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Potential effects of environmental contaminants on P450 aromatase activity and DNA damage in swallows from the Rio Grande and Somerville, Texas

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Abstract Cliff swallows (*Petrochelidon pyrrhonota*) and cave swallows (*P. fulva*) were sampled during the breeding season at several locations in the Rio Grande, Texas, to evaluate the potential effects of environmental contaminants on P450 aromatase activity in brain and gonads and DNA damage in blood cells. The tritiated water-release aromatase assay was used to measure aromatase activity and flow cytometry was used to measure DNA damage in nucleated blood cells. There were no significant differences in brain and gonadal aromatase activities or in estimates of DNA damage (HPCV values) among cave swallow colonies from the Lower Rio Grande Valley (LRGV) and Somerville. However, both brain and gonadal aromatase activities were significantly higher ($P < 0.05$) in male cliff swallows from Laredo than in those from Somerville. Also, DNA damage estimates were significantly higher ($P < 0.05$) in cliff swallows (males and females combined) from Laredo than in those from Somerville. Contaminants of current high use in the LRGV, such as atrazine, and some of the highly persistent organochlorines, such as toxaphene and DDE, could be potentially associated with modulation of aromatase activity in avian tissues. Previous studies have indicated possible DNA damage in cliff swallows. We did not observe any differences in aromatase activity or DNA damage in cave swallows that could be associated with contaminant exposure. Also, the differences in aromatase activity and DNA damage between male cliff swallows from Laredo and Somerville could not be explained by contaminants measured at each site in previous studies. Our study provides baseline information on brain and gonadal aromatase activity in swallows that could be useful in future studies.

Keywords Contaminants · DNA damage · Flow cytometry · Lower Rio Grande · Swallows · P450 aromatase

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Introduction

Population growth and economic expansion due to industry and agriculture have impacted natural resources and the quality of life in various regions of the Rio Grande (TNRCC 2002). The maquiladoras (assembly plants) established along the U.S.-Mexico border have often been reported of improperly disposing of hazardous waste, corrosive materials, organic solvents, acids, and metals (Varady et al. 2001). The Lower Rio Grande Valley (LRGV) comprises the counties of Starr, Hidalgo, Willacy and Cameron in south Texas, and is an important

agricultural region with citrus, sugarcane, cotton, corn, and sorghum crops (TNRCC 2002). Approximately 5.9 million pounds of insecticides and 8.7 million pounds of herbicides were applied to upland cotton crops in Texas in 2005 (USDA 2006). Pesticide runoff, untreated sewage, and industrial effluents commonly enter into agricultural irrigation reservoirs and tributaries of the Rio Grande (Mora and Wainwright 1998). Previous chemical analyses of carcasses of cliff swallows (*Petrochelidon pyrrhonota*) and cave swallows (*Petrochelidon fulva*) from the LRGV suggested that some persistent organochlorine and metal contaminants were still elevated and could potentially affect wildlife (Mora et al. 2005, 2006).

Wildlife species can be used as sentinels of contaminant exposure by analyzing physiological and biochemical changes to the organism. The endocrine system and DNA are two targets that can be damaged or altered by contaminant insult. Genetic material in both somatic and germ cells are two areas where environmental contaminants can produce mutagenesis. Germ line mutagenesis can lead to birth defects and transgenerational effects and can affect fitness in wildlife populations (Bickham et al. 2000). Irreversible genetic insult at the somatic cell level may develop into cytogenic problems, such as neoplasms, cancer, and/or genotoxic disease (Shugart et al. 1992). Exposure to environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) from petroleum sources and various industrial pollutants may facilitate clastogenic activity (chromosomal breakage) in the DNA molecule (Bickham et al. 1998; Matson et al. 2005). Flow cytometry is a technique used to measure clastogenesis and the subsequent unequal distribution of broken and rearranged chromosomes in daughter cells (Bickham 1990). Some known genotoxic chemicals, such as, chromium, mercury, and PAHs, have been detected in fish and wildlife in the Rio Grande (Mora and Wainwright 1998; IBWC 1998).

