FEMINIZATION IN COMMON TERNS (*STERNA HIRUNDO*): RELATIONSHIP TO PERSISTENT ORGANIC CONTAMINANTS

by

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B. A. Chemistry
College of St. Catherine, Saint Paul, MN 1989

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION

January, 1998

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This thesis is for my Dad,
Tom Hart

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ABSTRACT

Concern about skewed sex ratios and female-female pairings among endangered roseate terns (*Sterna dougallii*) on Bird Island in Buzzards Bay, Massachusetts prompted studies with common terns from the same site as a surrogate species. Over seventy percent (11/15) of male common tern embryos sampled from this site in 1993 had testes containing areas of ovarian cortical tissue (ovotestes), suggesting that terns may be affected by endocrine-disrupting contaminants. These terns are exposed to non-ortho PCBs which bind to the aryl hydrocarbon receptor (AhR), and lower chlorinated PCBs, hydroxy-metabolites of which bind to the estrogen receptor. Our objectives were to document the presence of ovotestes in common tern embryos from Bird Island and Nauset, a reference site, in 1994, and to determine the relationship between environmental contaminants and ovotestes development, as well as other health-related effects.

Pipping tern embryo gonads were examined histologically, and yolk sacs were extracted and analyzed for PCBs and chlorinated pesticides. Extracts also were analyzed for dioxin equivalents (TCDD-EQs) using a chick embryo hepatocyte (CEH) bioassay. Total PCBs were significantly higher in Bird Island tern embryos (mean 114 , range 17 - 663 ug/g lipid) than Nauset (mean 35, range 8 - 178 ug/g lipid); but were variable at both sites. Total PCBs were highly correlated with TCDD-EQs. Tern hepatic EROD activity was relatively insensitive to induction; only when TCDD-EQs were above 82 $\pm$ 26 ng/g lipid were EROD activities elevated. Levels of organic pesticides were below levels thought to be of toxicological significance. The percentage of male tern embryos with ovotestes at Nauset (60%) and Bird Island (78%) was high and not significantly different; ovotestes in terns from both sites ranged in severity from absent (1) to intersex (4). There was no significant relationship between ovotestes severity and any of the contaminants measured. However, the data suggested a contaminant level threshold of 100 ug/g lipid total PCBs and 30 ng/g lipid TCDD-EQs above which the formation of ovotestes in tern embryos is more likely to occur. Principal component analysis of PCB isomer patterns distinguished between Bird Island and Nauset sites, with Bird Island having relatively higher levels of lower chlorinated PCBs; however, there was no distinction between terns with ovotestes and those without. Common tern prefledglings and paired same-nest eggs were collected from Bird Island in 1995 to examine the persistence of ovotestes. Gonadal histology revealed no ovarian tissue on testes, indicating that the ovotestes do not persist after 3 weeks posthatch.

Our data suggested that: 1) ovotestes could be related to contaminants; 2) a background level of ovotestes may be present, but elevated by contaminant exposure; or 3) ovotestes could be normally present in male common tern embryos at hatching. Observations of the normal presence of persistent cortical tissue in the testes of several avian species suggest that concern about feminizing effects of contaminants on bird populations as measured by ovotestes development in hatching birds may be exaggerated. Before conclusions can be made about terns from Bird Island, it is necessary to establish whether persistent ovarian cortical areas in testes are normally present. Studies with common terns from a pristine site are currently underway.

Thesis supervisor: Mark E. Hahn, Associate Scientist, WHOI

ACKNOWLEDGMENTS

There are a great many people to whom I owe thanks for their continued involvement, support, and encouragement. First, I would like to thank my advisor Mark Hahn for his enthusiasm, patience, support, and abundance of literature. As Mark's first student, I believe we have both learned a lot over the years it has taken to complete this thesis, and it is a big accomplishment for us both. I have learned a great deal from the freedom Mark has allowed me, and have been able to develop confidence in myself through my own successes and failures.

My committee members have also been a great help toward completion of this thesis. John Stegeman has always been ready with input at just the right moment, be it invaluable advice or a corny joke. Judy McDowell has always been available with positive advice and support. Pete Dedon (M.I.T.) has been extremely helpful both with advice and a willingness to accommodate Woods Hole students by travel or satellite link. I benefited greatly from spending nine months in the laboratory of Sean Kennedy (Canadian Wildlife Service), and thank him for being my mentor and friend. Finally, this work would not have been possible without Ian Nisbet who allowed me to become a part of this project. I have enjoyed every visit to Bird Island, and look forward to more.

I would also like to thank Lauren Mullineaux for chairing my thesis defense, sharing the slidemaker with me, and helping to make the Jamaica trip very memorable. I also thank Larry Madin for chairing my thesis proposal defense and for somehow managing to fix one of the three non-working overhead projectors available.

I owe great thanks to Michael Moore, who initially encouraged my involvement in this project. He, along with Ian Nisbet, Jeremy Hatch, and D. Michael Fry started the 1994 embryo collections, and I thank them all for their support. This project has required research in numerous disciplines, and thus, has been a continuing challenge from which I have learned a tremendous amount, including the importance of looking at the older literature.

The gonadal histology on the 1994 pipping common tern embryos was carried out by D. Michael Fry at U.C. Davis. I thank both Michael Fry and Lela Preston (U.C. Davis) for completing this work and providing me with the data, as well as answering numerous questions. I would also like to thank Michelle McCafferty and Roxanne Smolowitz at the MBL for helping me to embed and section the prefledgling gonads. Michelle provided me with extremely high quality slides, and I thank her for all her help. Roxanne Smolowitz,

Michael Moore, D. Michael Fry, and Laird Shutt were all very helpful with interpretation and confirmation of gonadal histology.

Much of this work was completed at the Canadian Wildlife Service in Hull, Quebec, where I spent nine very busy months in Sean Kennedy's laboratory. Sean and the members of his lab, Len Bastien, Stephanie Jones, and Angela Lorenzen, were invaluable for completing the egg and yolk sac extractions and the chick embryo hepatocyte bioassay. Also at CWS, Henry Won was extremely helpful and generous in carrying out the chemical analysis on my samples. Debbie Ashby taught me how to prepare my samples for chemical analysis, and Henry provided me with proper materials, carried out the injections, provided the analysis reports and helped with interpretations. Abde Idrissi also provided many insights and advice for all steps of the extractions. Also very helpful were Dr. Tony Scheuhammer and the members of his laboratory, Della Bond and Elyse Routhier, who carried out the mercury analysis on common tern embryo tissues. I also want to thank Dr. Laird Shutt, Glen Fox, Dr. Ross Norstrom, Brian Collins, Courtney Sandau, Rob Letcher, Masresha Asrat, and Karen Timm who provided helpful discussions and materials for this work. I would particularly like to thank Glen Fox who has continually shown interest in this work and provided insights and literature from his vast collection. I would also like to thank Dr. Laird Shutt along with Angela Lorenzen; their work on common terns from Canadian sites and willingness to collaborate has provided and will continue to provide interesting data and results.

At WHOI, I want to thank all the members of Mark Hahn's lab who have provided daily help and support, including Bonnie Woodward, Sibel Karchner, Sue Bello, Brenda Jensen, Diana Franks, and Wade Powell. I am especially grateful for my long-time office mates and friends, Bonnie and Sibel who were always there to help, to listen, and to laugh with.

I also want to thank all the members of John Stegeman's lab who have also been a great help on a daily basis, including Michael Moore, Bruce Woodin, Margie Oleksiak, Eli Hesterman, Jennifer Schlezinger, Rachael Cox, Carolyn Miller, Shannon Bard, Stefanie Valentini, Stephanie Innis, Celine Godard, Michael Morss, Heather Handley, Joanna Wilson, Malin Celander, and Renee White. Bruce has been especially helpful with teaching lab techniques, finding long lost supplies and equipment, and providing numerous concert diversions. Rachael Cox has been a big inspiration during the last few months of trying to complete this thesis, as she has been doing the same (while having a baby). I want to thank Michael Morss for all his help, especially with histology and dissections.

Malin Celander has been a great friend, and taught me a lot- especially about Sweden and Idun (her dog). Eli Hesterman (who is also a semi-member of the Hahn lab) has been great, providing some clarity to sometimes confusing scientific and other discussions.

Many other people at WHOI have also been a help toward completing this work. They include Colleen Hurter, Judy Kleindinst, Bill Cruwys, and everyone in the Education Office, especially Julia Westwater and Jake Peirson. I especially want to thank Jake Peirson for all his support and his sense of humor. I also want to acknowledge the Chemical Oceanography Department where I spent my first year and a half in the Joint Program, and I especially thank Ellen Druffel for her encouragement.

I entered the Joint program with a group of people who became great friends, all of whom I have surpassed in "time spent as a graduate student". They included Joe LaCasce, Gary Jaroslow, Pascal LeGrand, Carl Wolfteich, and Javier Escartin. These people have suffered through the many ups and downs of graduate school with me, including nightmare courses at MIT, long weekly bus rides to WHOI, rat and fruitfly infestations at the Somerville house, and some incredible hangovers. Some of them even convinced Jake Peirson to call me in Canada and tell me I lost my fellowship (which he did quite convincingly, enjoying every minute). Even though most of them were in other countries for my defense, I heard from them all; most of them were careful to mention their surprise that I was finally going to be Dr. Konee (many of them have never mastered the proper pronounciation of my name). I especially want to thank Gary for distributing a highly accurate description of my defense talk. I can't imagine what these years would have been like without all of you.

I want to give a special thanks to Javier Escartin who has introduced me to all sorts of interesting Spanish ideas which became more clear once I could understand his accent (after a year or so). Javier taught me a fashion sense (believe it or not), and the importance of great vacations. He also recognized early on my qualities resembling Platero the stubborn donkey, but despite this he has remained a great and loyal friend through everything. Te quiero, Haby.

Margie Oleksiak, who fortunately arrived a year after me, has been my great friend and support since we met, and I want to thank her very much; I couldn't have done this without her. We have had lots of adventures together, starting in Jamaica with the goat, pufferfish, octopus, eel, and airport X-ray machine. We have managed to keep each other continually entertained with our abundant oddities; I can't imagine daily life without Margie. Definitely, the hardest part of finishing graduate school is knowing we aren't

going to see each other everyday. At least you can just get a rat terrier, how will I manage without you ?

There are also many other great friends I have met at WHOI. I want to thank all my housemates over the years, who have provided lots of entertainment and friendship. As the only female living with four men in a one bathroom house in Somerville, I was looked on as a font of cleaning wisdom (this is not a good thing) and discovered it is definitely a myth that men are quick in the bathroom. Among my Woods Hole housemates, I especially want to thank Cecily Wolfe and Kristen Fehlhaber who are both great and enlightening friends. I also want to thank all of my friend Margie's housemates, who saw a lot of me over the years, especially Ken Gartner and Joanne Briana. There has been a huge Spanish influence during my stay in graduate school, and I want to thank all these people (both the real Spaniards and the associates); especially Javier (the first, and the leader), Gorka Sancho (who looks better in my bathingsuit than I do, what a body), Dan Torres (who is crazy), Young-Gyu Park (who is also crazy), Gaspar Taroncher (who is the only person I know that hits his head on the emergency shower pulls eight feet above the floor), and Ea Lin Lim (who is deathly afraid of seacucumbers, to my great delight). I also want to thank the many other friends who have helped me get through graduate school, including Maureen Clayton, Cecilie Mauritzen and Johannes, Barb and Anne LaCasce, John Salzig, Alex Bocconcelli, and Mandy Hannaford.

In addition to friends at WHOI, I would like to thank the many people at the Canadian Wildlife Service who not only helped me with my thesis work but became my friends. Sean and his family welcomed me to Ottawa, and have continually provided friendship and humor. Len, who taught me how to make extractions, also was a great debate partner for numerous chemistry-related and other topics, helped me to fix my car which suffered through the harsh Canadian winter, and introduced me to moose. Stephanie interjected a lot of humor into the many long days she spent with me. We found out that we had a lot in common, including an ability to make "attractive" faces, an interest in gross things (some of which are now preserved in formalin), a fascination with what might fit in our nose and the edibility of cellulose packing chips. Angela continually amazed me with her efficiency and her wit. Dave Ward, a constant source of humor, also explained the ease of using Ottawa buses for transportation. Bruce and Lori Pauli have been great friends to me, feeding me numerous delicious meals and providing loads of entertainment. I also want to thank Bruce for finding that tiny teflon washer I dropped on the floor and suggesting I purchase a second one. I want to thank Tony Scheuhammer for

his entertaining antics, including having Michael Kasserra and I over for Canadian Thanksgiving where he enlightened us with "why aliens are real" (unfortunately Michael and I fell asleep during this fascinating video lecture). I also want to thank numerous other Canadians who provided friendship and entertainment including Karen Timm and Michael Kasserra (who introduced me to poutine), Abde Idrissi, Kim Williams, Dave Noble, Masresha Asrat, Debbie Ashby, Craig and Karen Hebert, and Claire Carver. I particularly want to thank Karen Timm, who was the first person to point out to me that there was some chemistry between Laird Shutt and I, and Kim Williams who takes particularly good care of Laird and without whom I am sure Laird would not have made it this far.

One of the best things to happen to me in Canada was meeting Laird Shutt, who will soon officially be part of my family. Although Laird started out as a colleague, cleverly inviting me to coffee in order to talk about terns, he soon became my best friend, and later my fiancé, despite the 450 mile distance between Ottawa and Woods Hole. We'll see if you still "love to drive" once we are in the same place! I appreciate all your support and patience during this continually lengthening period of "writing the thesis". I especially thank you for spending your entire Christmas vacation (plus lots more) miserably working all night (every night) along with me, to get the final product together. It has been hard on both of us, but since we made it through this, we should be able to make it through anything. I am looking forward to life after the thesis, with you.

Finally, I want to thank all my family and friends back home in Minnesota as well as my Mom and family in Washington. My friend since eighth grade, Mona, has always been there for me, and I couldn't have made it without her visits, letters, and expensive phone calls. Also, thanks to Mona's family, especially Dale and Annette, for being my second family. Kathy, my Dad's wife, has been a great friend and support, and has also kept me informed. I especially want to thank my Gramma and Grampa who have always meant "home" to me. Finally, I want to thank my Dad, Tom Hart, to whom this thesis is dedicated. He has helped me so much in so many ways, and I love him dearly.

This work was supported by an Environmental Protection Agency (EPA) Graduate Student Fellowship (91478201), the National Oceanic and Atmospheric Administration (NOAA) National Sea Grant College Program Office (NA46RG0470, R/P-58), a Woods Hole Oceanographic Institution Independent Study Award, and the Woods Hole Oceanographic Institution Education Program.

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ABBREVIATIONS

AHH:	aromatic hydrocarbon hydroxylase
AhR:	aromatic hydrocarbon receptor
ANOVA:	analysis of the variance
BSA:	bovine serum albumin
CEH:	chick embryo hepatocyte bioassay
cpm/dpm:	counts/ disintegrations per minute
CYP:	cytochrome P450
DCC:	Dextran-coated charcoal
DDD:	dichlorodiphenyldichloroethane
DDE:	diphenyldichloroethylene
DDT:	dichlorodiphenyltrichloroethane
DES:	diethylstilbestrol
DMSO:	dimethyl sulfoxide
dpm:	disintegrations per minute
EC:	epithelial capsule
EC50:	concentration of substrate (or ligand) required to reach half-maximal activity (or binding)
EROD:	ethoxyresorufin-O-deethylase
ER:	estrogen receptor
EtOH:	ethanol
GC/MS:	gas chromatography/mass spectroscopy
GLEMEDS:	Great Lakes embryo mortality, edema, and deformities syndrome
GPC:	Gel permeation chromatography
HCB:	hexachlorobiphenyl

ABBREVIATIONS, continued

H&E:	hematoxylin and eosin stain
Hg:	mercury
HPLC:	high pressure liquid chromatography
IgG:	immunoglobulin G
Kd:	Dissociation constant
kD(a):	kilodalton
MAb:	monoclonal antibody
MIS:	Mullerian inhibiting substance
MFO:	mixed function oxidase
mRNA:	messenger RNA
NADPH:	nicotinamide adenine dinucleotide phosphate
NSB:	nonspecific binding
o,p'-:	ortho, para-
PAH:	polycyclic aromatic hydrocarbons
PB:	phenobarbital
PBS:	phosphate buffered saline
PCB:	plychlorinated byphenyl
PCDD:	polychlorinated dibenzo-p-dioxins
PCDF:	polychlorinated dibenzofurans
PGC:	primordial germ cell
PLHC:	*Poeciliopsis lucida* hepatoma carcinoma
p,p'-:	para, para-
ppm:	parts per million
PROD:	pentoxyresorufin-O-dealkylase

ABBREVIATIONS, continued

SDS:	sodium dodecyl sulfate
SE:	standard error of the mean
SB:	specific binding
ST:	seminiferous tubule
TB:	total binding
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF:	2,3,7,8-tetrachlorodibenzofuran
TCDD-EQ	TCDD equivalents
u	micro
uCi	microCurie

CHAPTER ONE: INTRODUCTION

CHAPTER ONE: INTRODUCTION

INTRODUCTION

The objective of this thesis is to examine the relationship between persistent organic contaminants and feminization in common terns (*Sterna hirundo*), as measured by presence of ovotestes. Concern about terns breeding on Bird Island in Buzzards Bay, Massachusetts, began when endangered roseate terns (*Sterna dougallii*) showed abnormal reproductive behavior. The roseate tern breeding population exhibited a sex ratio skewed toward females, female-female pairing, and supernormal clutches. These same phenomena were observed in Western gulls breeding on the coast of California in the 1970s (Hunt and Hunt 1977; Hunt, Wingfield et al. 1980). Michael Fry and coworkers found that male gulls injected with DDT at levels similar to those found in the California sites developed feminized testes (Fry and Toone 1981; Fry, Toone et al. 1987). They suggested that contaminant exposure leading to feminization of male gulls could affect reproductive function, behavior and possibly survival of males. The observed female-biased sex ratio could then result through increased male mortality or male self exclusion from the breeding colony. This link between Western gull feminization and DDT exposure suggests that a contaminant could be causing reproductive abnormalities in the Bird Island terns as well, and similarly affect the breeding population. Because roseate terns are an endangered species, common terns from Bird Island were studied as a surrogate species. In a preliminary sampling of male common tern embryos in 1993, 73% of the gonads examined (11/15) were feminized, with ovarian-like tissue present in the testes (Nisbet, Fry et al. 1996). These terns breed and feed near New Bedford Harbor, a Superfund site highly contaminated with PCBs and heavy metals, and high levels of PCBs often are found in

eggs and tissues of these birds (Nisbet and Reynolds 1984; Weaver 1984; Pruell, Norwood et al. 1990; Lake, McKinney et al. 1995). The focus of this study was to examine whether PCBs, their metabolites or other contaminants could be causing feminization in the Bird Island common terns.

