August 2008

Organochlorine Pesticide Contamination and its Potential Effects on Eggshell Characteristics of Dickcissels (Spiza americana)

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Abstract:

Dickcissels (*Spiza americana*) are small, sparrow-like songbirds that nest in grasslands in the U.S. and winter in Venezuela. Farmers in Venezuela intentionally spray dickcissel flocks with organochlorine (OC) and other illegal pesticides in an attempt to kill them when they feed in both rice and sorghum fields. Previous studies have shown that organochlorine (OC) pesticide contaminants (e.g., DDT) have significantly reduced eggshell thickness in eagles and falcons. Although DDT was banned in the United States more than twenty years ago, OC compounds and their metabolites still persist in wildlife, possibly resulting in reduced reproductive success and in disrupted endocrine systems (Harper et al. 1996). Recent studies (e.g., Harper et al. 1996; Klemens et al. 2000; Bartuszegvige et al. 2002) have shown OC contamination in Neotropical migratory passerines (i.e., songbirds that breed in Canada and the United States and winter in Mexico and Central and South America.), including dickcissels. The purpose of this study is to determine the relationships between OC contamination on thickness and color (hue, saturation and brightness) of dickcissel eggshells. The ecological implications of contamination may include the effect of eggshell coloration on the amount of male parental investment, reduced hatching success of dickcissel eggs, as well as the success of dickcissels in rejecting brown-headed cowbird (*Molothrus ater*) eggs.

Key words: dickcissel, *Spiza americana*, male avian parental care, sexual selection, egg coloration, female condition, organochlorine pesticides

Introduction:

Organochlorine (OC) pesticides are endocrine-disrupting molecules that bind to intracellular receptors and interfere with hormone mediation within the cell, causing an array of macro affects on a given organism (Colborn et al. 1993). OC pesticides have been shown to be bioaccumulate in raptorial birds such as falcons and eagles (Cade et al. 1971; Peakall, 1974; Cromartie et al. 1975; Grier, 1982); but recently, it has been documented that passerines (i.e., songbirds) are also contaminated with OC pesticides (Harper et al. 1996; Klemens et al. 2000; Bartuszevige et al. 2002; Wiedenfeld et al. unpublished data). However, a consensus to the extent to which Neotropical migrant passerines are contaminated has not yet been reached. Klemens et al. (2000) found ubiquitous contamination among passerine samples that comprised 72 individuals of 11 species, as 66 of the birds were contaminated with OC pesticide residues (92%) with levels of contamination found between 1.36 ng/g and 391.43 ng/g. [Note: the terms ng/g and µg/g (eg, the concentration of pesticide in a given sample) are used interchangeably with parts per billion (ppb) and parts per million (ppm)]. Klemens et al. (2003) found thirteen of seventeen tested OC pesticides in 19 of 55 (35%) tested passerines. In contrast, Capparella et al. (2002) found a lack of widespread OC contamination in South American resident passerines (only 3% were contaminated with levels between 1.9 and 7.9 ng/g) but this study contained few individuals that lived near land slated for agricultural use.

Dickcissels (*Spiza americana)* are migratory Neotropical passerines that winter in Venezuela and breed in North American grasslands (Figure 1). Within the past forty years dickcissel native wintering areas have been converted from natural grasslands to agricultural sites (mainly rice and sorghum), and the dickcissels made an opportunistic conversion to utilizing cereal grains for

their winter diet (Basili and Temple, 1999). Due to their presence as crop pests, South American farmers began to attempt to kill them with toxic chemicals. Basili and Temple (1999) surveyed Venezuelan farmers in order to gauge pesticide use, and 14% of them admitted to using a variety of chemicals (both OC pesticides and other chemicals) in trying to kill dickcissels, either spraying their seed directly or dousing dickcissel roosts during the night. The latter technique is known to have killed hundreds of thousands of birds in under an hour, perhaps contributing to the population decline of 40% since their populations were first censused in 1966 (Sauer et al. 1966). The South American crop loss due to dickcissels is most dramatic during the period prior to migration; a physiological response to changes in photoperiod triggers hyperphagia (i.e., excessive eating) in order to build food reserves for the journey returning to North America (Zimmerman, 1965). In addition, since females are the last to leave wintering sites, they are the most vulnerable to chemical attack, possibly dramatically decreasing species' reproductive output (Basili and Temple, 1999).

OCs have numerous impacts on avian embryos and chicks. Egg-laying in all avian species is an involved process, although the energy expended by the female dickcissel during egg-laying is only between 26 and 42 percent of the energy demanded by nestling care (Krementz and Ankney, 1986). Additionally, although total body fat within the female declines, this decline is not linear and the source of fat used for the eggs is not completely understood (Krementz, 1984). This has possible implications for direct transfer of fat-soluble OC pesticides into the egg yolks of avian embryos (Ulman, 1972; World Health Organization, 1984; Fry, 1995 and others). Several OC pesticides have also been identified as directly estrogenic, and their presence affects the development of embryos within the egg, acting to feminize the embryos. Avian embryos are

particularly vulnerable to these affects since metabolite products are not excreted and continue circulation during incubation (Fry, 1995), an important consideration when attempting to measure contamination long after the egg was produced.