Endocrine-disrupting chemicals that mimic or antagonize endogenous hormones can cause detrimental physiological effects to an organism other than acute death (Colborn et al. 1993). Some endocrine disruptors inhibit and/or induce sex steroid biosynthesis and have been linked to reproductive abnormalities in wildlife (Guillette et al. 1994; Jobling et al. 1998; Reeder et al. 2005). Estrogen is a sex steroid that plays an essential role in the structural development and physiological function in all vertebrates. The P450 aromatase enzyme system is responsible for the conversion of C19 androgen steroids into phenol A ring estrogens (Simpson et al. 1994). The amino acid sequence of aromatase is encoded on the CYP19 gene and is highly conserved within the Phylum Chordata (Conley and Hinshelwood 2001) with cDNA derived avian amino acid sequence homologies being approximately 70% identical to human CYP19 (McPhaul et al. 1988). Aromatase expression is detected most frequently in gonads and brains of higher vertebrates (Conley and Hinshelwood 2001; Simpson et al. 2002) and is essential for differentiation and development of these organs, maintenance of reproductive tissues, and sexual behavior. Genetically female chicken embryos require estrogen to differentiate into phenotypic females (Bruggeman et al. 2002). Eggs treated in ovo with an aromatase inhibitor exhibit phenotypic sex-reversal, such that genetic females develop male testes that produce sperm (Elbrecht and Smith 1992). Estrogen is critical for maintenance of reproduction in birds by sustaining small prehierarchal follicles prior to fertilization, producing yolk and albumen, and forming the eggshell (Armstrong 1984; Nitta et al. 1991). Estrogen is also important for physiological processes in the male gonad and brain (Lephart 1996; O'Donnell et al. 2001). Aromatase activity has been positively correlated with increased aggressiveness in Japanese male quail, *Coturnix coturnix japonica* (Schlinger and Callard 1989) and depression of brain aromatase activity in females could affect breeding behaviors such as receptiveness for mating and parental care (Zala and Penn 2004).

The objective of this study was to evaluate the variation in DNA content of blood cells by flow cytometry and changes in P450 aromatase activity in gonads and brains of cliff and cave swallows as potential indicators of exposure to environmental pollution in the Rio Grande. Cliff and cave swallows can be exposed to a variety of contaminants through their diet and directly from sediment when acquiring mud for nest construction. Cliff and cave swallows are widespread and abundant in the Rio Grande and do not forage far from their nests (Brown and Brown 1995; West 1995); thus, both species were considered potential good indicators of exposure and effects of environmental contaminants on nesting insectivorous birds of the Rio Grande. Although the potential for alterations in P450 aromatase activity by environmental contaminants has been proposed (Ankley et al. 1998; Sanderson and van den Berg 2003), to our knowledge, there are no studies that document contaminant-aromatase activity interactions in wild birds.

Materials and methods

Sample collection

We collected adult cave swallows from five sites in the LRGV (Mission, McAllen, Pharr, Llano Grande, and Brownsville) during the breeding season in 2003 and in Somerville, Texas (approximately 500 km north of the LRGV) during 2003 and 2004 (Table 1). Also, we collected adult cliff swallows from Laredo, Texas (about 200 km northwest of the LRGV) in 2003 and Somerville in 2004 (Table 1). Somerville was

Table 1 Location of cave and cliff swallows collected in 2003 and 2004 near the Rio Grande and a reference site, Somerville, Texas

Species	Site	Males (*n*)	Females(*n*)	Coordinates	Description
Cave swallow	Mission	8	2	26°11.389′ N 98°19.825′ W	Culvert <1.0 mile south of Mission near WWTP[a]
	McAllen	5	5	26°11.180′ N 98°14.830′ W	McAllen main canal near Boeye reservoir and airport
	Pharr	1	9	26°09.846′ N 98°10.351′ W	Bridge by Pharr/San Juan WWTP; golf course to the southwest
	Llano Grande	5	5	26°07.207′ N 97°57.670′ W	Bridge on FM 1015 over Llano Grande Lake
	Brownsville	5	4	25°57.284′ N 97°27.307′ W	Bridge adjacent Robindale WWTP in Brownsville
	Somerville	6	5	30°24.994′ N 96°32.527′ W	Pastureland, bridge on FM 60 over Davidson Creek
Cliff swallow	Laredo	6	4	27°34.488′ N 99°30.336′ W	Bridge over Manadas Creek, industrial corridor
	Somerville	5	4	30°24.994′ N 96°32.527′ W	Same location as previously described

