashion. Also, reduced hatchability in the kestrels showed that the eggshell thinning properties of Dicofol are not as strong as those of DDE at similar doses.

Reproductive and behaviourial effects of Dicofol on progeny of exposed kestrels were studied by Fry *et al.* (1988). Male progeny of Kelthane(containing Dicofol) dosed birds (10 ppm) were less aggressive and more submissive compared to control males in an analysis of aggressive behaviour and dominance. The reproductive performance of second generation birds was reduced for the 10 ppm

Kelthane group with high infertility and low hatching success. Results were consistent with the hypothesis that exposure *in ovo* to o,p'-dicofol results in impaired development of male falcons (Fry *et al.* 1988).

Sex hormones acting early in the developmental stage are a major influence on or even a determinant of adult sex differences in avian reproduction (Ottinger *et al.* 1984). Thus, it is possible to achieve striking effects using only brief hormone treatments, provided they are given early in development. Irreversible changes in reproductive behaviour can only be produced when treatment is applied during critical periods. Ottinger *et al.* (1984) stated that in the few cases where both morphological and behaviourial studies of sexual differentiation have been conducted in the same avian species, it was striking to note that these two aspects occur in parallel and follow the same general pattern. When quail embryos are exposed to estradiol for instance, not only is the behaviour demasculinized, so is the size of the proctodeal gland (male-androgen-dependent structure) (Adkins 1975). Thus, data collected on morphology and behaviour alterations resulting from organochlorine effects would be useful.

LONG-TERM AND GENERATIONAL EFFECTS OF ORGANOCHLORINES, INCLUDING DICOFOL

Little information has been reported on the impact of organochlorines on the next generation and their ability to function normally even without further exposure to contaminants.

For example, if embryos are contaminated via the female, what impact might there be on their ability as adults to lay healthy eggs and produce normal young?

Three generations of Japanese quail were administered 15 ppm p,p'-DDT to measure adverse effects (Carnio and McQueen 1973). Generations one and three showed decreased egg production. In the third generation, fertility was greatly decreased, the production of abnormal eggs was increased, and the egg-residue load was increased. Thus, duration of exposure may be as important as the concentration of compound administered. This also raises the question as to whether birds laying eggs in past-exposure areas face equal or greater risks than those breeding in highly contaminated regions.

Long-term exposure of quail embryos to 1-10 mg of o,p'-DDT resulted in decreased survivability to 5 weeks post-hatch and reproductive behaviours were diminished in birds injected during development (Bryan *et al.* 1989). Quail exposed to DDT had decreased numbers of ovipositions, increased numbers of eggshell malformations, and reduced progeny of parents. Long-term estrogen-like effects on behaviour and hematology were noted, as well as altered primary feather development from birds receiving embryonic exposure (Bryan *et al.* 1989).

David and Lutz-Ostertag (1976) studied the effects of DDT on multiple generations of quail. They found that whether a point source or multiple applications of the pesticide was used throughout the 3 generation study, the results were decreased egg

and chick weights, lower productivity and delayed onset of laying.

As stated earlier, Fry *et al.* (1987) studied sex ratio skews and breeding patterns of gulls off the coast of California. Feminized male chicks were discovered, as well as abnormal sex skews on breeding grounds where male birds were few in number. The lack of suitable pairs led to female-female pairings, where each female laid infertile eggs in the nest, comprising a supernormal clutch of 4 to 6 eggs (Conover 1984). This sex-skew could be the result of decreased fitness of males hatched from contaminated eggs (Wingfield *et al.* 1980), or decreased fitness of juvenile (Burger and Gochfeld 1981) and/or adult male gulls exposed to the estrogenic effects of organochlorine pollutants (Conover and Hunt 1984).

In waterbirds from western Washington, significant eggshell thinning (up to 13%) was seen in eggs of great blue herons (*Ardea herodias*) from heronries in agricultural areas. Eggs from urban-industrial regions though, showed less (5-7%) thinning (Speich *et al.* 1992). Concentrations of DDT (0.49-1.19 ug/g) in the eggs was not sufficient enough to account for the thinned shells. These levels of contaminants and eggshell thinning were all currently below levels associated with reproductive impediment. It suggests that these results could be long-term residual effects from Kelthane (containing DDT compounds), expressed as persistence of right oviducts and developmentally induced eggshell thinning (Speich *et al.* 1992).

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CHAPTER 1

Reproductive and Morphological Effects of O,P'-Dicofol on Two Generations of Captive American Kestrels

INTRODUCTION

Dicofol (1,1-bis(p-chlorophenyl)-2.22-trichloroethanol) is the active ingredient in the pesticide marketed under the trade name Kelthane. The compound is registered for use in 196 different pesticide preparations (USEPA 1984), which account for the several million kilograms of Dicofol that are applied annually, primarily to combat cotton and citrus mites (Rohm and Haas 1984). Structurally, Dicofol is DDT with the addition of an oxygen atom. Despite the similarities of the compounds and the extensive studies of DDT, little is known about the effects of Dicofol on avian species.

During the 1970's most of the organochlorine insecticides were banned or heavily regulated, but it was not until the mid-1980's that the U.S. Environmental Protection Agency (USEPA) became concerned about the use of Dicofol products, which at the time contained up to 9% DDE, as well as 6% DDT and related compounds (DDT-r) (Clark and Krynitsky 1983). The DDT contaminants in Dicofol were augmenting the environmental load of DDT-r and thus increasing the risk to sensitive avian species. Regulations from the USEPA in 1988 effectively limited the amount of DDT-r in Kelthane to less than 0.1% DDT-r (Dolan 1986).

Research on pure Dicofol indicates that there are eggshell thinning responses comparable with those of DDE in the diet. Eggshell thinning in ring neck doves (*Streptopelia risoria*) fed diets containing 33 mg/kg pure Dicofol (Schwarzbach *et al.* 1988)

and American kestrels (*Falco sparverius*) fed 10 mg/kg pure Dicofol (Fry *et al.* 1988) showed responses similar to DDE-induced shell thinning. Eastern screech-owls (*Otus asio*) fed 10 ug/g Dicofol (wet weight) (Wiemeyer *et al.* 1989) and mallard ducks (*Anas platyrhynchos*) fed 5-93 mg/kg Dicofol (Bennett *et al.* 1990) showed eggshell thinning analogous to the effects of DDE.

The estrogenic effects of DDT have been recognized in male gull embryos showing developmental feminization (Fry and Toone 1981). As a result of effected males not entering the breeding population, abnormal female-female pairings have been documented during breeding seasons (Fry *et al.* 1987). Long-term exposure of o,p'-DDT to quail embryos resulted in attenuated reproductive behaviours, described as estrogen-like effects, as well as decreased egg-laying and increased eggshell malformations (Bryan *et al.* 1989).

Little research has addressed the generational effects of Dicofol on sensitive avian species. Generational effects of Kelthane have been reported in the house mouse (*Mus musculus*) fed 500 ppm (dry weight) in the diet for 5 generations (Brown 1972). Each successive generation showed decreased weight of young, decreased litter size and increased mortality. For all generations combined there was a reduction in fertility, viability, and lactation indices.

Fry *et al.* (1988) showed impairment of reproductive performance in male American kestrels hatched from females receiving 10 ppm Kelthane, i.e. high infertility, low hatchability, and decreased aggressive behaviour (Fry *et al.* 1988).

The objective of this study was to determine the potential effects of Dicofol on American kestrels, a species sensitive to DDT (Porter and Wiemeyer 1970) and closely related to the endangered Peregrine falcon (*F. peregrinus*). The study reports on two generations of kestrels, the dose generation and the resulting undosed offspring. In both generations, as a measure of reproductive performance, eggshell thinning, fertility, hatchability were examined in captive kestrels exposed to Dicofol. Gross and histological morphology of the gonads of hatchling chicks and adults of the second generation were examined to evaluate the ability of Dicofol to feminize male kestrels.

MATERIALS AND METHODS

In 1988, American kestrels were paired and placed in plywood breeding chambers approximately 1.25 m x 1.25 m x 2.50 m high at the Avian Science and Conservation Centre (ASCC), of McGill University, Ste. Anne de Bellevue, Quebec, according to procedures previously described by Bird (1982, 1985).

Thirty female birds were randomly selected from a pool of known breeders that had experienced at least one successful breeding season, and each was assigned to 1 of 3 treatment groups as follows:

(A) 0 ppm Dicofol (control group): 10 females were dosed with corn oil alone by oral gavage on a daily basis, prior to receiving a normal diet of frozen-thawed cockerels.

(B) 5 ppm Dicofol: 10 females were dosed with a corn oil vehicle containing 1.5 mg/kg body weight/day, prior to receiving a normal diet of frozen-thawed cockerels.

(C) 20 ppm Dicofol: 10 females were dosed with a corn oil vehicle containing 6.0 mg/kg b.w./day, prior to receiving a normal diet of frozen-thawed cockerels.

Treatment females were placed in breeding cages with males and dosing began on this date which was recorded as day one. The purity of the o,p'-dicofol used was 99.99% as supplied by the Rohm & Haas Co. (Philadelphia, PA.). The Dicofol was dissolved in corn oil and administered by oral gavage.