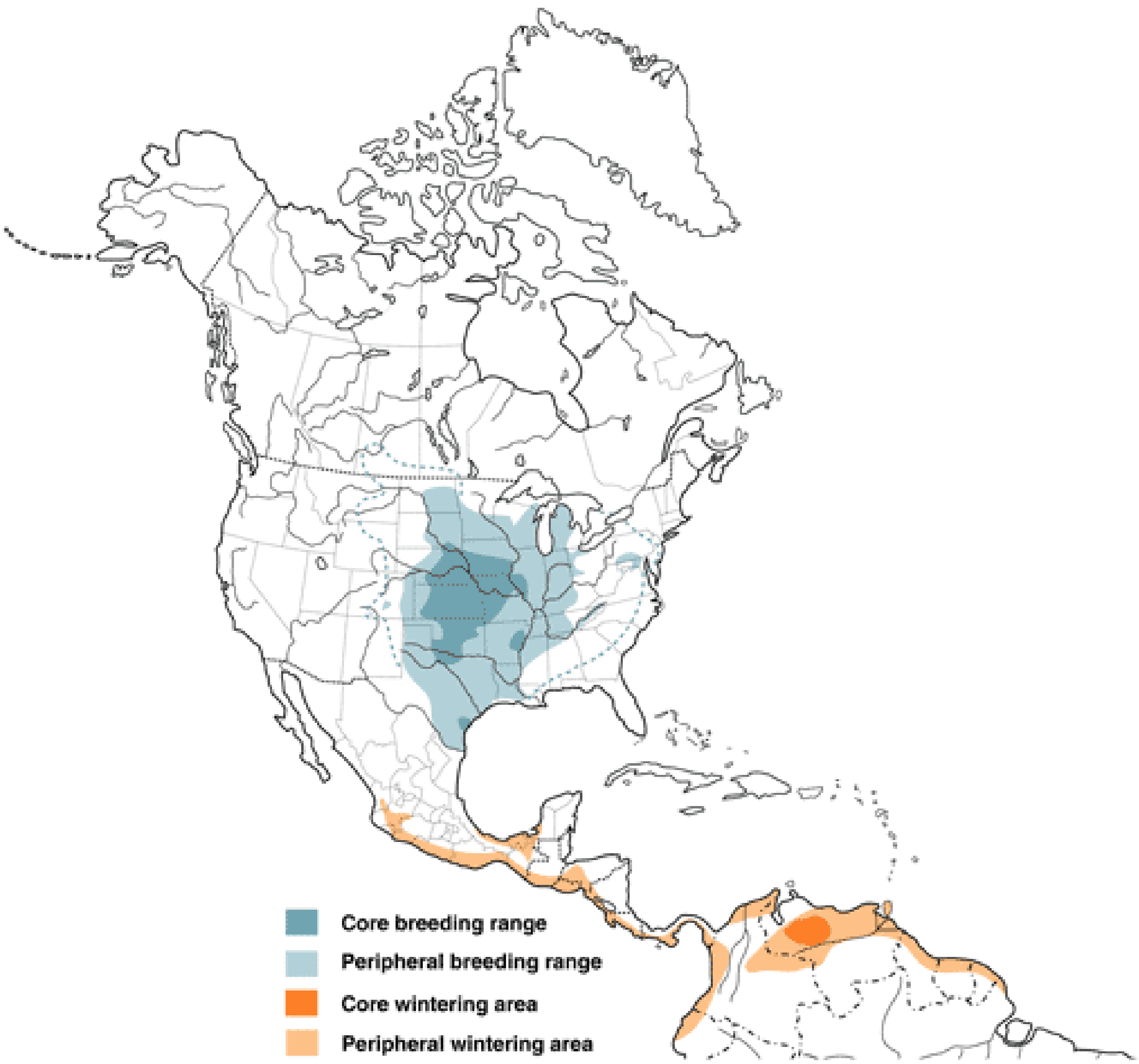

Figure 1: Range of the dickcissel (Temple, 2002)

It has been shown that OC contamination is linked with eggshell thickness in several avian species, including loggerhead shrikes (*Lanius ludovicianus*), woodcocks (*Philohela minor*), bald eagles (*Haliaeetus leucocephalus*), peregrine falcons (*Falco peregrinus),* and in many other predatory birds (Anderson and Duzan, 1978; Dilworth et al. 1972; Grubb et al. 1990; Cade et al. 1971; Peakall, 1074; Ratcliffe, 1970), but this effect has not been documented in passerines. More recently, the presence of DDE was confirmed to correspond to a significant decrease in eggshell thickness in Eurasion sparrowhawks, *Accipiter nisus* ($p = 0.03$ for regression of logDDE vs. eggshell thickness; Jagannath et al. 2008). Although the process by which the eggshell is formed within the oviduct is still not completely known, there is consensus in the literature that DDT and its metabolites inhibit carbonic anhydrase within the avian shell gland of female birds (Bitman et al. 1970; Miller and Peakall 1976). As carbonic anhydrase is the primary enzyme that incorporates carbonate ions into the eggshell, the primary protective element of the egg is weakened. Precise details of this mechanism are unknown. Also, other pollutants such as PCBs and organophosphates may result in a negatively additive effect (World Health Organization, 1984). The porosity of the eggshell, a key component in gaseous exchange as well as strength, is reduced by pollutants such as DDT (Cooke, 1979; Fox, 1976). In addition, it has been shown that DDE-induced decreases in magnesium concentration in eggshells alters the mechanical properties of the eggshell (Board and Scott, 1980).

The reduction of eggshell thickness and strength could affect dickcissels in a number of ways. First, eggshells with increased fragility are more likely to be damaged by the mother during brooding. Furthermore, the incubation process is further complicated by the brood parasitism by brown-headed cowbirds (*Molothrus ater*). Dickcissels have a complicated relationship with

cowbirds, and dickcissel reproductive fitness is diminished by cowbirds in two ways: laying eggs in dickcissel nests as well as the attempted removal of cowbird eggs by dickcissels from their nests (Zimmerman, 1983). Dickcissels are considered to be "acceptors" of cowbird eggs with a rejection rate of approximately 11% (Peer et al. 2000), yet this is perhaps surprising due to the stark color difference in the visible spectrum between dickcissel and brown-headed cowbird eggs (blue and white spotted brown, respectively). Possible reasons for this lack of rejection include a smaller dickcissel bill size, which makes puncturing or grasping the larger cowbird eggs difficult and the possibility of host egg damage during attempted ejection (Peer et al. 2000). Therefore, it has been postulated that costs of rejection might outweigh the benefits for many dickcissels, which raise on average 3.5 offspring, including both dickcissels and cowbirds (Zimmerman, 1983).

The color of a dickcissel egg is normally blue, yet there are some dickcissels which have white morphs, possibly due to a genetic mutation that disrupts the breakdown of porphyrin into biliverdin or prevents deposition of biliverdin into the eggshell (Siefferman et al. 2006). The predominantly blue color of dickcissel eggs is caused by the incorporation of the pigment biliverdin into the eggshell from the shell gland (Baird et al. 1975). Biliverdin, the first breakdown product of heme, plays a role as a hydrophilic antioxidant in birds (Stocker et al. 1987). Therefore, as mother birds are contaminated by pesticides, oxidative stress may contribute to a decrease in overall antioxidant titers and a subsequent decrease in available antioxidants to incorporate into offspring eggshells. Hence, biliverdin content in eggs may act as an indicator of female condition, a variable that could include OC pesticide contamination. Siefferman et al. (2005) assert that older female birds in better body condition may lay more

colorful eggs, while Moreno et al. (2005) found that older females lay less colorful eggs. However, it must be mentioned that human perception of color is not necessarily the most accurate in studies done on eggshell color in birds, especially given that birds possess highly advanced visual photosystems, which include the ability to see in the UV range (Goldsmith, 1990; Withgott, 2000). This signaling mechanism of antioxidant incorporation conferring brightness is very similar to many types of sexual signaling such as carotenoid deposition in male feathers (Lozano, 1994). Eggshell color is also correlated with the amount of maternal antibodies deposited into the yolk and subsequent fledgling success (Morales et al. 2006), as well as maternal immunocompetence (Moreno et al. 2005).