[a] WWTP is wastewater treatment plant

selected as a reference site because it had been used before as a reference site (Mora et al. 2006) and it was quite distant from Laredo and the LRGV. Adult birds were captured with mist nets deployed over bridges or culverts for 10 to 15 min. The birds were removed from the mist nets and confined in nylon hosiery for 10 to 20 min until a small volume of blood (<1 ml) was collected from the jugular vein with a sterile needle. Approximately 5 drops of blood were placed into cryogenic vials containing freezing medium (10% glycerol, 10% fetal bovine serum, and 1% penicillin/streptomycin in RPMI Medium 1640) and stored in liquid nitrogen for flow cytometry. After blood collection, swallows were euthanized by cervical dislocation and weighed to the nearest 0.1 g. The ovaries or testes were excised in the field with acetone cleaned scissors and tweezers, quickly transferred to cryogenic vials and stored in liquid nitrogen for aromatase analysis. Carcasses were wrapped in aluminum foil, placed in a marked plastic storage bag, and stored on dry ice. In the laboratory, the brain was quickly removed from each semi-frozen carcass, placed in aluminum foil, and immediately returned to the −80°C freezer until analyzed. All tissues were kept frozen at −80°C until used for various analyses. Swallows were collected and handled under approved protocol (AUP# 2003-99) by Texas A&M University Laboratory Animal Care Committee and according to guidelines to the use of wild birds in research (Gaunt and Oring 1997).

Flow cytometry

For the flow cytometry, random blood samples for each species were prepared and processed according to established techniques (Vindelov and Christiansen 1994). A reference domestic chicken *(Gallus domesticus*) erythrocyte sample with known DNA content was processed concurrently with each run as a control to assess potential instrument or procedural inaccuracies. Thawed blood samples (50 µl) contained in the freezing media were added to a trypsin/citrate buffer solution and gently homogenized with a Teflon dounce. After 10 min, trypsin inhibitor with ribonuclease was added to each sample to terminate the reaction and then left undisturbed for 10 min. The sample solution was filtered through 30 µm nylon mesh into a polypropylene tube followed by the addition of propidium iodide to stain nuclear suspensions. Nuclei were stained for 15 min and then analyzed using a Beckman Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, California) that measures the fluorescence of stained nuclei reflecting relative DNA content. From each sample, the DNA content of 10,000 nuclei from the G1, or resting phase of the cell cycle, was measured and presented as a normally distributed histogram with a corresponding coefficient of variation [CV = 100 × standard deviation/mean of the histogram peak]. The breadth of the distribution of peaks indicates CVs and increasing breadth indicates a wider range of DNA content. Half-peak coefficients of variation (HPCV) values were used for statistical analysis.

P450 aromatase activity

We used the *tritiated water-release* aromatase assay, which measures aromatase activity as a function of the quantity of $^{3}H_2O$ (tritiated water) released as an indirect estimate of

estrogen production (Lephart and Simpson 1991). Incubation of radiolabeled [1β-^{3}H] androstenedione substrate with aromatizing tissues creates a stereospecific loss of 1β-^{3}H (hydrogens) and production of a radioactively-unlabeled estrogen molecule. The radiolabeled hydrogens subsequently form ^3H_2O molecules, which are counted with a scintillation counter then calculated to an estrogen conversion rate, or more specifically, an aromatase activity.