The calculation of the Dicofol in dry food matter into a daily

oral dose was based on previous work on DDE (Bird *et al.* 1983), where it was determined that 10 ppm DDE in feed represented 3 mg DDE/kg body wt/day as a steady state dose. The actual levels of Dicofol used in this study were designed to emulate potential Dicofol loads in wild birds and were based on earlier research on kestrels and ring doves (*Streptopelia risoria*) (Schwarzbach *et al.* 1988). Unfortunately, exact levels of Dicofol in the corn oil administered by gavage could not be determined due to difficulties with the assay technique at Clemson University, South Carolina.

Each morning before feeding all females were captured using a hand net and dosed. On a weekly basis, they were weighed to the nearest gram and the doses were recalculated to adjust for changes in body weight throughout the breeding season. At the time of weighing, a 1 ml blood sample was removed from the antebrachial vein and the plasma was stored frozen for later residue analysis.

Nest boxes were checked on a daily basis and eggs were marked with nest number, clutch sequence, and date of laying. The clutch was removed, and dosing stopped, after 5 consecutive days without laying an egg. Artificial incubation removed any variation in incubation behaviour of the adult birds and also reduced the chances of egg breakage due to thinned shells.

Before being set in a Marsh Farms Roll-X incubator held at standard temperature and humidity (Bird 1985), the eggs were measured for length and width and candled to determine viability of the embryo. Afterward, all eggs were candled at 3-day intervals until pipping time (approximately 24 days).

The number of fertile eggs, as well as chicks hatched and fledged were recorded. Unhatched eggs were examined by dissecting microscope for signs of embryo development. Embryos and chicks that died at pipping were placed in 100% buffered formalin for later histological examination of the gonads. The contents of infertile eggs were stored frozen for later residue analysis.

Pairs remained coupled until the end of the incubation time necessary for those laying a second clutch in an attempt to get two clutches from each pair. Upon removal of the eggs from second clutches all laying females were sacrificed by cervical dislocation. Non-laying females were sacrificed when other laying females had completed their second clutches and it was apparent that these females would not attempt to nest. Liver and abdominal fat samples from the females were removed immediately, weighed and stored frozen for later analysis.

All eggshells were washed in water and allowed to air dry. Eggshell thickness was determined to the nearest mm^{-1} by a digital Mitoyou hand-held micrometer. Eggshell measurements were randomly chosen from 5 sites selected on the air cell end of the egg approximately 3-5 mm from the equator or pip line about the circumference of the shell.

Pipped eggs were placed in individual holding corrals in a standard hatcher (Bird 1985) until they hatched. Within 24 hours, chicks were transferred to a brooder and kept in groups of 4 to 5 nestlings at temperatures maximizing their comfort (Bird 1985). Individual chicks were identified by colour markings placed on the

wings and back with felt tip markers. Every 4 hours from 08:00 until 20:00 h, chicks were hand-fed a diet of day old cockerels ground in a food processor.

At 30 days of age the fledglings were sorted by sex, leg banded and placed in over-wintering pens (15 m x 10 m x 2.5 m; l x w x h) for the fall and winter season.

On 5 May 1989, 56 of the chicks hand-reared in 1988 and surviving to 1989 (hereafter referred to as the second generation) were paired with colony birds that had experienced at least one successful breeding season, and placed into the same breeding pen set up described earlier. The experimental design was as follows:

(A) Thirteen male and 8 female second generation chicks hatched from control dosed parents were paired with a proven colony breeder of the respective opposite sex.

(B) Twelve male and 13 female second generation chicks hatched from the 5 ppm dosed females were paired with proven colony breeders of the respective opposite sex.

(C) Four male and 6 female second generation chicks from the 20 ppm dosed female group were also paired with proven colony birds of the respective opposite sex.

Nest boxes were checked daily and the same data were collected as in 1988. In 1989, all pairs were allowed to complete their clutches, incubate the fertile eggs to hatching, and rear their young until fledging with minimal interference. Upon successful completion of fledging their young, the treatment bird of the pair was removed and sacrificed by cervical dislocation. A gross

dissection was completed immediately and gonads were photographed *in situ* using Ectachrome slide film, and then removed and placed in 10% buffered formalin for histological sectioning.

The embryonic gonads from the 1988 females, as well as their one-year old progeny paired in 1989 were removed from storage formalin and embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E). Serial sections of all left gonads were cut transverse to the long axis of the gonad and each section was scored for the presence of any primordial germ cells located in the cortex of the gonad. Histological preparation was completed by the Pathology Laboratory, University of California at Davis. Both embryonic and adult gonads were assigned a key for histopathological examination of the tissues. Each testis was divided into 8 equal pie-shaped pieces both on the longitudinal and cross-sectional cutting of the tissues to enable proper orientation and recording of the data. Embryonic testes sections exhibiting feminization were positively identified as males by the presence of seminiferous tubules in the medullary portion of the gonad. Adult reproductive tissues were evaluated for development of ovarian cortical tissue in the testes, and primordial germ cells (PGC) incorporated into the resulting abnormal cortex of the gonad. Serial sections of both adult and embryonic testes were examined. The location and number of primordial germ cells present in each gonad, presence/absence of a cortical ridge, as well as any other abnormalities were recorded and tallied over the total number of tissue sections cut per individual.

STATISTICAL ANALYSES:

Statistical analysis were performed using procedures from Systat (Wilkson 1990). All tests were performed at the 0.05 level of significance.

Dose Levels from 1988 Females Receiving Treatment

Dose calculations were based on weights of birds taken at weekly intervals, therefore the data were analyzed as repeated measures which were complete up to the 6 weekly weights/bird. The analyses for weights 7 and 8 were determined to see if the longer term effects were more important, even though some birds had already stopped being dosed. The time within subjects was tested using a multivariate test Wilk's lambda to determine the significance of the time period of the experiment.

Reproductive Data from 1988 Treated Females

Data from the 1988 females included days to lay first egg, number of eggs in the first clutch, days to complete first clutch, laying interval between clutches, number of eggs in the second clutch, days to complete second clutch, total eggs broken, total eggs fertile, total eggs hatched, proportion of eggs fertilized and the proportion of eggs hatched. These were analyzed by a 3-group Kruskal-Wallis test.

Reproductive Data from 1989 Treated Birds

Data from second generation males and females (paired with a colony breeder of the opposite sex) were individually analyzed by sex of the treatment bird for the following reproductive parameters: days to lay first egg, number of eggs in the first clutch, days to complete first clutch, number of eggs lost, total eggs fertile, eggs infertile, eggs hatched, dead embryos, dead-hatched, chicks fledged, proportion lost post-hatch, proportion fertile, proportion hatched, proportion fledged. These were analyzed by a 2-group Mann-Whitney U test. Data from the male 20 ppm group were not adequate to be included in the analysis, thus only control and 5 ppm dose groups were included in the analysis.

Eggshell Thickness Measurements

Pearson Correlation Matrix and the Matrix of Bonferoni Probabilities were carried out on the 5 measurements taken on each eggshell to determine significant differences in data collection. A Kruskal-Wallis one-way analysis of variance was then used to analyze the eggshell thickness measurements. Least Mean Square was used to calculate percentage of eggshell thinning.

Histology of chicks and adults hatched in 1988

The analysis of the serial section counts of the primordial germ cells within the gonads of the male embryos hatched from treated females were analyzed using classical nonparametric methods, i.e. Kruskal-Wallis test. Similar analyses were performed on male birds, hatched from treated females, which had reached sexual maturity and completed one breeding season.

Assays for Dicofol in eggs and carcasses of experimental birds

Assay techniques failed to provide Dicofol levels in eggs and carcasses of birds in the three experimental groups, despite several months of effort by laboratory technicians trained in the field at Clemson University. No other assay laboratories were willing to carry out the task.

RESULTS

DOSE-WEIGHT RELATIONSHIP FROM TREATMENT FEMALES GAVAGED IN 1988

Weekly doses were calculated for individual birds based on their weight taken once a week. The recorded weights for each bird were then analyzed as a repeated measures statistical test. Data were complete on all 30 birds up to week 6 of dosing, however there were no significant differences in weights among the treatment groups. Data were also analyzed for week 7 and 8 (although some birds had quit laying) to determine if longer term effects were evident, but both were non-significant. The time within subjects was tested using a multivariate Wilk's Lambda test. The time course of the experiment in terms of dosing the birds was significant ($p<0.05$), but the time*treatment interaction was not significant.

BREEDING DATA FROM 1988

During the 1988 breeding season 242 eggs were produced by the 30 females, i.e. 77 by the controls, 92 by the 5 ppm group, and 73 eggs by the 20 ppm group. Breeding parameters measured for these groups were calculated and probabilities are presented in Table 1. There was only marginal significance in the time to complete laying of the second clutch, i.e. 6.7 days for the controls, 8.0 days for the 5 ppm group and 8.8 days for the 20 ppm group. If success for these birds is measured in terms of total number hatched (by artificial incubation) divided by the total number of eggs laid,

results show 33.8% success in the control group, 29.3% in the 5 ppm and 20.5% in the 20 ppm group.

EGGSHELL THICKNESS MEASUREMENTS 1988

In 1988 228 eggshells were measured. A decrease in shell thickness, i.e. 5.45% in the 5 ppm group and 10.98% in the 20 ppm group, expressed as a decrease in percent of control thickness values, is shown in Figure 1. The shell thickness in the 20 ppm group was significantly different ($P<0.05$) from the control group.

