P450 AROMATASE ALTERATIONS AND DNA DAMAGE AS AVIAN POLLUTION BIOMARKERS IN CLIFF AND CAVE SWALLOW BREEDING NEAR THE RIO GRANDE REGION, TEXAS

A Thesis

by

MEGAN ANNETTE SITZLAR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Toxicology

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Approved by:

Co-Chairs of Committee,	Miguel A. Mora
	John W. Bickham
Committee Members,	K. C. Donnelly
Chair of Toxicology Faculty,	Robert Burghardt

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ABSTRACT

P450 Aromatase Alterations and DNA Damage as Avian Pollution Biomarkers in Cliff and Cave Swallow Breeding Near the Rio Grande Region, Texas. (December 2005)

Megan Annette Sitzlar, B.S., Tennessee Technological University

Co-Chairs of Advisory Committee: Dr. Miguel A. Mora
Dr. John W. Bickham

The endocrine system, specifically relating to sex hormones, and genetic material can be targets of environmental contaminants. Environmental contaminants in the Rio Grande region may originate from industrial or agricultural processes and growing populations lacking proper water and sewage infrastructure. Cliff (*Petrochelidon pyrrhonota*) and cave (*P. fulva*) swallows breeding near the Rio Grande were selected to monitor aromatase activity alterations and DNA damage. Swallows were sampled at six sites along the Rio Grande from Brownsville to Laredo, and a reference site (Somerville) 350 miles north of the Rio Grande. DNA damage, based on nuclear DNA content, was determined by flow cytometry. A significantly larger mean half peak coefficient of variation (HPCV) of DNA content in contaminated sites compared to a reference site reflects possible chromosomal damage. No detectable HPCV differences were observed in cave swallows among locations, notwithstanding the presence of mutagenic contaminants. Selenium may provide a protective role against genetic damage. However, cliff swallows from Laredo had significantly higher HPCV values than those from Somerville. DNA damage could be attributed to metals and polycyclic aromatic

hydrocarbons released near Laredo. Brains and gonads, two estrogen-dependent organs, were tested for aromatase activity with a tritiated water method. Brain aromatase activity was higher, though not always statistically, for male cave and male and female cliff swallows. Dichlorodiphenyldichloroethylene (DDE) may play a role in the increased activity. Female cave swallows in Llano Grande appeared to have a greatly depressed brain aromatase activity, possibly attributed to past human use of toxaphene. Testicular and ovarian aromatase activity in cliff and cave swallows from Rio Grande was higher than in those from Somerville, though not always significantly. DDE, atrazine, sewage treatment plant contaminants (phthalates, alkylphenols, ethynylestradiol), metals, or other pollutants could play a role in the increased gonadal activity. Increased aromatase activity, in association with contaminants, may be easier to detect in testes of male birds which normally exhibit low levels of estrogen. Site-related contaminants may be playing a role in DNA damage and aromatase alterations. This is the first known study which uses aromatase activity as an endocrine disruptor indicator in wild birds.

DEDICATION

To my mother - the wisest oak tree that ever stood. She taught me to always stand strong, no matter how many axes swung my way. She instilled in me listening to Nature would educate me more than being the babbling brook itself.

ACKNOWLEDGMENTS

I couldn't have made this goal possible without help from numerous people. My committee members, Drs. Miguel Mora, John Bickham, and K.C. Donnelly, have been instrumental in allowing me to develop my own critical thinking skills and a sense of ownership of the project. Thanks to Miguel for teaching me new things in the field and critically reviewing my thesis so I could become a better writer. I also appreciate the help when my mother was ill. Thanks to Dr. Bickham for running the flow cytometer and providing a teaching assistantship which introduced me to several fine undergraduates; even a few of the students embodied my idea of a true Aggie.

I'd like to thank the USGS for providing the project grant. I am grateful to the U.S. Fish and Wildlife Service for giving us excellent accommodations at the Santa Ana Wildlife Refuge. I appreciate the field volunteers (Tim Fredericks, Jennifer Nelson, and Megan Russell) who helped with mistnetting and other field activities. My labwork could have NEVER been completed without the generosity of Dr. Fuller Bazer and the patience of Dr. Jo-Ann Fleming. Dr. Bazer is the recipient of numerous awards and holds various academic titles, yet continues to be a humble and personable man. There are not enough words to express how highly I regard Jo-Ann. I knew nothing about a true biology lab, not even how to use a pipette, but Jo-Ann always made sure I understood. She unselfishly took time out of her schedule to train and mentor me. I hope I run into such a meticulous and brilliant scientist who I can learn from again.

Thanks to my lab mates, Mike, Catherine, and Deborah for letting me complain. Special thanks to Deborah for giving tons of great advice and all the special pick-me-

ups. I am very fortunate to have such wonderful friends (Laura, Melinda, Misty, and Danielle) which were never but a phone call away, especially Sharon who always knew when to send fun money. Thanks to Ben for providing relentless support, love, and patience. Now, let's have some fun. Thanks to my family, especially Mom, Amber, Andrea, and Trent, for helping me develop the trait of determination and inner strength. Finally, thanks to God for letting me use His birds and giving me the outdoors for my lifelong playground.

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INTRODUCTION

The Rio Grande bisects the Texas-Mexico border region beginning in El Paso with a distance of 1931 kilometers until reaching its confluence with the Gulf of Mexico (Eaton and Andersen 1987). Human populations along the transboundary region of the Rio Grande concentrate near the seven major sister cities of the Texas-Mexico border from north to south: El Paso/Ciudad Juarez; Presidio/Ojinaga; Del Rio/Ciudad Acuna; Eagle Pass/Piedras Negras; Laredo/Nuevo Laredo; McAllen-Edinburg-Mission/Reynosa; and Brownsville-Harlingen-San Benito/Matamoros (TNRCC 2002). Projected growth from counties that contain these cities is expected to rise from 2000 census reports of 1.9 million to an estimated 3.1 million by 2025 (TSDC and OSD 2004). Concomitantly, population and economic expansion strains "natural resources, environment, and quality of life" in the Rio Grande region (TNRCC 2002).

Employment opportunities from manufacturing industries have promoted settlement of thousands of people, especially from Mexico, to the fertile economic region of the Rio Grande. The inception of the Border Industrialization Program by the Mexican government circa 1965 resulted in employment by *maquiladoras*. Maquiladoras, foreign-owned (most notably the U.S.) assembly plants, are advantageous to companies due to tax-exempt import of raw materials and low labor costs with high productivity (Reed et al. 2000). Maquiladora operations increased after approval of

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North American Free Trade Agreement (NAFTA) in 1994 (TNRCC 2002). In 2003, approximately 3,230 maquiladoras were reported with 1,260 of those located along the Mexican states bordering Texas (http://www.texasep.org/html/wst/wst_4imn_brdr.html). Approximately 603,000 people were employed by maquiladoras during 2002 (TNRCC 2004).

Maquiladoras have been associated with production of hazardous wastes that could result in illegal or improper disposal and may potentially affect human health and natural resources of the Rio Grande region (Varady et al. 2001). Hazardous waste production and use of corrosive materials, organic solvents, acids, and heavy metals is attributed to manufacture of electronics, textiles, and automotive parts at maquiladora plants (Varady et al. 2001). As stated by the La Paz Agreement between Mexico and the U.S. in 1983, production waste must be returned to the country that originally imported the raw materials for process. Only 20-30% of hazardous waste from production is estimated to be returned to the home country with the remainder being improperly stored or illegally disposed (World Bank 1993; TNRCC 2004). Moreover, only 12% of approximately 8 million tons of hazardous wastes produced annually in Mexico is sufficiently treated and disposed (TNRCC 2004).

The Rio Grande Valley economy is also derived from the agricultural industry. Various regions along the border, especially the counties of Starr, Hidalgo, Willacy, and Cameron, collectively termed Lower Rio Grande Valley Region (LRGV), produce citrus, sugarcane, cotton, corn, and sorghum crops (TNRCC 2002). Hidalgo County accounts for 80% of the entire citrus production in Texas with Cameron and Willacy

contributing 15% and 5%, respectively (Sauls n.d.). The Texas Agricultural Statistics Service for 2002-2003 estimates 727,300 acres in the LRGV were cultivated for cotton, corn, sorghum, and sugarcane (USDA n.d.). In 1995, approximately 29 million pounds of pesticides were applied and $376 million spent on pesticides to maintain crop production value and volume in Texas (Gianessi and Anderson 1995).

Pesticides do not necessarily remain at the site of application but may be transported by drift or runoff to remote soils, atmosphere, surface water, and groundwater based on chemical properties of the biocide (vanLoon and Duffy 2000). Thus, agricultural use of pesticides may have the potential to contaminate other natural resources. In addition to pesticides, untreated sewage and industrial effluents are commonly released into agricultural irrigation reservoirs and other surface waters (Miyamoto et al. 1995). Besides pesticides, the accumulation of nitrogen, phosphorus, and various metals from fertilizer applications has been cited as a top source of water quality impairment (Zhang et al. 2004). Dependence on the Rio Grande for agricultural purposes, such as irrigation waters, has severely depleted water flow of the river, hence concentrating pollutants and further compromising water quality (TNRCC 2002).

Human population growth in major border cities, like Nuevo Laredo and Ciudad Juarez, has surpassed the rate of municipal infrastructure development (Miyamoto et al. 1995), thus limiting the services of electricity, trash collection, solid and hazardous waste management, and drinking, sewage, and wastewater treatment (TNRCC 2002). Municipal solid and hazardous waste leachates from illegal dumping or crowded landfills can contribute to degraded water quality. Improper sanitation of drinking water

and wastewater has elevated cases of water-borne diseases of hepatitis A and shigellosis to approximately three times that of the United States average (Miyamoto et al. 1995; TNRCC 2002). Approximately 450,000 low-income residents of colonias, small semi-rural substandard housing developments along the Texas border region, are potentially more susceptible to endemic agricultural, municipal, and industrial pollutants which are attributable to improper infrastructure and waste management systems (Tiefenbacher 2000; TNRCC 2002).