Thus, it has been proposed that the color of eggshells may act as a signaling mechanism of female quality to males, a type of post-mating selection. Male dickcissels arrive to breeding territory first, and stake their claims to their territory (0.3 – 1.1 ha; Gross, 1921). Dickcissels show resource-defense polygynous behavior, and females choose a place to nest based on quality of the location and mate based on the male that holds the best quality territory (Zimmerman, 1966; Schartz and Zimmerman, 1971). Therefore, male dickcissels may have zero to many breeding partners, and are able to contribute differentially to each female and her offspring. During brooding, eggs are able to be viewed by the male dickcissels, and thus have the potential to be a type of post-mating selection. This effect would be most pronounced in species where male parental investment is present but variable (Moreno and Osorno, 2003). The contribution of care a male dickcissel gives directly to his offspring is disputed: Zimmerman (1983), Gross (1921) and Long et al. (1965) assert that males take no part in parental care. In contrast, a more recent study (Rivers et al. 2003) indicates that dickcissels are in fact biparental.

Soler et al. (2005) proposed that egg color intensity is associated with parental investment in passerines, although this assertion is tempered by other possible explanations. First, eggshell pigment could be used directly by the embryo as an acquired antioxidant during gestation. Second, as the hormone (and oxidant) progesterone stimulates egg-laying, biliverdin deposition could be a self-protection mechanism used by the mother during the laying period and its presence in eggshells is therefore unintentional. Third, antibacterial properties of pigment are also useful in preventing pathogenic infections (Moreno and Osorno, 2003). However, if biliverdin deposition is a post-mating signal determining male parental investment, OC pesticides may act to diminish the viability of affected eggs.

The purpose of this study was to determine if there was a relationship between OC pesticide contamination in dickcissel eggs and eggshell thickness and eggshell color. We hypothesized that thickness would be negatively correlated with OC pesticide contamination, and that eggshell color would diminish with higher levels of pesticides. The changes in eggshell thickness due to pesticide contamination will possibly accelerate the rate of species decline via an increase of dickcissel egg fragility, which may be further magnified by the dickcissel's attempts at rejecting brown-headed cowbirds eggs. Diminished eggshell color may affect the parental care given to the offspring by male birds, a factor influencing chick viability.

Methods

[A note on personal contributions: Much of the data included in this paper has been obtained by a team of many students throughout the past four years (see acknowledgements). I have been a member of the Dickcissel group for the past two years, and my contributions have ranged from extracting pesticides to processing said extracts and injecting them in the gas chromatograph and analyzing results. Each egg takes approximately 2 hours to extract and analyze, yet this time estimate is not entirely accurate, as I also spent much time this past year to troubleshoot the GC, as well as helping to train a new student working in the group. In addition, background research has taken a considerable amount of time to complete. My efforts have been particularly concentrated on the variable of color, and I have worked with set up and determining protocol for the color determination of the eggshells, as well as taking all of the pictures in this project and analyzing them in Photoshop.]

Sample collection and storage: The dickcissel eggs used for analysis were collected from nests in Indiana, Illinois, and Iowa from 2004 - 2007, under all necessary permits. They were stored in a -80 °C freezer until thawed and analyzed.

Determination of Eggshell Thickness: Three approximately equally-sized pieces of eggshell (excluding the eggshell membrane) each approximately 0.5 cm in diameter were removed after the egg thawed and were air-dried for at least six months. Previous studies have dried eggshells for 1 month to three months, and some incorporate oven-drying (Laporte, 1982; Ohlendorf et al. 1988). Shell thicknesses (in mm) were determined using a Starrett Thickness Indicator. Each of

the three eggshell pieces was measured three times, and the mean of these nine measurements per egg was utilized in this study.

Method of Pesticide Extraction: The remaining eggshell and its contents were weighed, homogenized, mixed with sodium sulfate (a varied amount depending on the amount of water present in the egg), and Soxhlet-extracted with approximately 200 mL pesticide-grade hexane (Fisher) for 18-24 hours into a round bottom flask. The extract was concentrated to about 3 mL, and transferred to a 0.15 m chromatography column containing Florisil (20 g, activated at 130ºC for at least 24 hours) and sodium sulfate (1-2 cm flanking the Florisil). The concentrated pesticide extract was eluted using three washes with increasing polarities: 6% diethyl ether in hexane, 15% diethyl ether in hexane, and 50% diethyl ether in hexane. Samples were concentrated using a rotary evaporator to approximately 3 mL and rediluted with hexane to 10 mL in a volumetric flask (Frick et al. 1998; Klemens et al. 2000).

Method of Pesticide Residue Analysis: All prepared specimens were tested for the presence and levels of 17 different OC compounds and metabolites using gas chromatography (Harper et al. 1996; Frick et al. 1998). Each fraction was analyzed with a Hewlett Packard (HP) 6890 series gas chromatograph. Within this apparatus, two Ni63 electron capture detectors operated at 300ºC. Microliter injections were made using an autosampler into a splitless injector operated at 230ºC. Helium was used as the carrier gas as the sample was separated on two different silica capillary gas chromatograph columns. Two columns were used to ensure presence of pesticides: 30-m DB-35 (0.32 mm inside diameter) and 30-m DB-1701 (0.32 mm inside diameter). The oven temperature was raised from an initial temperature of 150-200 ºC at a rate of 8ºC/min, from

200-290ºC at 4ºC/min then maintained at 290ºC for seven minutes (total time on the column = 35.75 minutes). Data were collected using HP ECD software, and the peak areas from 8 different pesticide standards was used to calculate presence and amount of a given pesticide (Table 1).

Table 1: Detection limit is specified in terms of micrograms (µg) of pesticide per gram (g) of eggshell and egg material used in the extraction process. [note: µg/g is equivalent to ppm]

Compound assayed	Detection limit (in µg/g)
Aldrin	0.012-0.020
alpha-hexachlorocyclohexane (α -BHC)	0.004-0.0065
beta-hexachlorocyclohexane (β -BHC)	0.004-0.0065
gamma-hexachlorocyclohexane (γ -BHC)	0.004-0.0065
delta-hexachlorocyclohexane (δ -BHC)	0.004-0.0065
2,2-Bis(4-chlorophenyl)-1,1-dichloroethane (p,p'-DDD)	0.004-0.0065
2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene (p,p'-DDE)	0.004-0.0065
2,2-Bis(4-chlorophenyl)-1,1-thrichloroethane (p,p'-DDT)	0.004-0.0065
dieldrin	0.004-0.0065
endosulfan I	0.012-0.020
endosulfan II	0.004-0.0065
endosulfan sulfate	0.040-0.065
endrin	0.004-0.0065
endrin aldehyde	0.004-0.0065
heptachlor	0.008-0.013
heptachlor epoxide	0.004-0.0065
methoxychlor	0.004-0.0065

Pesticides were positively identified when sample retention times were within 0.05 min of the average retention time of the calibration standards on both columns. All concentrations that were below detection limits were treated as zeros. Mean pesticide levels were calculated as a concentration of the amount of pesticide to the amount of mass per egg (e.g., ng/g; Klemens et al. 2000). Linear regressions, t-tests, and p tests (Sokal and Rohlf, 1994) were calculated using Excel software.