HISTOLOGY OF EMBRYONIC GONADS FROM 1988

Twenty-seven chicks from the 0, 5 and 20 ppm groups were grossly dissected and gonads removed for histology. Data concerning *in situ* condition of the gonads showed slight visible abnormalities in the 5 ppm and the 20 ppm dose groups. The left testes in several embryos appeared elongated and flattened over the underlying adrenal tissue, and in some cases the testicular tissue was translucent and very thin. Normally, testicular tissue has a somewhat kidney bean shape and is well formed on the periphery. Normal testicular anatomy was noted in all control testes and most of the right testes (not analyzed) of the dosed chicks.

Female chicks from the 0, 5 and 20 ppm groups were dissected and evaluated for the presence of right oviducts and/or ovaries. Normally, females only develop the left oviduct and corresponding ovary. Five control females with normal gonadal anatomy were sectioned and examined. Of two 5 ppm females that were dissected,

one showed the presence of a small right oviduct and the other was severely autolysed and not sectioned. Seven 20 ppm chicks were examined grossly, revealing the following: normal appearance (1), a bi-lobed left gonad (1), a very translucent gonad (1), partial right oviduct (2), a right gonad or a vestigial pronephros (2). The age of these latter two chicks made it difficult to determine whether the gonad was forming or the pronephros was regressing at this time. One of these birds also had a peculiar fat-body-like formation in the region where the left gonad would lie if the bird had developed one.

Histological sectioning of the female gonads revealed normal morphology in the control chicks. No sections were analyzed on the 5 ppm females because of degeneration of the tissue. Females from the 20 ppm group revealed 2 normal females, one unable to be discerned due to autolysis of the tissue. Five of the 20 ppm females exhibited ovarian tissue, however there was abnormal morphology present that suggested interference. The ovarian cortical tissue in these cases was arranged into loosely structured rings or tubules. Most did not possess a uniform pattern or have distinct empty tubule rings characterized by male gonads, however similarities were discernible. One other gonad which was feminine in appearance possessed a bilobed structure with distinct tubules in the lower portion of the gonad. Further evidence could not be gathered due to the condition of the tissue. The anatomy described for this tissue shows a trend toward increasing order in the

tubules of the male portion of the gonad, which is evidence of a hermaphroditic condition.

Male chicks analyzed for histological gonad sections are reported from the 3 dose groups in Figure 2. Figure 2A depicts normal testicular morphology of seminiferous tubules made up of narrow columns of seminiferous epithelial cells. Testicular sections with cortical ridges present on both testes may be noted in Figures 2B and C taken from the 5 ppm dose group. A prominent cortical ridge of primordial germ cells along the testicular organ limit of a 20 ppm bird is shown in Figures 2D and E. Numerous large cells located throughout the gonad, localized in the upper right corner, and replacing normal seminiferous tubules are representative of birds from the 20 ppm dose group (Fig. 2F). Figures 2G, H and I illustrate an abnormally shaped gonad that appears to have a large section of female-like tissue emanating from the gonad or is attached to the male gonad.

In all, 570 serial sections were analyzed from male chicks hatched from dosed females in 1988. Table 2 shows the serial sections that were cut from control (12), 5 ppm (7), and 20 ppm (8) chicks, respectively. There was a highly significant increase ($p<0.05$) in the number of primordial germ cells (PGC) per serial section, the number of PGC per sector analyzed, and an increase in the number of sectors positive for PGC per serial section analyzed, when compared to the control groups. Sectors per serial section cut increased with an increase in treatment dosage. Similarly, the number of PGC per section increased with the higher dosage. The

number of PGC per cortical ridge sector was greatest in the 20 ppm dosage group followed by the 5 ppm group and the fewest were recorded in the control group.

BREEDING DATA FROM 1989

From the chicks produced in the 1988 breeding season, 7 female and 12 male control kestrels survived to sexual maturity in 1989, 12 females and 12 males survived in the 5 ppm group, and 6 females and 4 males survived in the 20 ppm group. These birds and their colony mate of the respective opposite sex produced 146 eggs in the 1989 breeding season. Data for female birds are summarized in Table 3 and male data are in Table 4 (due to the low number of 20 ppm birds surviving, only the control and 5 ppm groups were used to generate accurate statistical comparisons). Hereafter the second generation males and females will be referred to as control, 5 ppm, and 20 ppm groups, respectively.

Female 5 ppm birds showed a significant number of eggs lost ($P<0.05$), as well as a significant decrease in the proportion of total eggs laid ($P<0.05$) when compared to the control group. Eggs were considered lost if they were no longer in the nest box. It could not be determined if the lost eggs were eaten by the pair, or removed from the nest box and lost through the wire bottomed cage.

With respect to second generation males, a significant difference ($P< 0.05$) in the number of chicks dead after hatching was noted between the control and 5 ppm groups. For dead or missing chicks it could not be accurately determined whether the

parents ate the chick, fed it to the remaining hatchlings, or whether it was removed and lost through the wire floor to predators.

EGGSHELL THICKNESS MEASUREMENTS 1989

Thirty-three eggshells from second generation females measured in 1989 showed no significant ($P>0.05$) difference when compared between treatments and with the other females laying in 1989.

Eggshell thickness of the 57 shells collected from established colony breeding females paired with second generation males were not significantly ($P>0.05$) different. Compared with those from second generation females, there were no significant ($P>0.05$) differences between the two groups of females.

GROSS MORPHOLOGY AND HISTOLOGY OF ADULT GONADS FROM 1989

Second generation males were sacrificed and their gonads were photographed *in situ*. A typical male testis at the end of a breeding season, i.e. not at maximum size and after gonadal regression has begun, is depicted in Figure 3A. This is similar to the testes in the spring when it begins to increase in size for the onset of breeding. Normally the left testis is more developed than the right, i.e. 12% larger, and situated more on top of the adrenal gland than the corresponding right testis. Figure 3B illustrates a typical testis from a 5 ppm male where both gonads were enlarged when compared to the control testis. However, the left testis is approximately 30% larger than the corresponding right gonad. These gonads not only appeared larger but were flattened in shape.

In a gonad from a 20 ppm male with similar characteristics to that of the 5 ppm group, the left testis was 23% larger than the right and was also flattened (Fig. 3C).

Two hundred gonadal sections were cut from the testes of males at the end of the breeding season and those that were suitable (i.e. not autolyzed) were examined histologically. Fifty of the 72 sections cut from testes from 9 control males showed normal testicular morphology. Two control males had regressed seminiferous tubules indicating that they had finished their breeding season. Seven control males showed active testes with open lumens in the seminiferous tubules containing cellular and precipitated protein strands from fixation of fluid contents. Spermatozoa appeared active in some and both prophase and metaphase stages of cellular division were observed. Variations in the stage of testicular activity can be attributed to the timing of the onset of pair-bonding (or lack thereof), the size of brood raised, and whether the parents were successful in fledging their nestlings.

Sixty-eight of the 96 sections cut from 12 individual 5 ppm males were examined histologically for abnormalities. Two testes from the 5 ppm group were necrotic and could not be clearly denoted, and 2 appeared normal with regressing lumens characteristic of the end of the breeding season. Eight of the testes showed large cells in the periphery of the organ just under the smooth muscle layer. One individual showed large vacuoles and fat cells throughout the gonad. Thirty of the 32 sections cut from four 20 ppm males were examined and testes in regression were

noted, indicating that the birds had completed their clutches earlier than the other groups or had not attempted to breed.

The presence of primordial germ cells per gonad was significantly higher ($P<0.05$) in the 5 ppm group when compared to the control group. The 20 ppm group could not be included due to the lack of appropriate samples.

DISCUSSION

Adult females gavaged daily in 1988 showed variable changes in body weights as expected during the breeding season (Romanoff and Romanoff 1949), and hence, their doses were altered accordingly over the course of the experiment. The weights of the control and dosed females did not differ significantly ($P>0.05$) and all birds received approximately the same amount of corn oil with or without Dicofol. Hence, variations in results could not be attributed to heavier birds receiving potentially more Dicofol.

Breeding data parameters measured from the gavaged females revealed no significant ($P>0.05$) differences among the 3 groups, except that a trend in time to renest was noted. Bird and Laguë (1982) found that older experienced females tend to renest faster than inexperienced birds and that second clutches are generally smaller than the first. All females in the study were approximately the same age and laid fewer eggs in the second clutch, although fewer 20 ppm birds attempted to lay first and second clutches. Wiemeyer *et al.* (1989) determined that Kelthane with or without DDT did not affect laying date, clutch size or incidence of cracked or broken eggs in screech owls. However, first year breeding females did show a failure to incubate eggs.

Twenty ppm o,p'-dicofol did significantly lower kestrel shell thickness (Fig. 1). The lower 5 ppm Dicofol dose yielded only a 5.45% decrease in thickness compared to control eggshells, indicating a lack of a linear relationship between Dicofol dosage level and shell thickness.

Eggshells retrieved from the nests of second generation birds laying in 1989 showed no thinning effects.