BACKGROUND

Wildlife Toxicology

Exposure to chemicals in the environment

The widespread manufacture and use of synthetic organic chemicals began in the 1950's, resulting in the spread of these chemicals into the environment. Prior to the 1950's, high incidences of developmental abnormalities and dramatic population declines among wildlife populations were not observed (Colborn, vom Saal et al. 1993). Since the 1950's, chemical contamination of the environment has continued and spread to every corner of the globe. Billions of pounds of synthetic chemical compounds are produced annually for use in industry and agriculture. Portions of these chemicals are released deliberately and unintentionally into the environment, along with millions more pounds of chemicals that are unintended byproducts of the synthetic chemicals (Bason and Colborn 1992; Stegeman and Hahn 1994). Many of these compounds, such as chlorinated hydrocarbons, are very resistant to degradation, and thus persist in the environment and bioaccumulate in tissues of organisms. The abundance and persistence of these chemicals has resulted in their widespread distribution into all environments, particularly aquatic environments. Chemicals often are dumped directly into rivers, lakes and oceans, and also

are transported indirectly through hydrologic and atmospheric processes. The eventual transport of most chemicals to aquatic environments and the dependence of both wildlife and humans on these environments poses an important risk of exposure. In addition to the cancer risks posed by many of these chemicals, many persistent contaminants also have the potential to disrupt the endocrine systems of animals and humans (Colborn and Clement 1992). Exposure to endocrine disrupting pollutants during development can be detrimental to an organism, and if enough individuals are exposed, entire populations may be affected.

Endocrine effects of contaminant exposure in wildlife and humans

Many wildlife populations show developmental and reproductive abnormalities that likely are due to disruption of the endocrine system by chemical contaminants. For example, alligators living in Lake Apopka, Florida, the site of an extensive pesticide spill in 1980, have experienced developmental and gonadal abnormalities including small penises in males, altered levels of sex steroids, and population declines (Guillette Jr., Gross et al. 1994). Snapping turtles from Lake Ontario show increased deformities and decreased hatching success associated with the presence of PCBs (Bishop, Brooks et al. 1991). Masculinization of female mosquito fish in Eleven Mile Creek, Florida clearly is linked with effluent from kraft pulp mills (Davis and Bortone 1992). Declining populations of gastropods along marine coasts show development of male gonads in the females (imposex) which clearly is associated with organo-tin exposure from antifouling paints (Gibbs, Spencer et al. 1991).

In addition to effects in wildlife, more controversial relationships have been suggested between contaminant exposure and effects in humans. These include relationships such as increased breast cancer incidence associated with increased DDE levels (Wolff, Toniolo et al. 1993), decreases in sperm counts associated with exposure to

estrogenic compounds (Sharpe and Skakkebaek 1993), and developmental/behavioral effects in children associated with high levels of PCBs in mothers (Fein, Jacobson et al. 1984). Many specific cases of effects in humans also clearly are related to chemical exposures. These include residents of the Love Canal area who were exposed to high levels of dioxins and whose children were born with high incidences of birth defects (Francis 1994), as well as the Yusho and Yu-Cheng poisoning episodes in which mothers exposed to rice oil contaminated with PCBs gave birth to infants with a high incidence of developmental abnormalities (Hsu, Ma et al. 1985).

Effects of contaminant exposure in aquatic birds

Aquatic birds appear to be particularly vulnerable to effects from environmental contaminants, and thus are a good marker species for environmental quality and potential health risks to humans and wildlife. Dramatic population declines and collapses of Great Lakes fish-eating birds, including cormorants and bald eagles, were observed first in the 1950s and 60s when widespread environmental contamination first began. These declines were associated with severe egg shell thinning caused by DDE, a degradation product of DDT. This egg shell thinning resulted in poor hatchability and chick survival (Gilbertson, Kubiak et al. 1991; Giesy, Ludwig et al. 1994). DDT is no longer used in the U.S. or Canada, but despite the lessening of DDT contamination and eggshell thinning, reproductive impairment of the Great Lakes fish-eating birds continues among many species including cormorants, herons, bald eagles, gulls, and terns. These observed effects have been termed the Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) which is similar to "chick-edema disease", a disease found in poultry exposed to dioxins, polychlorinated dibenzofurans, and PCBs. Elevated environmental levels of dioxin-like compounds including planar PCBs, polychlorinated

dibenzo-*p*-dioxins, and dibenzofurans, are strongly implicated as the causative factors in GLEMEDS. Some of the characteristics of GLEMEDS affected bird populations include eggshell thinning, poor hatchability, embryo and chick mortality, growth retardation, edema and deformities. Deformities may include crossed bills, missing eyes, club feet, and duplications of feet at the joint of the femur and tibia, exencephaly, defective feathering, and spina bifida. In addition, breeding birds may show abnormal parental behavior associated with prolonged incubation periods, egg loss, and nest inattentiveness. At the physiological level there are indications of altered thyroid function, increased aryl hydrocarbon hydroxylase activity, altered vitamin A homeostasis, and altered heme biosyntheses (porphyria), as well as hepatomegaly, necrosis, and fatty degeneration of the liver in embryos (Gilbertson 1983; Gilbertson, Kubiak et al. 1991; Sanderson, Norstrom et al. 1994).

In addition to the GLEMEDS effects observed among aquatic birds, other abnormalities affecting populations also have been observed, particularly among gull and tern populations. Beginning in the late 1960s and early 1970s, nests have been observed containing supernormal clutches of 5 or 6 eggs instead of the normal 2 to 3 eggs. Prior to this time, supernormal clutches were a very rare phenomena (Harper 1971; Hunt and Hunt 1973; Fox 1992). The supernormal clutches are due to multiple females engaging in a shared breeding attempt, where the female-female pairings are likely a behavioral response to a female-biased operational sex ratio. A female-biased sex ratio can result from range expansion and colonization because females tend to disperse from their natal breeding site more readily than males (Greenwood 1980); however, the populations of concern in the cases cited were in decline. A sex ratio biased towards females also could result from high differential male mortality and/or possibly embryonic feminization as a result of local environmental factors (Fry 1995). Contaminant related embryonic feminization of males might result in suppression of sexual behavior and self-exclusion from the breeding

colony, biasing the sex ratio toward females (Fry 1995). Regularly occurring adult mortality with most of the dead being males has been observed in some contaminated colonies, suggesting that males could be more susceptible to some contaminant related effects (Hunt and Hunt 1977; Fry and Toone 1981; Fry, Toone et al. 1987; Fox 1992). A strong correlation exists between high contaminant levels in breeding colonies and the observations of a female-biased sex ratio, female-female pairing, supernormal clutches, and increased male mortality, suggesting that contaminants affect male reproductive capabilities (Fry, Toone et al. 1987).

In a specific case, a declining population of gulls on Santa Barbara Island, California, showed abnormal breeding behavior, female-female pairing, and supernormal clutches. These gulls also were exposed to high levels of DDT, and therefore egg injection studies were carried out with DDT levels similar to those found at the Californian site. These studies showed that embryonic exposure to DDT metabolites, estradiol, and methoxychlor could result in feminization of male gull hatchlings, which included development of ovotestes and oviducts. It was proposed that the Californian male gulls were feminized by exposure to DDT metabolites acting as estrogens, and that these males were unable to breed, possessed abnormal behavior, and had an increased incidence of mortality (Fry and Toone 1981; Fry, Toone et al. 1987).

Data from tern colonies in Europe and North America suggests that terns are sensitive to contaminant related effects. Decreased hatching success of common terns (*Sterna hirundo*) in the Elbe estuary, Germany, was correlated with elevated levels of PCBs in eggs (Becker, Schuhmann et al. 1993). Common tern colonies in the Netherlands have shown several effects correlating to increasing levels of PCBs and "TCDD equivalents" (defined later). These effects included delayed egg laying, prolonged incubation periods, smaller chicks and eggs, and elevated hepatic ethoxyresorufin-O-deethylase (EROD) and pentoxyresoufin-O-dealkylase (PROD) activites (Murk, Boudewijn

et al. 1996). These elevated contaminant levels or EROD or PROD activities also were associated with increased plasma thyroid hormones, hepatic T4-glucuronyltransferase activity, and plasma retinol and yolk sac retinoid levels (Murk, Bosveld et al. 1994). In North America, declining populations of both common and Forster's terns (*Sterna forsteri*) are found on the Great Lakes. Common terns from industrialized areas of the Great Lakes showed elevated PCB concentrations, decreased hatching success, developmental anomalies, decreased femur-length-to-body-weight ratios, and elevated hepatic microsomal aryl hydrocarbon hydroxylase (AHH) activities compared to common terns from less contaminated Great Lakes sites (Hoffman, Smith et al. 1993). Forster's terns from contaminated Green Bay on Lake Michigan showed elevated levels of PCBs and TCDD-equivalents, which were related to impaired reproductive output. Hatchability of eggs was greatly decreased at the Green Bay site, and hatchlings had decreased weights, and increased liver weight to body weight ratios. Decreased reproductive success also may have been related to contaminant effects on adults that decreased parental attentiveness and resulted in nest abandonment and egg disappearance (Kubiak, Harris et al. 1989). Among Caspian terns (*Hydroprogne caspia*) in the Great Lakes, decreased hatching and increased deformity rates also were correlated with increased concentrations of PCBs and TCDD-equivalents (Ludwig, Kurita-Matsuba et al. 1996). These data suggest that terns are aquatic bird species that may be experiencing impaired reproductive success related to contaminant exposure.

Avian Biology

General ecology

The common and roseate terns breeding on Bird Island in Buzzards Bay Massachusetts overwinter in South America and arrive at Bird Island to breed from May through July. The common terns generally lay a single clutch of 2-3 eggs, while the roseate terns lay a clutch of 2 eggs and often are more successful at fledging their young. On Bird Island, fledging success is usually food-limited, although weather and owl predation may play a role. The common terns feed shallowly within a range of about 15 km and consume mainly Atlantic silversides (*Menidia menidia*), herring (*Clupea harengus*), sand lance (*Ammodytes americanus*) , and crustaceans (various species). The roseate terns forage deeper in the water column and travel further, ranging up to 30 km, and consume mainly herring and sandlance (Nisbet 1978; Nisbet and Reynolds 1984; Nisbet 1997).

Birds are capable of quickly depositing and mobilizing large amounts of fat necessary for both migration and egg-laying. Common terns migrate to South America over winter, leaving in September to November with fat deposits acquired in their summer feeding grounds. By the time they reach their wintering grounds, they have flown thousands of kilometers and utilized nearly all of their fat reserves. Over the winter, they acquire the necessary reserves for their spring migration to the breeding grounds, and upon arrival in May, they again have utilized nearly all of their reserves. Thus, prior to egg laying, they must acquire the necessary energy and lipids from food near their breeding grounds (Burger, Nisbet et al. 1992; Gill 1995).

Female terns mobilize their newly replenished lipid reservoirs during egg laying and deposit most of their contaminant burden into eggs along with the lipids. Since terns are migrating birds, contaminants in their eggs could reflect their wintering and migratory

grounds as well as their breeding areas. Bird Island terns generally have contaminant burdens in their eggs that reflect those of prey in their feeding grounds (Nisbet and Reynolds 1984), likely because the wintering grounds are much less contaminated. This is evidenced by studies showing lower levels of heavy metals acquired at the wintering grounds than at Bird Island during the breeding season. Levels of other contaminants at the winter and breeding grounds have not been compared (Nisbet and Reynolds 1984; Burger, Nisbet et al. 1992). A lowered contribution of contaminants from wintering grounds may also occur because part of the contaminant burden acquired over the winter may be metabolized and excreted as it is mobilized along with lipid reservoirs during migration.

During the egg laying period, the female terns reduce their food intake and rapidly deplete their lipid reserves, laying eggs with increasing contaminant burdens. This suggests that the first egg incorporates proportionally more lipids that have been recently ingested in food, and therefore has lower organochlorine contaminant levels. However, later laid eggs likely require the female to draw on lipid reserves in her body that have higher concentrations of contaminants (Nisbet 1982). Although there are differences in contaminant burdens in consecutive eggs within a clutch, there is generally a much greater difference in contaminant burdens among clutches within a breeding colony (Custer, Pendleton et al. 1990; Custer, Custer et al. 1997). Egg contaminant burdens reflect the diet of the laying female (Nisbet 1982), and so can differ with the female's feeding grounds. There is a range in contamination of feeding grounds within 10-30 km of Bird Island, which can lead to wide variations in contaminant burdens of eggs among clutches.

Fish-eating aquatic bird populations, including the tern populations examined in this study, are particularly susceptible to contaminant effects as a result of several factors. First, their habitat and feeding grounds often include highly polluted waters, and their position as top predators in the food chain allows significant bioaccumulation of lipophilic

contaminants. Bird Island terns are particularly vulnerable to contaminant exposure since they feed grounds near New Bedford Harbor, a Superfund site highly contaminated with PCBs and heavy metals. Extremely high contaminant concentrations have been measured in eggs and tissues of Bird Island terns, with some adults clearly dying from PCB poisoning as diagnosed by elevated brain PCB levels (Nisbet and Reynolds 1984; Weaver 1984; Pruell, Norwood et al. 1990; Lake, McKinney et al. 1995).

Embryonic exposure is another factor increasing susceptibility of piscivorous birds to contaminant effects. High concentrations of contaminants bioaccumulated in female lipids are transferred to the yolk during egg formation, thus exposing the developing embryo to elevated contaminant levels. Developing embryos can be greatly affected by contaminant exposure, and birds may be particularly susceptible for several reasons. In the last half of development the embryonic liver has active mixed function oxidases which are inducible and metabolize contaminants. Metabolism generally makes contaminants more water soluble and excretable; however, in the egg these water soluble compounds remain in circulation. This occurs because water is recycled within the egg, while nitrogenous wastes are sequestered as semi-solid urates in the allantois. Thus, exposure to potentially toxic or hormonally active metabolites can occur during development. Embryonic contaminant exposure can be very high since most of the female's contaminant burden will be incorporated into her eggs, and effects in the developing embryo can be severe, resulting in mortality, impaired development, or reduced reproductive capacity (Fry 1995).

Avian Sexual Differentiation

Sexual Differentiation - Genetic versus hormonal

In both birds and mammals, sexual differentiation of the gonad is linked to the heterogametic sex, while hormones produced by the developing gonads control differentiation of the accessory and secondary sex characteristics. In mammals, males are the heterogametic sex (XY sex chromosomes), and a gene on the Y chromosome contains a testis-determining factor causing the gonad to develop into the male testis. Hormones produced by the testis, testosterone and Mullerian inhibiting substance (MIS), result in development of the Wolffian ducts into vas deferens and regression of the Mullerian ducts. Gonadal testosterone production also results in male development of the phallus, the brain, and other male characteristics. In birds, the female is the heterogametic sex (ZW) and the presence of the W chromosome results in development of the undifferentiated gonad into an ovary. However, a gene homologous to that containing a testis-determining factor in mammals has not yet been found in birds. The avian ovary produces estrogens, which allows development of the Mullerian ducts into oviducts, as well as female development of the genitals, brain, and other secondary sex characteristics. The homogametic sex, female (XX) in mammals and males (ZZ) in birds, is the default sex, and without hormonal influences, the embryo will develop into the homogametic sex. Thus, in mammals, a female will develop unless the Y chromosome is present to determine testis formation and production of testosterone and other hormones produced by the testis. In birds, the male will develop unless the W chromosome is present to determine ovarian development and production of estrogen (Ottinger, Adkins-Regan et al. 1984; Wachtel, Wachtel et al. 1991; Behringer, Finegold et al. 1994; Fry 1995; Yoshida, Shimada et al. 1996; Andrews, Smith et al. 1997).