Determination of Eggshell Hue, Density, and Brightness: Eggshell fragments were photographed under 60W equivalent fluorescent lighting approximately 0.35 m above the specimen using a ambient fluorescent lighting in a room without natural light using a Sony Cyber-shot 3.2 mega pixel digital camera. Photos were analyzed in Adobe Photoshop for hue, saturation, and brightness. Hue is quantified in degrees from 0-360, saturation ranges from 0-100 (grey to fully colored), and brightness ranges from 0-100 (black to white). Measurements were taken from three parts of the eggshell fragments and the mean was used in comparing color to pesticide content (Soler et al. 2005; Villafuerte and Negro, 1998).

In order to confirm the reproducibility of our method using Adobe Photoshop, six pictures were taken of a single eggshell sample and analyzed as per the above protocol, except that 10 different sites on each eggshell picture were tested in Photoshop for hue, saturation and brightness (thus, sixty total measurements were taken). A Q test was performed on each set of ten measurements in order to determine the presence of outliers. Since ten data points were analyzed, if our confidence level is set to 95%, the critical Q value will be 0.466 (Shoemaker et al 1974). Only one point of a total of 180 values was thrown out as an outlier, and the Q tests for both individual pictures and the data set as a whole found no other outliers.

Results and Discussion:

A total of 103 eggs were analyzed for pesticide content in this study, 94 eggs were analyzed for eggshell thickness, and 59 were analyzed for eggshell color. However, during analysis of the entire original data set, it was noticed that there were three distinct groupings of samples within the data (Figure 2). Upon further study, it was found that the top 13 of the top 15 contaminated eggs (containing over 400 ng/g of OC pesticides) were run on the GC in December of 2006 on the same date. In addition, these eggs were primarily contaminated with beta-BHC and heptachlor in high amounts, two compounds that had not been detected prior to this analysis date. There are several possible hypotheses for this observation: first, these eggs (all gathered in the summer of 2006) experienced a more toxic exposure to both heptachlor and beta-BHC while in South and Central America that past winter. Second, there was an error within the GC's hardware or software which caused for the actual amounts present of these two compounds to be increased by one or more orders of magnitude. The second hypothesis can be tested by re-injecting the particular samples from December 2006. As the validity of these samples is therefore under question, this set of data was excluded from this analysis, leaving 77 eggs analyzed for eggshell thickness and pesticide content, and 46 analyzed for eggshell color and pesticide content.

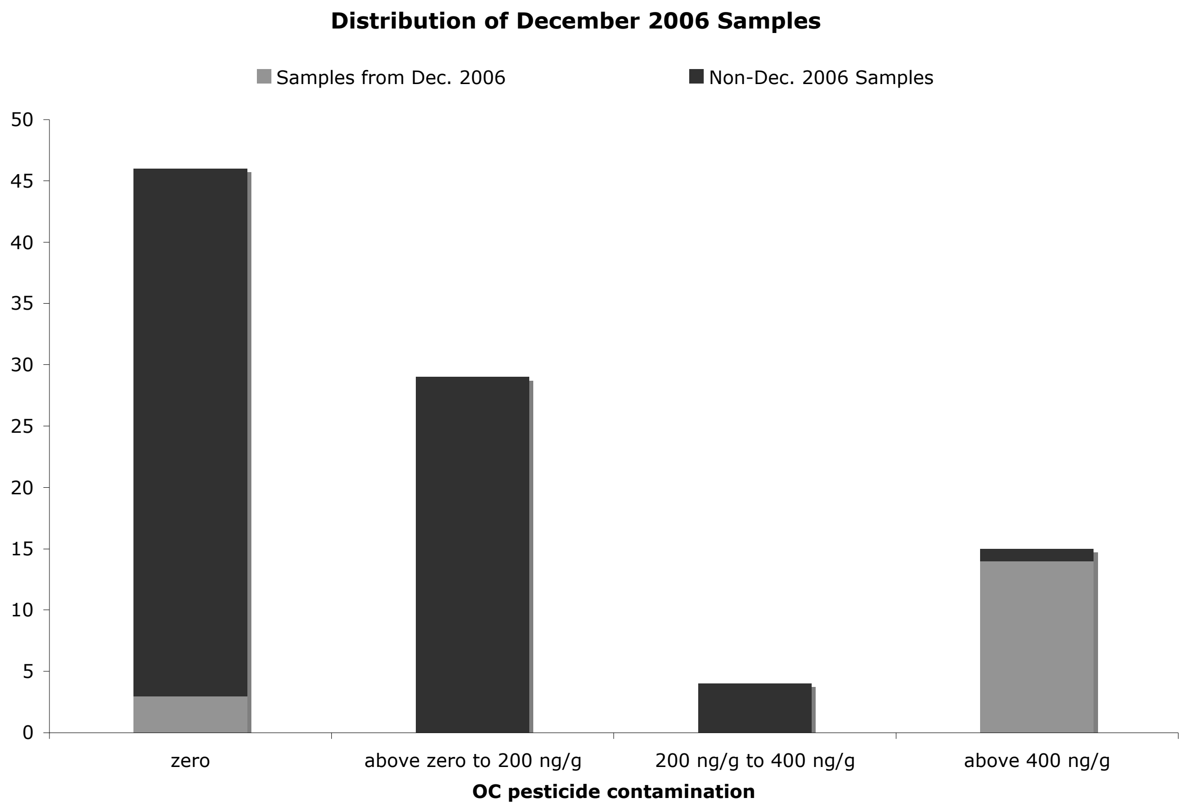

Figure 2: Contamination levels in complete data set with representation of December 2006 samples.