Eggshell thinning in American kestrels caused by organochlorines has been documented by Wiemeyer and Porter (1970) and Peakall (1975). Eggshell thinning by Kelthane and Dicofol has been documented by Bennett *et al.* (1990) who found Dicofol's magnitude of shell thinning in mallards to be similar to that of DDE. Captive American kestrels fed Kelthane showed reduced shell thickness and weight, when exposed to dietary levels of 1 to 30 ug/g (wet weight) (Clark *et al.* 1990). In ring doves, the incidence of broken eggs were significantly higher in birds exposed to p,p'-dicofol compared to control and DDE-fed ones (Schwarzbach *et al.* 1988). Similarly, screech owls exposed to Kelthane with and without DDT-r produced shell weights and thicknesses that were significantly lower for both dosed groups than for controls (Wiemeyer *et al.* 1989).

The causal relationship between DDT contamination and eggshell thinning in various populations of birds has been well documented (Ratcliffe 1967, Hickey and Anderson 1968, Berger *et al.* 1970). When eggshell thinning exceeds 20%, reproductive success is not high enough to maintain a population (Ratcliffe 1967, Hickey and Anderson 1968, Peakall 1975, Springer *et al.* 1984).

Recent data from waterbirds exposed to organochlorine contaminants showed DDT concentrations in eggs to be too low to account for the observed eggshell thinning and it was suggested that long-term residual effects of exposure to Kelthane containing

DDT may be expressed as persistent right oviducts and developmentally induced shell thinning (Speich *et al.* 1992).

Histological sectioning of male embryonic gonads revealed abnormal morphology in the 5 and 20 ppm treatment groups. Evidence of feminization in both treatment levels ranged from testes with approximately normal morphology to those with a thickened ovary-like cortex in the left testis composed of primordial germ cells. PGC's were found singly or in clusters throughout the cortex or were more commonly located at the ventral border of the gonad as a cortical ridge composed of cuboidal or columnar epithelium. The number of PGC's per cortical ridge was greatest in the 20 ppm dose group. Normal testicular tissue can be differentiated by its thin squamous cell epithelial cortex and localization of PGC's to the seminiferous tubules. Positive identification of feminized male gonads is made certain by the presence within the cortex of PGC's that enter into meiotic prophase, as they do in the ovary. The large ovarian-type germ cells in prophase with vacuolar eosinophilic cytoplasm provide a distinct marker (Fry and Toone 1981). The left gonad primordium, early in development, typically possesses an incipient ovarian cortex surrounding the medulla, whereas the right primordium consists of a medullary component with trace amounts of incipient cortex (Romanoff 1960). Therefore, the right gonad possesses more potential for becoming male and the left gonad has increased potential for bisexuality. In the female the right gonad thus disappears altogether. The outcome of sexual development is the predominance of one sex by the degeneration of

one component with concurrent differentiation of the other. Romanoff (1960) stated that gonadal development is controlled by hormones which are acted upon by genes, since the appearance of hormones in development is associated with the time of sexual differentiation in the avian embryo. Genetic male embryos receiving female hormones or related estrogenic compounds develop an ovotestis characterized by a cortex of ovarian type tissue with variable degrees of thickness, covering the testicular medulla in the left gonad while the right gonad remains normal (Romanoff 1960).

The amount of feminization in this study was variable and did not always correlate with parental Dicofol dose, but it does agree with what was found by Fry *et al.* (1989) who studied feminization in American kestrels fed technical Kelthane and p,p'-dicofol. Morphological results were also similar to feminization occurring in DDT-exposed chicken and quail (Lutz-Ostertag and David 1973) and gull embryos (Fry and Toone 1981).

Dicofol treated second generation females paired with known colony breeding males in 1989 showed a significant loss of eggs laid. Due to attempts to minimize stress and allow the birds to breed without disruption it was often difficult to determine why and how this occurred. The author did on several occasions witness (unseen by the birds) females picking eggs out of the nest and dropping them on the cage floor or attempting to place them on perches or even eating them. Because breeding pens were randomized with treatments, stress unrelated to the treatments would have

likely shown up in a particular block of pens regardless of treatment. This was not the case. Treatment-related stress in dosed females or behaviour alterations may have caused the loss of the eggs. Alternatively, aberrant behaviour by the colony male may have been responsible, although no direct evidence of this was noted.

Second generation males paired with known colony breeding females showed a decreasing trend in the proportion of chicks hatched from eggs laid, as well as a significant increase in chicks lost after hatching. The former could be related to decreased nest attentiveness and incubation behaviour since male kestrels are responsible for some incubation (Bird 1988).

Mineau *et al.* (1984) proposed that declining gull populations from Lake Ontario resulted from pollutant-induced endocrine dysfunction. Alterations in hormonal levels were postulated to induce behavioural anomalies such as abnormal nest construction, altered breeding synchrony, and decreased incubation attentiveness and parental care, which have been reported in organochlorine-exposed wild bird populations (Rattner *et al.* 1984).

Chicks were lost after hatching in several ways. Birds reported to have lost chicks typically removed them as they hatched, or waited for the complete clutch and then began removing them. Parents were also seen killing chicks in the nest and partially eating them or feeding them to the remaining hatchlings. Alternatively, they dropped them on to the cage floor where they would perish or be lost to predators. Due to the routine *ad*

libitum feeding of cockerels, food was never a limiting factor. Thus, altered behaviour of the treated males was most likely the basis for chicks being lost.

Gross morphological results are in accordance with feminization reported in domesticated avian species (Romanoff 1960), gulls (Fry and Toone 1981, Fry *et al.* 1987) and American kestrels (Fry *et al.* 1989). Treated birds showed a gross enlargement of the left testis which appeared cream-coloured with translucent borders and rested on or adjacent to the adrenals on the cranial portion of the kidney. Normal morphology of a kestrel testis shows ellipsoid organs of approximately the same size, although the left testis may be slightly larger and more cranial (Romanoff 1960). Gross morphological alterations included very flattened, enlarged and translucent ovotestis (Figures 3A, B & C). One 5 ppm female bird had a black terminal tail band, which is typically a male characteristic. This female was paired with a colony male, but produced no eggs. Upon sacrifice, the female was noted to contain testes with no ovarian-like structures present. Unfortunately its gonads were destroyed, precluding histological analysis. Bryan *et al.* (1989) reported altered feather morphology in Japanese quail exposed to o,p'-DDT, although no alterations in gonadal morphology were observed.

The timing of histological evaluation of adult testes at the end of breeding season imposed constraints on just what could be evaluated. Most testes showed regressing testicular lumens with few spermatozoa present. Suspect PGCs were located in the

periphery of the organ in the smooth muscle layer and were significantly different from the control testes sampled.

Since only 4 chicks produced by 20 ppm males in 1988 survived to breed, it was difficult to evaluate their reproductive capability. Of the eggs laid by colony females paired to these birds, all were fertile. Perhaps the most important impact on the second generation birds was the lack of surviving high dose males to breeding age. Sex-ratio skews in gull populations have been hypothesized to result from selective mortality of males (Hunt *et al.* 1980). Fry *et al.* (1987) noted sex-skews in breeding populations of gulls exposed to estrogenic compounds. Where female-female pairing was evident, feminization of male chicks and abnormal female development was present. The origins of homosexual female pairing and abnormal sex ratios have been theorized to be caused by decreased survival of male gulls from contaminated eggs as opposed to females, or by feminization from the estrogenic effects of the compounds resulting in males that do not appear on breeding grounds or do not participate in sexual pairing (Wingfield *et al.* 1980, Burger and Gochfeld 1981, Conover 1984, Conover and Hunt 1984, Fry *et al.* 1987).

Data from this study suggest both better survival of female embryos and feminization of males. Feminization of male chicks has been proven to distinctly alter reproductive behaviour of mature birds (Adkins 1975, Whisett *et al.* 1977). Thus, the chances of populations exposed to estrogenic compounds successfully fledging normal clutches may be greatly reduced. It can only be

speculated that the results of such long-term exposure alter populations in a negative manner.

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Table 1: Summary of 1988 breeding data for female American kestrels exposed to 0, 5, 20 ppm o,p'-dicofol. Data are presented as means.

PARAMETER MEASURED	CONTROL	5 PPM	20 PPM
Days to lay 1st egg	14.7 (4.1)	14.4 (4.2)	11.8 (4.3)
Number eggs 1st clutch	5.0 (0.6)	4.7 (0.4)	5.1 (1.8)
Days to complete clutch 1	9.6 (2.2)	8.7 (0.7)	10.1 (1.2)
Interclutch time	11.4 (3.0)	11.2 (0.5)	7.4 (0.6)
Number eggs 2nd clutch	3.9 (0.7)	4.5 (0.5)	4.6 (0.7)
Days to complete clutch 2	6.7 (1.6)	8.7 (1.3)	8.9 (1.5)
Total eggs broken*	7.0 (0.3)	4.0 (0.6)	8.0 (0.5)
Total eggs fertile*	40.0 (2.2)	41.0 (3.3)	31.0 (3.2)
Total eggs hatched*	26.0 (1.5)	27.0 (2.2)	15.0 (1.6)
Percent fertile eggs*	51.9 (0.5)	44.5 (0.4)	42.5 (0.7)
Percent fertile/hatched*	65.0 (0.6)	65.8 (0.7)	48.8 (0.3)

() +/- standard error

* Combined score for both clutches laid

** Significant at the $p<0.05$ level

Table 2: Histological sections of male embryonic gonads harvested from chicks hatched from female American kestrels exposed to 0,5,20 ppm o,p'-dicofol in 1988.