In addition to being classified as one of the most endangered rivers in North America due to extensive degradation from anthropogenic activities, in 1993 the Rio Grande was considered to create "a greater threat to human health than any other river system" (American Rivers 1993). Industrial, agricultural, and municipal contaminants in the Rio Grande are allegedly contributing to the demise of human health in the border region (Schmidt 2000). Hazardous industrial solvents, such as methylene chloride (MC), have been detected in water samples from the Rio Grande (IBWC 1994); MC is considered to be a "probable cancer-causing agent in humans" by the USEPA (ATSDR 2000a). In 1994, concern for human ingestion of fish containing high levels of polychlorinated biphenyls (PCBs) prompted the Texas Department of Health to issue a fish consumption ban in the Donna Reservoir (TNRCC 1994). PCBs were previously used as hydraulic and electrical lubricants and coolants (ATSDR 2000b). Consumption of fish with high levels of PCBs is linked to memory and learning impairment in adults (Schantz et al. 2001). Metals from various sources (e.g. mining, natural weathering, industry, and pesticides) have been commonly reported in sediments from several

tributaries and the main stem of the Rio Grande (IBWC 1994). Metals, such as chromium and mercury, have the potential to cause various chronic and acute toxicity responses such as kidney damage and neurological effects (Goyer and Clarkson 2001). Metals are also of concern because of their persistence and non-biodegradability in the environment (Goyer and Clarkson 2001).

Pesticide application in the border region is of interest to human health as pesticide runoff may directly enter or percolate through soils into drinking water sources (TCPS 1999a) or come in contact with humans through other mechanisms (e.g. ingestion, inhalation, or dermal exposure). Atrazine, an herbicide extensively used on corn and sorghum in Texas, is the most frequently detected pesticide in Texas drinking water supplies. Atrazine is confirmed to cause early onset of mammary gland tumors and other lymphomas in mice (TCPS 1999b). The USEPA classifies atrazine as a "possible human carcinogen", and it has been recently banned in Europe (TCPS 1999b). Organophosphate metabolites were detected in urine from children and infants residing in proximity to agricultural fields near a colonia in the Rio Grande valley. Agricultural pesticide drift and track-in were indicated as sources of exposure and nondietary ingestion designated as the primary route of exposure (Shalat et al. 2002; Freeman et al. 2004). Although insecticide use in Texas has shifted from persistent organochlorines compounds (OCs), like DDT (1,1,1-trichloro-2,2,bis(p-chlorophenyl)ethane) to more highly neurotoxic organophosphates and carbarmates, OCs are persistent and continue to be a potential health hazard (Gianessi and Anderson 1995). For instance, DDE, a metabolite of DDT, was reported at measurable levels (20-60 parts per billion) in soil

from elementary school yards along the Texas-Mexico border (Miersma et al. 2003). Moreover, farmworkers may also come into direct contact with pesticides through application processes. Pesticides may increase the risk of many cancers (e.g. non-Hodgkin's lymphoma, myeloma, prostate, and brain) in farmers (TCPS 1999a). Interestingly, many pesticide toxicities, especially for chronic exposure, have not been sufficiently investigated (TCPS 1999a).

Although humans in the Rio Grande region are a likely receptor of contaminants, wildlife targets are in more direct contact with their environment and may experience higher exposures. In addition to habitat fragmentation, agricultural and industrial contaminants are cited as souces which may have contributed to 126 endangered and threatened species in Rio Grande basin of Texas (Mac 1998; Gil and Wilkins 2003). Chemical pollutants have been detected in all wildlife taxa, especially in the LRGV. For instance, DDE concentrations of 10 ug/g ww were found in fish from four resacas (oxbows) in the LRGV (Mora et al. 2001). Levels of toxaphene (90 to 312 ng/g ww), a hazardous insecticide primarily used for cotton and banned in 1990, were reported in fish from the Donna and Pharr settling basins in Hidalgo County (Eisler and Jacknow 1985, Wainwright et al. 2001). In 1998, body burden concentrations of DDE residues (< 0.5 ug/g ww), were detected in spotted whiptail lizard (*Cnemidophorus gularis*) carcasses in Hidalgo and Cameron counties (Clark et al. 1995). DDE, PCBs, and mercury (Hg) residues were reported in addled aplomado falcon eggs collected in 1995-1996 near Laguna Atascosa National Wildlife Refuge in the LRGV (Mora et al. 1997). A study performed in 1999-2000 detected DDE, PCBs, toxaphene, nickel, chromium,

and selenium in cliff and cave swallows nesting along the Rio Grande (Mora unpublished data). Blood and hair of the endangered ocelot (*Felis pardalis*) from the LRGV contained low levels of DDE, PCBs, and mercury (Mora et al. 2000). DDE, selenium, and mercury were the most frequently documented contaminants in all wildlife taxa reported in a contaminant assessment during 1965-1995 (Mora and Wainright 1997), however various concentrations of many other contaminants were detected.

Wildlife, including birds, from the Rio Grande region can serve as biomonitors of environmental contamination. Birds have become an increasingly useful wildlife model in toxicological research (Scanes and McNabb 2003). For this study, cliff (*Petrochelidon pyrrhonota*) and cave (*Petrochelidon fulva*) swallows were chosen as sentinel species because they are widespread, abundant, and occupy an insectivorous trophic position (Brown and Brown 1995; West 1995). Insectivorous birds have a tendency to bioaccumulate contaminants via emergent aquatic insects living in contaminated sediments (Larsson 1984; Klemens et al. 2000). Swallows may be exposed to sediment contaminants when acquiring mud for nest construction. Additionally, during the breeding season, swallows do not forage far (<1.5 km radius) from their nests (Brown and Brown 1995), thereby creating a localized area for contaminant exposure.

The tree swallow (*Tachycineta bicolor*), which shares characteristics (i.e. ubiquitous, abundant population, and insectivorous) with cave and cliff swallows, is used extensively in ecotoxicology studies. Studies which concern a range of

contaminants, such as polycyclic aromatic hydrocarbons (Custer et al. 2001); PCBs (Secord et al. 1999; McCarty and Secord 2000; Stapleton et al. 2001); pesticides (Burgess et al. 1999), and metals (Bishop et al. 1995; Custer et al. 2003) use tree swallows as bioindicator species. Wildlife can serve as sentinel species of contaminant exposure through the presence of chemicals in their tissue, as well as occurrence of physiological and biochemical detriment (i.e. genetic damage, endocrine disruption, immunosuppression, deformities, lack of reproductive successes, etc).

Exposure to environmental contaminants, including metals, PAHs, some organochlorines, and radiation can result in DNA damage (genotoxicity) (Hebert and Luiker 1996; Shugart et al. 2003). PAHs are primarily formed from the incomplete combustion of fossil fuels and petroleum products. PAHs may be released into the environment from a variety of sources including petroleum spills/seepages, industrial/municipal wastewater, stormwater runoff, and atomospheric deposition (Albers 1995). Representatives (i.e. chromium, mercury, and PAHs) of these confirmed genotoxic chemicals have been detected in wildlife and abiotic sources in the Rio Grande region (IBWC 1994; Mora and Wainwright 1997; IBWC 1998). Genetic insult from exposure to chemical contaminants may lead to a number of effects which weakens the structural integrity of the DNA molecule (Shugart et al. 2003). Both somatic and reproductive tissues are targets for mutagenicity from exposure to environmental contaminants (Bickham 1990). Shugart et al. (1992) identified the primary effects of interactions with a genotoxic agent to produce "adducts, strand breakage, or chemically altered bases." Improper repair of the damaged DNA molecule may be subsequently

transferred to actively replicating daughter cells and manifest itself as irreversible cytogenetic effects or mutations with deleterious physiological consequences (Shugart et al. 1992; 2003).

Cytogenetic effects can occur as structurally or numerically abnormal chromosomes with aberrations (breakages and rearrangement), aneuploidy, or polyploidy appearing in the karyotype. Unrepaired genetic mutations may alter the conservative DNA nucleotide sequence. Nucleotide alterations are due to point and frameshift mutations, duplications, additions and deletions, translocations and others (Hebert and Luiker 1996; Shugart et al. 2003). Most genetic mutations, whether by sequence alterations or physical disruption of the DNA molecule, adversely affect fitness in wildlife populations (Bickham et al. 2000).

Various techniques, based on the type of genetic perturbation, are employed to evaluate genotoxic exposure in ecotoxicology. Flow cytometry is a popular and sensitive cytogenetic assay which detects DNA content variation from a large population of suspended cells (Bickham 1990). Variation among cells, which can be caused from exposure to clastogenic contaminants in the environment, is detected from the unequal distribution of broken and rearranged chromosomes in daughter cells (Bickham 1990; Dallas and Evans 1990). This nuclear DNA content variability is generally quantified as either the full-peak or half-peak coefficient of variation (FPCV or HPCV). Besides the benefit of evaluating a large cellular population, flow cytometry is a rapid and facile test with extreme sensitivity (Otto and Oldigies 1980; Bickham 1990).

Flow cytometry provides a measure of DNA that is fragmented and/or has rearranged chromosomal material (Shugart et al. 2003). This approach will be used to assess DNA damage in swallows. The procedure applies the technique of specifically staining DNA with a stochiometrically-binding fluorochrome, namely propidium iodide. A laser within a flow cytometer excites the dyed DNA and each cell emits a particular level or intensity of fluorescence which is subsequently measured with a photometer. The measurement of each cell, which corresponds to individual DNA content, is presented as a histogram printout with relative DNA content on the abscissa and number of cells on the ordinate. A flow cytometer has the capability to distinguish variation between cells "by as little as 1% in DNA content" (Bickham 1990). Additionally, relative DNA content is also indicative of various phases of the cell cycle (G1,S, G2 and M). The G1 phase or resting portion of the cell cycle, which contains the largest amount of cells, produces a normally distributed histogram. A coefficient of variation ((CV) = 100 x standard deviation/mean of the peak) calculated from the histogram represents the width of the distribution and corresponds to variability of DNA content. Larger or broader distribution peaks, hence higher CVs, indicate a wider range of DNA content. Organisms have a uniform amount of DNA in each cell and derivation from this may indicate damaged DNA and clastogenic activity (Bickham 1990; Rabinovitch 1994).