Eggshell Thickness

At least one OC pesticide was detected in 34 of 77 eggs analyzed for both eggshell thickness and total OC pesticide content, and DDE was the most commonly detected compound (13 of 77 eggs; Table 3). There was a significant negative relationship between eggshell thickness and levels of total OC compounds (Figure 3). An ANOVA test was not attempted because the number of compounds detected per egg and the number of times each compound was detected were too small of sample sizes. However, a follow-up t-test indicated that the mean eggshell thickness in eggs with no OCs present was significantly greater than the thickness of eggshells with OCs present (Table 4).

Table 3: Values of OC pesticides are given in parts per billion (ng pesticide/g sample)

Compound	Number of eggs with detection	Minimum detected	Maximum detected	Mean	Standard Deviation
Aldrin	0	--	--	--	--
α-BHC	0	--	--	--	--
β-BHC	1	116.94	116.94	116.94	--
γ-BHC	0	--	--	--	--
δ-BHC	5	90.48	197.53	152.20	42.79
p,p'-DDD	1	287.23	287.23	287.23	--
p,p'-DDE	13	43.29	417.97	105.83	99.53
p,p'-DDT	1	91.6	91.6	91.6	--
Dieldrin	4	48.03	89.17	67.29	18.81
Endosulfan I	0	--	--	--	--
Endosulfan II	0	--	--	--	--
Endosulfan Sulfate	0	--	--	--	--
Endrin	4	81.45	130.43	101.29	21.15
Endrin Aldehyde	4	45.90	104.65	78.52	30.05
Heptachlor	4	46.30	269.50	179.10	102.71
Heptachlor Epoxide	2	39.43	74.47	56.95	24.78
Methoxychlor	2	104.07	284.21	194.14	97.45

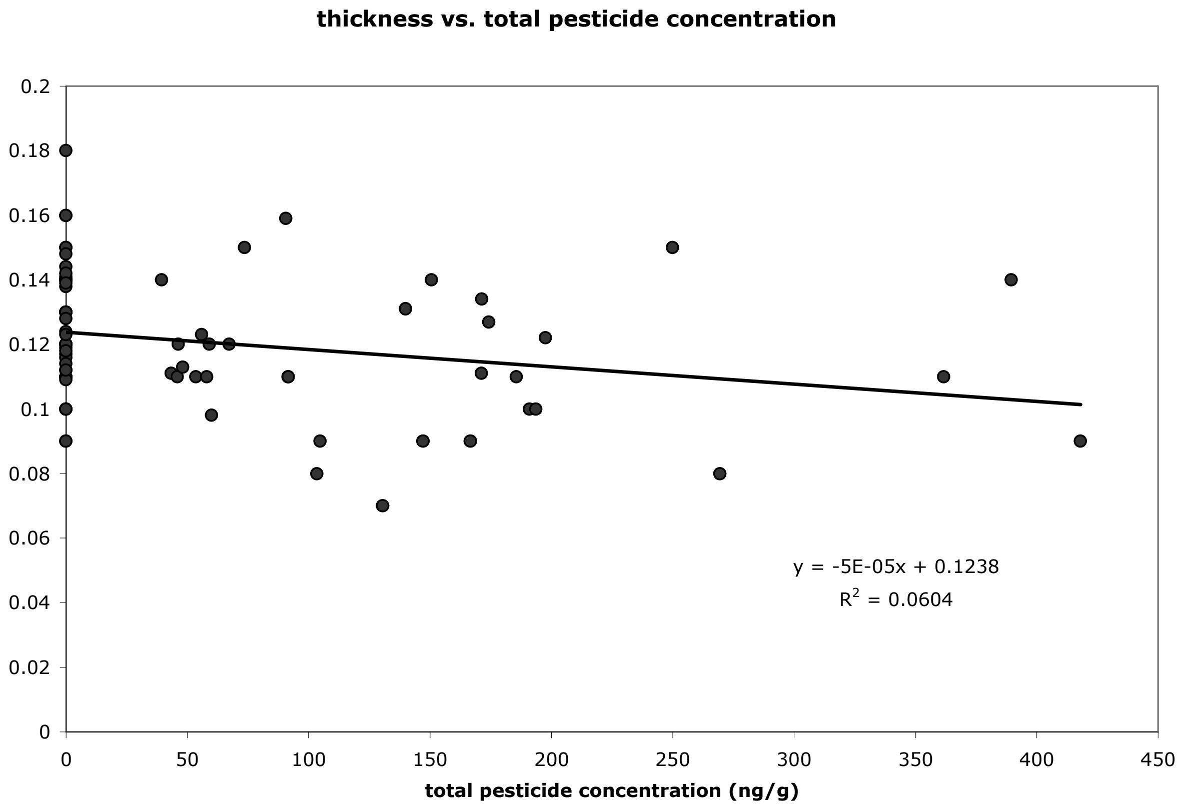

Figure 3: Linear regression comparing eggshell thickness and total OC pesticide contamination; non-contaminated eggshells have a range of thicknesses from 0.090 to 0.180 mm, while contaminated eggshells have a range of thicknesses from 0.070 to 0.159 mm.

Table 4: t test results comparing eggshell thickness in contaminated vs. non-contaminated eggs

Mean and standard deviation of thickness (mm) of **contaminated** samples (n=34)	Mean and standard deviation of thickness (mm) of **non-contaminated** samples (n=43)	t	t critical (95% c.l.)	p
0.114 ± 0.0214	0.126 ± 0.0195	2.54	1.99	0.0130*

The starred p value indicates a 1.3% chance that difference in the means of eggshell thickness in contaminated eggshells and non-contaminated eggshells is due to random error. Although our regression finds a low R^2 value, this simply means that there are other factors involved which help determine eggshell thickness: the significant negative slope does indicate a relationship

between OC pesticide contamination and eggshell thickness. In addition, the significant t test confirms that pesticide contamination is a determinant of eggshell thickness.

These results suggest that OCs may be a factor contributing to a decrease in the thickness of dickcissel eggshells. Similar findings have been found in a number of avian species classified as top predators. For instance, a study on loggerhead shrikes determined after measuring 104 eggshell samples that the only variability within pre-World War II eggs (i.e., unexposed to DDT and other organochlorines) was eggshell thickness (Anderson and Duzan, 1978). Even though the eggshell thickness declined only 2.6%, the standard deviation was so low that this was a statistically significant change in thickness that was hypothesized to be caused by DDT. Another study reputed that woodcock eggs collected in 1971 were 2.9% thinner than the mean thickness of eggshells collected prior to 1925, a statistically significant finding (Dilworth et al. 1972). In addition, a study on bald eagle eggshell fragments in the southwest (Grubb et al. 1990) found that eggshell thickness decreased by 8.8% in comparison to pre-World War II eggs, also a significant decrease.