Briefly, samples, buffers, and cofactors were kept on ice at 4°C while processing. Brain and ovary samples were considered as a whole and testes samples were processed as left and right testes. We randomly selected 6 to 10 samples of the same tissue type to run in a batch. Tissue weights were measured (0.1 g) using an analytical balance. Tissue samples and the positive control (a sample of chicken ovary of similar weight) were homogenized in 445 µl of the appropriate buffer: brain buffer-10 mM potassium phosphate (KPO_4), 100 mM potassium chloride (KCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM glucose-6-phosphate (G-6-P), and 10 mM dithiothreitol or gonad buffer-50 mM KPO_4, 1 mM EDTA, and 10 mM G-6-P adjusted to pH 7.4–7.6. Homogenates were incubated with cofactors of 1 mM NADP and 0.5 units/500 µl final reaction volume G-6-P dehydrogenase and 100 nM [1β-^{3}H] androstenedione substrate (specific activity 25.3 Ci/mmol; Perkin Elmer Sciences, Inc., Boston, MA) for brain homogenates or 50 nM of substrate for gonad samples. A blank sample consisted of 2 mg/ml bovine serum albumin in buffer, cofactors, and 50 nM or 100 nM substrate, depending on the tissue type incubated. All extraction efficiencies (1000 × unreacted tritiated water in buffer for a 500 µl final volume; specific activity 1.0 mCi/g; Sigma Co, St. Louis, MO) for intra-assay validation were calculated at greater than 98%. Incubation conditions were 15 min for brain and 3 h for gonads in 95% O_2:5% CO_2 at 37°C and were placed on ice for 5 min to stop enzymatic reactions. After controls and samples were centrifuged at 21,000 *g* for 2 min, supernatant (200 µl) was transferred to eppendorf tubes with the remainder supernatant reserved for protein determination (Bradford 1976). Chloroform ($CHCl_3$; 500 µl) was added to the extract and centrifuged at 21,000 *g* for 2 min, then $CHCl_3$ was removed, and all were centrifuged again. The supernatant (100 µl) was mixed with 100 µl of dextran-coated charcoal (DCC; 0.5%:5% w/w) and centrifuged again at 21,000 *g* for 15 min to form a DCC pellet. Supernatant (125 µl) was added to polyethylene scintillation vials (Beckman Coulter, Fullerton, CA) prepared with 4 ml of Scintiverse® scintillation cocktail (Fisher Scientific, Pittsburgh, PA) and analyzed using a Model LS 6500 Multipurpose Scintillation counter (Beckman Coulter, Fullerton, CA). After subtracting the disintegrations per minute (DPM) detected in blanks, each sample was multiplied by a dilution factor of 8. Aromatase activity was expressed in femtomoles of androstenedione converted per hour per milligram protein (fmole/h/mg protein).

Statistical analysis

Flow cytometry and P450 aromatase data were analyzed for normality by the Shapiro-Wilk's test and evaluated for homogeneity of variances using the Levene's test. The HPCV data were normally distributed, the aromatase data were not. Two sample comparison procedures (Mann-Whitney and *t*-test) were used to compare differences in HPCV and P-450 activities between sexes within locations, and between locations (cliff swallows). The GLM procedure with ranked data (similar to a non-parametric analysis of variance) was used to determine differences in brain and gonad aromatase activities between seasons and among locations for cave and cliff swallows. All statistical analyses were performed with SAS® Software (SAS 9.1 for Windows, Cary, NC, USA). Significance levels were established at $P \leq 0.05$.

Results

Gonadal and brain aromatase activity

There were significant differences between sexes in gonadal but not in brain aromatase activity of adult cave swallows ($F = 74.7$, $P < 0.0001$); these differences were affected by location ($F = 2.4$, $P = 0.049$, Table 2). Gonadal aromatase activity was significantly higher in adult female than in adult male cave swallows from most locations ($P < 0.001$). However, brain and gonadal aromatase activities were not significantly different among locations for male or female cave swallows ($P > 0.05$).

In cliff swallows, there were significant differences between sexes ($F = 75.2$, $P < 0.0001$) and locations ($F = 16.5$, $P = 0.001$) in gonad aromatase activity (Table 2). Within locations, gonadal aromatase activity was significantly higher in female than in male cliff swallows ($P < 0.001$), but there were no differences in brain aromatase activity between sexes. Between locations, there were no significant differences in brain or gonadal activites of females; but gonadal and brain activites of males were significantly higher in Laredo than in Somerville ($F = 11.6$, $P = 0.0078$ and $F = 4.9$, $P = 0.05$, respectively).