Many wildlife studies have used the flow cytometry method (FCM) as a valuable tool in detecting clastogenic activity from contaminants, such as pesticides (Parker 2002), PAHs, and petroleum (Bickham et al. 1998a; Custer et al. 2000). FCM to monitor DNA damage has been investigated in all vertebrate classes as reported in the

following: fishes (Bickham et al. 1998b; Lingenfelser et al. 1997); reptiles (Lamb et al. 1995; Matson et al. 2005a); amphibians (Lowcock et al. 1997; Parker 2002; Matson et al. 2005b); birds (Custer et al. 1994; Custer et al. 2000; Musquiz 2003; Matson et al. 2004) and mammals, including humans (McBee and Bickham 1988; Bickham et al. 1998a; Yañez et al. 2004). Specific to the Rio Grande region, Parker (2002) found increased HPCVs in frogs exposed to undetermined contaminants. Musquiz (2003) found significantly higher HPCV values for cliff swallows in El Paso than in Somerville.

The endocrine system is also a target for insult from endocrine-disrupting chemicals, specifically those that mimic or antagonize endogenous hormones (Colborn et al. 1993). Investigation of endocrine-disrupting effects allows for an in depth physiological approach to exposed animals rather than assessment of gross endpoints, such as deformities or acute death (Colborn et al. 1993). Many studies have shown disruption of sexual hormonal homeostasis attributed to estrogen mimics, antiestrogens, or antiandrogens originating from synthetic or natural compounds in the environment. Several studies strongly suggest reproductive abnormalities resulting from environmental contaminants and include: the increased occurrence of intersex frogs (Reeder et al. 2005); observation of demasculinized males and superfeminized female alligators (Guillette et al. 1994); presence of ovotestis in male herring gulls (Fry and Toone 1981); and widespread detection of intersexed fish (Jobling et al. 1998).

Though improper balance of estrogen may contribute to imposexed wildlife, vaginal and uterine tumors in mice and humans and testicular tumors in mice, under normal circumstances estrogen plays essential structural and functional physiological

roles in all vertebrates. Estrogen results from the enzymatic conversion by P450 aromatase of C19 androgen steroids into phenol A ring estrogens (Simpson et al. 1994). P450 aromatase belongs to the cytochrome P450 superfamily, but is only remotely similar in comparison with cDNA sequences of other microsomal enzymes. The amino acid sequence for aromatase is highly conserved within the Phylum Chordata (Simpson et al. 1994; Conley and Hinshelwood 2001). For example, avian P450 aromatase nucleotide sequence is 70% identical to its human counterpart (McPhaul et al. 1988). Both the preserved amino acid coding region, which is located on the CYP19 gene, and the expressed aromatase protein are analogous among tissues that express aromatase (Conley and Hinshelwood 2001; Simpson et al. 2002). The main difference among tissue expression of aromatase is the regulation of the promoter region of the CYP19 gene (Simpson et al. 1994, 2002; Conley and Hinshelwood 2001).

Aromatase expression in gonads and brains is common in most vertebrates; however, more extensive tissue distribution (e.g. adipose, placenta, bone, and fetal liver) occurs in higher vertebrates, such as humans and primates (Conley and Hinshelwood 2001). Aromatase expression, and subsequent conversion into estrogens, is a critical component of differentiation and development of gonads and brain, maintenance of reproductive tissues, and sexual behavior.

In avian species, genetic female chicken embryos require estrogen production to differentiate into a phenotypic female with development of a left gonad. On the other hand, primary sexual differentiation in mammals is genetically controlled with little influence from estrogen, but more so the presence of testosterone (Nakabayashi et al.

1997; Bruggeman et al. 2002). Aromatase appears in female chicken embryonic tissues at incubation Day 6.5 and remains elevated after hatching (Yoshida et al. 1996). Estrogen is critical in the maintenance of reproduction by sustaining small prehierarchal follicles (Armstrong 1984; Nitta et al. 1991), which develop into postovulatory follicles and the eventual embryo. Egg production requires estrogen for production of yolk and albumen and formation of the egg shell (Nitta et al. 1991).

Estrogen is not solely a female hormone, but it is also important in conducting various physiological processes in the male gonad and brain (Lephart 1996; O'Donnell et al. 2001). *In situ* hybridization does not detect aromatase mRNA in the gonads of male chicken embryos during the critical period (Days 6-9) of differentiation; however, mRNA levels increase between incubation Day 10 and 7 days posthatch (Yoshida et al. 1996). However, a transient estrogen receptor is present (Days 7-10) in male chicken embryos which could be sensitive to estrogenic substances giving rise to phenotypic sex reversal (Etches and Kagami 1997; Nakabayashi et al. 1997). In adult roosters, aromatase mRNA expression and aromatase activity were discovered in pachytene spermatocytes, round and elongated mature spermatids, and also in the flagella of a spermatozoa. In addition to establishing an estrogen source in the adult rooster, estrogen receptors were detected in various structures of the epididymal region of the roosters, thereby suggesting an action site for estrogen (Bahr et al. 1997). O'Donnell et al. (2001) summarize numerous studies which recognize estrogen as an essential hormone in the reproductive maintenance of spermatogenesis and male fertility.

Androgen metabolism by aromatase in the brain influences sexual behavior, particularly male performance. Estrogen formation affects differentiation of neural structures with several nuclei being sexually dimorphic (Lephart 1996). Aromatase activity has been correlated with aggressiveness in quail (Schlinger and Callard 1989) and aromatase is highly expressed in the telencephalon of songbirds, especially zebra finches (Silverin et al. 2000).

Several studies, mostly with murine species, have examined the morphological, physiological, and behavioral effects of mice and rats with inactive aromatase genes (aromatase knockout ArKO) which produce no estrogen. Similarly, other studies have assessed the detrimental biological effects of the application of an aromatase inhibitor. Infertile ArKO female mice do not produce many viable follicles and lack the gonadal estrogens for ovulation (Britt et al. 2001). Administration of fadrozole hydrochloride, a synthetic aromatase inhibitor, to pregnant rats impeded the uterine tissue framework required for the latter stages of pregnancy and resulted in malformed fetuses (Tamada et al. 2004). Male ArKO mice displayed arrested spermatogenesis and impaired fertility (Robertson et al. 1999), as well as disrupted sexual behaviors, such as motivation, partner preference and performance. ArKO mice exhibit reduced sexual mounts and rare ejaculations (Bakker et al. 2002). In relation to avian species, eggs treated *in ovo* with an aromatase inhibitor resulted in a phenotypic sex-reversal of a genetic female possessing the morphology of male testes with spermatogenic capabilities (Elbrecht and Smith 1992).

As previously mentioned, aromatase is responsible for the conversion of androgens to estrogens in multiple tissues (ovarian, testicular, adipose, and brain) and the simplest and quickest way to measure androgen conversion by aromatase is with the *tritiated water-release* aromatase assay (Lephart and Simpson 1991). The principle behind the tritiated water aromatase assay is to measure aromatase activity, hence an indirect method of estrogen production, by the quantification of $^{3}H_2O$ (tritiated water). Various aromatizing tissues are incubated with a radiolabeled androgenic substrate, [1β-^{3}H] androstenedione in an aqueous phase to create a stereospecific loss of 1β-^{3}H (hydrogens) along with the production of a radioactively-unlabeled estrogen molecule. The radiolabeled hydrogens subsequently form $^{3}H_2O$ molecules. Energy from the reacted radiolabeled $^{3}H_2O$ is measured with a scintillation counter in disintegrations per minute (dpm) to establish the amount of androstenedione substrate to estrogen which aromatase converts.

This study includes the following specific objectives:

1. To determine if there was any DNA damage (flow cytometry) associated with contaminants in blood of swallows from the Rio Grande and a reference site;
2. To determine the presence and baseline levels of aromatase activity in two estrogen-dependent tissues (brain and gonad) in cliff and cave swallows;
3. To determine if either induced or suppressed aromatase activity occurred in association with contaminants in gonads (testes or ovary) and brain of swallows from the Rio Grande and a reference site; and

4. To associate potential DNA damage and P-450 aromatase alterations with possible contaminant sources.

MATERIALS AND METHODS

Field Collection Sites and Description

Six sites were selected near the Rio Grande, of which five were located in the LRGV, specifically Mission, McAllen, Pharr, Llano Grande, and Brownsville. The sixth site was located in Webb County approximately 210 km northwest from the LRGV and northwest of Falcon reservoir (Figure 1, Table 1). Sites along the Rio Grande were selected based on the location of swallow colonies and proximity to areas with contaminants of concern, such as municipal wastewater treatment plants, agriculturally contaminated sites, or industrial facilities. Somerville, a reference location roughly 560 kilometers north of the Rio Grande, was also selected (Figure 1). Somerville was far away from the Rio Grande to allow for comparisons with Rio Grande sites, and also was different to the Rio Grande because it was not close to municipal or industrial sources and was somewhat close (8-15 km) to agricultural activity.

Each colony site was located on a bridge or culvert with nesting colonies and mud nests positioned beneath in crevices to the sides or along concrete beams spanning the structure. Locations with numerous nesting colonies, with at least 100 nests, were chosen. Birds were collected during May 2003 in the LRGV and Laredo and during June 2003 and 2004 in Somerville.