As DDT and metabolites are the only OC pesticides in which eggshell thinning has been confirmed via mechanism of action, a t test was used to see if there was any relationship between the presence of DDT and metabolites and eggshell thickness (Bitman et al. 1970; Miller and Peakall, 1976; Table 5).

Table 5: Results of t tests comparing DDT and metabolite contamination to eggshell thickness

Mean and standard deviation of thickness of samples (mm)					
DDT/metabolites	Other pesticides	Non-contaminated	t	t critical	p
0.119 ± 0.020	0.121 ± 0.021		0.291	1.99	0.772
0.119 ± 0.020	---	0.126 ± 0.020	1.13	2.00	0.264
0.119 ± 0.020	0.110 ± 0.022	---	1.27	2.04	0.212

These tests reveal no significant relationship between DDT and metabolites and eggshell thickness in dickcissels. However, our sample size most likely too small to analyze this variable fully.

However, according to Pocker et al. (1971), the method of action that pesticides reduce carbonic anhydrase activity is merely occlusion. This means that a presence of any amount of pesticides crystallizing out of solution will block activity of carbonic anhydrase and also block calcium transfer into the eggshell.

In addition, falcons and other raptors have also experienced significant eggshell thickness decreases: DDE levels were significantly negatively correlated with eggshell thickness of peregrine falcons in several locations in Alaska. After environmental exposure to OC pesticides, Alaskan tundra peregrines had a shell thickness reduced by 21.7%, taiga peregrines had a shell thickness reduced by 16.8%, and Aleutian peregrines had an eggshell thickness reduction of 7.5%. This amounted to DDE concentrations of between 22.5 and 673 ppm, as well decreased fledgling viability (Cade et al. 1971). Ratcliffe also published a review on increases of egg breakage frequency, decreases in eggshell thickness, and diminishing breeding success in peregrines and sparrowhawks (1970).

However, the range of eggshell thicknesses is wide across species, and thus other studies remain unhelpful as species variation in eggshell thickness of dickcissels has not yet been measured. However, in a study of eggshell thickness in cowbirds and other passerines (not including dickcissels) by Spaw and Rohwer (1987), the standard deviation for eggshell thickness ranged from 0.001 mm (in hooded orioles, *Icterus cucullatus trochiloides*) to 0.022 mm (in *Psarocolius montezuma*). However, the mean standard deviation among 33 passerine species from over 300 egg samples was 0.008 mm. Therefore, according to this study (and making the assumption that dickcissels have similar eggshell thickness variance) the eggs we collected experienced a broadened range of eggshell thicknesses that could be consistent with persistent pesticide exposure in some individuals.

A decrease in eggshell thickness of dickcissels may also have ecological implications. Eggshells may be more likely to be damaged accidentally by their mother during brooding, but also damaged in the act of attempting to remove brown-headed cowbird eggs from their nests. Although dickcissels are commonly known as "acceptors" of brown-headed cowbird eggs, approximately 11% eject the eggs from their nest (Peer et al. 2000). Egg rejection occurs when eggs are knocked out of the nest, punctured, buried, or otherwise rendered when the embryo is unable to survive (Peer et al. 2000). Although dickcissels have a smaller bill size, the most common method of ejection for dickcissels is pecking a hole in a brown-headed cowbird egg. Therefore, if dickcissel eggshells are indeed thinner due to pesticide contamination, this method of ejection could result in damage to their own eggs (their bill could glance off the cowbird egg puncture their own egg). Thus, because their own eggs are being destroyed when they employ their only "known" rejection method, rejecting brown-headed cowbird eggs could be more

expensive than actually raising these brown-headed cowbird chicks to adulthood, even with the added energy costs of raising extra chicks (brown-headed cowbirds are larger than dickcissels and thus their fledglings require more food and out-compete dickcissel chicks).

Eggshell thickness is known to be affected by many other factors, including a lack of essential nutrients (manganese, vitamin D and calcium), and shortage of food or a specific type of food usually used to glean these particular nutrients could prove detrimental to eggshell thickness (Sturkie, 1965). Another valid concern is the soil pH's effect on calcium acquisition, a variable condition caused by acid rain deposition. However, according to the 2006 study from National Atmospheric Deposition Program and National Trends Network, pH in Illinois and the central grasslands ranges from 4.8 to 6.1, ranges generally not known for their extreme inability to provide nutrients (in comparison to the pH values from 4.3 to 4.5 in the Appalachian states and the eastern coast of the United States; see Hames et al. 2002). In addition, it has been shown that heavy metals, especially Strontium-90, decrease eggshell thickness in passerines (Mora, 2003).

Eggshell Color

Forty-six eggs were tested for both OC pesticide contamination and eggshell color. There was no significant relationship between total OC levels and eggshell hue or saturation (Table 6). However, there was a significant negative relationship between total OC levels and eggshell brightness as determined by linear regression (Figure 4). A follow-up t-test indicated that the mean eggshell brightness in eggs with no OCs present was significantly greater than the brightness of eggshells with OCs present. These results suggest the OCs may influence the relative lightness or darkness of the color of the dickcissel eggshell.