Flow cytometry

Within locations, there were no significant differences in HPCV values for DNA content between male and female

Table 2 Median values and range for brain and gonad aromatase activity (fmole/hr/mg protein) in cave and cliff swallows collected in 2003 and 2004 from the Rio Grande and a reference location, Somerville, Texas[a]

Species	Site	Brain		Gonad	
		Adult males	Adult females	Adult males	Adult females
Cave swallow	Mission	10.9 A (6.7–52.0)	10.7 A (4.7–16.7)	0.7 A (0.5–1.9)	26.5 A (23.9–29.0)
	McAllen	14.3 A (6.6–35.7)	15.0 A (10.9–22.1)	0.8 A (0.4–1.2)	37.3 A (6.5–49.2)
	Pharr	16.4 A	16.1 A (9.1–27.9)	1.0 A	21.6 A (10.1–112.5)
	Llano Grande	13.8 A (13.5–25.9)	8.1 A (2.9–13.9)	1.0 A (0.6–1.3)	26.5 A (14.9–80.4)
	Brownsville	10.1 A (5.1–20.8)	13.0 A (9.4–15.3)	1.7 A (0.7–1.9)	9.4 A (5.5–23.3)
	Somerville	8.1 A (5.0–16.0)	16.5 A (13.6–20.5)	0.4 A (0.3–0.9)	10.5 A (3.0–17.8)
Cliff swallow	Laredo	16.1 A (8.1–24.6)	11.0 A (9.1–13.8)	1.2 A (0.6–2.6)	38.2 A (17.3–70.0)
	Somerville	8.3 B (4.5–17.8)	9.0 A (2.9–13.7)	0.4 B (0.2–0.7)	11.7 A (5.2–23.0)

[a] Number analyzed is the same as in Table 1. Within columns, median values not sharing the same letter are significantly different

Table 3 Half peak coefficient of variation (HPCV, means ± SD) of DNA content in blood cells of adult cave swallows (*Petrochelidon fulva*) and cliff swallows (*Petrochelidon pyrrhonota*) collected in 2003 and 2004 from the Rio Grande and a reference location, Somerville, Texas

Species	Site	*n*	HPCV (Mean ± SD)
Cave swallow	Mission	10	3.40 ± 0.45 A
	McAllen	10	3.22 ± 0.40 A
	Pharr	10	3.11 ± 0.29 A
	Llano Grande	10	3.29 ± 0.26 A
	Brownsville	9	3.00 ± 0.43 A
	Somerville	11	3.31 ± 0.48 A
Cliff swallow	Laredo	10	3.34 ± 0.35 A
	Somerville	9	2.85 ± 1.00 B

Means not sharing the same letter are significantly different

Increasing HPCV values are representative of increased DNA damage

swallows in the Rio Grande Valley or in the Somerville reference site ($P > 0.05$). Among locations, there were no significant differences in HPCV values for adult cave swallows (Table 3, $P > 0.05$). However, HPCV values for adult cliff swallows from Laredo were greater than those for cliff swallows from Somerville (Table 3, $P = 0.018$).

Discussion

The lack of differences in brain and gonadal aromatase activities in female and male cave swallows among locations in the Rio Grande and the reference site in Somerville suggest that there may not be an association with environmental contaminants or that the contamination is similar among all sites studied. However, male cliff swallows from Laredo had higher levels of brain and gonadal aromatase activity and HPCV values than those from Somerville. Whether such differences between these two sites are associated with contaminants cannot be established by using data from previous studies. During 1999–2000, concentrations of DDE and inorganic elements were significantly greater in swallows from El Paso than from other locations of the Rio Grande, except for Pharr and Llano Grande (Mora et al. 2006). Notwithstanding, concentrations of OCs and inorganic elements were not significantly different between cave swallows collected in Laredo in 1999 and cliff swallows collected in Somerville in 2000 (Mora et al. 2006). Cave swallows collected during 1999 near Laredo had lower HPCV values (mean = 2.2, $n = 20$, Mora et al. 2006) than cliff swallows from this study. However, the swallows collected during 1999 were from east of Laredo outside of the city whereas the cliff swallows collected in 2003 where from Manadas Creek, a region with heavy vehicle traffic and industrial activity within the city. Thus, location could help explain the differences between the two Laredo sites, although differences between species and collection years could not be excluded.