Bird and Blood/Tissue Collection

The following cave swallow males (M) and females (F) were collected at each location: Brownville (5M;4F), Mission (8M;2F), McAllen (5M;5F), Pharr/San Juan

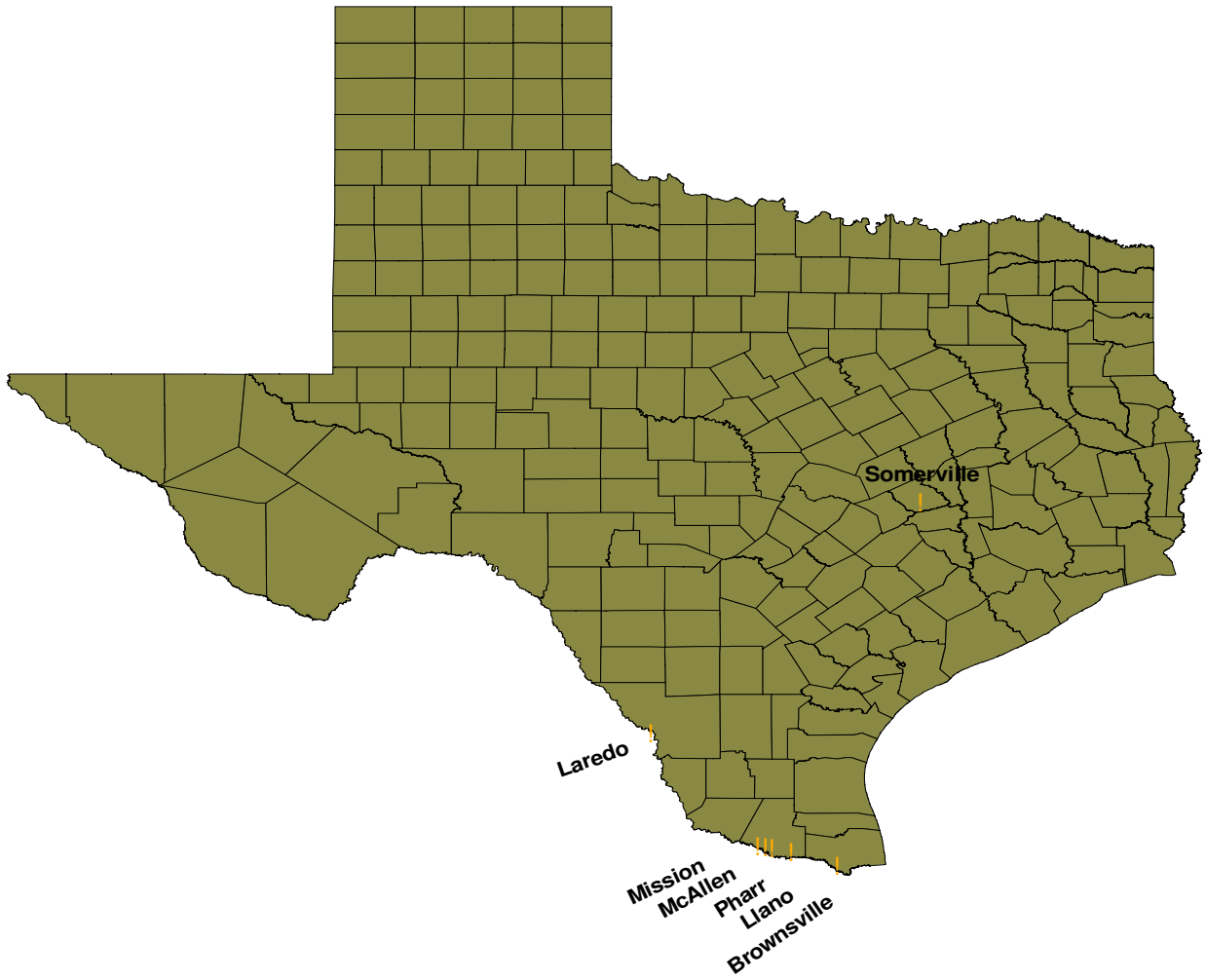

Figure 1. Location map of cave and cliff swallow colonies near the Rio Grande border and a reference site, Texas.

Table 1. Location of cave and cliff swallow colonies near the Rio Grande border and a reference site, Texas.

Site Name	Coordinates		Description
Mission	26˚11.389’ N	98˚19.825’ W	Culvert <1.0 mile south of Mission Wastewater Treatment Plant
McAllen	26˚11.180’ N	98˚14.830’ W	McAllen main canal adjacent Boeye reservoir and airport
Pharr	26˚09.846’ N	98˚10.351’ W	Bridge bordering Pharr/San Juan Wastewater Treatment Plant and small oil refinery, golf course located to southwest
Llano Grande	26˚07.207’ N	97˚57.670’ W	Bridge over Llano Grande Lake
Brownsville	25˚57.284’ N	97˚27.307’ W	Bridge adjacent North Brownsville/Robindale Wastewater Treatment Plant
Laredo	27˚34.488’ N	99˚30.336’ W	Bridge across Manadas Creek, Industrial areas in proximity
Somerville	30˚24.994’ N	96˚32.527’ W	Pastureland

(1M;9F), Llano Grande (5M;5F), 2003 Somerville (1M;4F) and 2004 Somerville (5M;1F).

Cliff swallows were collected only in Laredo (6M;4F) and 2004 Somerville (4M;5F). All adult birds were captured with mist nets thrown over the open space below the bridge or culvert structure where the birds were nesting. The mist nests were left open for no longer than 15 minutes. The birds were removed from the mist net and were carefully placed in nylon hosiery for a few minutes until time of sample processing. All birds were collected and processed under an approved Animal Use Protocol (AUP #2003-99) by the University Laboratory Animal Care Committee (ULACC) at Texas A&M University.

Nestling chicks were removed by hand from nests that contained at least three nestlings at selected sites. Cave and cliff swallow chicks were collected in Brownsville (8 cave swallows), Pharr (5 cave swallows), Laredo (4 cliff swallows), and Somerville (5 cave swallows). Chicks were contained in nylon hosiery similar to the adults before blood collection.

Immediately after collection, approximately 0.1 ml of blood was drawn from the jugular vein from each individual bird for flow cytometry analysis. Blood was collected with a 1 ml latex-free syringe equipped with a 25 gauge ⅝ PrecisionGlide® needle containing sodium heparin to prevent coagulation. The blood sample was released into Corning® 2.0 ml cryogenic vials containing approximately 1.0 ml of freezing media.

Freezing media consisted of 10% glycerol, 10% fetal bovine serum, and 1% penicillin/streptomycin mixed into RPMI Medium 1640. Blood and media were mixed

for approximately 10 seconds with the vials temporarily stored in a portable liquid nitrogen tank. Upon return to the laboratory, frozen samples were stored in a -80˚C freezer until flow cytometry analysis.

Immediately after blood collection, birds were sacrificed by cervical dislocation. Thereafter, bird mass was measured with a portable Ohaus® microbalance and measurements recorded to a tenth of gram. Adult birds were investigated for presence of brood patch and cloacal protuberance. Excision of gonads from adult swallows was the only surgical procedure performed during field collection. Removal of internal organs began with an incision directly below the ribcage cutting from the bird's left to right. The entire ribcage was lifted to expose internal organs and intestines were pushed aside to reveal either ovarian follicles or paired testes. Sex and condition of gonads were recorded for both males and females. Gonads were detached with sterile scissors and removed with stainless steel tweezers. Each gonad was placed in a labeled 1.5 ml cryogenic vial for future aromatase assay analysis. Carcasses were wrapped in labeled aluminum foil, placed in a marked plastic storage bag, and stored in dry ice until taken to the lab where they were stored at -80˚C. After transportation to the laboratory, the brain of both chicks and adults were removed. Brain samples were kept cold and did not thaw completely during dissection. An incision was made near the base of the neck and skin pulled forward to reveal the skull. Two cuts were made directly above the eye sockets into the skull; the incision was carried to the back of the skull. The skullcap was peeled back to reveal the soft brain tissue. A dissecting knife was placed under the cerebral hemispheres and gently lifted posteriorly to the brain stem where a final incision was

made. The entire brain for each bird was wrapped in labeled foil and stored at -80˚C until aromatase analysis.

Flow Cytometry

Flow cytometry analysis was performed on a single day to minimize variation. Samples were randomly processed to prevent investigator bias. No intermingling of species or developmental stage (i.e. adult or nestling) occurred within an individual run. A blood sample with known DNA content from a domestic chicken *(Gallus domesticus*) was similarly processed and included in each run to investigate potential machine or procedural inaccuracies. The cryogenic vials containing blood samples were completely thawed and gently inverted to assure uniform samples. Both adult and chick swallow blood samples were processed similar to previously developed techniques (Vindelov and Christensen 1994). Briefly, aliquots of 450 µl of trypsin digestion solution were dispensed in empty, labeled 1.5 ml eppendorf tubes followed by 50 µl citrate buffer. Next, 50 µl of blood sample was added to the trypsin/citrate buffer mixture which was then homogenized thoroughly 3-5 times with a Teflon dounce. Eppendorf tubes were securely closed, inverted three times to allow mixing, and left undisturbed for 10 minutes. An aliquot of 375 µl of trypsin inhibitor with ribonuclease (RNase) was added to each sample and again left undisturbed for 10 minutes. The entire sample mixture was filtered through 30 µm nylon mesh into a Falcon® polypropylene tube and a final amount of propidium iodide (375 µl) added to stain nuclear suspensions. Samples were stained for 15 minutes before being analyzed with the Beckman Coulter Epics Elite flow cytometer (Beckman Coulter, Fullterton, California). From each sample, the DNA

content of 10,000 nuclei was measured and presented on a histogram with corresponding CV. The more conservative half-peak coefficient of variation (HPCV), which minimizes influences from noise and cellular debris, was used for statistical analysis.

Tritiated Aromatase Assay

Several quality control/quality assurance (QA/QC) pretests were performed to optimize this test. These included capped versus open eppendorf tubes, incubation conditions, and assay interferences. Because oxygen is involved in the hydroxylation of androgen to estrogen, a QA/QC experiment was conducted to detect oxygen deficiencies between a capped (closed oxygen environment) and open (open oxygen environment) eppendorf. No differences in aromatase activity were detected.

Current literature indicates optimal saturating substrate concentration and incubation time for gonads and brain samples are 50 nM of [1β-^{3}H] androstenedione (specific activity 25.3 Ci/mmol; Perkin Elmer Sciences, Inc., Boston, MA) for 3 h (Lephart and Simpson 1991) and 100 nM of [1β-^{3}H] androstenedione for 15 min (Foidart et al. 1998; Soma et al. 1999; Silverin et al. 2000), respectively. A time course and substrate concentration experiment was performed on chicken ovary surrogates to confirm the incubation conditions.

It is possible that cofactors, buffers, or even the tissue itself may interfere or conceal the final product of $^{3}H_2O$. DPM results for five increasing known concentrations of "unreacted" tritiated water (specific activity 1.0 mCi/g; Sigma Co, St. Louis, MO) were plotted against DPM results for the same five concentrations, but with 125 µl of ovary homogenate containing buffer and cofactors added. Both sets of plotted

values formed a similar straight line; therefore, nothing in the homogenate preparation provided interference with the final results.