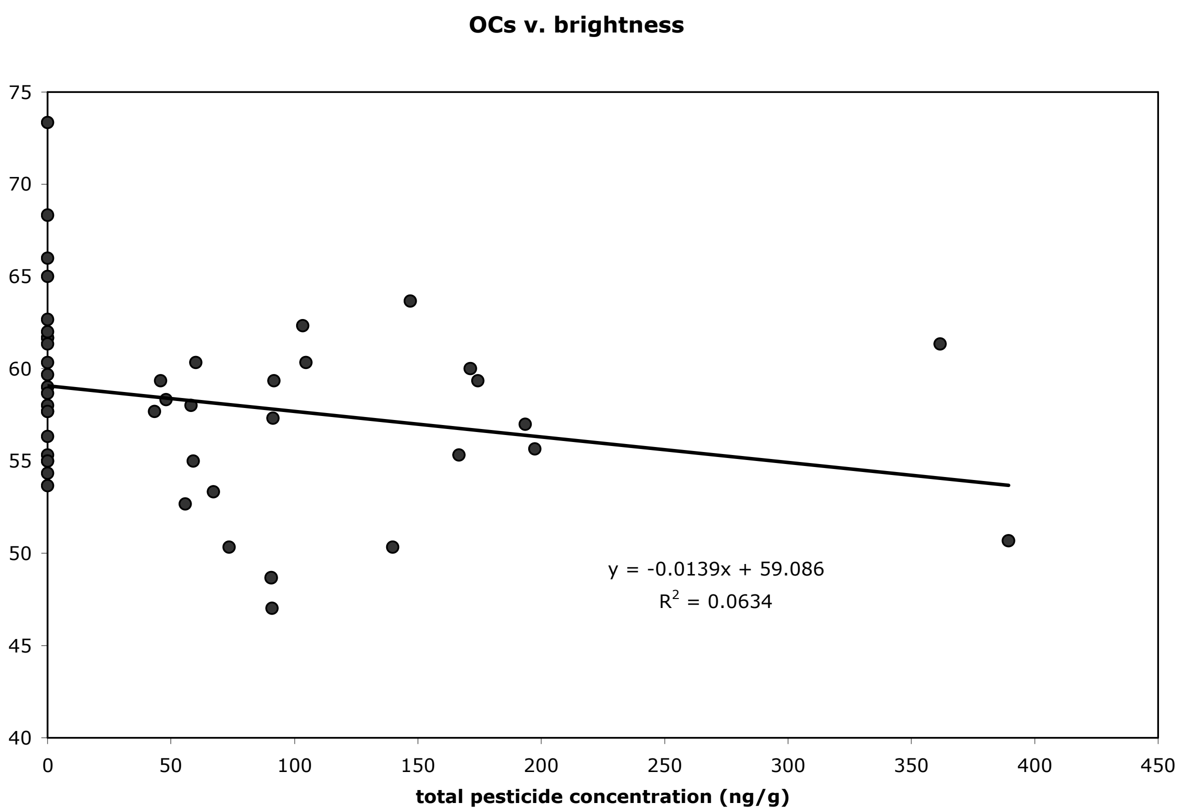

Figure 4: Linear regression comparing eggshell brightness and total OC pesticide contamination; non-contaminated eggshells have a range of brightness values from 53.67 to 73.33, while contaminated eggshells have a range of brightness values from 47.00 to 63.67.

Table 6: t test and p values of the comparison between contaminated and non-contaminated samples. Brightness has both a significant t value and p value (starred)

	Mean and standard deviation of color variable in **contaminated** samples	Mean and standard deviation of color variable in **non-contaminated** samples	t	t-critical (95% c.l.)	p
Hue	55.5 ± 2.7	54.7 ± 2.4	0.99	2.02	0.33
Saturation	75.7 ± 5.6	74.5 ± 5.6	0.82	2.00	0.42
Brightness	56.4 ± 4.5	60.1 ± 4.9	2.68*	2.02	0.01*

It is unknown exactly how biliverdin concentration manifests itself within the eggshell (e.g., how to quantify its contribution to hue, saturation, or brightness). The hue of an eggshell often varies among females (Siefferman et al. 2005). Unfortunately, the photochemical characteristics of biliverdin seem to be unknown, as is the exact mechanism for incorporation within the shell gland (e.g., how evenly biliverdin is spread throughout the entire egg, the proximity of biliverdin molecules to pores, and possible changes in biliverdin concentration for adjacent eggs depending on timing of laying). However, it is known that progesterone acts as an oxidant within female birds during laying and therefore reduces antioxidant concentration, but predicting fluctuation of progesterone levels in genetically different females has not yet been attempted (von Schantz et al. 1999). Thus, isolating details how an individual female's eggshell thickness is effected by her progesterone levels should be investigated.

Even though a complete understanding of biliverdin has not yet been reached, this significant negative relationship between total OC levels and eggshell brightness could have implications on male parental investment. The presence of more biliverdin in eggshells could act as a visual signal of female quality, and male dickcissels may be using this trait of eggshell color to determine, in the case of polygyny, where their efforts will result in the healthiest chicks with the

greatest chance of passing on their genes (Zimmerman, 1966). It is important to note that not all male dickcissels have more than one mate, and therefore, even if signaling is to take place in these dickcissels with only one brood to choose from, this may result in varying degrees of parental care. This could explain the confusion on the exact nature of parental care of dickcissels referenced in Zimmerman (1983), Gross (1921), Long et al. (1965) and Rivers et al. (2003).

Additionally, a recent study by Jagannath et al. (2008) assessed that eggshell pigmentation is an indicator of pesticide contamination in sparrowhawks (primarily DDT and its metabolites). However, the eggs of sparrowhawks they studied also contained eggshell spotting with protoporphyrin (a pigment present both on the egg and within the egg), and DDE was negatively correlated with eggshell thickness in only unspotted eggs. Thus, their results show that pigmentation reflects the level of DDE contamination in sparrowhawk eggs, yet it is independent of eggshell thickness.

Thickness vs. Brightness

We hypothesized that higher contamination leads to thinner and dimmer eggs, so we would expect a positive correlation between thickness and brightness (e.g., non-contaminated eggs would be thicker and brighter; contaminated eggs would be thinner and dimmer). However, a comparison of both these variables yielded a negative correlation (Figure 5). It is interesting to note that in comparing the two sets, the samples do follow this general hypothesis, yet within each set, there is actually a negative relationship between thickness and brightness. At this point, this relationship remains unexplained and deserves further thought.