	SECTIONS ANALYZED (n)	SECTORS SECTION	#P.G.C. SECTION	#P.G.C. SECTOR
CONTROL	259 (12)	0.33a	0.06b	0.18c
5 PPM	141 (7)	1.61a	1.27b	0.79c
20 PPM	170 (8)	2.91a	1.74b	0.60c

section = histologic cut through the gonad

sector = 8 equal sized 45 degree wedges of gonad

P.G.C = number of primordial germ cells

n= number of birds

a= $p<0.001$ (2 df)

b= $p<0.000$ (2 df)

c= $p<0.004$ (2 df)

Table 3: Breeding data summary for second generation female American kestrels hatched from females previously exposed to o,p'-dicofol and laying eggs in 1989. Data presented as means.

PARAMETERS MEASURED	CONTROL	5 PPM
Days to lay 1st egg	17.3 (4.6)	12.7 (2.2)
Number eggs 1st clutch	4.9 (0.2)	5.2 (0.8)
Time to lay 1st clutch	9.3 (0.9)	10.1 (2.0)
Total eggs lost	1.2 (0.6)	1.4 (1.3)*
Total eggs fertile	3.4 (1.5)	3.4 (1.3)
Total eggs infertile	1.4 (0.7)	1.7 (0.8)
Total eggs hatched	2.7 (1.2)	2.8 (1.7)
Number of dead embryos	0.4 (0.2)	0.3 (0.2)
Number dead post-hatch	0.5 (0.4)	1.2 (0.6)
Number fledged	2.1 (1.3)	1.6 (1.5)
Percent lost	11.8 (1.7)	23.8 (4.2)*
Percent fertile	70.6 (7.1)	65.1 (6.4)
Percent hatched	79.2 (11.)	82.9 (6.9)
Percent fledged	78.9 (7.2)	55.9 (3.8)

() +/- standard error

* Significant at 0.05 level

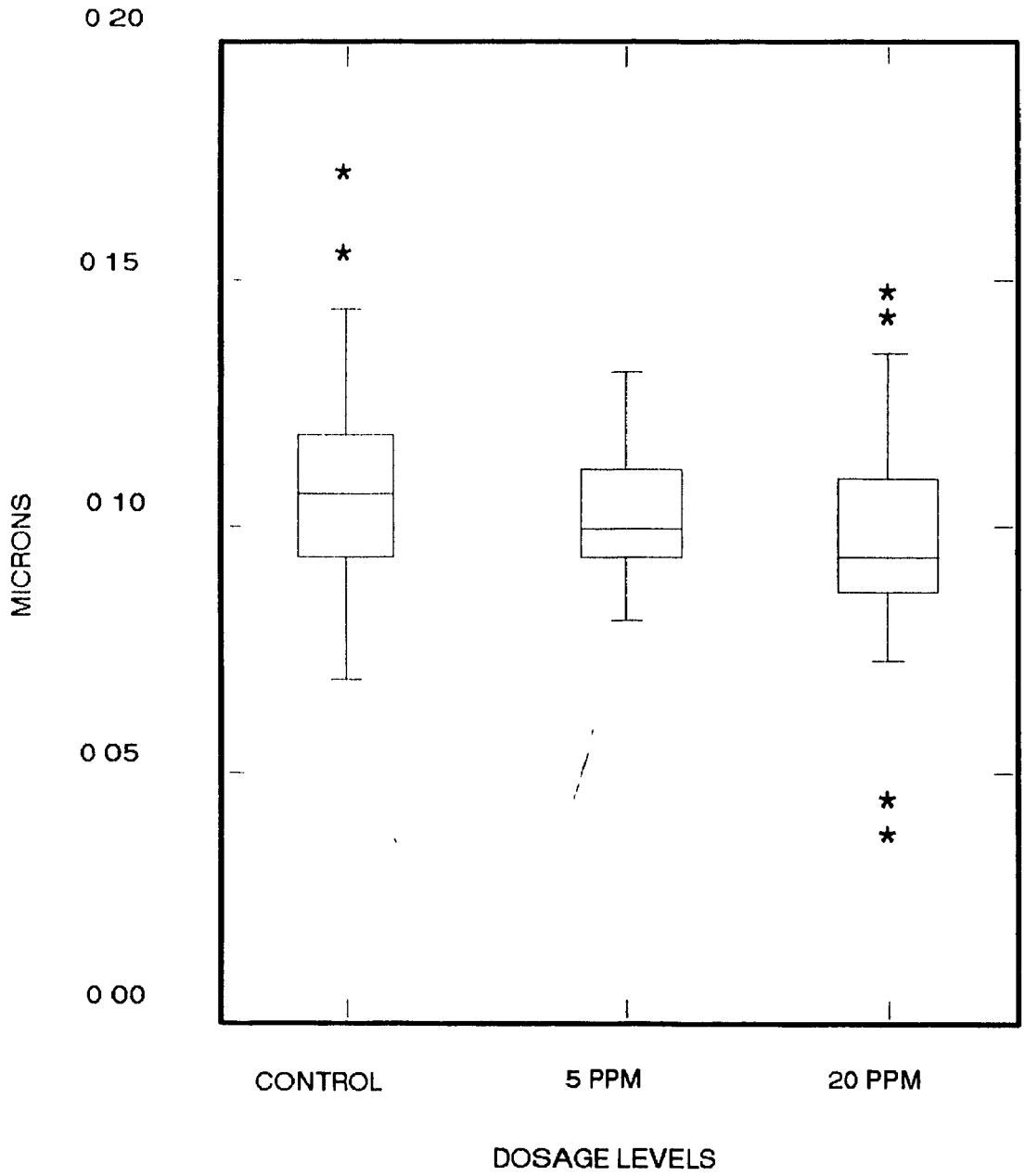
Table 4: Breeding data summary for second generation male American kestrels hatched from females previously exposed to 0, 5, 20 ppm o,p'-dicofol and laying eggs with an established breeding female in 1989. Data presented as means.

PARAMETERS MEASURED	CONTROL	5 PPM
Days to lay 1st egg	17.8 (5.8)	17.4 (7.4)
Number eggs 1st clutch	5.4 (0.8)	5.3 (0.8)
Time to lay 1st clutch	10.8 (1.6)	9.8 (1.1)
Total eggs lost	1.8 (1.6)	1.7 (1.0)
Total eggs fertile	3.2 (2.2)	3.5 (1.5)
Total eggs infertile	1.8 (0.7)	1.8 (1.0)
Total eggs hatched	1.2 (0.8)	2.6 (0.9)
Number of dead embryos	0.6 (0.3)	0.4 (0.3)
Number dead post-hatch	0.08 (.02)	0.9 (0.5)*
Number fledged	1.1 (0.8)	1.7 (0.8)
Percent lost	33.8 (0.1)	31.2 (2.2)
Percent fertile	58.5 (5.8)	65.6 (7.0)
Percent hatched	36.8 (2.3)	73.8 (6.5)
Percent fledged	92.8 (3.2)	64.5 (4.1)

() +/- standard error

* Significant at 0.05 level

Fig. 1: Eggshell thickness measurements from female American kestrels exposed to 0, 5, 20 ppm o,p'-dicofol in 1988. All measurements are recorded in microns. Shown is the mean ± standard error (vertical rectangle) and the + 95 % confidence interval (vertical line). (*) = extreme values.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DOSAGE LEVELS

Fig. 2: Histological sections from embryos and chicks hatched from female American kestrels exposed to 0, 5, 20 ppm of o,p'-dicofol in the 1988 breeding season. (A) Section of normal testis of an American kestrel chick hatched from control female. The gonad cortex (C) is composed of squamous epithelial cells; (ST) normal seminiferous tubule (80X). (B) kestrel testis from a chick hatched to a 5 ppm dicofol exposed female. Arrows indicate ridge of cortical tissue, (ST) = seminiferous tubule (100X). (C) Similar 5 ppm dicofol treatment chick showing a cortical ridge of tissue, composed of primordial germ cells (80X). (D) Prominent cortical ridge indicated by arrows on a 20 ppm dicofol chick (32X). (E) Higher magnification of Figure D, showing localization of PGC (80X). (F) 20 ppm male testis with large PGC (*) located throughout the cortex and ventral portion of the gonad (80X). (G) Abnormal shaped 20 ppm male gonad; identifiable male tissue is located between the arrows (12.5X). (H) Higher magnification of Figure G; shows seminiferous tubules in the male portion of the gonad (between the arrows) and loosely arranged cells of the second half resembling ovarian tissue (32X). (I) Higher magnification of Figure G; shows loose connection of male tissue (between the arrows) to the second portion of the gonad which resembles ovarian-like cells (80X).