In addition to the fact that males are heterogametic in mammals and females are heterogametic in birds, several other differences occur in sexual differentiation between mammals and birds. First, the mammalian Y chromosome is strongly male-determining, but the avian W chromosome is not as strongly female-determining. Thus, mammalian XXY embryos become males, but triploid ZZW avian embryos are intersexes. Second, in mammals the development of the ovary or testis is genetically determined, while secondary sexual differentiation is under hormonal control. Influence of hormones during development normally will not convert an ovary to a testis, or a testis to an ovary in mammals. In birds, however, embryonic gonads can be sex-reversed with exogenous hormones. For example, testis can develop into ovotestes when exposed to estrogens, and a loss of estrogen production in the ovary can result in development of a functioning testis. Third, unlike mammals, the gonads of most birds develop asymmetrically. In the female, the right gonad and oviduct regresses and only the left ovary and oviduct is functional. In most species the right gonad has more potential to develop into a testis than into an ovary, and if the left ovary is removed, the right residual gonad will develop into a testis (Wachtel, Wachtel et al. 1991).

General description and Control of avian gonadal differentiation

The development of the gonad into a testis or an ovary occurs through development of either the cortical (female) or medullary (male) gonadal tissue, and the degeneration of the other. In most bird species, the differentiating right gonad is made up almost entirely of the medullary tissue and has very little potential to develop cortical tissue. This corresponds to the ability of the right gonad to develop into a testis in males but to degenerate in most female species. Thus, in most avian species the differentiated gonads of

females consist of only the left ovary, while those of males consist of both the left and right testes. Similarly only the left Mullerian duct in the females develops into an oviduct, while both the right and left Wolffian ducts in the males develop into vas deferens. The left ovary in the female is larger and flatter than the testis, and the surface of the ovary is wavy and granular. The ovary contains a large number of cortical sex cords containing the primordial germ cells, which differentiate into the oogonia. The cortical sex cords and the primordial germ cells of the ovary develop in the cortical area of the undifferentiated gonad, while the medullary area degenerates. The testes in the male are both of similar size (or the left may be larger), are oval shaped, and are surrounded by an epithelial capsule giving a smooth appearance. Within the testes are seminiferous tubules containing the primordial germ cells, which differentiate into the spermatagonia. The seminiferous tubules and primordial germ cells of the testes develop in the medullary area of the undifferentiated gonad, while the cortical area degenerates (Romanoff 1960; Wachtel, Wachtel et al. 1991).

In birds, control of gonadal differentiation is influenced by hormones. The production of estrogens in the gonad by P450 aromatase (which converts androgens to estrogens) is important for female sexual differentiation. While both male and female gonads produce androgens during the period of sexual differentiation, the female gonad produces much higher levels of estrogens. This difference in estrogen production correlates with the presence of P450 aromatase in the medullary area of the female gonad, and very little or no P450 aromatase in the male gonad (Yoshida, Shimada et al. 1996; Andrews, Smith et al. 1997; Smith, Andrews et al. 1997). During the period of sexual differentiation, P450 17α -hydroxylase (which converts gestagens to androgens) is present at higher levels in the female than the male. This may correspond to the need for increased androgen substrate for conversion to estrogen by P450 aromatase in the female. In support of this idea, after gonadal differentiation, P450 17α–hydroxylase increases in the male but becomes lower in the female (Yoshida, Shimada et al. 1996). Studies with aromatase

inhibitors provide further evidence that P450 aromatase and estrogens play a role in female gonadal differentiation (Elbrecht and Smith 1992; Wartenberg, Lenz et al. 1992). Chickens exposed to aromatase inhibitors developed into phenotypic males with testes. When the female chickens were exposed to both aromatase inhibitors and estrogens, they were able to develop into phenotypic females with ovaries (Elbrecht and Smith 1992). This study strongly suggests that production of estrogens by P450 aromatase is necessary for avian gonadal differentiation and further female development.

The responsiveness of the gonads and other sexually differentiating organs to estrogen is also important in sexual differentiation. The presence of estrogen receptor in target tissues can indicate estrogen responsiveness. In differentiating female gonads, estrogen receptor was found in the medullary area of both the left and right gonad, but was greater in the cortical area of the left gonad compared to the right. This decreased espression of estrogen receptor in the right ovary is consistent with the lack of cortical differentiation in the right ovary (Gasc 1980; Andrews, Smith et al. 1997). In differentiating male gonads, the location (both the medullary areas and the left cortical area) and levels of estrogen receptor were similar to that of females as measured by messenger RNA levels and incorporation of radiolabled estrogen (Gasc 1980; Andrews, Smith et al. 1997). Estrogen receptor protein was not found in male gonads in one study (Andrews, Smith et al. 1997); however, this is not consistent with the ability of radiolabled estrogen to be incorporated into cell nuclei in male gonads (Gasc 1980). Furthermore, the ability of male gonads to be feminized (develop ovotestes) in response to estrogens suggests that estrogen receptor proteinss are located in the testis (Gasc 1980; Andrews, Smith et al. 1997). Studies of estrogen receptors by measuring incorporation of radiolabled estrogen suggested that their location is different in males and females after gonadal sexual differentiation. These studies showed that during sexual differentiation in chickens (day 5.5-7 of incubation), males and females both showed estrogen targets in medullary and left

cortical areas; however, by day 10 (post gonadal differentiation) only the left cortical area of male gonads showed incorporation of estrogen (Gasc 1980).

Detailed description of avian gonadal development

The first stage of gonadal development in the avian embryo shows no morphological distinction between the sexes. This stage lasts until the time of gonadal differentiation, which occurs in chickens from day 5.5-6.5 of their 21 day incubation period. During the first days of embryonic development, primordial germ cells appear and migrate to the area of the urogenital ridge and the future gonad. In the chicken embryo this migration is completed by day 4-5, and in almost all species more primordial germ cells are located on the left developing gonad. Also during this stage, the primary sex cords proliferate from the germinal epithelium, forming straight cords containing primordial germ cells. These cords fill most of the gonadal area, and their growth and proliferation contribute to the enlargement of the gonad. These primary sex cords eventually develop into the seminiferous tubules in the male. In the female, they are the medullary cords, which will degenerate and become nonfunctional (Swift 1914; Romanoff 1960; Meyer 1964) .

After the sexually indifferent stage, embryonic development of the gonad continues according to the sex. In the left gonad of the female, the germinal epithelium (from which the primary sex cords have proliferated) is several cell layers thick, forming a cortical layer in which primordial germ cells are evenly distributed. The primordial germ cells also begin pre-meiotic division to form oogonia. From this cortical layer, a second proliferation of sex cords containing primordial germ cells forms. These cortical cords proliferate, while the epithelial layer reduces to a single layer of cuboidal cells. The characteristic large cortical area of the female is then formed, and is made up of the secondary sex cords and

primordial germ cells. The primary sex cords still are located in the medullary area of the developing ovary, but they transform to distended tubules, isolated cord cells, degenerating cells, and isolated clusters of primordial germ cells. The right female gonad in most species is very reduced in size due to lack of cortical tissue. Because this gonad lacks the capability to develop a cortical area, it is unable to differentiate into an ovary and thus degenerates. Small right ovaries can occur occasionally in some species including the herring gull, pigeon, and chickens. In some species, such as hawks, the right ovary has the potential to develop a cortical area and, therefore, is able to develop into an ovary equivalent in size to the left ovary (Swift 1915; Stanley 1937; Lewis 1946; Romanoff 1960; Taber 1964) .

In the males, the testes are developed mainly from the medullary area of the differentiating gonad, and therefore both the right and left gonads can develop into a functional testis. The primary or medullary sex cords containing primordial germ cells develop into seminiferous tubules in the male, unlike in the females where they largely degenerate. The straight cords first become wavy, and are convoluted by day 11 in chickens. By hatching, the seminiferous tubules lined with primordial germ cells are massive, resulting in the increased size of the testes. Cells separating the cortical germinal epithelial layer and the inner seminiferous tubule mass proliferate to form the tunica albuginea, which becomes the fibroblastic capsule encasing each testis in the adult. The cortical germinal epithelium, which is several layers thick and forms the cortical layer of sex cords in the females, becomes thinner and flatter and no longer proliferates sex cords in the males. However, in the left testis this cortical layer containing primordial germ cells may persist for a time, and may give the left testis the appearance of an ovotestis. Normally this is a transient structure present during development of most bird species including the chicken, robin, sparrow, tern, and duck. In chicken embryos it usually persists until 14 to 10 days before hatching, in pheasants until 12 to 7 days before

hatching, and in pigeons until 2-1 days before hatching. In other species including quail, ducks, ring doves, and hawks it may persist until hatching and sometimes longer. The presence of this cortical epithelial layer represents a potential ovarian cortex, and can be stimulated during development with hormones to proliferate secondary cortical sex cords which will develop into an ovarian cortex (Laulanie 1886; Swift 1916; Stanley 1937; Riddle and Dunham 1942; Lahr and Riddle 1945; Lewis 1946; Romanoff 1960; Taber 1964; Haffen, Scheib et al. 1975).

Avian development of accessory sex organs

In addition to the differentiation of the gonads, hormonal control is important in differentiation of the accessory organs. These organs include the ducts that are essential for carrying the germ cells from the gonads out of the body. During early embryonic stages, both the Wolffian and Mullerian ducts are present in both sexes. In the males, the Wolffian ducts persist and develop into the vas deferens, while the Mullerian ducts degenerate. Degeneration of the Mullerian ducts in males is largely completed by about day 9-13 of incubation in chickens. In the females, the Wolffian ducts persist only as a vestigial structure, while the Mullerian ducts develop. In females of most species only the left Mullerian duct develops into a functioning oviduct while the right oviduct stops developing and regresses to just a vestigial duct (Gaarenstroom 1939; Romanoff 1960; Taber 1964).

Both Mullerian inhibiting substance (MIS) and estrogens appear to be involved in differentiation of the Mullerian ducts, although the mechanisms are unclear. While MIS is only secreted from the male testis in embryonic mammals, it is secreted from both the male and female gonads in avian embryos. It has been suggested that MIS will cause regression of Mullerian ducts in both males and females, unless activity is inhibited by the action of estrogens. Furthermore the action of estrogens may be modulated by androgens

(MacLaughlin, Hutson et al. 1983). Estrogen receptor has been found in both the left and right Mullerian duct of males and females, suggesting that both ducts are responsive to estrogens (MacLaughlin, Hutson et al. 1983; Andrews, Smith et al. 1997). This is supported by studies showing that estrogen administered at low levels during development results in preservation of these ducts in males, and at higher levels estrogen results in preservation of the right duct in females (MacLaughlin, Hutson et al. 1983). Some differences in estrogen receptor types or levels may be present between the left and right duct of both males and females, which may account for the regression of the right duct and preservation of the left duct in females (MacLaughlin, Hutson et al. 1983).

Avian development of secondary sex characteristics

In addition to accessory sex organs, secondary sex characteristics also may be under hormonal control, although sexually dimorphic characteristics may vary widely with bird species. Estrogen receptor targets have been found in the genital tubercle, the cloacal area, the bursa of Fabricius, and areas of the brain suggesting that these organs can respond to estrogen (Martinez-Vargas, Gibson et al. 1975; Gasc 1980; MacLaughlin, Hutson et al. 1983; Andrews, Smith et al. 1997). Development of the penis, which is initially present in both sexes as a genital tubercle, is affected by hormones. The genital tubercle retrogresses in the female with exposure to estrogens and further develops to form the penis in males of some species, particularly those in the order Anseriformes (Romanoff 1960; Taber 1964). In birds, another accessory organ affected by sex hormones is the syrinx, or voice organ, which is an enlargement at the bifurcation of the bronchi from the trachea. The syrinx is quite large and asymmetrical in the male of some species, and smaller and symmetrical in the female. Estrogens in the developing female prevent development of the male form of the syrinx (Romanoff 1960; Taber 1964). Other sex

differences, such as feather characteristics, also may be present, and may be under a variety of hormonal and genetic control. Some bird species show no sexual dimorphism in plumage, others show sexual dimorphism only during the breeding season, and still others show permanent sexual dimorphism. In species with permanently sexually dimorphic plumage, the differences are usually under hormonal control where the male form is suppressed by female estrogens. Other sex differences may include bill coloring, leg and feet coloring, body size, presence of combs or wattles, and behavioral characteristics. Some of these characteristics may be controlled by estrogens and others by androgens, and may be differently controlled among different bird species (Noble and Wurm 1940; Taber 1964).

Avian behavioral sexual differentiation

In addition to developing functioning gonads and accessory sex organs, sexually dimorphic behaviors are also important for successful sexual reproduction. These include male and female copulatory behaviors, which are necessary for successful fertilization of eggs. Other mating behaviors such as courtship displays or songs may be required for attracting a mate. Development of parental behaviors also may be necessary for successful raising of young. Nest attentiveness and feeding behaviors during incubation and chick raising may be very important.

In birds, like in mammals, gonadal hormone secretions of the heterogametic sex are important for organizing sexually dimorphic reproductive behaviors. In birds, development of copulatory behavior parallels gonadal development. The homogametic sex is the neutral sex and develops unless organizing hormones produced by the gonad of the heterogametic sex are present. In mammals, where the male is heterogametic, testosterone produced by the testis tends to masculinize and defeminize copulatory behavior. In birds,

where the female is the heterogametic sex, estrogen production by the ovaries tends to feminize and demasculinize copulatory behavior. It is actually estrogen that is responsible for sexual differentiation of copulatory behavior in both mammals and birds, because testosterone must be converted to estrogen by P450 aromatase in the brain of mammals in order to have a masculinizing effect. Thus, in both mammals and birds testosterone or estrogen exposure will result in development of copulatory behavior corresponding to the heterogametic sex (Adkins 1978; Adkins 1979; Ottinger, Adkins-Regan et al. 1984).

Sexual differentiation of the copulatory behavior occurs in the hypothalamus of the brain during a critical period. In both chicken (21 day incubation period) and quail (16-18 day incubation period) the critical period of development for copulatory behavior occurs prior to day 13 of incubation (Wilson and Glick 1970; Adkins 1979). There is evidence in pigeons, a less precocious bird species, that the critical period for this behavior may be early posthatching (Orcutt 1971). Exposure to estrogens or higher doses of aromatizable androgens affects hypothalamic differentiation, resulting in feminization and demasculinization of behaviors (Adkins 1979). The hypothalamus of the brain is responsive to estrogens during this period as suggested by the presence of estrogen receptors by the day 10 of incubation in the chicken embryo (Martinez-Vargas, Gibson et al. 1975). Exposure of female birds to antiestrogens during this critical period prevents feminization of behavior and results in masculinization of behavior. This further supports the necessity of estrogen action for feminization and demasculinization of behavior in birds (Adkins 1976).

In male passerine song birds, such as the zebra finch, there is an exception to the normal avian pattern of sexually dimorphic brain and reproductive behavior development. As just described, estrogen (or aromatizable androgen) normally results in demasculinization and feminization of reproductive behavior. However, in song birds, the action of estrogen on the brain is essential for masculine development and expression of

song. Male finches are able to sing a courtship song that females lack. Males also have a much larger and more developed telencephalon, the brain region that controls song, compared to females. The critical period for masculinization of song occurs from 4-7 days posthatch in zebra finches, and females exposed to estrogen during this critical period can develop male song patterns. Estrogen appears to be produced locally in brain regions of males by P450 aromatase, allowing masculinization of song differentiation. However, females also possess aromatizable androgens and P450 aromatase in song controlling brain regions, so it is unclear why female song patterns are not masculinized (Gurney and Konishi 1980; Schlinger and Arnold 1991; Schlinger and Arnold 1992; Schlinger and Arnold 1992).

Effects of hormonal exposure on sexual differentiation

Numerous studies have examined the effects of hormone exposure on the development of sexual organs in the avian embryo. Importantly, without hormone exposure, the neutral or default development is that of the male, the homogametic sex in birds. Thus, with no hormones the gonads develop into testes, and the genital tubercle and syrinx assume the male form. Unlike a typical male, however, the Mullerian ducts persist since the testes are not producing MIS, and secondary sex characteristics requiring androgens for expression will not appear (Romanoff 1960; Taber 1964; Ottinger, Adkins-Regan et al. 1984; Wachtel, Wachtel et al. 1991).

Exposure to Estrogens

Exposure to female hormones, or estrogens, has been widely examined. Estrogens generally have little effect in the female, but high exposure to estrogens can result in hyperfeminization. Effects can include enlargement of the cortical area of the left and/or

right ovary with increased proliferation of primordial germ cells and oogonia. Effects also can include over development of the left and/or right oviducts along with retention of the right oviduct (Boss and Witschi 1947; Gaarenstroom 1939; Lewis 1946; Romanoff 1960; Snedecor 1949; Willier, Gallagher et al. 1937).

In the males, exposure to female hormones has strong feminizing effects on the reproductive tract. The left testis may develop into an ovotestis, which is characterized by an ovarian-like cortex of varying thickness over a testicular medulla. Macroscopically, the testis may appear flattened and granular like a normal ovary, it may have only small areas or ridges of cortex visible, or it may have the appearance of a normal testis until examined histologically. In addition to the development of ovotestes, male exposure to estrogens often results in persistence of the Mullerian ducts, particularly the left duct. The Mullerian ducts may partially or completely develop into oviducts, or show cyst-like developments. The feminizing effects in the males exposed to estrogens have been observed with all bird species examined, including chickens, quails, ducks, doves, pigeons, and gulls (Kozelka and Gallagher 1934; Gaarenstroom 1937; Willier, Gallagher et al. 1937; Domm 1939; Gaarenstroom 1939; Danforth 1942; Riddle and Dunham 1942; Lewis 1946; Boss and Witschi 1947; Snedecor 1949; Romanoff 1960; Pincus and Erickson 1962; Taber 1964; Asayama 1965; Miyamori 1966; Haffen, Scheib et al. 1975; Fry and Toone 1981).