In addition to QA/QC, internal interassay and intraassay techniques were used with each sample run to ensure experimenter and instrument consistency. Chicken ovary was used as an interassay positive control. The follicles in the chicken ovary created highly variable aromatase activity results, therefore extraction efficiency on pre- and post-test DPM results for unreacted tritiated water served as an intraassay validation. All extraction efficiency calculations were >98%. Bovine serum albumin (BSA) was used for the blank sample.

Several trial runs of the assay were conducted on a laying hen ovary, gonad (male and female) samples from cliff and cave swallows from another study, and various passerine brain samples to establish and test proper laboratory procedures. Tissue specific samples were randomized.

Methods to measure aromatase activity were adapted from those described by Lephart and Simpson (1991) with slight modifications. Briefly, all samples, buffers, and cofactors were kept at 4˚C on ice before incubation period. Frozen tissues were transferred from cryogenic vials into eppendorfs and masses were measured with a Sartoris® analytical balance (eppendorf w/tissue-eppendorf empty). Each sample, including blank and inter/intraassay controls, had a final reaction volume of 500 µl. Field samples were homogenized in 445 µl of the appropriate buffer (e.g., brain: 10 m*M* potassium phosphate KPO_4, 100 m*M* potassium chloride KCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* glucose-6-phosphate G-6-P, and 10 m*M*

dithiothreitol; gonad: 50 mM KPO_4, 1 m*M* EDTA, and 10 m*M* G-6-P) with pH adjusted to 7.4-7.6. For the reaction to occur, cofactors of 50 µl of 10 mM NADP (1 mM final) and 5 µl of 100 unit/ml G-6-P dehydrogenase (0.5 units/500µl final reaction volume) were added to samples. Brain homogenate and cofactors were incubated with 1.26 µl of [1β-^{3}H] androstenedione for a 100 n*M* final concentration; gonad samples were treated with 0.63 µl of the substrate for a 50 n*M* final reaction concentration. A chicken ovary was used as a positive control and homogenized in 445 µl of gonad buffer. The blank sample consisted of 2 mg/ml BSA (20µl) in 425 µl of appropriate 1X buffer solution with no tissue added. Cofactors were added to both the positive control and blank sample. The extraction efficiency contained 20 µl of 1000X "unreacted" tritiated water and 480 µl of buffer with no tissue or cofactors included. Samples were gently vortexed for approximately 10 seconds. All samples and controls were incubated in an atmosphere of 95% O_2: 5% CO_2 at 37˚C. Incubation times for field gonad and brain samples were 3 h and 0.25 h, respectively. Enzymatic reactions were stopped by placing samples on ice. After 5 min, all samples were centrifuged at 21,000X g with two aliquots of 200 µl of supernatant transferred to new eppendorf tubes. Approximately 10 µl of remaining supernatant was reserved for subsequent protein concentration determination. To remove remaining insoluble substrate and cellular debris, 500 µl of chloroform ($CHCl_3$) were added to each aliquot. Chloroform samples were vortexed for 30 sec and centrifuged for 2 min at 21,000X g. The centrifuged samples formed a top aqueous layer with ^3H_2O and a bottom insoluble layer with residual substrate and debris. Most (400 µl) of the $CHCl_3$ was removed from the bottom and respun for 1 min. at

21,000X g. Removing the $CHCl_3$ made it easier to aliquot 100µl of the aqueous supernantant into new tubes containing 100µl of dextran-coated charcoal (DCC 0.5%; 5%: w/w) which also removed insolubles. Samples were vortexed for 60 sec and centrifuged for 15 min at 21,000X g to form a DCC pellet. The remaning supernantant (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 with a Beckman Coulter Model LS 6500 Multipurpose Scintillation counter. Protein concentrations were determined with the Bradford protein assay using 2 mg/ml bovine serum albumin standards (Bradford 1976). All DPM for blank samples were subtracted from each sample and multipled by a dilution factor before final aromatase activity was calculated. Final aromatase activity was expressed as femtomoles/incubation time (h)/mg protein.

Statistical Analysis

Flow cytometry and P-450 aromatase data were tested for normality by the Shapiro-Wilk's test and for homogeneity of variances with Levene's test. If the data were not normally distributed, they were log transformed and retested with Shapiro-Wilk's and Levene's test prior to data analysis. All significance levels were established at $p \leq 0.05$. For flow cytometry, comparison of HPCV among locations was analyzed by one-way analysis of variance (ANOVA) with a Fisher's Least Square Difference (LSD) post-hoc comparison. A t-test was used for comparison between HPCV values from Laredo and Somerville.

When appropriate (>3) sample size was available, a t-test was performed on log-transformed data for sex comparisons within each site for GSI and brain and gonad aromatase activity. At sites where male, female, and chicks were available (Brownsville and Somerville), an ANOVA test was used to compare brain aromatase activity among the three entities. A t-test was used to detect differences between female and chick brain aromatase activity at Pharr.

For the analysis of differences among locations in GSI and brain and gonad aromatase activity, an ANOVA on log-transformed data with a Fisher's (LSD) post-hoc comparison was used. If no significant differences among locations were established, then cave swallows from the LRGV were pooled to increase regional sample size and compared to Somerville. A t-test on log-transformed data was used for comparison between Pooled LRGV and Somerville for male brain and male and female gonad aromatase activity. A t-test on log-transformed data was used to evaluate differences between Laredo and Somerville for GSI and brain and aromatase activity.

All statistical analyses were performed with Version 11.5.2 SPSS statistical package (SPSS Inc., Chicago, IL).

RESULTS

Flow Cytometry

Cave swallows

There were no significant differences in HPCV values between male and female swallows, therefore HPCV data were pooled for both sexes to evaluate significant differences among sites. No significant differences (Table 2, $p > 0.05$) in mean HPCV values were detected in adult cave swallows among sites. Additionally, HPCV values were not different between swallows collected in Somerville during 2003 and 2004 or for both years combined and the LRGV sites. In chicks, there were no significant differences in HPCV values (Table 2, $p > 0.05$) between those from Brownsville and Somerville, the only two locations from which enough chicks were collected to allow for statistical comparisons. HPCV values were 9-26% higher in chicks than in adults at each corresponding location; however, the data were not statistically compared between age classes (Table 2).

Cliff swallows

HPCV values in adult cliff swallows from Laredo were significantly greater (Table 3, Mann-Whitney U test, $p = 0.018$) than those collected in Somerville during 2004. Blood of chicks from Laredo and Somerville from 2003 was not analyzed due to small sample size.

Table 2. Half peak coefficient of variation (HPCV) in blood cells of cave swallows (*Petrochelidon fulva*).

Age	Site	Sample size	HPCV Mean ± SD[a]
Adult	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 2003	5	3.42 ± 0.54 A
	Somerville 2004	6	3.22 ± 0.45 A
	Pooled Reference	11	3.31 ± 0.48 A
	Pooled Rio Grande	49	3.21 ± 0.38 A
	Pooled Reference	11	3.31 ± 0.48 A
Chick	Brownsville	8	3.58 ± 0.22 A
	Somerville 2003	5	3.63 ± 0.37 A
Adult		9	3.00 ± 0.43[b]
Chick	Brownsville	8	3.58 ± 0.22
		10	3.11 ± 0.29
	Pharr	4	4.22 ± 1.30
		11	3.31 ± 0.48
	Somerville	5	3.63 ± 0.37

[a] Denotes the abbreviation for standard deviation. [b] No statistical comparisons were made between age classes.

Table 3. Half peak coefficient of variation (HPCV) in blood cells of adult cliff swallows (*Petrochelidon pyrronata*).

Age	Site	Sample size	HPCV Median
Adults	Laredo	10	3.37 A
	Somerville 2004	9	2.62 B

Gonadosomatic Index (GSI)

Cave swallows

GSI comparisons between sexes were possible only at those sites where more than three specimens per sex were collected. Geometric mean and range of GSI values for each site and gender are presented in Table 4. At each location, cave swallow males had significantly higher ($p < 0.05$) GSI values than females. GSI values in males were 5-9.6 times greater than those in females.

There were significant differences in GSI values of males among locations (Table 4; ANOVA, $p = 0.003$). GSI was significantly higher in Rio Grande sites (Mission, McAllen, and Llano Grande) than in Somerville, except for Brownsville. Among LRGV sites, mean GSI values in swallows from Brownsville were similar to those from Mission, but were different from those in Llano Grande and McAllen. GSI values in male swallows from Llano Grande, McAllen, and Mission were similar. GSI values in females were similar among locations ($p > 0.05$).

Cliff swallows

GSI values in males were statistically greater than in females (Table 4; $p < 0.05$). GSI values in males from Laredo were 10.6 times greater than in females; whereas, males from Somerville were 12.5 times higher than females.

GSI values in male and female cliff swallows from Laredo were not significantly different than Somerville (Table 4; $p > 0.05$).

Table 4. Gonadosomatic index (GSI) for cave and cliff swallows from the Rio Grande and a reference location (Somerville).

Species	Site	Sample size		Geometric Mean and Range of Gonadosomatic Index	
		Male	Female	Male	Female
Cave swallow	Mission	8	N/A[a]	1.69 (1.11-2.29) BC	N/A
	McAllen	5	5	2.22 (1.83-2.90) C	0.23 (0.18-0.32) A
	Pharr	N/A	9	N/A	0.21 (0.10-0.51) A
	Llano Grande	5	5	1.83 (1.26-2.28) C	0.28 (0.15-0.50) A
	Brownsville	5	4	1.27 (0.72-1.94) AB	0.25 (0.15-0.64) A
	Somerville 2003	N/A	4[b]	N/A	0.14 (0.09-0.21) A
	Somerville 2004	5	N/A	1.21 (0.89-1.41) A	N/A
Cliff swallow	Laredo	6	4	1.81 (1.29-2.43) A	0.17 (0.11-0.25) A
	Somerville 2004	5	4	1.88 (1.27-2.39) A	0.15 (0.12-0.19) A

[a] Less than three birds were collected in Mission and Pharr, therefore were omitted from comparison. [b] Somerville cave swallows were mostly collected in 2004, except for females collected in 2003. At each location, GSI for male swallows was always significantly higher ($p < 0.05$) than females, therefore between column letters were omitted. Statistical significance ($p < 0.05$) among locations for each species is indicated by different letters within columns.