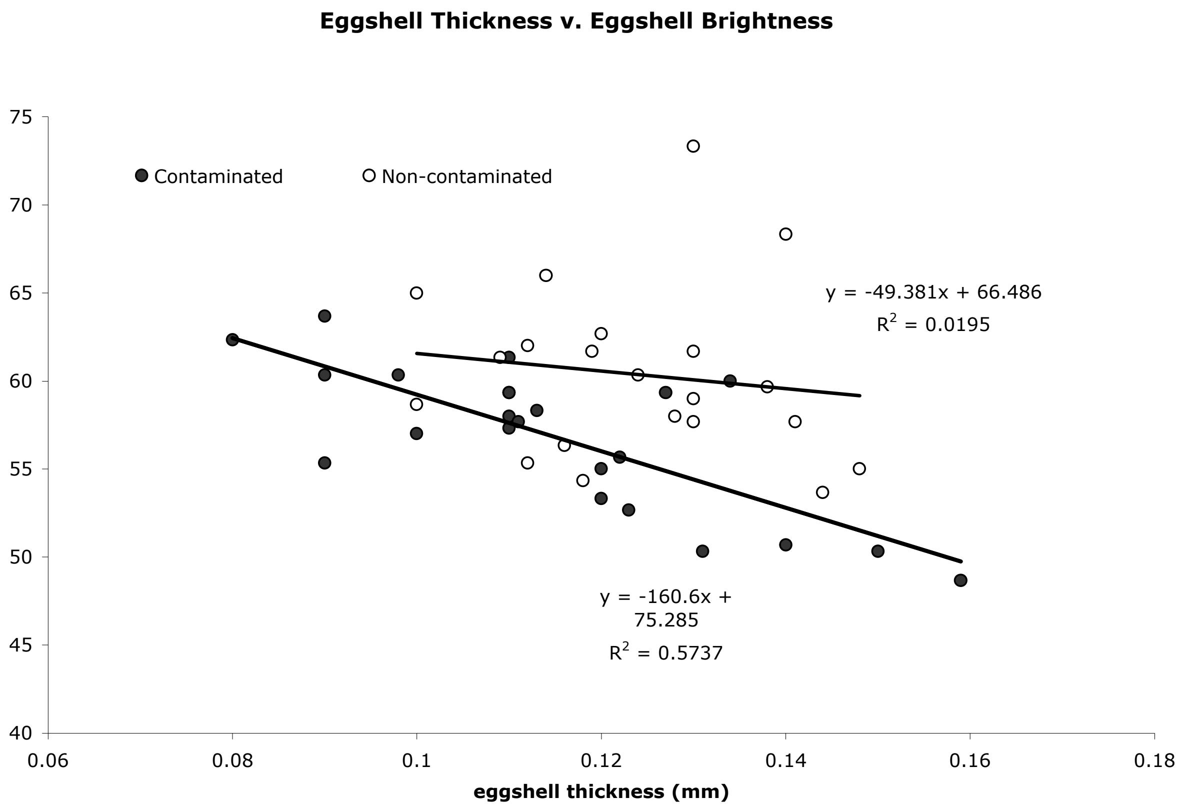

Figure 5: Comparison of eggshell thickness and eggshell brightness.

Conservation Implications:

Dickcissels are migratory songbirds that have seen a dramatic population decline in the past 40 years, and as many scientists have asserted, biodiversity loss can have a profound impact on many other unrelated species as well (Naeem 2002; Lugo 1995). Loss of habitat and pesticide contamination are linked to agricultural practices in South and Central America, and more land is continuing to become contaminated as increased pesticide use in fields spreads to non-agriculturally used lands (LeNoir et al. 1999).

Latin American countries have drastically expanded their production and sale of cash crops for export to support their economy, supplementing traditional crops with non-traditional foods such as broccoli, snow peas, and cantaloupes (Stutchbury, 2007). However, these non-native species quickly developed pest problems, leading farmers to pesticides, which they spray as a pre-emptive measure to ensure that these valuable crops are not in danger of being destroyed by pests. To minimize labor costs, farmers spray pesticide cocktails instead of following the advice from pesticide companies and spraying one at a time to avoid dangerously toxic chemical reactions. Ironically, many of these cash crops cannot be sold in the United States because they fail randomized tests which measure pesticide residues. However, they are still sold in many other countries which do not have as stringent of regulations as the United States imposes on food for human consumption. In addition, pesticides are a huge investment for a farmer, so they are usually stored inside a farmer's house to prevent robbery of such precious compounds (Stutchbury, 2007), increasing the now rampant human cases of pesticide poisoning in Latin America.

In effect, the decrease in the dickcissel population can be tied to a malfunctioning economic and social justice-related issue, wherein the solution lies going far beyond the current agricultural techniques employed by many South and Central American countries.

Possible solutions to alleviate the burden of pesticide contamination on dickcissels and other organisms include subsidies for not using chemical methods, or encouraging farmers to set aside land specifically for dickcissels and other native species (Lugo 1995). These efforts could be joint partnerships between groups such as the World Wildlife Fund, or The Nature Conservancy, as well as with local and national governments. In addition, changing the harvest time to just one week later could substantially decrease the amount of crop loss: if crops are not ripe in time for the dickcissel's pre-migratory period of hyperphagy, dickcissels would have to find nutrition elsewhere, mostly in what little native habitat is left (Basili and Temple, 1999).

Consumer-based solutions include buying shade-grown coffee, a crop which increases native habitat for not only dickcissels, but also for other tropical birds and organisms. In addition, due to the higher consumer cost of this coffee, these cooperatives often provide substantial economic incentives for farmers over slash and burn agriculture and pesticide usage. Buying organic products from Latin America will also reduce pesticide usage, and if there is enough demand for organic products, farming methods will have to be organic in order for farmers to make the highest profit. Decreasing overall pesticide usage is beneficial for not only birds, but for the safety and health of both farmers and the consumers in North America (Stutchbury, 2007).

However, no one solution in isolation will fix the problem of biodiversity loss: solutions must be used in tandem, with farmers, economists, politicians, and consumers working together to encourage more sustainable farming techniques in order to positively impact species diversity.

Future Studies:

Future studies should include comparing our results with dickcissel eggshells in museums that were collected before OC compounds were used in the U.S. In addition, the presence of heavy metals (e.g., lead, cadmium, and mercury) will be tested to determine if there is any relationship between these compounds and the thickness and color of dickcissel eggshells. In order to better understand the signaling function of dickcissel eggshell color, the use of a spectrophotometer to determine UV signatures would be beneficial. Also, it would be prudent to test dickcissel eggs for internalized protoporphyrin spotting and see if this is also causing changes in eggshell thickness. To gauge male response to eggshell brightness and confirm the correlation between brightness and male parental care, fieldwork to measure egg color and corresponding male care would be pertinent. In addition, further observational field studies to better measure dickcissel male parental care without researcher interference would be prudent. To further elucidate the physiological mechanisms of female egg reproduction, a multi-generational study of dickcissels either affected or not affected by pesticides would allow for a more comprehensive and controlled environment in which to probe both eggshell thickness and color.

Acknowledgements:

Much thanks to:

-My thesis committee here at Illinois Wesleyan University: Dr. Given Harper, Dr. Stephen Hoffmann, Dr. Will Jaeckle, Dr. Jeff Frick, and Dr. Stephen Press. Your time and effort with this project was much appreciated.

-My research partners through the years as well as the many IWU students who have contributed to this project since its inception (Jason Koval, Stephanie Ross, Toritseju Eshedagho, Matt Anderson, Emma Wear, Brandy Blackwell, Jill Raabe)

-The biology program and professors here at IWU: I don't know how I could ever thank you for your probing questions, excellent lectures, caring encouragement, and presence in all of my classes. Thank you.

-Those who have encouraged my love of science from the very beginning: from my mom to my grandfather to Marie Curie to Ms. Bizarro to Ms. Mielke to Alexandria Sams to Linda Griffith…you all pushed me to be my very best and always ask questions of the world around me.

-Dr. Brian Peer of Western Illinois University for his help with field work collecting dickcissel eggs.

-Dr. Stephanie Doucet of the University of Windsor (Canada) for her assistance in developing color protocol.

-the National Science Foundation for helping to purchase the gas chromatograph (grant BIR-9601523)

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