Exposure to Androgens

There are few effects of exposure to male hormones on the developing reproductive tract of male and female birds, except at very high doses. In females, exposure to high levels of androgens may have a weak masculinizing effect. Even with exposure to masculinizing hormones, the development of ovarian cortex still occurs in females. However, the regression of the ovarian medulla is prevented, and with very high androgen doses, testicular cords can develop. Androgens can alter the normal development of

oviducts in females, and even can cause regression of the Mullerian ducts in some cases. Also, the normally reduced Wolffian ducts in females can be induced to develop with androgens (Willier, Gallagher et al. 1935; Willier 1937; Gaarenstroom 1939; Romanoff 1960; Scheib 1983).

In males, the effect of exposure to androgens in some cases is not hypermasculinization, but rather feminization, although effects are much weaker than those from estrogens. Feminizing effects are similar to those described for estrogen exposure including development of an ovarian cortex in the left testis, or ovotestis, and the development of Mullerian ducts. The reason for this is not known; however, it may be due to conversion of androgens to estrogens by very low levels of P450 aromatase in males, resulting in circulating estrogens. In addition to feminizing effects, the Wolffian ducts may hypertrophy with androgen exposure (Willier, Gallagher et al. 1935; Willier 1937; Gaarenstroom 1939; Romanoff 1960; Scheib 1983).

Method of Exposure

The hormone exposure studies raise several important points concerning method of exposure, timing and period of exposure, dose response relationships, and the persistence of the effects. Similar effects have been seen in studies using different methods of exposure, including injection of eggs into either the air cell, albumen, or yolk, dipping of shelled eggs into hormone solutions, and injection of the mother bird prior to egg laying (Kozelka and Gallagher 1934; Willier, Gallagher et al. 1935; Gaarenstroom 1937; Willier 1937; Willier, Gallagher et al. 1937; Domm 1939; Gaarenstroom 1939; Danforth 1942; Riddle and Dunham 1942; Lewis 1946; Boss and Witschi 1947; Snedecor 1949; Romanoff 1960; Pincus and Erickson 1962; Asayama 1965; Miyamori 1966; Fry and Toone 1981). Riddle and Dunham injected laying female ring doves intramuscularly with estrogens dissolved in sesame oil. They found that eggs from these injected females produced

feminized male chicks. Almost all the males examined between ages 6 and 26 days after hatching had a left ovotestes and some oviduct development (Riddle and Dunham 1942). Similarly, in other studies where laying female chickens were injected with estrogens, male hatchlings were feminized (Taber 1964). This suggests that hormonal substances from laying female birds can be transferred to the yolk of the developing egg and result in exposure of the avian embryo during development. This supports the idea that in wildlife, hormone-like environmental chemicals can be transferred from the mother to the egg, resulting in exposure of avian embryos.

Timing of Exposure

The timing of hormonal dose is important for determining the degree of effect on the sex organs and secondary sexual characteristics of the developing embryo. In general, there is increased sensitivity of tissues and organs to chemical substances closer to the time of morphological differentiation (Pincus and Erickson 1962; vom Saal, Montano et al. 1992). In chickens, hormone exposure beginning before the time of gonadal sexual differentiation (before the seventh day of incubation) appears to produce the greatest effects on gonads. Furthermore, when comparing exposure from day 0 to day 7 of incubation in chickens, increased sensitivity to the influence of hormone-like substances occurs closest to the time of gonadal sexual differentiation (days 4.5-7) (Gaarenstroom 1937; Lewis 1946; Lewis and Domm 1946; Snedecor 1949; Pincus and Erickson 1962). As discussed earlier, during a particular time period of development in the male, the left gonad shows development of ovarian-like cortical areas even without external hormonal influence. Particularly in bird species with late persisting ovarian cortical areas, the presence of these areas may lengthen the time this potential cortex is susceptible to further stimulation when exposed to estrogens (Romanoff 1960; Haffen, Scheib et al. 1975; Wachtel, Wachtel et al. 1991). Differentiation of Mullerian ducts, sexually dimorphic areas of the brain, and other

sexually dimorphic characteristics are also most sensitive to effects close to and before their time of differentiation (Gaarenstroom 1939; Boss and Witschi 1947; Adkins 1979).

When the developing embryo is first exposed to a hormone during a critical period of development, and then exposed to multiple doses, either before or after hatching, the effects appear to be increased and more persistent (Lewis 1946; Boss and Witschi 1947). In wildlife, the developing embryo potentially could be exposed to hormonally active chemicals more than once or even continuously as the yolk sac is utilized. In its first few days post hatch the chick could experience exposure to a large dose as the remaining yolk sac is absorbed. Both timing and period of exposure might depend on the amount and location of chemicals in the yolk, and on how absorption of chemicals from the yolk occurred during embryonic development.

Dose-Response Relationship

In most studies males show increased feminization with increasing dose of estrogenic compound. In general, lower doses of estrogens result in development of ovarian cortical tissue in the testes; intermediate doses result in further ovotestes development and persistent oviducts, particularly the left oviduct; highest doses result in even further development of ovotestes that may appear ovarian macroscopically, as well as further hypertrophy and swelling of oviducts (Willier, Gallagher et al. 1937; Pincus and Erickson 1962). However, even though definite dose response relationships are observed, individual birds show a wide range of responses to the same dose, ranging from the slightest to most extreme responses (Willier, Gallagher et al. 1937; Gaarenstroom 1939; Lewis 1946; Snedecor 1949; Miyamori 1966; Fry and Toone 1981). This variation in response may be due to individual differences in response to the dose and/or differences in timing and amount of dose reaching the embryo (Willier, Gallagher et al. 1937). In injection or dipping studies, as in the natural egg, hormones or chemicals in the yolk may

be absorbed or incorporated into the embryo at different times and in different amounts. This might depend on how and where the chemical is incorporated into the egg, as well as differences among embryos in utilization of the yolk during development. Thus the same amount of chemical in the egg may not result in the same exposure of the embryo to the chemical.

Persistence of Effects

The persistence of feminization in males has varied with different studies and among different species. The ovotesticular gonads of feminized male chickens have been shown to lose cortical areas and revert back to functioning testes in several studies. The time period for chicken ovotestes to revert back to testes varied from 12 weeks to 9 months, although reversion can begin very soon after hatching (Snedecor 1949; Pincus and Erickson 1962; Taber 1964; Scheib 1983). Along with the gonads, feminized secondary sexual characteristics such as plumage and comb growth also reverted back to the male forms, although Mullerian ducts tended to persist. Other studies of feminized male chickens suggested that ovotestes and other feminized characteristics persisted for over two years (Domm 1939; Snedecor 1949; Taber 1964). Two to three year old male chickens with persistent feminization had a left ovotestes with an ovarian cortex and testicular tubules containing sperm underneath. In one case the cortex showed scattered follicles containing normal ova with yolk sac granules, although in most cases the cortical area was not so developed. Oviducts were present in most of these birds, and plumage and head furnishings also appeared female. The copulatory behavior of these males was feminized, ranging from neutral or inactive behavior to normal masculine behavior. Although some fertile copulations occurred, many were infertile (Domm 1939; Domm 1940; Domm and Davis 1941).

In species other than chickens, feminization of males has tended to be more persistent. Feminization of gonads in quail tend to be more advanced and persistent than chickens, but will eventually revert back to testes (Haffen, Scheib et al. 1975). Turkeys treated prior to sexual differentiation and killed 26-46 weeks posthatching all showed feminization of gonads. Left testes were ovotestes or extremely ovarian, and right testes were small or almost nonexistent (Taber 1964). Dove embryos exposed through estrogens in the mothers' lipids showed ovotestes persisting until approximately 30 days posthatch, but by 2-7 months posthatch no ovotestes were observed, although the remaining sample size consisted of only two birds (Riddle and Dunham 1942). Studies in gulls showed persistence of ovotestes up to 4 years, but these birds were injected during day 6 of incubation and then after hatching were given maintenance injections weekly for up to two years. The total amount of hormone (diethylstilbestrol) injected into the bird over the two years was about 500 ug, and even with no dosing from 2-4 years of age the 4-year-old birds still had distinctive ovotestes (Boss and Witschi 1947). In all species, oviducts that had developed in hormone exposed males persisted after hatch and did not appear to regress over time.

Although estrogen dosed females usually appear normal, some effects become apparent once the birds are reproductively mature. In nine month old female chickens dosed with estrogens, only 2/9 females laid eggs normally, while the rest produced small and yolkless eggs. Ova had been released, but apparently could not get into the oviduct, indicating that the oviducts were malformed (Snedecor 1949). Similar effects were observed in another case of estrogen dosed female chickens. Females showed normal plumage, head furnishings, and behavior, but began laying eggs later than normal. Some eggs were abnormal; small, shelless, or yolkless. Yolks were found in the abdominal cavity, and some had right oviduct development and distended oviducts. This study also

suggested that estrogen exposure resulted in malformation of oviducts in the females, and could result in permanent effects (Domm 1940; Domm and Davis 1941).

Effects of xenobiotic chemical exposure on gonadal development

Environmental chemical contaminants can alter avian gonadal differentiation similarly to estrogens. Contaminants that have shown feminizing actions in male birds include DDT and its metabolites, as well as methoxychlor and its estrogenic metabolite HPTE. Exposure of quail and chickens during incubation to DDT resulted in feminization at doses of 2,3,5, and 10 parts per thousand. At all doses males developed ovotestes and retained Mullerian ducts, while females retained the right Mullerian duct. Quails appeared to be slightly more sensitive than chickens since, at the lowest doses, more males retained Mullerian ducts than in chickens (Lutz-Ostertag and David 1973). Western and California gulls injected with DDT metabolites during incubation were 10-50 times more sensitive to feminization than either chickens or quail. At doses as low as 2 ppm of o,p'-DDT, males developed areas of ovarian-like cortical tissue on their testes, and with 5 ppm they developed left and right oviducts as well. Other DDT metabolites including p,p'-DDE and a 4:1 mixture of p,p'-DDE:p,p'-DDT similarly induced feminization at doses of 20 ppm and 50 ppm respectively. Gulls also were sensitive to feminization by methoxychlor and its metabolite HPTE at levels of 2 ppm (ovotestis development) and 50 ppm (oviduct development). Female gulls exposed to the higher doses of feminizing contaminants tended to develop right oviducts, and thus also were sensitive (Fry and Toone 1981; Fry, Toone et al. 1987).

Another study examining effects of DDT exposure on gonadal development in chickens found effects different from feminization. Chicken embryos exposed to 5, 10, or 20 mg DDT prior to incubation were examined at day 12 of their 21 day incubation period.

Males showed testes developing with mainly stroma, and fewer seminiferous cords. Females showed ovaries with distended medullary cords located nearer to the surface of the gonad. Differences in DDT metabolites present, stage of development, sensitivity of the chicken species, or dosing might account for differences in the effects observed in this study (Swartz 1984).

Another environmental contaminant affecting gonadal development is chlordecone (Kepone), which is known to have estrogenic activities. Feeding studies of young chickens at levels of 50 ppm in food caused males to develop female plumage and resemble females (Eroschenko 1981). This suggests that chlordecone exposure during embryonic development may result in feminization of male gonads. The female reproductive tract also was affected by chlordecone exposure. Effects included those usually induced with estrogens such as secretory activity in certain cells of the oviduct, increased numbers of primary oocytes, and rapid oviduct weight gain (Eroschenko 1981).

Possible Mechanisms of Endocrine Disruption

Contaminants are known to act as endocrine disrupters in some wildlife populations and also may be affecting terns on Bird Island. Endocrine disrupting chemicals act by many mechanisms, including: mimicking the effects of natural hormones by recognizing their binding sites on hormone receptors and transport proteins; antagonizing the effect of these hormones by blocking their interaction with their binding sites; altering the activity of steroidogenic enzymes and thus, altering the natural pattern for synthesis and degradation of hormones; altering hormone receptor levels; altering the hypothalamic-pituitary axis of endocrine control (Colborn and Clement 1992; Crain and Guillette Jr. 1997). This study examined individual contaminants and also focused on two groups of contaminants that can

have endocrine disrupting effects, estrogenic compounds and dioxin-like compounds. Bioassays were used to measure cumulative effects from the two contaminant groups; results helped address questions of mechanisms and possible causative agents resulting in feminization and other effects in common terns.

Estrogenic Compounds:

As previously discussed, development of ovotestes in birds is known to occur through exposure to estrogens; therefore, we wanted to investigate the estrogenic activity of contaminants to which common terns were exposed. Estrogenic compounds that mimic effects of the steroid hormone estrogen often are able to bind to the estrogen receptor (ER). Thus, estrogen receptor binding was used as an indication of estrogenic potency, although other mechanisms may be involved in producing estrogenic effects that would not be measured by receptor binding. A wide variety of structures are known to bind to the estrogen receptor, including organochlorine pesticides and/or their metabolites, certain PCB metabolites, other organochlorines, plasticizers, pharmaceutical agents, and phytoestrogens (Soto, Justicia et al. 1991; Soto, Lin et al. 1992; Klotz, Beckman et al. 1996; Vonier, Crain et al. 1996; Coldham, Dave et al. 1997; Harris, Henttu et al. 1997). Many compounds are able to bind directly to the estrogen receptor including the synthetic estrogen diethylstilbestrol (DES), the pesticides o,p'-DDT and chlordecone, and components of plastics such as nonylphenol. Other compounds must be metabolized before they bind well to the estrogen receptor; these include the pesticide methoxychlor, which is demethylated to a more estrogenic bisphenolic compound, and some PCBs, which are hydroxylated before they act as estrogens (Bulger, Mucitelli et al. 1978; Korach, Sarver et al. 1987).

Although some synthetic compounds, such as DES, have an affinity for the estrogen receptor equivalent to that of estradiol, most environmental contaminants

(pesticides, PCBs, plasticizers) have an estrogen receptor binding affinity at least a thousand-fold less than estradiol (Soto, Justicia et al. 1991; Soto, Lin et al. 1992; Klotz, Beckman et al. 1996; Vonier, Crain et al. 1996; Coldham, Dave et al. 1997; Harris, Henttu et al. 1997). However, *in vivo* other factors may affect the estrogenic potency of a compound, including binding to non-receptor hormone binding proteins in plasma or cytosol. One function of estrogen binding plasma proteins, such as sex steroid binding globulin in humans, is to restrict entry of endogenous estrogen into cells. As a result, only a small fraction of the total endogenous estrogen in blood is able to pass into cells (Sheehan and Young 1979; vom Saal, Montano et al. 1992). The man-made estrogen DES does not bind strongly to estrogen-binding plasma proteins (Sheehan and Young 1979). Therefore, DES can freely enter cells resulting in increased biological activity relative to similar blood concentrations of endogenous estrogen, most of which is prevented from entering cells (Sheehan and Young 1979; vom Saal, Montano et al. 1992). Similarly to DES, some environmental contaminants also show low or no binding affinity to non-receptor estrogen binding proteins. This may contribute to the *in vivo* effectiveness of these pollutants, possibly making their estrogenic potencies much greater than their estrogen receptor binding affinities indicate (vom Saal, Montano et al. 1992; Crain and Guillette Jr. 1997). Some environmental contaminants can bind to non-receptor hormone binding proteins, and this property may affect estrogen (or other hormone) regulation as well by altering the binding of the natural hormone to these proteins (Danzo 1997).

An ER agonist can enter a cell, bind to the nuclear ER and elicit a spectrum of biological effects that would normally occur only in response to the natural hormone; or alternatively, in the case of an ER antagonist, receptor binding could prevent normal responses. When estrogen or another active ligand binds to ERs, the ERs can be activated to bind to estrogen response elements (EREs) on genes, and alter transcription. There are at least two types of ERs; additional response elements activated by ligand binding to the

ER, and many interactive pathways involved in estrogen receptor signaling, indicating the multitude of ways estrogen is involved in regulation of processes affecting growth, differentiation, and functioning of target tissues (Katzenellenbogen 1996; Yang, Venugopalan et al. 1996; Kuiper and Gustafsson 1997; Paech, Webb et al. 1997). Estrogen is involved in synthesis of vitellogenin protein and zona radiata proteins, induction of cellular growth, viability of sperm, sexual differentiation of the brain and reproductive tract, bone maintenance, and cardiovascular protection (Copeland, Sumpter et al. 1986; Hines, Alsum et al. 1987; Soto, Justicia et al. 1991; Pelissero, Flouriot et al. 1993; Arukwe, Knudsen et al. 1997; Hess, Bunick et al. 1997). As already reviewed, exposure to estrogen or estrogenic compounds can extensively affect development, function, and behavior in birds.