Brain and Gonad Aromatase Activity

Cave swallows

Table 5 presents geometric mean and ranges for brain aromatase activity. There were no differences in brain aromatase activity between male and female cave swallows from McAllen and Llano Grande. However, at Llano Grande the differences between males and females approached significance ($p = 0.056$) with males having brain aromatase activity almost twice that of females. There were no significant differences ($p > 0.05$) among brain aromatase activity among male, female, and chick swallows collected in Brownsville and Somerville. Brain aromatase activity in female swallows from Pharr was similar to chicks at that location ($p > 0.05$). No significances between the sexes or among adults and chick were detected; therefore, letters to distinguish differences were omitted.

Among locations, there were no significant differences (Table 5; $p > 0.05$) in brain aromatase activity of male swallows. Brain aromatase activity was statistically similar ($p > 0.05$) between male cave swallows from pooled LRGV sites and those from Somerville. However, brain aromatase activity tended to be greater in male cave swallows from the LRGV than in those from Somerville. In contrast, brain aromatase activity of female cave swallows was different among locations (Table 5, ANOVA, $p = 0.021$). Brain aromatase activity did not differ at most locations, except for Llano Grande which was only similar to Brownsville. Brain aromatase activity tended to be lower in female cave swallows from the LRGV, especially in swallows from Brownsville and Llano, than in those from Somerville.

Table 5. Brain aromatase activity of cave and cliff swallows collected from the Rio Grande and a reference location (Somerville).

Species	Site	Sample size			Geometric Mean and Range for Brain Aromatase Activity (fmol/incubation(hr)/mg protein)		
		Male	Female	Chick	Adult Males	Adult Females	Chicks (both sexes)
Cave swallow	Mission	8	N/A[a]	N/A[b]	12.78 (6.73-51.98) A	---	---
	McAllen	5	5	N/A	14.62 (6.57-35.69) A	15.78 (10.90-22.05) A	---
	Pharr	N/A	9	5	---	15.69 (9.09-27.94) A	14.75 (8.96-26.95) A
	Llano Grande	5	5	N/A	15.58 (13.46-25.94) A	7.95 (2.86-13.93) B	---
	Brownsville	5	4	6	10.99 (5.07-20.80) A	12.48 (9.43-15.34) AB	12.05 (2.63-31.21) A
	Somerville	5	4	5	9.21 (5.03-16.03) A	17.15 (13.63-20.45) A	12.06 (4.79-18.96) A
	Pooled LRGV	23	23	N/A	13.29 (6.73-51.98) A	---	---
Cliff swallow	Laredo	6	4	N/A	16.35 (8.11-24.56) B	11.09 (9.13-13.81) A	---
	Somerville	5	4	N/A	8.01 (4.54-17.87) A	7.23 (2.89-13.68) A	---

[a] At some sites (Mission and Pharr), less than 3 birds were collected or [b] no sample was taken (chicks at Mission, McAllen, Llano Grande, Laredo, and Somerville (cliffs)) and omitted from comparison. Statistical significance ($p < 0.05$) of brain aromatase activity for adults and chicks among locations is indicated by different letters within columns.

Table 6 presents geometric mean and ranges for gonad aromatase activity. Gonad aromatase activity was significantly higher in female cave swallows than in males at each location (Table 6, $p < 0.05$). Aromatase activity in females from Brownsville and Somerville was 9 to 35 greater in females than in males.

Gonad aromatase activity in male cave swallows was overall significantly different among locations (Table 6, ANOVA, $p = 0.047$. Somerville was only similar to McAllen and Llano Grande. Brownsville, Llano Grande, and Mission were similar, but McAllen was only similar to Mission. In female cave swallows, differences in gonad aromatase activity among locations approached significance at 0.076 (ANOVA). Gonad aromatase activity was significantly greater (Table 6) in male (t-test, $p = 0.019$) and female (t-test, $p = 0.015$) cave swallows from LRGV sites pooled than in those from Somerville.

Cliff swallows

Comparison of brain aromatase activity was only possible between adult males and females from Laredo and Somerville; small sample size limited inclusion of chicks for statistical comparison. There were no differences in brain aromatase activity between males and females at either Laredo or Somerville.

Brain aromatase activity was significantly higher (Table 5, t-test, $p = 0.037$) in males from Laredo than in those from Somerville; however, brain aromatase activity was similar in females from both sites (Table 5, $p > 0.05$).

Gonad aromatase activity was significantly greater in female cliff swallows from Laredo and Somerville than in males (Table 6 $p < 0.05$). Gonad aromatase activity in females from Laredo was approximately 25 times higher than in males.

Gonad aromatase activity was significantly greater (Table 6, t-test $p = 0.007$) in male cliff swallows from Laredo than in those from Somerville. Gonad aromatase activity in females from Laredo was approximately three times higher than in females from Somerville and the geometric means approached significance ($p = 0.061$).

Table 6. Gonad aromatase activity of cave and cliff swallows collected from the Rio Grande and a reference location (Somerville).

Species	Site	Sample size		Geometric Mean and Range for Gonad Aromatase Activity fmol/incubation(hr)/mg protein	
		Male	Female	Male	Female
Cave swallows	Mission	8	N/A[a]	0.96 (0.54-1.87) BC	---
	McAllen	5	5	0.70 (0.36-1.15) AC	26.79 (6.53-49.22) A
	Pharr	N/A	8	---	26.46 (10.06-112.46) A
	Llano Grande	5	5	0.88 (0.58-1.33) AB	29.34 (14.86-80.36) A
	Brownsville	5	3	1.31 (0.71-1.88) B	11.98 (5.54-23.30) A
	Somerville	5	4	0.52 (0.33-0.93) A	8.16 (3.00-17.79) A
	Pooled LRGV	23	21	0.94 (0.36-1.88) B	24.28 (5.54-112.46) B
Cliff swallows	Laredo	6	4	1.26 (0.57-2.57) B	34.40 (17.30-69.97) A
	Somerville	5	4	0.43 (0.23-0.71) A	10.54 (5.18-22.96) A

[a] At some sites (Mission and Pharr), less than 3 birds were collected and omitted from comparison. Within columns, values not sharing the same letter are significantly different.

DISCUSSION

DNA damage is suspected when the exposed population shows higher HPCV values than a corresponding reference population (Custer et al. 2003). The results of my flow cytometry analysis suggest that there was no indication of DNA damage in swallows from the Lower Rio Grande Valley relative to the reference site. In contrast, Musquiz et al. (2003) reported HPCV differences and possible DNA damage in cave swallows collected in 1999/2000 among locations along the Rio Grande.

Despite the fact that contaminant residues were not analyzed in this study, historical reports and a visual assessment of the area can give an idea of sources of concern. The LRGV is a region of intense agricultural production and heavy use of pesticides. Mora and Wainwright (1998) indicated a steady decline of DDE concentrations in biota from the Rio Grande region, but recent studies have found levels higher than detected for over 20 years (Mora et al. 2005). DDE persists at potentially deleterious levels (> 3 ug/g) (Blus 1996) which could affect reproduction (i.e., eggshell thinning, reproductive success) of avian species in the LRGV and may have the ability to affect DNA. Lowcock et al. (1997) associated genetic damage with high HPCV values in frogs exposed to pesticides applied to corn and potato fields. Parker (2002) found variable HPCV levels in Rio Grande leopard frogs in five sites near the Rio Grande; and suggested that genetic damage occurred in frogs least likely to be exposed to pesticides. It should be noted that susceptibility of contaminants to amphibians is greater than birds because permeable skin and direct contact with contaminated media (soil, water, or sediment) creates a greater pathway of contaminant exposure. Organochlorine

pesticides, such as α-hexachlorocyclohexane, aldrin, dieldrin, p,p'-DDT are known to induce micronuclei formation in green-lipped mussels (Siu et al. 2004). Yanez et al. (2004) suggested that there was a connection between chromosomal damage in Mexican women and residing in a region sprayed with DDT for control of malaria vectors. In contrast, Bolognesi (2003) reviewed several epidemiological studies where it is indicated that there is no correlation between genotoxicity and pesticide exposure. Rakitsky et al. (2000) indicate that the majority of pesticides are weakly genotoxic and require high toxic doses to reach this state. There is little concern of PCBs affecting DNA in swallows at my study sites since PCBs have been in decline in Rio Grande biota (Wainwright et al. 2001). Moreover, PCBs did not increase DNA mutations in tree swallows near capacitor manufacturing plants (Stapleton et al. 2001).

Along with pesticide inputs, intensive agricultural practices contribute to occurrence of inorganic elements, such as arsenic and selenium which have been reported in LRGV biota (Schmitt et al. 2004). Arsenic and some metals act through a mechanism which interferes with DNA repair, but not by inducing chromosomal aberrations directly (Beyersmann 2002). However, if these elements which cause improper DNA repair occur with clastogenic metals, the likelihood of permanent DNA damage increases. Selenium concentrations were similar among LRGV locations and the reference site in 1999/2000 swallows (0.7-1.8 ug/g dry wt) (Mora et al. 2005). Selenium has been shown to exhibit antigenotoxic properties and display a protective role against genetic damage and cancer (Rizki et al. 2001, El-Bayoumy 2001). The antigenotoxic properties of selenium and the prevalence of selenium from agricultural

practices (disturbed soils and erosion) may explain the lack of significant findings from my reference and LRGV locations. Musquiz (2003) suggested that chromium was a causative source of genotoxicity in some LRGV sites. Schmitt et al. (2004) found detectable levels of chromium in LRGV fish collected in 1997 (1.08-28.51 µg/g wet wt) and chromium ranged from 8.2-9.5 µg/g in swallows from the Rio Grande region (Mora unpublished data). Chromium has the capability to induce DNA-strand breaks (Tsuzuki et al. 1994). Mercury is another element reported in biota of the Rio Grande from 1965-1995 (Mora and Wainwright 1998). Mercury has been shown to induce DNA damage in beluga whales (*Delphinapterus leucas*) (Gauthier et al. 1999). Notwithstanding, the lack of differences in HPCV values among locations suggest that neither chromium nor mercury could be associated with any DNA damage in swallows from the Rio Grande.