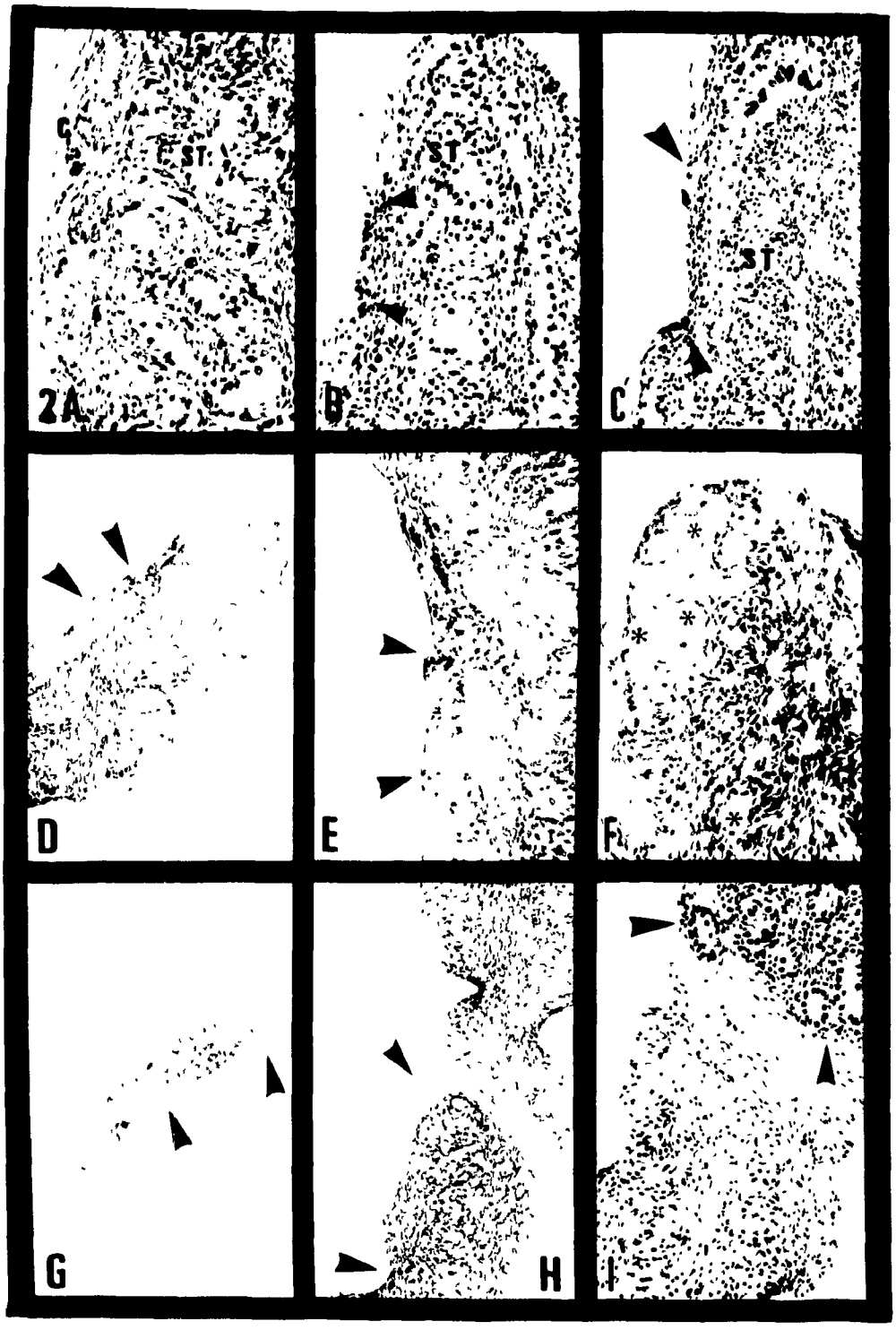
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Fig. 3: Photographed *in situ* gonads from second generation male American kestrels hatched from females exposed to 0, 5, 20 ppm o,p'-dicofol. Birds were sacrificed post-breeding season. (A) Reproductive organs of a partially eviscerated male American kestrel hatched from a control female. Testes (R) right and (L) left are of normal shape. Single arrow (right) and double arrows (left) indicate vas deferens. (*) = adrenal gland. (B) Reproductive organs from a male hatched from a 5 ppm dicofol exposed female. Note gross enlargement and flattening of the left testis (L) compared to the right (R). Arrow indicates right and double arrow indicates left vas deferens which appear thickened. (*) = adrenal gland. (C) Reproductive organs from a male hatched from a 20 ppm dicofol exposed female. There is a gross difference in size and shape of the testis, where the right (R) testis is smaller than the enlarged and flattened left (L) testis. The left gonad is thinned, translucent and partially draped over the adrenal gland, indicative of an ovotestis on gross morphology. (*) = adrenal gland.


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CONNECTING STATEMENT

Chapter 1 identified the effects of o,p'-dicofol on the reproductive performance of two generations of captive American Kestrels. Differences in eggshell thickness from the Dicofol exposed females and morphological alterations in the reproductive organs of the second generation birds hatched from thin-shelled eggs were noted in Chapter 1. Differences noted in the reproductive performances of the second generation birds may be attributed to morphological alterations of the gonad and behavioral anomalies.

Chapter 2 describes the effects of the parental dose on the aggressive tendencies of the second generation male birds and the pair bonding abilities of both sexes when matched with an established breeder of the opposite sex.

CHAPTER 2

Behaviour of Captive American Kestrels Hatched from O,P'-Dicofol Exposed Females

INTRODUCTION

Organochlorines are widespread environmental contaminants. Many investigators have studied the effects of DDT and related compounds on the behaviour of birds (Fry and Toone 1981). For example, delayed ovulation and abnormally aggressive behaviour toward mates and chicks resulted from dietary loads of DDT fed to Bengalese finches (*Lonchura striata*) (Jefferies 1967, 1971). Mallard (*Anas platyrhynchos*) ducklings hatched from parents fed 3 ppm DDE displayed hyper-responsive reactions to maternal calls and decreased flight distances from fright stimuli (Heinz 1976).

Dicofol (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethanol) is the active ingredient in an organochlorine pesticide marketed under the trade name Kelthane which is used as an acaricide mainly against cotton and citrus mites. Dicofol has been examined by the U.S. Environmental Protection Agency for its DDT-related contaminants, but more recently the deleterious effects of Dicofol itself have been questioned (Speich *et al.* 1992).

Reproductive and behavioral studies of progeny hatched from American kestrels (*Falco sparverius*) showed male progeny to be less aggressive and more submissive to control birds (Fry *et al.* 1988), suggesting that *in ovo* exposure to Dicofol effectively impairs the development of male falcons.

Behavioral effects of organochlorine compounds have been little studied compared to anticholinesterase agents. However, because of the persistence of organochlorine compounds in the environment,

knowledge concerning behavioral effects extended over generations is becoming important. Few cues are currently available to understand the mechanisms by which these compounds affect behavioral development (Bignami 1976). Many reproductive and non-reproductive behaviours are known to be influenced by hormonal treatments during critical periods in development, so that appropriate behavioral responses occur later during maturation and in the adult (Ottinger *et al.* 1984).

This study was prompted by the alterations in reproductive parameters measured on birds hatched from females exposed to 5 and 20 ppm o,p'-dicofol (Chapter 1). My objective was to determine whether normal levels of aggressiveness, which are critical to proper pair bonding and subsequent production of offspring, occur in these males hatched from treated females. This was accomplished by placing males hatched from 5 and 20 ppm Dicofol exposed females, in competitive situations with untreated conspecifics of similar size and age. Pair interaction was also studied between second generation hatched birds and a known colony breeder of the opposite sex in an attempt to determine if treated birds could form traditional pair bonds.

MATERIALS AND METHODS

American kestrels (referred to as the second generation) hatched from females exposed to 0, 5, 20 ppm o,p'-dicofol daily throughout the breeding season were hand-reared to fledging at the Avian Science and Conservation Centre of McGill University. The birds were then separated by sex, placed in overwinter flight pens (20 x 20 x 3.5 m) and maintained on a diet of day-old cockerels (see Bird 1985 for description of the kestrel colony management). In the spring of the following season, the birds approached sexual maturity and were manipulated in the following manner:

DOMINANCE TRIALS 1989

In March 1989, the second generation male birds were measured for tarsus and antebrachium bone lengths as an indication of body size. The birds, having been exposed to treatment levels of 0, 5, and 20 ppm o,p'-dicofol, were then placed into groups according to size (i.e. small, medium and large), determined from a combined measurement of the tarsus and antebrachial length of each bird. The experimental set-up consisted of a flight pen measuring 1.5 m x 2.5 m x 2.5 m with a slanted rope perch, making one end of the perch much higher than the other (approx 0.45 m). Thus, there was a limited number of optimal perch sites. Theoretically, the more aggressive bird selects the highest perch close to the wall, which is considered optimal for detection of "predators" and food (K. MacLellan unpubl. data). Also, to further induce competitive

behaviour, food was restricted to a third of each bird's daily ration, and only one food item was placed in the pen at a time. Therefore, by holding size, and age or level of experience constant, any effects of the organochlorine on the bird's aggressive tendencies would be evident in its ability to compete and maintain a rank in the presence of conspecifics when resources were limited.

Birds of similar size and varying treatment levels were introduced into the pen and observed for one hour in the morning and then again for one hour in the afternoon, with the introduction of one day-old cockerel at the beginning of the afternoon observation period. Data were collected on encounters included: frequency, cause of the interaction and winner. This was continued for 4 consecutive days, or until a ranking could be discerned.