Ah-Receptor Agonists (Dioxin-like Compounds):

Dioxin-like compounds also are known to have numerous endocrine disrupting effects and were expected to be present in high quantities in Bird Island tern samples. Thus, this group of contaminants also was examined. The AhR agonists, or dioxin-like compounds, are grouped together because many of their effects are mediated through the aryl hydrocarbon-receptor (AhR) for which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most biologically potent ligand (Landers and Bunce 1991). TCDD is just one member of the polychlorinated dibenzo-p-dioxin (PCDD) family, which has 75 possible congeners. In addition to certain dioxins, some polychlorinated dibenzofurans (PCDFs), which have 135 possible congeners, and polychlorinated biphenyls (PCBs), which have 209 possible congeners, are able to bind to the AhR. The AhR binding affinity of congeners in all three of these polychlorinated hydrocarbon families depends on the location of the chlorine atoms. The congeners most similar to the planar configuration of TCDD are the most

potent ligands (Landers and Bunce 1991). Among the PCBs, congeners with chlorines occupying the lateral para-positions, and one or both of the meta-positions of each ring, but lacking chlorines in the ortho-positions are most Ah-receptor active (McKinney, Chae et al. 1976; Yoshimura, Yoshihara et al. 1979). In addition to the synthetic organochlorine compounds, polycyclic aromatic hydrocarbons (PAHs), including benzo[a]pyrene, bind to the Ah-receptor. PAHs form when organic material is combusted incompletely, and sources include fossil fuel combustion, crude oils, and refined petroleum products (Brunstrom 1990). In general, compounds with a high affinity for the AhR are planar, non-polar ligands and include a wide variety of persistent and ubiquitous compounds (Landers and Bunce 1991).

The Ah-receptor mechanism involves entry of the dioxin-like compound into the cells, binding of the compound to the cytosolic Ah-receptor, and translocation of the receptor-ligand complex into the nucleus. In the nucleus, the activated ligand-receptor complexes bind to specific sequences of DNA referred to as dioxin response elements (DREs). This binding results in changes in protein expression through altered gene transcription and may affect the physiological state of the organism (Landers and Bunce 1991). One well known Ah-receptor mediated response is the induction of cytochrome P4501A (CYP1A) which results in increased CYP1A protein and activity. There is also evidence that the Ah-receptor mechanism is involved in the ability of dioxin-like compounds to produce the structural malformations of cleft palate and hydronephrosis in mice, as well as in their anti-estrogenic action (Peterson, Moore et al. 1992). Both *in vitro* and *in vivo* evidence suggest that the anti-estrogenic effects are mediated by the Ah-receptor, and although the exact mechanism is not certain, it may involve both or one of two processes. One process may be increased metabolism of estrogen due to Ah-receptor mediated enzyme induction, in which CYP1A1 catalyzes the C-2, C-15α, and C6α hydroxylations of 17β-estradiol. The second process may be down regulation of estrogen

receptors within the target cell, leading to a decreased responsiveness to estrogen (Peterson, Theobald et al. 1993). It is important to note that dioxins do not compete with radiolabeled estrogens for binding to estrogen receptors, and that estrogens do not bind to the Ah-receptor or compete with radiolabeled TCDD for binding (Peterson, Theobald et al. 1993).

The Ah-receptor's role in producing many other signs of developmental and reproductive toxicity elicited by dioxin-like compounds, including female reproductive toxicity (hormonal irregularities in the estrous cycle, reduced litter size, and reduced fertility), male reproductive toxicity (altered regulation of LH secretion, reduced testicular steroidogenesis, reduced plasma androgen concentrations, reduced testis and accessory sex organ weights, abnormal testis morphology, decreased spermatogenesis, and reduced fertility), and developmental toxicity (fetal/neonatal growth retardation and death) is unclear (Peterson, Moore et al. 1992). Thus, although all dioxin-like compounds bind to the Ah-receptor, some effects of dioxin-like compounds may not be Ah-receptor mediated (Peterson, Theobald et al. 1993).

In birds, injection of TCDD and other dioxin-like compounds into adults and embryos elicits characteristic developmental effects, which appear to be mediated by the Ah-receptor. Embryos are always more sensitive to dioxin-like toxicity than adults, and sensitivity varies widely among different bird species. The chicken embryo is the most sensitive bird species studied, while turkey, ring-headed pheasant, mallard duck, domestic duck, domestic goose, golden-eye, common tern, herring gull, black-headed gull, and eastern bluebird embryos are considerably less sensitive to dioxin-like compounds (Peterson, Theobald et al. 1993; Kennedy, Lorenzen et al. 1996; Lorenzen, Shutt et al. 1997). The signs of developmental toxicity elicited by particular dioxin-like compounds also vary among different bird species, and include some or all of the developmental effects observed in chicken embryos. In chicken embryos, injection of dioxin-like compounds

causes a toxicity syndrome characterized by pericardial and subcutaneous edema, liver lesions, inhibition of lymphoid development in the thymus and bursa of Fabricius, microophthalmia, beak deformities, cardiovascular malformations, and mortality (Peterson, Theobald et al. 1993). In contrast, turkey embryos injected with a dose high enough to cause microophthalmia, beak deformities, and embryo mortality did not produce liver lesions, edema, or thymic hyperplasia, all hallmark signs of TCDD toxicity in the chicken embryo. In pheasant embryos TCDD caused embryo mortality, but edema, liver lesions, thymic hypoplasia, and structural malformations did not occur (Peterson, Theobald et al. 1993). Dioxin-like compounds result in embryo mortality across bird species, and may include other characteristics of chicken embryo TCDD-toxicity.

Cytochrome P450s (CYPs):

Cytochrome P450 (CYP) proteins may play important roles in effects of both Ah-receptor and estrogen receptor mediated effects, and may also be used as biomarkers of exposure to these contaminant groups. The cytochrome P450 monooxygenases are a superfamily of membrane bound heme proteins with over 400 genes, which catalyze the oxidative metabolism of their substrates. The general scheme for monooxygenase reactions is:

$$R + NADPH + O_2 + H \rightarrow RO + NADP^+ + H_2O$$

where R represents the substrate and RO the oxygenated product. CYPs are important for the oxidative metabolism of a host of endogenous and exogenous compounds, catalyzing hydroxylations, epoxidations, and alkylations. Many CYP forms function in the synthesis and metabolism of endogenous compounds such as steroid hormones, fatty acids, prostaglandins, and fat soluble vitamins (Parkinson and Safe 1987). Many CYPs

(particularly CYP families 1, 2, and 3) also metabolize foreign compounds, including those with estrogen receptor and Ah-receptor agonist properties (Gonzalez 1992). Foreign compound metabolism generally converts hydrophobic substrates into oxygenated metabolites, which are more readily excreted by the organism. This process also may activate the compound to a more reactive metabolite; thus CYPs play an important role in determining toxicity of xenobiotics, including endocrine disrupters. For example, PCBs and some pesticides such as methoxychlor may be hydroxylated by CYPs to form estrogenic metabolites (Bulger, Mucitelli, et al. 1978). In addition, many CYPS are inducible, often by the same substrate they metabolize. Induction can drastically alter the amounts and activities of CYPs, and thus affect the metabolism of a large number of substrates, both endogenous and exogenous. For example, as already discussed, exposure to AhR agonists may result in increased metabolism of estrogen resulting in anti-estrogenic effects (Peterson, Theobald et al. 1993). Clearly, changes in amounts and activities of CYPs can dramatically affect regulation of many processes including the endocrine system.

Members of CYP1A and CYP2 subfamilies often are used as biomarkers of contaminant exposure. As mentioned, CYP1A forms are induced by and metabolize dioxin-like compounds, and CYP1A induction can be used as a biomarker of dioxin-like contaminant exposure and Ah-receptor mediated toxic effects. CYP1A induction in liver and other tissues can be indicated by elevated amounts of CYP1A protein and elevated ethoxyresorufin-O-deethylase (EROD) and aryl-hydrocarbon hydroxylase (AHH) activities, which are catalyzed by CYP1A (Stegeman and Hahn 1994). The CYP1A inducing potency of individual compounds or a contaminant mixture also can be measured in a bioassay. Cells in culture, often liver cells, can be exposed to the compounds of interest and also to TCDD. Markers of AhR activity (CYP1A protein, EROD activity, porphyrin accumulation) can be measured and compared to those of TCDD to determine the dioxin-like potency of the compounds or the dioxin-equivalents (TCDD-EQs) (Giesy,

Ludwig et al. 1994; Kennedy, Jones et al. 1995). CYP2 forms may metabolize and be induced by phenobarbital, ortho-substituted (noncoplanar) polyhalogenated biphenyls, and chlorinated pesticides. Thus, in organisms where the CYP2 is inducible, this can be a biomarker of exposure to CYP2 substrates, which include many estrogenic contaminants. Elevated levels of CYP2 proteins and pentoxyresorufin-O-dealkylase activity may indicate exposure to CYP2 substrates (Stegeman and Hahn 1994).

Thesis Research:

The presence of abnormal reproductive behavior in roseate terns and ovotestes in male common terns at Bird Island may indicate contaminant induced endocrine disruption of this population. To address this possibility, this thesis examines the relationship of contaminants to the presence of ovotestes in common terns as well as to other contaminant related effects. In chapter two, pipping common tern embryos from contaminated Bird Island are compared to terns from a control site, Nauset. Comparisons include ovotestes presence and severity, necropsy data including egg, body, and organ weights, deformities, hepatic CYP1A protein levels, hepatic EROD and PROD activities, and PCB levels in embryo yolk sacs. Chapter three examines exposure of tern embryos to contaminants in much greater detail. A suite of PCB congeners and organochlorine pesticides were measured in yolk sac extracts, and extracts also were used in a chick embryo hepatocyte bioassay to determine dioxin-equivalents (TCDD-EQs). Total mercury levels, indicative of methyl mercury exposure, were measured in the common tern embryo tissues. The relationship of individual contaminants and contaminant groups to each other, and to deformities, EROD activities, and ovotestes presence was examined. Chapter four examines the persistence of ovotestes in common tern prefledglings from Bird Island.

Eggs collected from the same nest as the prefledglings were used to examine the relationship of contaminants to ovotestes persistence. Chapter five addresses the estrogenic potency of yolk sac extracts from common tern embryos. Finally, in chapter six the thesis results are summarized and future directions are discussed.

CHAPTER 2: MORPHOLOGICAL, HISTOLOGICAL, AND BIOCHEMICAL CHARACTERIZATION OF COMMON TERNS COLLECTED IN 1994 FROM BIRD ISLAND AND NAUSET, MASSACHUSETTS.

Chapter 2: Morphological, Histological, and Biochemical Characterization of Common Terns collected in 1994 from Bird Island and Nauset, Massachusetts.

Introduction

Terns and other fish eating birds are particularly susceptible to contaminant exposure. They live and breed in aquatic habitats, which are often highly contaminated, and they are at the top of the food chain, which results in increased bioaccumulation of contaminants. Furthermore, these birds can be highly exposed during development, a period particularly sensitive to toxic effects. As the embryo utilizes the yolk for nutrition, it is exposed to contaminants deposited in the yolk by the female. Much of the laying female's contaminant burden is deposited in the yolk, and therefore developmental exposure to contaminants can be very high and may result in permanent impairment. If developmental exposure is widespread, whole populations can be affected. Because of their susceptibility to contaminant related effects, terns and other aquatic bird populations can serve as a sensitive indicator of poor environmental quality, and therefore are important species to monitor.

Many aquatic bird populations have shown reproductive and developmental problems which have been associated with contaminants including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs), and organic pesticides. Problems have included impaired reproduction, growth retardation, morphological abnormalities, behavioral changes, and alterations of hormone metabolism (Gilbertson, Kubiak et al. 1991; Giesy, Ludwig et al. 1994; Sanderson, Norstrom et al. 1994; Murk, Boudewijn et al. 1996). Cormorants, black-crowned night-herons, gulls, great blue herons, and terns all have been affected. Terns on

the Great Lakes and in the Netherlands have shown impaired reproduction, reduced body weight and femur length, increased liver to body weight ratios, increases in monooxygenase activities, and impaired behavior, all of which were associated with high levels of PCBs (Kubiak, Harris et al. 1989; Hoffman, Smith et al. 1993; Ludwig, Kurita-Matsuba et al. 1996; Murk, Boudewijn et al. 1996). High levels of DDT, PCBs and other contaminants also have been associated with a sex ratio skewed toward females, female-female pairing, and supernormal clutches. These phenomena were observed in Western and California gulls breeding on the west coast of California in the 1970s, as well as in Great Lakes Herring gulls. Michael Fry et al. found that hatching male gulls developed feminized testes when eggs were injected with DDT at levels comparable to those found in eggs from contaminated California sites. It was suggested that feminization of male gulls may reduce their presence at the breeding colonies, resulting in the abnormal breeding behavior (Fry and Toone 1981; Fry, Toone et al. 1987).

This study is concerned with terns breeding near Bird Island in Buzzards Bay, Massachusetts, along the eastern coast of the United States. These terns breed and feed near New Bedford Harbor, a Superfund site highly contaminated with PCBs and heavy metals, and high levels of PCBs often are found in eggs and tissues of these birds (Nisbet and Reynolds 1984; Weaver 1984; Pruell, Norwood et al. 1990; Lake, McKinney et al. 1995). Roseate terns breeding on Bird Island exhibited abnormal reproductive behavior similar to gulls in California in the 1970's, including a sex ratio skewed toward females, female-female pairing, and supernormal clutches. The Californian gull studies suggested the possibility that, like the gulls, contaminants may be causing the reproductive abnormalities observed in the Bird Island terns. Because roseate terns are an endangered species, common terns from Bird Island were examined as a surrogate species. In a preliminary sampling of male common tern embryos in 1993, 73% of the gonads examined (11/15) were feminized, defined as possessing ovarian-like tissue in the testes (Nisbet, Fry

et al. 1996). The 1993 study also examined the relationship between severity of feminization and organochlorine contamination in embryo livers. No clear relationship between the severity of feminization and contaminants was found, although further investigation was suggested for PCB congener 29 (Nisbet, Fry et al. 1996).

The high percentage of feminized male common tern embryos at Bird Island in 1993 (73%) prompted this further examination of the relationship of feminization to contaminants. We wanted to examine whether PCBs, their metabolites or other contaminants could be causing feminization in the Bird Island common terns. To help address this question, site differences in feminization of male common terns were compared between Bird Island and a less contaminated site. Comparisons also were made between feminization, other contaminant related effects, and biomarkers of exposure to particular groups of contaminants.

This chapter compares two populations of common terns, one breeding on Bird Island, close to highly contaminated feeding grounds, and the other breeding at Nauset on outer Cape Cod, selected as a control site because of the surrounding clean feeding grounds (Nisbet and Reynolds 1984). In order to examine the relationship between effects and contaminants, comparisons between the two sites included measurements that have been related to contaminant effects in terns and other aquatic birds, including examination of the male gonads for feminization, and measurements of chemical contaminants, groups of contaminants, and biomarkers of contaminant exposure. Gross measurements were made on eggs and embryos, and embryos were examined for the presence of deformities. Elevated levels of deformities, decreases in body weights and egg volumes, and changes in organ weights have been associated with elevated organochlorine contaminant levels (Kubiak, Harris et al. 1989; Ludwig, Kurita-Matsuba et al. 1996; Murk, Boudewijn et al. 1996). Liver fluorescence was used as an indicator of porphyria since exposure to PCBs and dioxins has been correlated with elevated porphyrin levels in livers of birds (Kennedy,

Fox et al. 1998). Reproductive tracts were examined for abnormalities, and male gonads were examined histologically for feminization. Hepatic mixed function oxidase activities (EROD and PROD) and protein levels (CYP1A) were examined as they may indicate contaminant induced cytochrome P450 proteins (Murk, Bosveld et al. 1994; Murk, Boudewijn et al. 1996). Induction of EROD and PROD can indicate exposure to dioxin-like compounds and phenobarbital-like compounds, respectively, and we hoped to use these activities as biomarkers of exposure to the respective groups of chemicals. Yolk sacs also were used to measure exposure to lipophilic chemicals, since contaminants deposited by the female are sequestered largely in the yolk, and embryos are exposed to these contaminants as they use the yolk sac for nourishment (Custer, Custer et al. 1997). Yolk sacs were used both for chemical analysis to measure individual contaminants, and for bioassays to measure activities of chemical groups. Bioassays to measure dioxin-like compounds and estrogenic compounds were included. The chemical data, including the bioassay for dioxin-like compounds and its relationship to observed effects, is discussed in further detail in Chapter Three, and the estrogenic bioassay is discussed in Chapter Five.

Methods

Collections: Common tern embryos were sampled from tern colonies on Bird Island in Buzzards Bay, MA (41° 40' 10" N, 70° 43' 00" W), and from Nauset on outer Cape Cod, MA (41° 48' 50" N, 69° 57' 00" W), in 1994 (Figure 1). Fully developed embryos at the stage of starring, pipping, or hatching were sampled both at early hatching (June 15-16) and late hatching (July 12-21) from Bird Island and once (July 7-8) from Nauset. Approximately 30 embryos were sampled during each of the three collection periods.