Temporal differences between the 1999/2000 (Musquiz 2003) and 2003/2004 collections may help explain the differences observed between the HPCV values found in my cave swallows and those detected in the Musquiz study. Environmental factors such as increased rainfall and dilution of contaminants, sediment migration, influx/outflow of municipal and agricultural runoff, and changes in pesticide application rates are a few events that could have affected HPCV values. The swallows in these study sites may have not been exposed to the same intensity level or type of contaminants associated with clastogenic effects than those collected in 1999/2000 (Musquiz 2003). Also, other than chromosome breakage/rearrangements, flow cytometry may not be sensitive enough to detect lower-level contaminant exposure effects, such as a changes in DNA strand-breaks.

In contrast to cave swallows, cliff swallows from Laredo exhibited higher HPCV values than those from Somerville, suggesting birds from Laredo may be exposed to clastogenic agents in their surrounding environment. The city of Laredo is situated in an industrial area with several manufacturing and warehousing facilities which store, manufacture, transport, and release hazardous materials. The International Boundary and Water Commission toxic substance study classified Manadas Creek as a "high concern" tributary due to the potential for toxic substance effects (IBWC 1998). Clastogenic metals, such as chromium and mercury, may occur from industrial outputs as well and be causative sources of genotoxicity.

Laredo is also the busiest inland port of entry for international commerce with nearly 8,000 commercial trucks arriving each day (TNRCC 2002). Thus, idling trucks and tailpipe emissions can contribute to PAH releases. Additionally, 20% of the natural gas produced in Texas is supplied by the oil and gas industry of Webb and Zapata Counties (TNRCC 2002). Flow cytometry analysis is very sensitive to petroleum and PAH-induced genetic damage (Bickham et al. 1998a). Genotoxic effects were detected in mink and sea otters exposed to petroleum, a major source of PAHs (Bickham et al. 1998a). Elevated concentrations of PAHs in winter scaup carcasses were significantly correlated with chromosomal damage in blood cells (Custer et al. 2000). PAHs also were suggested as a probable source of higher HPCV values in common eiders nesting in the Baltic Sea (Matson et al. 2004). It is reasonable to suggest that birds from Laredo are exposed to PAH related compounds and that PAHs could have been associated with the elevated HPCV values in swallows from this site.

HPCV values tended to be higher in chicks than adult swallows, even at the reference site. The increased levels of HPCV in chicks may be from increased cellular processes during the developmental stage. Gene amplification for differentiation and development of extremities and organs during development was indicated as the source of DNA content variation in developing *Xenopus laevis* (Fritz et al. 1990). To my knowledge, this is the only study that recognizes a pattern of higher HPCV values in chicks than adults that is not associated with contaminants.

However, chick swallows from Pharr/San Juan had the highest HPCV among chicks in the LRGV, suggesting possible exposure to clastogenic agents. Chicks have been described as a better model of local contamination (Bishop et al. 1995; Secord et al. 1999) because they consume aquatic and terrestrial insects near their nesting site instead of wintering and migratory grounds as in adults (Secord et al. 1999).

Gonadosomatic index was greater in male cave and cliff swallows than in females at each location. Thus, males bear larger gonadal weights per body weight compared to females. Results from another study shows that males had GSI values approximately 5 to 20 times greater than females (Musquiz 2003). In this study, GSI values were only only 5 to 13 times higher than females. Though gross morphology of ovarian tissue was observed to be rather large in reproductively active females, testicular tissue may be denser and weigh more creating a higher GSI. GSI was similar among locations for female cave and cliff swallows and for male cliff swallows. GSI of male cave swallows varied among locations and it was greater at most LRGV sites than in Somerville. These GSI values are similar to those obtained in swallows previously

collected in 1999 and 2000 (Musquiz 2003). The differences in GSI among male cave swallows could be explained because of differences in reproductive anatomy within a species. Lombardo (2001) discovered that the cloacal protuberance, the external genitalia which stores sperm, had both individual and breeding season variation in tree swallows. Increased testes size is also linked to large colony size because of the advantages of increased sperm for extrapair and within-pair copulations which occur in large competitive breeding-colonies (Brown and Brown 2003). Brown and Brown (2003) reported cliff swallows 1 and 2 years of age exhibit smaller testis and have a higher propensity of being caught at larger colonies (Brown and Brown 1996). In this study, we did not assess age distribution, therefore it is difficult to establish whether lower GSI values could be reflective of younger birds not excluded from the sample size. Wainwright et al. (2001) found the greatest GSI and plasma testosterone levels in male carp at an oxbow lake contaminated with DDE and toxaphene. It was suggested that DDE's antagonistic behavior on the androgenic negative-feedback loop of the hypothalamo-pituitary-gonadal axis created more circulating testosterone and larger testes (Wainwright et al. 2001). It is difficult to ascertain whether differences among GSI values of male cave swallows could be related to contaminant loads or are the result of other ecological variables. My reference site in Somerville had the lowest GSI values and the least variation.

An overall trend was observed in tissue–specific aromatase activity in cave and cliff swallows. Male and female brain aromatase activity was similar. Aromatase activity in ovaries was higher than in brain. Ovarian aromatase consistently exceeded

both male and female brain aromatase in several studies (Schlinger and Arnold 1991; Saldanha and Schlinger 1997; Saldanha et al. 2000). The lowest activity levels were found in male testes of the swallows. This study examined aromatase activity in various tissues of a highly colonial bird and contributes to understanding of the distribution of aromatase in another songbird taxa (Silverin et al. 2000).

Aromatase activity has been determined primarily on chickens (G*allus domesticus*), quail (*Coturnix cotumix japonica*), and zebra finch (*Taeniopygia guttata)* (Foidart et al. 1998), and a few more studies have examined the role of aromatase in other passerine songbirds (Saldanha and Schlinger 1997; Foidart et al. 1998; Silverin et al. 2000; Riters et al. 2001) and a sub-oscine passerine (Saldanha et al. 2000). The only other study confirming presence of aromatase activity in songbird testes was by Saldanha and Schlinger (1997) in brown-headed cowbirds (*Molothrus ater*). Aromatase activity in testes of zebra finches and other songbirds was nondetectable (Schlinger and Arnold 1991; Silverin et al. 2000). However, the method used in other studies is different from the one used in this research. Bahr et al. (1997) were able to detect aromatase activity in sperm of the rooster using the tritiated water release method similar to mine. The presence of P450 aromatase in testes of male swallows is not surprising because of increasing evidence in support of the role of aromatase in the maintenance of spermatogenesis (O'Donnell et al. 2001).

Expectedly, high levels of aromatase activity were expressed in the ovary of female swallows during the reproductive season. Estrogen synthesis in the ovary during the reproductive phase in the chicken is dominated by aromatase activity in the small

follicles, pre-follicular hierarchy (Armstrong 1984). Many smaller follicles were observed in both cliff and cave swallows.

High levels of aromatase activity were detected in brain of male and female swallows. These findings agree with trends obtained for male and female zebra finches (Schlinger and Arnold 1991) and brown-headed cowbirds (Saldanha and Schlinger 1997). Equal or greater estrogen amounts in male brain tissue than in female brain is possibly derived from the greater amount of circulating aromatizable androgens (Schlinger and Arnold 1991). The presence of brain aromatase activity in male and female swallows is supported by the notion that aromatase found in diencephalic brain regions is implicated in the modulation of copulatory and reproductive behaviors during the breeding season, whereas aromatase found in the telencephalic region has more involvement with spatial memory and is present throughout the annual cycle (Foidart et al. 1998).

Attention has focused on the capability of various environmental chemicals to cause endocrine disrupting effects, such as the inhibition or induction of sex steroid biosynthesis (Sanderson and van den Berg 2003). The present study not only provides baseline levels for future contaminant studies, but appears to be one of the only avian wildlife studies to associate brain and gonad aromatase activity with potential contaminants.

In both cliff and cave swallows, aromatase activity in testes and ovary was distinctly higher in Rio Grande sites than in Somerville. Caution should be given to the elevated levels of female gonad aromatase due to potential confounding effects from the

ovulatory cycle. Small white and small yellow follicles produce the highest aromatase activity compared to preovulatory follicles (Armstrong 1984), however, I considered the ovary as a whole and did not assess whether follicular stages were equal among females. A subtle imbalance in the hormonal environment of both male and female gonads has the ability to cause deleterious effects to reproductive tissues. Disrupted steroid concentrations (increased estradiol-17B) resulted in polyovular follicles and polynuclear oocytes in ovarian tissue and poorly organized testes and decreased phallus size in male gonads of American alligator (*Alligator mississippiensis*) inhabiting a contaminated environment (Guillette et al. 1994). Though gross observation of gonad tissues revealed no obvious signs of malformed genitalia, future studies should consider histological examination of reproductive organs. Suppressed ability to reproduce may decrease reproductive fitness and lower population numbers.

Brain aromatase activity among locations was more variable. Villeneuve et al. (2004) showed different dose-response relationships between brain and gonad aromatase activity in fathead minnows (*Pimephales promelas*) exposed to fadrozole, an aromatase inhibitor. For both cliff and cave swallows, brain aromatase was elevated in the Rio Grande in relation to Somerville, however, it was not significantly different in most locations. Female brain aromatase activity was greatly depressed in Llano Grande and to a lesser extent in Brownsville, thus suggesting that location may be a factor in explaining site plays a role in the differences which were detected. There may be contaminant hotspots in the LRGV where chemicals alter aromatase activity in bird tissues. Brain aromatase is crucial for regulation of estrogen-dependent reproductive

behaviors in both male and female. For instance, male Japanese quail dosed with ethinylestradiol and diethylstilbestrol exhibited decreased sexual behaviors, such as neck grab, mount attempt, and cloacal contact movement (Halldin et al. 1999). Depression of brain aromatase in females could affect breeding behaviors such as receptiveness of mating and parental care (Zala and Penn 2004). My study did not involve behavioral observations at the colony.