Several contaminants previously reported in wildlife in the LRGV are known as potential inducers of P450 aromatase activity in birds. Maruya et al. (2005) reported toxaphene residue levels of 5.9 ± 1.6 μg/g lipid weight in swallows from Llano Grande, 5.0 ± 1.6 μg/g in swallows from Pharr, and 2.7 ± 0.16 μg/g in those from Mission. Toxaphene has been reported to affect aromatase activity through possible antagonistic effects with the estrogen-related orphan receptor (ERR) α-1 (Yang and Chen 1999). Also toxaphene exposure (10 μM; about 4 ppm) decreased aromatase activity in JEG-3 cells likely through the down-regulation of aromatase expression (Laville et al. 2006). Mora et al. (2006) reported elevated concentrations of DDE in carcasses of swallows from Llano Grande (6.6 μg/g ww) and Pharr (7.4 μg/g ww). You et al. (2001) reported that p,p′-DDE (100 ng/ml) induced hepatic microsomal aromatase in male rats. DDE had a synergistic effect with a follicle stimulating hormone and induced aromatase activity

in human ovarian granulosa cells (Younglai et al. 2004). We assumed that concentrations of persistent OCs, particularly DDE, in swallows from our study were similar, or fairly close to those reported by Mora et al. (2006). Swallows are known to exhibit high fidelity and tend to return to the same nesting area from previous years. DDE is persistent in the environment and takes many years to eliminate (estimated half-life for DDT is 2–15 year in soil and approximately 150 year in aquatic environments; http://npic.orst.edu/factsheets/ddtgen.pdf, April 17, 2008). Other contaminants possibly associated with effects on aromatase activity include atrazine, chemicals present in sewage effluents, polybrominated diphenyl ether (PBDEs), and metals (Sanderson et al. 2001; Hayes et al. 2003, Huggett et al. 2003; Laville et al. 2006). The results from our study do not support the hypothesis that the possible elevated levels of toxaphene, DDE, or other contaminants in the LRGV are associated with increases in aromatase activity in swallows.

The HPCV results also indicate that there were no alterations of the DNA content in blood cells of adult cave swallows relative to those observed at the reference site in Somerville. However, HPCV values between cliff swallows from Laredo and those from Somerville were different, supporting the results from the aromatase activity. These differences also are supported by a previous study in which HPCV values were greater in cliff swallows from three Rio Grande locations than in those from Somerville (Mora et al. 2006). Mercury, chromium, and cadmium, have been associated with increased DNA-strand breaks (Tsuzuki et al. 1994; Bolognesi et al. 1999; Codina et al. 2000). Cliff swallows from Laredo seemed the most affected by an undetermined environmental factor as reflected by increased brain and gonadal aromatase activity in males and increased variation in DNA content of both males and females relative to the reference site. The Manadas Creek sampling site in Laredo was exposed to PBDEs released into the environment by a chemical industry located near the watershed (USEPA n.d.). There were also reported releases of antimony, arsenic, lead, and zinc into this watershed (USEPA n.d.). Laredo is the busiest inland port of entry for international commerce with nearly 8,000 idling commercial trucks (TNRCC 2002) contributing to tailpipe emissions of PAHs each day.

To our knowledge, this study is the first to address aromatase activity in brain and gonads of avian species in the Rio Grande region and their potential association with environmental contaminants. However, only the data from cliff swallows suggest that some contaminants potentially occurring in Laredo site are probably associated with increased aromatase activity and potential DNA damage. Male swallows had lower and less variable background levels of aromatase in their testes during the breeding season than females. We suggest that males may be a better model to detect increases in aromatase activity as females seem to exhibit considerable variation. More field studies are needed to further understand the potential effects of contaminants on avian aromatase activity.

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