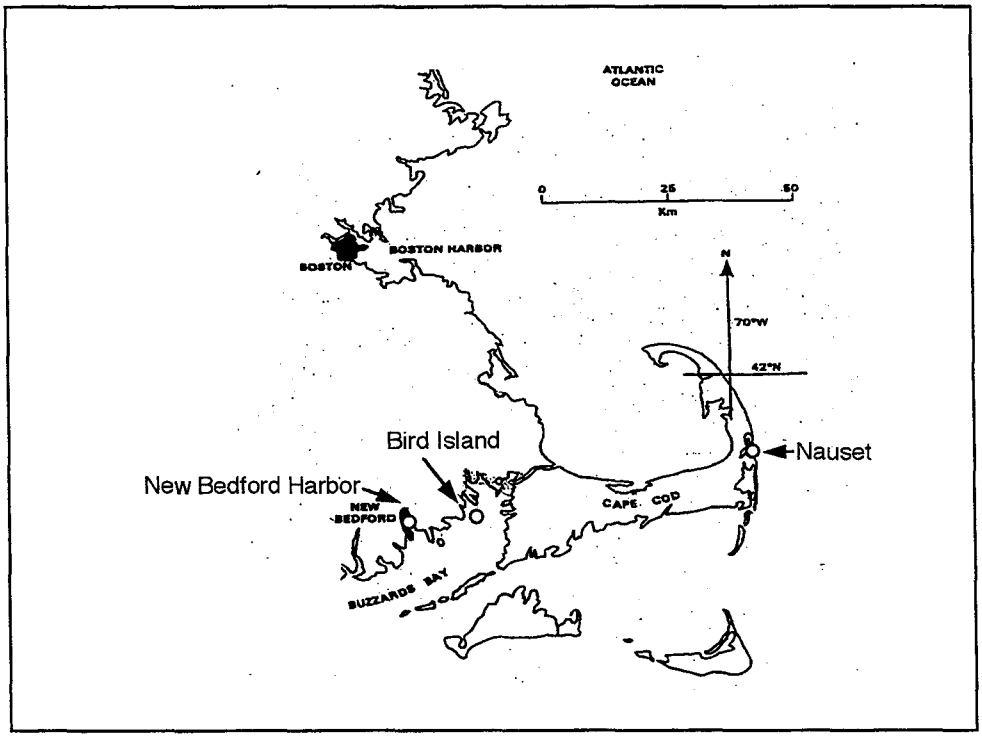


Figure 1. Locations of study sites. Bird Island is located in Buzzards Bay (41° 40' 10 " N, 70° 43' 00" W) approximately 18 km from New Bedford Harbor. Nauset is located on outer Cape Cod (41° 48' 50" N, 69° 57' 00"W).

Terns rarely fledge more than 1-2 chicks per nest. Therefore eggs were taken from three egg clutches in order to minimize the impact of sampling on the breeding population.

Reproductive success: Productivity of common terns at Bird Island and Nauset was assessed by counting the number of active nests in the breeding season and estimating the number of fledged young produced per nest. Productivity was assessed by Ian Nisbet.

Processing of embryo samples at Necropsy: Each egg was weighed and measured. The embryo was removed and examined for deformities. Then it was decapitated and blood was collected; a small volume of whole blood was stored in 70% ethanol for genetic sexing, and the remainder was centrifuged and the serum stored frozen. The yolk sac was removed, weighed, and frozen in acid washed vials for later extraction of chemical contaminants. The liver was removed, weighed, and screened with a UV lamp for red fluorescence in order to check for porphyria. Liver subsamples were stored in liquid nitrogen (2/3 liver) and 10% neutral buffered formalin (NBF) (1/3 liver). The remaining tissues were fixed in 10% NBF, with one leg fixed in 70% ethanol.

Microsome preparation: Microsomes were prepared from frozen livers by differential centrifugation as described previously (Hahn and Stegeman 1994). Proteins were determined using a fluorescent protein assay (Lorenzen and Kennedy 1993).

Ethoxyresorufin-O-deethylase (EROD) activity and pentoxyresorufin-O-dealkylase (PROD) activity: EROD and PROD activity of tern embryo hepatic microsomes was measured with a fluorescent kinetic assay (Hahn, Lamb et al. 1993; Kennedy, Lorenzen et al. 1993). Ethoxy-resorufin or pentoxy-resorufin substrate was added to microsomes and formation of resorufin product was measured at room temperature.

Immunoblotting: Western blotting techniques were carried out with antibodies against CYP1A using the monoclonal anti-scup 1A (Kloepper-Sams, Park et al. 1987; Stegeman, Smolowitz et al. 1991).

Gonadal histology: D. Michael Fry, Ph.D. at UC Davis carried out gross and histological examination of the tern embryo reproductive tracts for abnormalities of male and female characteristics. Gonadal tissues were embedded in paraffin, sectioned at 3-5 um intervals yielding at least 20 sections, and stained with hematoxylin and eosin. The severity of feminization of male testes was assessed from the extent of ovarian-like cortex and primordial germ cells (PGCs) located outside the seminiferous tubules. A severity index of feminization among male embryos was assigned, with 1 being normal and 4 being intersex. Details of the severity index can be seen in Table 3. More detailed description of gonadal analysis has been described previously (Fry, Toone et al. 1987; Nisbet, Fry et al. 1996).

Statistics: Data were analyzed using analysis of variance (ANOVA). Fisher's Exact test was used to detect significant differences among sites. Spearman's rank correlation coefficient was used to test correlations. Level of significance was $p \leq 0.05$.

Results

Results of necropsies performed on pipping tern embryos from Bird Island and Nauset are given in Table 1. Egg volume, fresh egg mass, egg mass at pipping, body mass, yolk-free body mass, yolk mass, liver mass, and relative liver mass did not differ significantly among sites. Beak length of embryos at the early Bird Island collection was significantly shorter than at the late Bird Island and Nauset collections. Spleen mass and

relative spleen mass were significantly greater at Nauset than at Bird Island. Liver fluorescence was not observed in embryos from any of the sites suggesting that porphyria may not be occurring. Edema was infrequently observed with only very slight edema found in one bird from Bird Island. Crossed beaks were observed on two embryos, one at Nauset and one at early Bird Island.

Results of hepatic biochemistry for common tern embryos are given in Table 2 and in Figure 2. The mean EROD activities from embryos collected at both early and late Bird Island were significantly greater and more variable (19.6 ± 15.7 SD, 21.8 ± 15.1 SD pmol/min/mg) than those from Nauset (11.1 ± 3.7 SD) (Table 2 and Figure 2). PROD activity was slightly greater in embryos from the late Bird Island collection than at either the early Bird Island or Nauset collections, although PROD values from all sites were extremely low, ranging from 0.7-6.1 pmol/mg/min (Table 2 and Figure 2). The EROD activities were highly and significantly correlated with the CYP1A protein levels (Figure 3) (r^2=0.94, p=0.0002), consistent with the expectation that CYP1A catalyzes EROD activity. PROD activities also were correlated with CYP1A protein levels (Figure 3) (r^2=0.34, p=0.01), indicating that CYP1A could be responsible for catalyzing the low levels of PROD activity, or that inducers of PROD activity covary with inducers of EROD activity. Also consistent with CYP1A as the catalyst for PROD activity, the EROD and PROD activities were significantly correlated (Figure 4) (r^2=0.45, p=0.0001).

Common tern embryos were sexed at necropsy and the sexes were confirmed histologically. The percent of males at the sites were 42, 59, 37, for Bird Island early, late, and Nauset, respectively, as detailed in Table 3. The presence of oviduct development was not observed in reproductive tracts of male common tern embryos. The gonads of male common tern embryos were examined for the presence of ovarian-like tissues in the testes (ovotestes), and the results are shown in Table 3. Feminization of male testes, including ovarian-like cortical areas and/or primordial germ cells located outside the seminiferous

Table 1. Necropsy data for common tern embryos collected at Bird Island and Nauset, Massachusetts. Mean ± standard error (n).

	Bird Island early collection (June 15-16, '94)	**Bird Island late collection** (July 12-21, '94)	**Nauset** (July 7-8, '94)
fresh egg mass(g)[a]	20.10 ± 0.32 (29) A	19.69 ± 0.68 (19) A	20.17 ± 0.37 (27) A
egg mass at pip (g)	16.61 ± 0.25 (30)A	15.68 ± 0.35 (22) A	16.59 ± 0.32 (29) A
egg length (mm)	41.82 ± 0.25 (29) A	41.15 ± 0.30 (19) A	42.34 ± 0.45 (27) A
egg width (mm)	30.11 ± 0.19 (29) A	30.01 ± 0.47 (19) A	29.98 ± 0.18 (27) A
egg volume(cm^3)[b]	18.24 ± 0.29 (29) A	17.87 ± 0.61 (19) A	18.30 ± 0.33 (27) A
body mass (g)	14.18 ± 0.22 (30) A	14.25 ± 0.58 (26) A	14.22 ± 0.27 (30) A
yolk mass (g)	1.36 ± 0.08 (31) A	1.18 ± 0.11 (27) A	1.21 ± 0.09 (30) A
yolk-free body mass (g)	12.79 ± 0.22 (30) A	13.12 ± 0.56 (26) A	13.01 ± 0.22 (30) A
beak length (mm)	9.80 ± 0.13 (30) A	10.18 ± 0.01 (27) B	10.15 ± 0.08 (27) B
liver mass (g)	0.34 ± 0.01 (31) A	0.32 ± 0.01 (27) A	0.35 ± 0.01 (30) A
liver mass/yolk-free body mass	0.027 ±0.001 (30)A	0.025 ±0.001 (26)A	0.027±0.0005(30)A
spleen mass (mg)	5.39 ± 0.28 (30) A	5.40 ± 0.32 (24) A	6.67 ± 0.42 (29) B
spleen mass/ yolk free body mass	0.42 ± 0.02 (29) A	0.41 ± 0.03 (24) A	0.52 ± 0.04 (29) B
liver fluorescence[c]	0/31	0/27	0/30
crossed beaks	1/31	0 /27	1/30

[a] Fresh egg mass is calculated from the formula (0.529g/cm3)(length)$(width)^2$, as determined by I.C.T. Nisbet.

[b] Egg volume is calculated fro the formula 0.48(length)$(width)^2$ according to Davis (1975) Ibis 117: 460-473.

[c] liver uv fluorescence is an indicator of porphyria

A. Within rows, means with the same letter are not significantly different at $p<0.05$.

Table 2. Hepatic biochemical data for common tern embryos collected at Bird Island and Nauset, Massachusetts. Mean ± standard error (n), (range).

	Bird Island early collection (June 15-16, '94)	**Bird Island late collection** (July 12-21, '94)	**Nauset** (July 7-8, '94)
liver mass/yolk-free body mass	0.027 ±0.001(30)A	0.025 ± 0.001(26)A	0.027 ± 0.001(30)A
microsomal yield (mg protein/g liver)	16.6 ± 0.8 (31)A	15.9 ±0.6 (27)AB	14.3 ± 0.5 (30)B
EROD (pmol/min/mg)	19.6 ± 2.3 (31)A (7.2 - 86.2)	21.8 ± 2.9 (27)A (7.8 - 49.8)	11.1 ± 0.7 (30)B (0.5 - 18.1)
PROD (pmol/min/mg)	2.1 ± 0.2 (31)A (0.8 - 5.2)	2.9 ± 0.2 (27)B (1.4 - 6.1)	2.1 ± 0.1 (30)A (1.1 - 3.4)
CYP1A (pmol 1A1/100ug)	0.06 ± 0.02 (6) A (0.01 - 0.19)	0.05 ± 0.01 (6) A (0.01 - 0.11)	0.02 ± 0.01 (7) A (0.001- 0.03)

A. Within rows, means with the same letter are not significantly different at $p<0.05$.

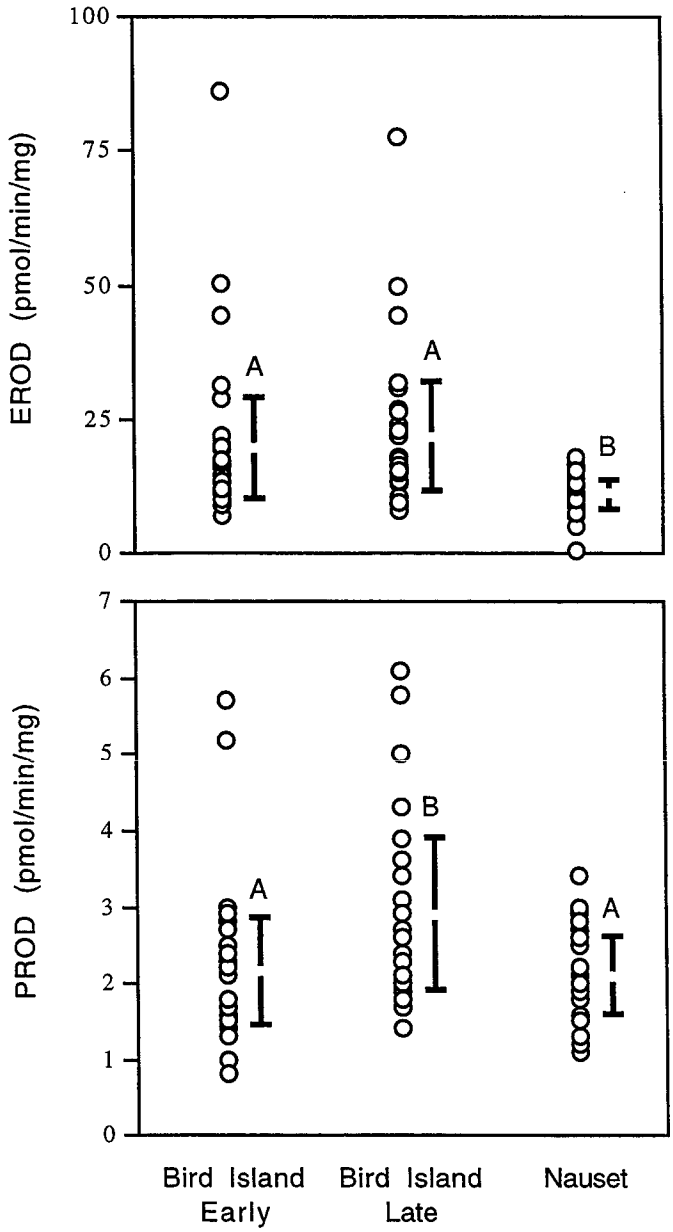


Figure 2. Pipping common tern embryo EROD and PROD activities by collection site. Breaks in standard error bars indicate site means. Bars with the same letter are not significantly different at p= 0.05.

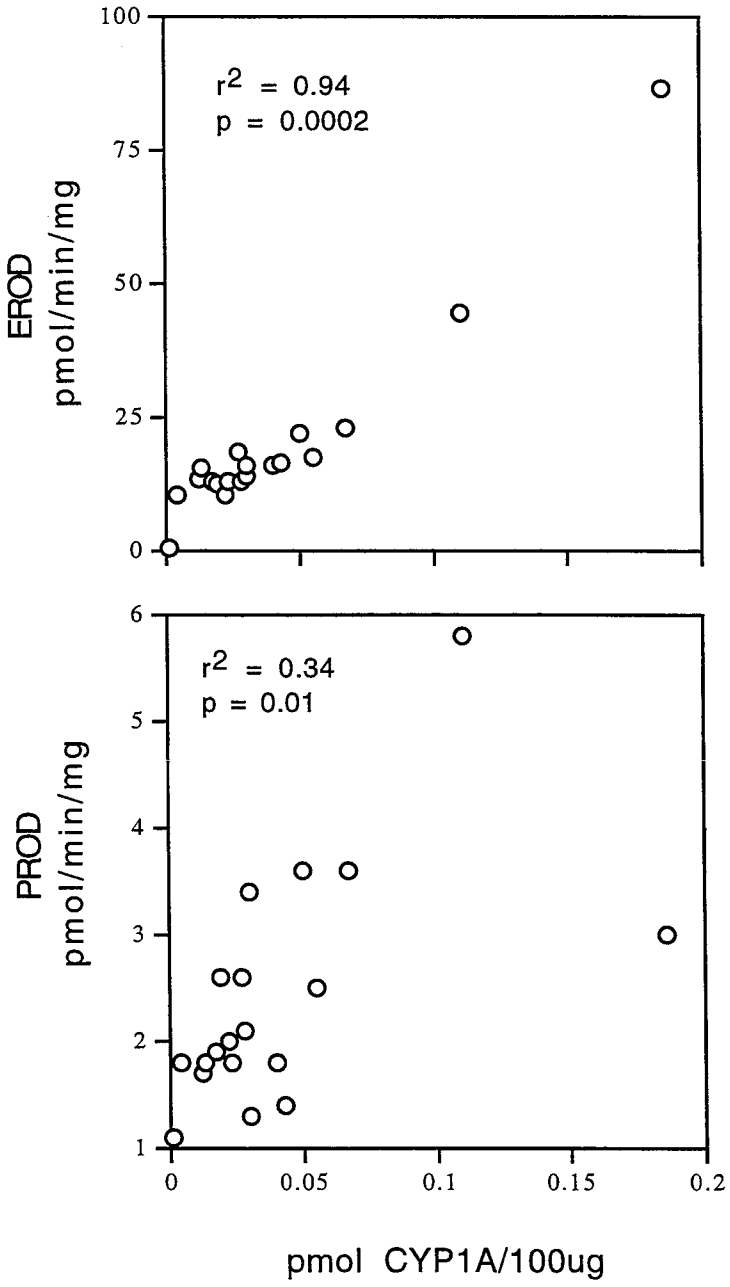


Figure 3. Hepatic EROD and PROD activities versus CYP1A protein in pipping common tern embryos collected from Bird Island and Nauset in 1994.