It is difficult to assess whether some endocrine active substances are possibly affecting aromatase levels because (a) swallows are exposed to complex mixtures in the environment and (b) a limited number of chemicals have been reported to cause aromatase induction/inhibition. Currently, the Endocrine Disruptor Screening Program and Advisory Committee developed by the USEPA identifies 85,000 substances to be considered for endocrine-disruption screening, including alterations in aromatase activity (Fenner-Crisp et al. 2000).

A limited number of studies associates environmental chemicals with aromatase activity alterations; some of these environmental contaminants, such as DDE, toxaphene, alkylphenols, atrazine, metals, are suspected to be present near the swallows' breeding sites. You et al. (2001) discovered that p,p'-DDE could induce hepatic microsomal aromatase in male rats. Likewise, DDE concentrations similar to those found in follicular fluids in women, produced a synergistic increase of FSH-stimulated aromatase activity in human granulose cell lines (Younglai et al. 2004). During 1999/2000, p,p'-DDE was detected at high concentrations in swallow carcasses from the LRGV, such as 6.6 ug/g wet wt at Llano Grande and 7.4 ug/g wet wt at Pharr, whereas only 0.3 ug/g wet

wt was detected in Somerville (Mora et al. 2005). One could argue that swallows may be exposed to contaminants on their wintering grounds, however stable isotope analysis has strongly correlated exposed to local breeding sources in the Rio Grande (Mora et al. 2005). Presence of DDE in the agriculturally-dominated region of the LRGV could play a role in alterations/inductions of steroidogenesis.

Chlordane and toxaphene are two other organochlorine pesticides that are no longer used in the LRGV, but still detected in biota (Mora unpublished data) and thus warrant concern. Toxaphene suppressed aromatase activity in vitro through antagonistic behavior of the estrogen-related orphan receptor (ERR) α-1, which is homologous to the estrogen receptor α (Yang and Chen 1999). Swallows from Llano Grande had the highest toxaphene residue levels (5000 ± 1600 ng/g lipid) detected in 1999/2000 (Maruya et al. 2005); therefore, because of its continued presence toxaphene may have suppressed female brain aromatase activity in wildlife collected at Llano Grande. However, toxaphene had no effect on aromatase activity in human choriocarcinoma JEG-3 cell line (Drenth et al. 1998).

Atrazine, and other triazine compounds, are herbicides heavily used on sorghum and sugarcane in Texas (http://aggie-horticulture.tamu.edu/extension/cropbriefs/sorghum.html). Detection of atrazine is ubiquitous in LRGV water samples collected by the USGS National Stream Quality Accounting Network (USGS n.d.). Moreover, there is a strong correlation with atrazine exposure and cancers in human and rodent female reproductive tissues (Kettles et al. 1997, Sanderson and van den Berg 2003). Estrogenic effects of triazine herbicides may result from their increased induction of aromatase

(Sanderson and van den Berg 2003). Sanderson et al. (2000) found that 2-chloro-s-triazines induced aromatase 2.5-fold in H295R human adrenocortical carcinoma cell line. Hayes et al. (2002, 2003) proposed that the aromatase induction by atrazines contributed to the hermaphroditic, demasculinized frog occurrence in both field and laboratory studies. It may be possible that triazine herbicides may be influencing increased aromatase activity in LRGV swallows.

Several of my sites (i.e. Mission, Pharr, Brownsville) were located near municipal wastewater treatment plants. The incidence of intersexed feminization of male fish has been linked to estrogenic substances in sewage treatment plants. Possible chemicals responsible for these effects include: natural and synthetic (birth control) estrogens; alkylphenolic compounds (surfactants); phthalates (plastics); and bisphenol A (polycarbonate resins) (Jobling et al. 1998). Although some of these chemicals are ligands of the estrogen receptor, other mechanisms of estrogenicity are not as clear. Another possible mechanism of increased estrogenic effects is through aromatase expression (Hugget et al. 2003). Short incubation times with bisphenol-A transfected human cell lines enhanced aromatase activity; whereas, an 18 hour incubation inhibited aromatase (Nativelle-Serpentini et al. 2003).

Aromatase activity could be elevated at some sites because of the regional differences in land-use practices, such as in Somerville and Laredo. Somerville is located in a minimally impacted agricultural region, whereas Laredo is more industrial and contaminants associated with industrial processes could be affecting aromatase. Brominated flame retardants (BFR) are ubiquitous in wildlife and in breast milk in

humans worldwide and little is known about their toxicity, hence there is raised concern over BFR as potential endocrine disruptors (Birnbaum and Staskal 2004). Though studies are limited, one experiment reported that several polybrominated diphenyl ethers (PBDE) produced estrogenic effects in breast cancer cells (Meerts et al. 2001). An undisclosed company located near Manadas Creek, listed on the EPA's Toxic Release Inventory program, releases a PBDE into the environment. Though the bulk of the product is disposed of in another city, some of the PBDE is released through air stack emissions (USEPA n.d.). The same company also has released numerous metals (antimony, arsenic, lead, and zinc) in varying quantities (5-250 pounds) to Manadas Creek in the past (USEPA n.d.). Many other companies in the area could be responsible for contributing to industrial waste releases.

PAHs are likely to be present in environmental media near Laredo. These compounds also may be altering aromatase activity. Based on the PAH structure, Fang et al. (2001) found that certain compounds can bind to the ER and have weakly estrogenic effects. PAHs inhibit aromatase *in vitro* in fish ovarian tissues (Monteiro et al. 2000). However, no other studies of PAH effects on aromatase activity were found.

Numerous metals have been detected in sediment, water, and biota samples in both Webb County and the LRGV (USGS n.d.; Schmitt 2004). Martin et al. (2003) reported that divalent cadmium, copper, cobalt, nickel, lead, mercury, tin, and chromium interacted with ER-α to induce cell proliferation in human breast cancer cells. Future evidence may confirm metals to have endocrine-disrupting effects through other mechanisms, perhaps altering aromatase activity.

It is incorrect to assume only agricultural chemicals could be affecting aromatase activity in LRGV swallows and industrial contaminants in Laredo. It is more likely that the effects of aromatase altering substances overlap. Many chemicals were considered that could contribute to the increases (DDE, atrazine, alkylphenols, metals) or decreases (toxaphene) in aromatase activity observed in the current study. Tissue analysis to quantify contaminants present in swallows would be beneficial to better establish a more direct cause-effect relationship. There are many chemicals and mixtures of chemicals, which have not undergone evaluation for endocrine-disruptor status. Also, there are many short-lived organophosphate and carbamate pesticides used persistently in the LRGV with unstudied steroidogenic or ER/AR-mediated effects. An overlooked area of endocrine disruption on steroid hormones is the potency of agricultural fertilizers as well as human wastes, such as ethynylestradiol from birth control and human urine which may contain endogenous testosterone or estrogen. In addition to prior research focused on estrogen/androgen receptor affinity, aromatase activity provides another valuable assay to assess interactions with sex steroid hormone synthesis.

CONCLUSIONS

Flow cytometry was used in the current study to detect clastogenic DNA damage in wildlife. The HPCV values detected in cliff swallows collected from Laredo were higher than for those collected in Somerville suggesting possible DNA damage in swallows from Laredo. Nonetheless, HPCV values in cave swallows from the LRGV were similar to those from Somerville suggesting no DNA damage in other regions. My results were somewhat different from those of a previous study (Musquiz 2003) suggesting that contaminant exposure to cave swallows in my study may have been different, either in level or types of contaminants, to that observed in previous years.

The present study appears to be the first which measured aromatase activity in gonads and brain to demonstrate endocrine disruption in wild birds. In addition, data were obtained on baseline levels of P450 aromatase in two estrogen-dependent tissues from different geographical regions in Texas. Testicular and ovarian aromatase activity in cliff and cave swallows from the Rio Grande was higher than in swallows from Somerville. In most cases, brain aromatase activity also was elevated in the Rio Grande in relation to Somerville, but it was not always significant. Aromatase activity in female brain was notably depressed in female brain of swallow from Llano Grande. There may be contaminant hotspots, such as impounded lakes, in the LRGV where chemicals exert aromatase inhibition, instead of induction.

As there are suspected changes to the hormonal environment of adult swallows, alterations in brain and gonad aromatase may also be affecting embryos at a critical stage of gonad and neural differentiation and development. Because the avian endocrine

system and function is essentially similar to other vertebrates (Norris 1996), it is suspected that alterations observed in birds could also be occurring in other vertebrate classes. My results suggest that it is possible that the observed differences in aromatase activity in swallows from the Rio Grande are related to environmental contaminants and further in-depth studies would be useful to gain more knowledge in this regard. My field experiment considered that swallow exposure constituted *in vivo* exposure to environmentally relevant concentrations of contaminants instead of a dosage experiment which sometimes uses environmentally unrealistic concentrations.

There are several recommendations for future research. Only one species and one sex with a higher samples size at locations should be collected. Testes of swallows, instead of the ovary should be targeted to detect presence of elevated levels of aromatase instead of reproductively cyclic female swallows. Although brain tissue was used in this study relating to sex steroid synthesis alterations, future studies may use brain tissue to determine endocrine disruption in the form of cholinesterase inhibition, thereby having two endocrine biomarkers for one bird sample. Future sampling should encompass a broader spatial evaluation of sites along the Rio Grande, including samples as far north as El Paso. Environmental media, such as water or sediment, should be collected and evaluated for contaminants in conjunction with tissue sampling dates. Finally, tissue contaminant residues should be analyzed in swallow samples to determine body burden.

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VITA

Megan Annette Sitzlar received her Bachelor of Science degree in Biology from Tennessee Technological University in Cookeville, Tennessee in December 1999. She chose Wildlife and Fisheries as her minor. She moved to Nashville where she pursued a career in the private environmental consulting industry. Ms. Sitzlar entered the Toxicology program at Texas A&M University in the fall of 2002, and she received her Master of Science degree in December 2005. Drs. Miguel Mora and John Bickham from the Department of Wildlife and Fisheries were her advisors. Her research focused on contaminants and their effects on wildlife, and she plans to have this research published in a professional journal.

Ms. Sitzlar may be contacted at 620 Ridge Road, Madisonville, Tennessee 37354. Her email address is msitzlar@hotmail.com.