SECOND GENERATION PAIR BEHAVIOUR

On 5 May 1989, 56 of the hand-reared kestrels that had overwintered and reached sexual maturity (including those males that participated in the aggression trials), were paired with colony birds that had experienced at least one successful breeding season, and placed into the breeding pens described in Chapter 1. Treatment birds, paired with a proven colony breeder of the opposite sex were included in the following experimental set-up:

(A) Thirteen male and 8 female second generation chicks hatched from control dosed parents;

(B) Twelve male and 13 female second generation chicks hatched from the 5 ppm dosed females;

(C) Four male and 6 female second generation birds from the 20 ppm dosed group.

Nest boxes were checked daily for egg-laying and hatching date, (see Chapter 1). All pairs were allowed to complete their clutches, incubate eggs to hatching, and rear their young until fledging.

A sample of 10 pairs composed of 5 second generation females and 5 second generation males, were chosen (using a table of random numbers) from the control group and from the 5 ppm treatment group. In the 20 ppm group, the smaller sample size meant that (4 second generation males and 6 second generation females) were included in daily observations.

Pairs to be observed were randomly drawn from a hat each morning, and behavioural observations were conducted between the hours of 08:00 and 12:00 for a 10-min period per pair. At the beginning and every 30 sec thereafter, the following behavioural classifications were recorded for each bird of a pair: perching; eating; chirring (calls made by either sex when copulating or exchanging food items); kleeing (general calls made by either sex); preening (grooming behaviours); flying; copulating; attempted copulation (when the male mounted the female but did not complete the act of copulation); food transfer (when one bird of the pair presented the other with a morsel of food and the second bird accepted it); attempted food transfer (when the second bird did not

take the offered food item); on the nest box (perched on the nest box roof); inside the box; or perched on the lip of the nest box opening; and attempts to land on one of these nest box locations. Aggression (overt physical contact using beak and/or talons on the other member of the pair) and a category of miscellaneous behaviours, e.g. watching insects, picking at the wall, etc., were also recorded.

Observations ceased 7 days after a female completed her clutch and began incubating. In the case of birds that did not lay, observations ceased when the last successful pair completed laying its clutch.

STATISTICAL ANALYSIS

Statistical analysis were performed using procedures of Systat (Wilkson 1990).

Dominance Trials

Second generation males were placed in groups of 3 birds of different treatments and assessed for dominance or rank order based on their level of aggressive behaviours. The data were analyzed as a blocked partial Spearman correlation (the partial correlation was calculated between the behaviour and Dicofol level given the block) (Lehman 1975).

Pair Behaviour

The behavioural data collected on the paired birds, consisting of a second generation bird and a colony bird with at least one successful breeding season were analyzed as considering means of the 4 groups: second generation males, second generation females, colony males, and colony females. For behaviours involving both birds in a pair e.g. copulation, food transfer, the tests of the second generation and colony birds are duplicates. The test statistic used was the Kruskal-Wallis one- way analysis of variance (Wilkson 1990).

RESULTS

Ordered ranks i.e. dominance, were found to exist in each of the trials conducted using aggressive behaviours as determining parameters. The ranks were not found to be consistently stable or linear and codominance occurred involing several different levels of aggressive individuals. Results of the partial blocked Spearman Rank correlation (Table 1) show a highly significant correlation between the level of aggressiveness and rank holding ability associated with prenatal Nicofol dose, indicating that the males hatched from treated eggs were less likely to attain and maintain a high ranking position in the presence of conspecifics.

The bird's position on the perch was found to change with the presence or absence of food, thus making certain regions of the perch more desirable at different times. Dominant individuals were found to prefer the higher regions of the perch (against the wall) during non-feeding times. Perch sites proximate to the food were favoured when food items were present.

The highest point of the perch was preferred when food was absent because it served as an optimal observation point and the wall likely functioned as added protection or cover. Perch sites directly across from the food were preferred when food items were present because they allowed the bird to be as close to the food item as possible without leaving the safety of the perch and then landing on the floor mesh where they were more vulnerable to predators.

Thus, a bird's rank did not change significantly with the presence or absence of food items, although perch ranks changed with the presence or absence of food items. Typical perch rankings of birds when food was absent are depicted in Fig. 1A , and in Fig. 1B when food was present. Lower ranking birds were displaced from optimal perch sites by higher ranking individuals that hovered above the subordinate until it simply moved or was displaced by force.

Perch rank order was much more stable than feeding hierarchy. The order in which the birds initiated feeding was not as important as the ability to maintain control over the food item. Dominant individuals ate later in the feeding order by simply removing the food item from the possession of a subordinate. This was accomplished by a spectrum of encounters that ranged from submission and avoidance to overt bodily force and injury. The lower ranked birds often initiated the feeding behaviour. Since the aggressive birds captured a larger share of food though, the subordinate birds were more eager to initiate feeding behaviour because they were hungry. More overt agonistic encounters were associated with feeding than perching as the aggressive motivation of the lower-ranked birds increased with hunger.

Aggressive interactions were observed for short periods of time, usually lasting less than 60 sec. Actual physical encounters never resulted in serious injury and were more often avoided by the birds mantling their food, i.e. covering it with their body and wings to indicate possession. This behaviour has been noted in numerous

other species studied by Eible-Eibsfeldt (1961) as a means of avoiding potentially damaging fights.

Behavioural data were collected on 10 breeding pairs per dose group. Second generation females showed a significant difference from control birds in the category of miscellaneous behaviours ($P<0.05$). These behaviours included picking at the rope perch, watching flies, laying down and other such displacement activities.

The colony mates of the second generation females of the 5 ppm and 20 ppm groups spent more time at the nest box door than the second generation control mates.

Second generation 5 ppm males completed significantly less numbers of copulations ($P<0.05$) than the corresponding control birds. The colony females paired with the second generation males showed a decreased number of copulations ($P<0.05$). This is a duplicate test of the male data since the copulation was scored simultaneously for both birds involved in the activity. The number of attempted food transfers was also significantly higher ($P<0.05$) in the second generation 5 ppm group.

DISCUSSION

Experimental trials involving second generation males hatched from 0, 5 and 20 ppm Dicofol treated females showed alteration in aggressive behaviours when placed in a competitive environment.

The overt agonistic encounters involved food items and perch sites. High ranking birds most often initiated agonistic encounters and were able to dominate lower ranked individuals. Similar results were found by Patterson (1977) in both captive and wild populations of shelducks (*Tadorna tadorna*), and by Feare and Inglis (1979) in starlings (*Sturnus vulgaris*).

Lesser ranked kestrels initiated feeding bouts more often, possibly in an attempt to eat before the higher ranked individuals chose to. Higher ranked individuals are afforded the luxury of eating when they wanted to, whereas the lesser ranked birds were forced to eat soon after the presentation of the food items since waiting includes the risk of no food being left.

Avoidance and submission were more common than fighting, and likely served as a means of conserving energy and reducing injuries. The number of fights was also reduced because such acts were mainly initiated by high ranking individuals. In other words, if rank order had not been established, many more potentially harmful fights may have occurred. Aggressive interactions occur when the outcome of such an event is not certain to the individuals involved, and the rank order can be considered unstable. Guhl

(1956) noted that birds within an unstable rank fought more, ate less and gained less weight.

In the breeding pairs, second generation females from the 5 ppm group displayed a significantly greater amount of displacement actions when compared to control birds, perhaps indicating that these females were less interested in pair bonding and raising of progeny normally undertaken by breeding pairs. Colony males paired with 5 ppm treated females also showed an increased trend in nest box door attentiveness, but this was not statistically significant. It could reflect the altered female behaviour, i.e. increased nest attentiveness by the male.

The second generation males from the 5 ppm group showed a significant decrease in the number of copulations and an increased number of incomplete food transfers when compared with control males. This may be concluded as a lack of stimulation and sex drive on the part of the treated male, or may be indicative of rejection by their females.

Long-term effects of quail embryos exposed to o,p,'-DDT resulted in decreased survivability of chicks to 5 weeks, decreased number of ovipositions, increased number of eggshell malformations, and an increase in estrogen-like effects on reproductive behaviours (Bryan *et al.* 1989). Corresponding interferences with gender specific behaviours were reported with *in ovo* injections of synthetic estrogens (Whitsett *et al.* 1977, Gildersleeve *et al.* 1987). Alterations in behaviours were noted by Carnio and McQueen (1973) who administered 15 ppm p,p'-DDT to 3 generations of Japanese quail

and caused decreased fertility and a substantially decreased percentage of fertile eggs. Heinz (1976), who exposed mallard (*Anas platyrhynchos*) parents to 3 ppm DDE, found their offspring to be hyper-responsive to avoidance calls and showed decreased flight distances to a frightening stimulus.

The permanent effects of early exposure to estrogenic compounds have been reported in herring gulls (*Larus argentatus*) (Boss 1943, Boss and Witschi 1947). Fry and Toone (1981) reported DDT-induced feminization of gull embryos. Feminization of male avian embryos has been reported to markedly alter adult reproductive behaviours (Adkins 1975, Whitsett *et al.* 1977). Suppression of male reproductive behaviours may result in sex-skewed breeding colonies and altered populations as proposed by Burger and Gochfeld (1981) and Fry *et al.* (1987). The occurrence of super-normal clutches (Conover 1984) and homosexual female pairing have been thought to originate from populations exposed to estrogenic contaminants. Hormonal changes that ultimately induce behavioural anomalies such as abnormal courtship and nest construction, altered breeding synchrony and decreased parental care have been reported in wild bird populations from heavily contaminated organochlorine areas (Rattner *et al.* 1984). Thus, abnormal development of birds exposed to organochlorine contaminants could still be a persistent problem after the cessation of pesticide applications. Reproductive population failures could result from developmental suppression and alteration of male gender-specific behaviours, or alterations in maternal care abilities of the female.