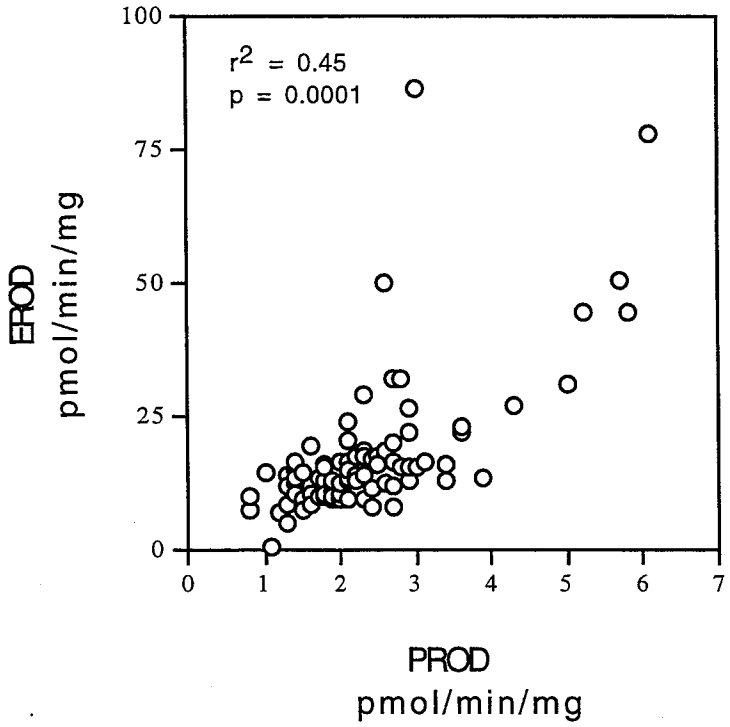


Figure 4. Hepatic EROD activity versus PROD activity in pipping common tern embryos collected from Nauset and Bird Island in 1994.

Table 3. Gonadal histology for common tern embryos collected at Bird Island and Nauset, Massachusetts.

	Bird Island early collection (June 15-16, '94)	**Bird Island late collection** (July 12-21, '94)	**Nauset** (July 7-8, '94)
embryos collected	31	27	30
sex from histology			
females	18 [a]	11	17
males	13	16	10
unidentified	0	0	3
%males	42 %	59 %	37 %
males analyzed for gonad histology[b]	9 (6)[c]	14 (6)	10 (7)
number of males per ovotestes category			
1- normal [d]	1 (1)[c]	4 (3)	4 (3)
2	4 (2)	2 (0)	0 (0)
3	3 (2)	7 (2)	5 (3)
4- intersex	1 (1)	1 (1)	1 (1)
total ovotestes (categories 2-4)	8 (5)[c]	10 (3)	6 (4)
total normal testes (category 1)	1 (1)[c]	4 (3)	4 (3)
% males analyzed with ovotestes	89[e] %	71 %	60 %

[a] Sex data from early Bird Island includes sexing at necropsy.
[b] Gonadal histology was evaluated by D.M. Fry, U.C. Davis.
[c] In parenthesis are the number of males for which contaminants were measured in yolk sacs.
[d] Male gonadal histology ovotestis index (adapted from D.M. Fry's severity ranking). Severity of ovotestis development increases with number:
1: normal: no ovotestis development; no primordial germ cells (PGCs) outside seminiferous tubules (ST), and no cortical ridge (CR).
2: presence of PGCs outside ST, and/or small clumps of PGCs
3: small cortical ridge (CR), PGCs present
4: intersex: extensive ovarian cortical ridge containing PGCs
[e] Proportion of abnormal gonads detected at both Bird Island collections are not significantly different from Nauset using a Chi-square test, $p < 0.46$.

Figure 5. Feminized testis of a male pipping common tern embryo; ovotestis of severity level 4 (intersex). An extensive area of ovarian-like cortical tissue is present rather than a smooth epithelial capsule. Primordial germ cells are located within this cortical area.

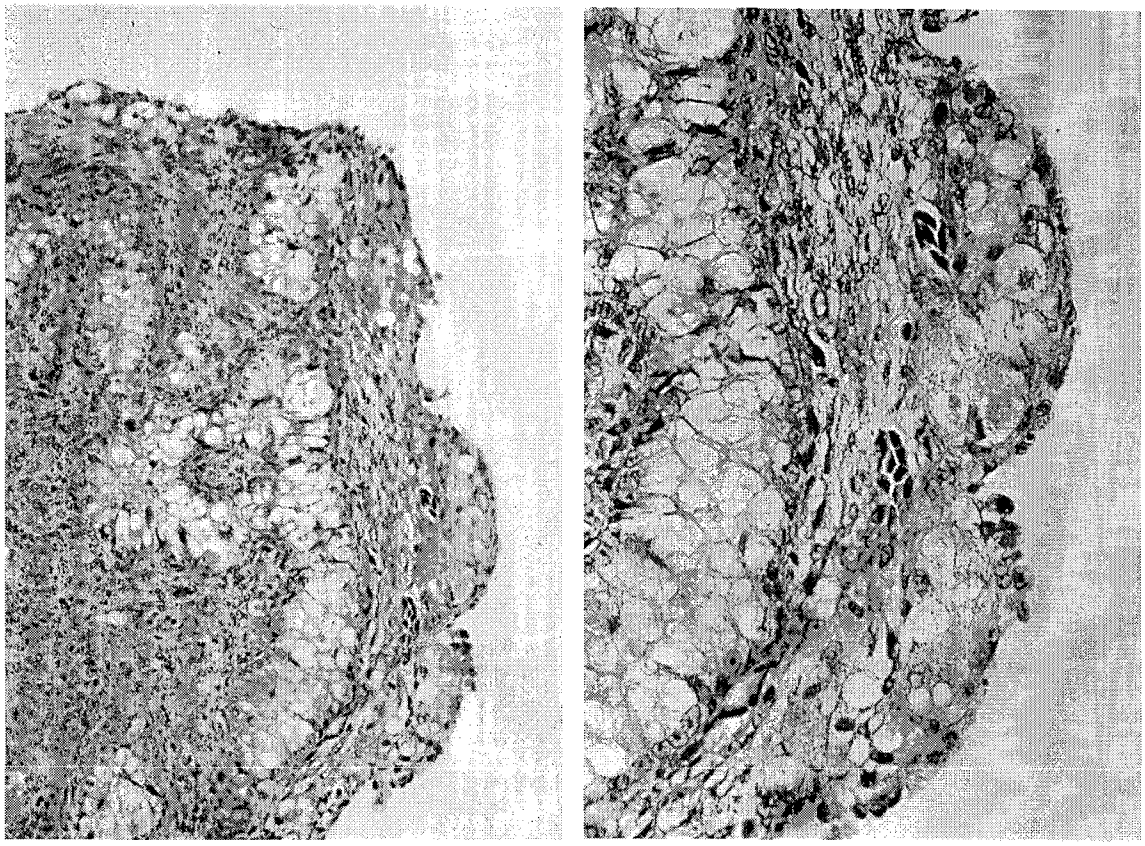
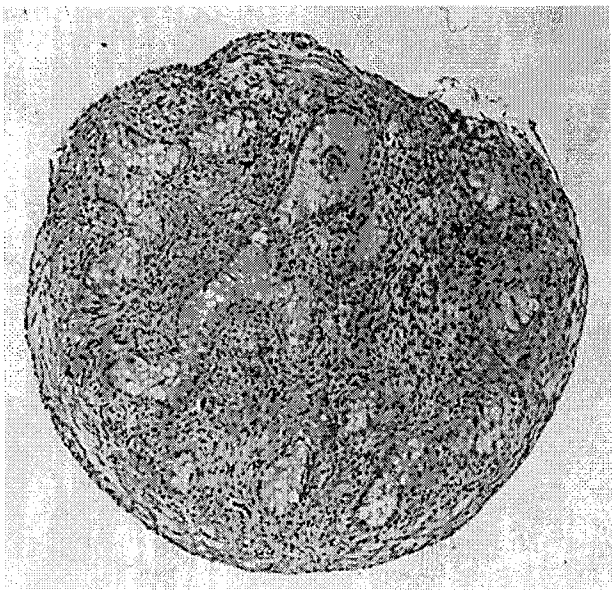


Figure 6. Normal testis of a male pipping common tern embryo. A smooth epithelial capsule (tunica albuginea) surrounds the testis. Seminiferous tubules are located in the medullary area of the gonad, and primordial germ cells are located within the seminiferous tubules.



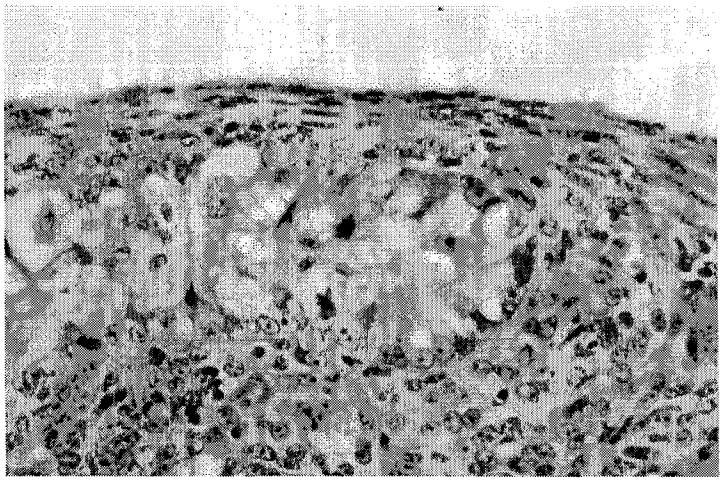
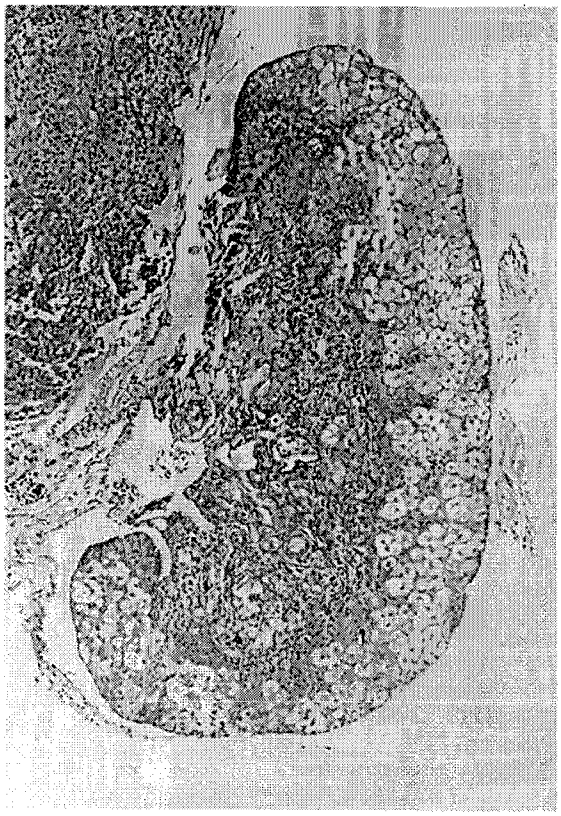
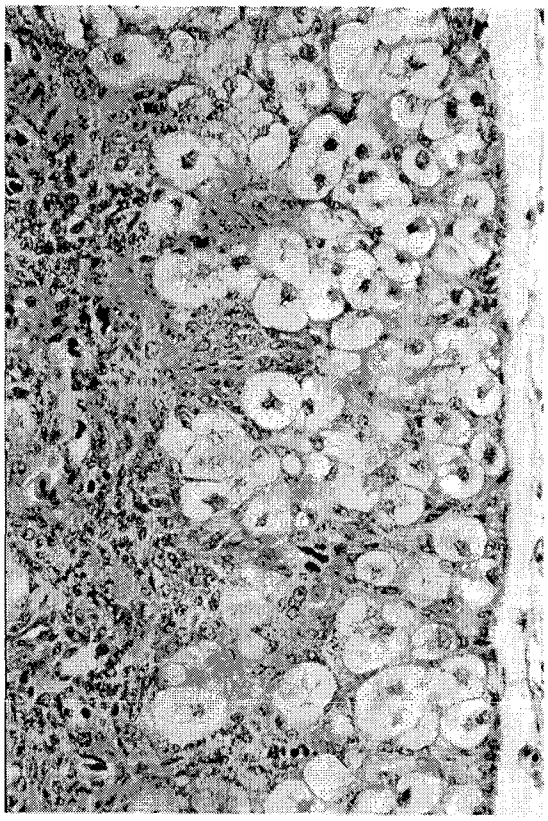


Figure 7. Normal ovary of a female pipping common tern embryo. The ovary shows a clearly differentiated cortex and medulla. Primordial germ cells are located within the cortex, and are distinct with their large size and vacular cytoplasm.





tubules, was found in embryos from both Bird Island and Nauset. The histological appearance of an intersex male common tern embryo testis is shown in Figure 5.a. For comparison, a normal male common tern embryo testis (Figure 5.b) and a normal female common tern embryo ovary (Figure 5.c) are shown. The normal male testis is surrounded by an epithelial capsule, and the primordial germ cells (PGCs) are located within the seminiferous tubules. The normal female ovary shows the extended cortical area within which the PGCs are located. The intersex male shows an extensive cortical ridge instead of the smooth epithelial capsule, and PGCs are located throughout the cortical area rather than entirely within the seminiferous tubules.

The prevalence of feminization at Bird Island (89% and 71%) was not statistically significantly higher than at Nauset (60%) among the males examined (Table 3); however, 10-30% more birds were feminized at Bird Island. The feminization was given a severity index with 1 being normal, 4 being intersex, and 2-3 increasing levels of severity (see Table 3 for more detail). Birds with level 3 and 4 severity of ovotestes were observed at both Bird Island and Nauset.

Discussion

Bird Island Early compared to Bird Island Late collections

Bird Island common tern embryos were collected during both an early and late hatching period. Relationships have been observed between elevated contaminant levels in eggs and elevated EROD activities, later egg laying dates and prolonged incubation periods (Kubiak, Harris et al. 1989; Murk, Boudewijn et al. 1996). Contaminated eggs tend to be smaller and produce smaller chicks (Kubiak, Harris et al. 1989; Murk, Boudewijn et al. 1996). A lengthened incubation period might result from retarded development and/or

parental inattentiveness, both of which may be related to a high contaminant burden in the embryo or adult. Later nesting dates may result from inexperience or poorer condition of the breeding birds; the late nesting could be related to contaminant levels if inexperienced birds feed in contaminated areas or if elevated contaminant levels have resulted in poorer condition. The embryos collected at the later hatching period at Bird Island were likely from later nesting birds.

There were no significant differences in embryo health or contaminant burdens between the early and late Bird Island collections. The statistically significantly shorter beak length measured at early Bird Island collections likely is due to investigator bias in measuring, since all the early collection beaks were measured by a different scientist than the late Bird Island and Nauset collections. The higher PROD activities observed in the late Bird Island collection, although statistically significant, are still very low, and probably not a good indicator of contaminant exposure, as will be discussed in a later section. There were no differences between early and late Bird Island embryo collections in egg masses, egg volumes, yolk-free body mass, liver mass, relative liver mass, or EROD activity (Tables 1 and 2). There was also no statistically significant difference in the contaminant burdens between Bird Island early and late embryos (Table 4). Although the total PCB means suggest that Bird Island early has higher total PCB levels, the range of total PCB levels is similar for both Bird Island early and late; there is only one extremely contaminated sample at Bird Island early, which raises the mean. These results indicate embryo health and contaminant levels are consistent throughout both collection periods, and suggests that data from both early and late collections can be combined into one Bird Island collection for comparison with the Nauset site in later chapters.

Table 4. Comparison of hepatic EROD activity and yolk sac PCB and PCDD/F levels in pipping common tern embryos from Massachusetts, the Canadian Atlantic coast and Great Lakes/St. Lawrence River, and the Netherlands.

SITE	EROD[a] pmol/min/mg	total PCBs[b] ug/g lipid	PCB 153[b] ug/g lipid	PCB 118[b] ug/g lipid	total PCDD/F[b] pg/g lipid
Bird Island-early. 1994	19.6 ± 2.8	133.15 (31.6-663.4)	15.5 (6.1-45.4)	14.4 (4.3-58.3)	N.A.[d]
Bird Island-late. 1994	21.8 ± 2.9	89.8 (17.2 -244.6)	14.2 (2.9-32.0)	13.2 (1.9-34.9)	N.A.
Bird Island 1996	N.A.	209.0 (16.5-658.7)	34.1 (3.1-115.9)	30.5 (1.6-100.8)	633.7 (225.3-1,146)
Nauset 1994	11.1 ± 0.7	35.4 (8.4-178.4)	7.01 (1.8-34.9)	5.29 (0.9-31.4)	N.A.
New Brunswick[1]	56.6 ± 10.7	3.9 (1.5-6.1)	0.8 (0.3-1.2)	0.3 (0.12-0.42)	78.3 (47.33-99.77)
Georgian Bay[2]	59.8 ± 8.1	23.8 (21.4-26.2)	3.5 (3.0-3.7)	1.3 (1.2-1.3)	406.9 (376.9-436.2)
St. Lawrence River, Site 1[3]	52.7 ± 7.0	42.31 n=1	4.7	3.3	12.2
St. Lawrence River, Site 2[4]	84.5 ± 12.5	80.0 (43.8-116.2)	7.4 (3.9-11.0)	2.9 (2.6-3.2)	226.7 (62.1-391.3)
Hamilton Harbour[5]	100.3 ± 13.4	112.7 (88.1-137.2)	17.1 (14.3-19.9)	6.5 (5.4-7.6)	726.7 (468.5-984.8)
Zeewolde, Netherlands[6,7,8]	137 ± 47[c]	N.A.	10.8 (0.3-31.2)	2.7 (1.0-4.4)	489 (225-1,669)
Westplaat, Netherlands[6,7,8]	315 ± 266[c]	N.A.	83.1 (54.2-119.4)	15.1 (9.7-21.4)	729 (256-1,559)
Slijkplaat, Netherlands[6,7,8]	491 ± 292[c]	N.A.	142.2 (91.8-205.7)	21.3 (11.8-33.7)	1511 (1,232-2,257)

a Mean ± s.e.; due to different methodologies, EROD activities may not be directly comparable among Massachusetts, Canadian and Netherlands studies.
b Mean (range).
c Mean ± s.d.
d N.A. - not available.
1 Kouchibouguac National Park, New Brunswick, Canada. Reference site.
2 North Limestone Island, Georgian Bay, Lake Huron, Canada. Reference site.
3 Cat Island navigation marker, St. Lawrence River, Ontario, Canada. Contaminated site.
4 Long Sault Island, St. Lawrence River, Ontario, Canada. Contaminated site.
5 Hamilton Harbour, Lake Ontario, Canada. Contaminated site.
6 From Bosveld, et.al. (1995) Environ. Tox. and Chem. 14: 99-115.
7 Common terns from the Netherlands sites were analyzed within 12 hours of hatching.
8 Zeewolde is a reference site. Westplaat and Sliijkplaat are contaminated sites.