This study indicates that parental exposure to Dicofol can have a significant effect on aggressive behaviour of captive kestrels. In wild birds, this could mean that organochlorine-exposed chicks might develop less aggressive tendencies and have a decreased ability to form a pair bond or provide adequately for a resulting brood should a pair bond form and fertile eggs are laid. Influences on behavioural development may vary in a wide variety of ways, ranging from direct hormonal effects at the time of exposure to altered mother-offspring interactions at a time when the compound is no longer present (Bignami 1976). Such effects could result in decreased reproductive fitness, increased energetic costs, and altered reproductive success in organochlorine contaminated wild bird populations.

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Table 1: 1989 analysis of aggressiveness and dominance of male second generation kestrels hatched from females exposed to 0, 5, 20 ppm o,p'-dicofol. Rank evaluated in groups of three size-matched birds.

TRIAL NUMBER	TREATMENT GROUP RANK			SPEARMAN RANK
	CONTROL	5 PPM	20 PPM	
1	1	2	3	1.0
2	1	2	3	1.0
3	1	2	3	1.0
4	2	1	3	0.5
5	1	2	3	1.0
6	2	3	1	-0.5
7	1	2	3	1.0

N = number of birds

B = number of trials

Mean is $x = 5.0$

Variance is $s^2 = 3.5$

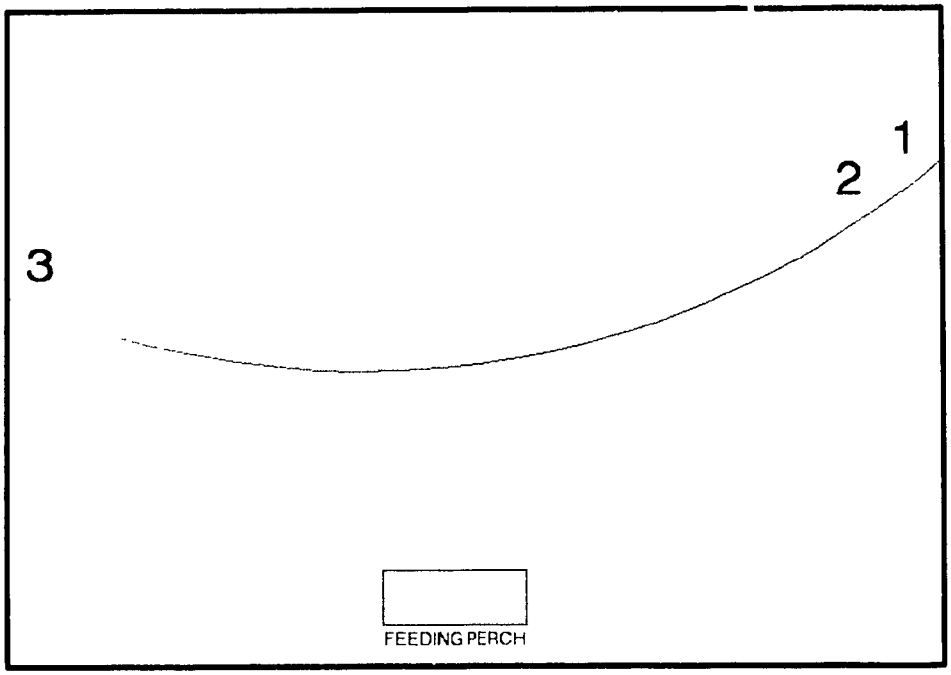
Z statistic summed across blocks $z = 2.67$

Z probability $p = 0.0038$* (* Significant at the 0.05 level).

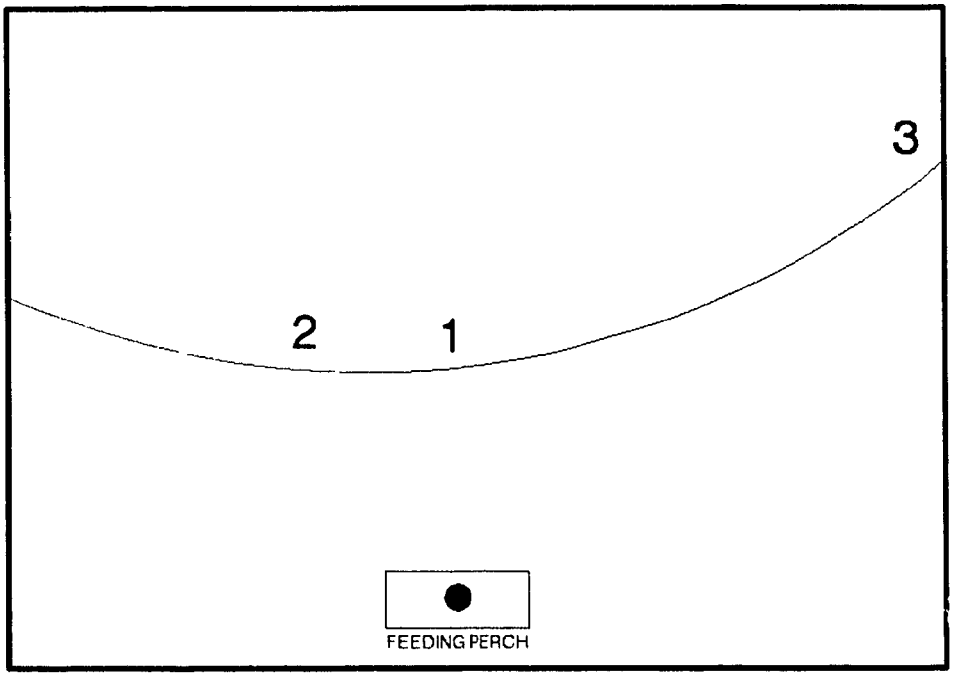
Fig 1: A) 1989 adult second generation male American kestrel dominance trial indicating bird rank-order placement on the perch in the absence of food-items. Numbers 1, 2 and 3 indicate a bird's rank and placement on the perch.

B) Bird rank-order placement in the presence of food items.

A) RANK ORDER WHEN FOOD IS ABSENT



B) RANK ORDER WHEN FOOD IS PRESENT



GENERAL CONCLUSIONS

Female American kestrels were exposed to 0, 5, 20 ppm o,p'-dicofol during the 1988 breeding season. Reproductive success measured as the total number of eggs hatched (artificially) divided by the total clutch number was 33.8%, 29.3% and 20.5% for the control, 5 ppm and 20 ppm groups respectively. Eggshell thickness was reduced 5.45% in the 5 ppm group and significantly so ($P<0.05$) by 10.98% in the 20 ppm females.

Histological sampling of the male reproductive gonads harvested from chicks produced by the exposed females demonstrated alterations in morphological anatomy. Male embryos were feminized by the estrogenic effects of o,p'-dicofol and this was evident by the presence of primordial germ cells in the gonadal cortex. Both 5 and 20 ppm of o,p'-dicofol resulted in feminization of the male gonad, although the degree of feminization did not always correlate with the maternal dose. A significantly ($P<0.005$) higher number of germ cells were noted in the 20 ppm gonads when compared to the control testes.

Male chicks from the same dose groups were sacrificed at sexual maturity and testes of males arising from maternal dose groups of 5 and 20 ppm o,p'-dicofol demonstrated alterations in gross morphology. Testicular size and shape were enlarged and flattened, resembling a thinly contoured testicle.

Adult birds of the second generation, exposed only via the maternal dose of o,p'-dicofol, displayed negatively correlated changes in reproductive success grouped by maternal dose. Females from the 5 ppm dose group lost significantly ($P<0.05$) more eggs after laying than did the controls. Similarly, the 5 ppm dose males paired with proven female breeders had significantly ($P<0.05$) more dead or missing chicks after hatch when compared to the control males.

Behavioral abnormalities were also found to exist among the second generation adult birds. Male birds separated by size were entered into a competitive arena to assess dominance of birds based on maternal dose. The estrogenic effects of the compound proved to be significantly ($P<0.05$) correlated with a bird's rank and aggressive tendencies, where increasing maternal dose, i.e. 5 ppm and 20 ppm Dicofol, resulted in decreasing rank order.

Pair behaviour of second generation adults mated with a proven breeder of the opposite sex were also significantly ($P<0.05$) different from controls. Males of the 5 ppm dose group had significantly lower copulation rates and significantly ($P<0.05$) higher failed attempts at pair bonding activities such as food-transfers to their respective mates. Treated females also exhibited significant ($P<0.05$) behaviour changes that resulted in these birds spending less time participating in pair-bonding and raising of progeny.

The results of this avian study add further evidence that birds of prey are at risk in areas where o,p'-dicofol is used in the

environment. Not only are birds at immediate risk when laying eggs in contaminated regions, there is a potential for their future generations to exhibit decreased reproductive success through morphological and behavioural alterations caused by point source maternal exposure during egg-laying to organochlorines.

Thus, while DDT and related organochlorines have been banned from use in North America, their persistence in the environment, their mobility (they are still used extensively in third world countries), and now their ability to reach across generations through the maternal-offspring interface promotes their continued role as major chemical contaminants in the environment today.