Bird Island compared to Nauset

Gross measurements

There were few differences in gross measurements between tern embryos from Bird Island and Nauset (Table 1). Decreased egg volumes, lower yolk-free body weights, and increased relative liver weights have been associated with aquatic bird populations affected by contaminants (Kubiak, Harris et al. 1989; Murk, Boudewijn et al. 1996); however, none of these differences were observed between Nauset and Bird Island. Results of other studies, both in field and injection studies, have shown no difference in the same gross measurements, even though there were clear differences in contaminant levels, biochemical activities, and hatching success (Rattner, Melancon et al. 1993).

Spleen weight and relative spleen weight were lower in Bird Island embryos than in those from Nauset (Table 1). Dioxins and PCBs have been associated with impaired immune function, and a lowered spleen weight at Bird Island could indicate impaired immune development. Treatment of kestrels and chickens with PCBs has been shown to reduce spleen weights and result in lymphoid depletion (Harris, Cecil et al. 1976; Hoffman, Melancon et al. 1996). Exposure to estrogens also has been shown to result in spleen atrophy and lymphocyte depletion in pigeons (John 1994). In some studies on developing chicken embryos, exposure to PCBs and dioxins has resulted in either splenomegally or had no effect on spleen weights (Powell 1996; Powell, Aulerich et al. 1996). In addition, splenomegally can occur as part of an immune response, and there is a period of rapid spleen growth just after hatching (John 1994). It is difficult to draw conclusions concerning the differences in spleen weights because of the variability in responses of spleen weights to different stimuli and the lack of information on contaminant effects on the avian spleen. Histological examination of spleens may provide insights to the observed differences in spleen weights between Bird Island and Nauset.

Two terns with crossed beaks were found, one at Bird Island and one at Nauset. Terns have higher incidence of crossed beaks relative to some other piscivorous birds such as cormorants (Ludwig, Kurita-Matsuba et al. 1996). Although the cause of crossed beaks is uncertain, it has been suggested that increased incidence of crossed beaks is correlated with elevated levels of planar PCBs and TCDD-EQs (2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents), both of which are aryl hydrocarbon receptor agonists (Ludwig, Kurita-Matsuba et al. 1996). Since a tern with a crossed beak was found at both sites, it could be indicative of a deformity causing agent present at both sites, or a naturally occurring incidence of beak deformities.

Biochemical data:

EROD data:

Induction of EROD activity can indicate exposure to contaminants that are aryl-hydrocarbon-receptor (AhR) agonists, such as PCBs, dioxins and furans. In terns, black-crowned night herons, and other aquatic birds, EROD activity has correlated well with levels of some contaminants, particularly total PCBs and dioxin equivalents, as well as some individual PCB congeners (Hoffman, Smith et al. 1993; Bosveld, Gradener et al. 1995).

The mean EROD activity was greater and had a higher standard deviation in terns from Bird Island than from Nauset, reflecting the wider range of individual variation in EROD at Bird Island as shown in Figure 2. The CYP1A protein levels measured in the common tern livers correlated well with the EROD activities (Figure 3), indicating that the increased activity is due to increased levels of CYP1A protein. This is consistent with other studies in which EROD activities are correlated with CYP1A levels (Hoffman, Smith et al. 1993; Bosveld, Gradener et al. 1995).

The relationship between the EROD activities and total PCBs for terns collected in 1994 from Bird Island early, late and Nauset is included in Table 4. The higher ERODs in early and late terns from Bird Island correspond with higher total PCB levels. The range of contaminant levels is much greater in both early and late terns from Bird Island than in terns from Nauset, similar to the EROD activity results.

The levels of EROD activity measured in terns in this study are lower than those measured in common terns in the Netherlands and the Canadian East Coast and Great Lakes/St. Lawrence River areas (Table 4). The levels of total PCBs at Bird Island, however, are near to or higher than those of the most contaminated Canadian sites, the St. Lawrence River 2 and Hamilton Harbor, which show EROD activities 4-5 times as great as Bird Island terns. The EROD activities measured at the contaminated Netherlands sites are 10-25 times greater than those at Bird Island. While the levels of PCB 153 at Bird Island are much lower than those at the most contaminated Netherlands sites, Westplaat and Slijkplaat, PCB 118 levels are similar. Likely, PCB congener patterns are quite different between Bird Island and the Netherlands sites. Although differences in AhR agonists, such as non-ortho PCBs and dioxins, or differences in sensitivity of terns to EROD induction at the different sites could be important, the contaminant data, particularly from the Great Lakes, suggests that higher EROD activities would be expected in Bird Island terns. The lower EROD activities observed in this study could be due largely to differences in the method of EROD measurements, particularly since incubations were at room temperature rather that at 37°C as in some of the other studies. In Bird Island compared to Nauset tern embryos, EROD activity is 2 times greater, while contaminants are 2-4 times greater. In Hamilton Harbor terns compared to the cleaner New Brunswick and Georgian Bay Canadian terns, EROD activity is elevated 2 times while contaminants are elevated 5-30 times. Similarly, the contaminated Westplaat and Slijkplaat terns compared to the cleaner Zeewolde terns in the Netherlands show EROD activity elevated 2-3 times while

contaminants are elevated 8-14 times. Thus, although the absolute EROD activities in terns from the three areas may not be comparable because of different techniques, the relationship between EROD activity and contaminant levels is similar.

PROD data:

PROD activity was measured as a biomarker of exposure to compounds such as organic pesticides and ortho substituted PCBs, which are known to induce the CYP2 subfamily enzymes in mammals. The very low levels of PROD activity in all terns examined here suggest that PROD may be unsuitable as a biomarker, or that exposure to CYP2 inducers was low. PROD activity was correlated with CYP1A protein levels and with EROD activities (Figures 3 and 4), and this in combination with the low activities suggests the PROD activities measured here likely were catalyzed by CYP1A rather than CYP2 proteins. This has been observed with other birds as well. Bosveld et al. found PROD activities in terns to be low and correlated with EROD activity, and suggested PROD activity was catalyzed by CYP1A (Bosveld, Gradener et al. 1995). In addition, CYP2-like protein levels were not elevated, suggesting that tern CYP2-like proteins were not induced or were not detected by the antibody used (Bosveld, Gradener et al. 1995). A further study using selective CYP isoenzyme inhibitors confirmed the hypothesis that PROD and EROD activity were catalyzed by the same enzyme in common terns (Bosveld, de Bont et al. 1995). Furthermore, bird injection studies with phenobarbital (PB), a typical CYP2B inducer in mammals, have failed to induce PROD activity or CYP2 protein levels in ducks and other seabirds (Walker and Ronis 1989). However, although Rattner et al. found only very low PROD activities in phenobarbital-injected black-crowned night herons, they detected elevated CYP2B-like proteins with polyclonal antibodies for CYP2B, suggesting the possibility that CYP2B-like proteins are inducible in aquatic birds (Rattner, Melancon et al. 1993). In all these studies, PROD activity appears to be very low in piscivorous avian

species, suggesting that it is not suitable as a biomarker for specific contaminant exposure. Elevated levels of CYP2 protein may be a more suitable marker; however, detection and elevation varies depending on bird species.

Contaminant Data:

Contaminant levels in yolk sacs of common tern embryos were compared from two populations: Bird Island, which has historically higher contaminant levels, and Nauset, which has historically lower contaminant levels. Although terns are migrating birds, the contaminants in embryo yolk sacs at Bird Island tend to reflect the contaminants in the breeding grounds (Nisbet and Reynolds 1984). In part, this is because lipid reservoirs have been depleted during migration from South American wintering grounds, and they must be replenished near the breeding grounds prior to egg laying. Accumulation of contaminants occurs with lipid accumulation; thus, contaminant burdens deposited in the eggs reflect those of the prey in the breeding grounds. It is also likely that contaminants reflect those near breeding grounds because wintering areas are less contaminated than the Bird Island area. Studies examining mercury in Bird Island common tern feathers showed that the burden acquired in the breeding grounds was much greater than that acquired over winter in South America (Burger, Nisbet et al. 1992; Burger, Nisbet et al. 1994). This indicated that wintering areas are cleaner than the Bird Island breeding grounds with respect to mercury and possibly other contaminants.

Our results support the expectation that Nauset is a cleaner breeding colony than Bird Island. As with the EROD activities, total PCB levels were significantly lower at Nauset than at Bird Island (Table 4). However, there is wide individual variation in total PCB levels among the terns at both Nauset and Bird Island. Total PCB levels range from 17.2 to 663.4 ug/g lipid at Bird Island, and from 8.4 to 178.4 ug/g lipid at Nauset. The wide range of contaminant burdens is not surprising since the contaminant burden will

depend on the feeding grounds utilized, and feeding ranges are from 10-20 km for common terns. Although Bird Island terns are located near a contaminated area, not all birds feed in the highly polluted sites, and cleaner feeding grounds are available surrounding Bird Island. The feeding grounds near Nauset are largely unpolluted, due to the cold water current running along its shore, but terns can feed in polluted areas prior to arriving at the Nauset breeding site. They may stop for a period of time at another nesting area, including areas at or near Bird Island, before finally settling at their final breeding grounds. Although Nauset is cleaner than Bird Island on a site basis, individuals with highly elevated contaminant levels were found at Nauset. The highest contaminant levels in individual terns from Nauset were comparable to intermediate contaminant levels in Bird Island terns and were higher than levels at the contaminated Hamilton Harbor, Great Lakes site (Table 4). This suggests that a cleaner site is needed. Furthermore, it may be more revealing to examine the relationship between contaminants and gonadal histology on an individual basis rather than a site basis, and this will be addressed in Chapter Three. More extensive contaminant data including PCB congeners, pesticides, mercury, and bioassay-derived dioxin equivalents also will be included in Chapter Three.

Gonadal Histology:

The prevalence of testes with ovarian cortical tissue containing primordial germ cells (ovotestes) among male common tern embryos was 60% at Nauset compared to 88.9% and 71.4 % at Bird Island early and late, respectively (Table 3). The trend for Nauset birds to show 10-30% less feminization than Bird Island terns may be consistent with a contaminant related effect, since Nauset birds have significantly lower EROD activity and total PCB levels than Bird Island terns. However, the prevalence of ovotestes at Nauset was still very high, and was not statistically significantly different from the

prevalence at Bird Island. This does not strongly support a contaminant related effect. There are several possible explanations for the high percentage of abnormalities at Nauset. First, ovotestes may be normal; second, some ovotestes development may be normal but increased by contaminants; finally, ovotestes may be related to contaminants other than those determined here.

Ovotestes may be normal:

One explanation for the high percentage of ovotestes at both Nauset and Bird Island is that the presence of ovotestes in male common tern embryos is normal. During normal development, the testis of the male embryo undergoes an "ovotestis" stage, in which the testis is surrounded by an ovarian-like cortex that may contain primordial germ cells (Laulanie 1886; Swift 1916; Romanoff 1960). In chickens, the gonad generally is differentiated completely into a typical testis by 14-10 days before hatching. However, some avian species show late persistence of these ovarian like cortical areas, which can still be present at the time of hatching. Testes of male doves, hawks, ducks, and quail all have been observed with ovarian cortical areas containing primordial germ cells at the time of hatching. Generally these areas disappear within a few days to weeks posthatching, but have been known to persist for several months in some species including hawks and ducks (Stanley 1937; Riddle and Dunham 1942; Lewis 1946; Haffen, Scheib et al. 1975). It is possible that terns are an avian species that normally possess persistent ovarian cortical areas at hatching.

Studies have been carried out in which chicken, duck, quail, dove, herring gull, and turkey eggs were dosed with estrogens and gonads were examined for developmental abnormalities. These studies generally showed no ovotestes presence among controls and 30-100% ovotestes among dosed males, in which the percentage of abnormal males increased with increasing dose, and also is affected by the timing of dosing during

incubation. Among birds where ovotestes have been observed normally, ovotestes development in estrogen dosed birds is more extensive, and often results in development of oviducts as well. No oviduct development was observed in male common tern embryos from Bird Island or Nauset.

In order to address the possibility that ovotestes are normal in terns at hatching, terns from several more sites, particularly uncontaminated sites, need to be examined at hatch, and/or during different stages of development. In addition, older hatchlings between several days old to fledgling age should be examined for the presence of ovotestes. This will be addressed in Chapter Four.

Some ovotestes development normal, but increased by contaminants:

A second possibility for the development of ovotestes in these birds is that some incidence of ovotestes is normal, but elevated contaminant levels, or exposure to a particular contaminant increases the incidence of ovotestes. This is supported by these data showing that among the 33 male tern embryo gonads examined, all the terns with EROD activities above 25 pmol/min/mg have ovotestes at some level of severity (Figure 8). The terns with EROD activities below 25 pmol/min/mg contain a mix of normal testes and ovotestes of severity 1-4. The incidence of ovotestes in Bird Island early and late embryos combined is 78%, compared to the incidence of 60% in Nauset embryos. However, male tern embryos with EROD activities lower than 25 pmol/min/mg have an incidence of ovotestes of 56% at Bird Island and of 60% at Nauset. The similarity in the percentage of ovotestes at lower EROD levels could mean a background level of ovotestes normally is observed, but contaminants increase the incidence of ovotestes. A normal background level of ovotestes could mask effects due to contaminants, making results more difficult to interpret.

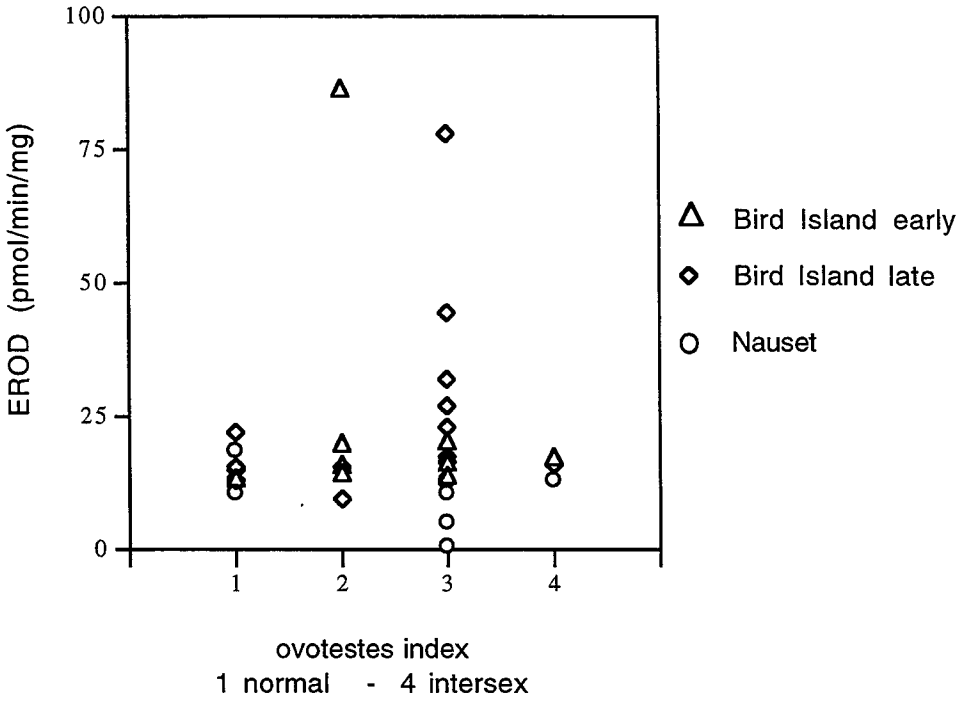


Figure 8. Hepatic EROD activity versus gonadal ovotestes index in pipping male common tern embryos from Bird Island and Nauset, collected in 1994.

Contaminants could affect the incidence of ovotestes in terns in at least two ways. They may act as a hormone, similar to a steroid hormone, or alter hormonal control of gonadal differentiation. Contaminants also could affect the incidence of ovotestes by slowing development, so that gonads are not fully differentiated at the time of hatch, and/or still posses persistent areas of ovarian cortex. Contaminants are known to lengthen incubation period (Kubiak, Harris et al. 1989; Murk, Boudewijn et al. 1996), suggesting that development is slowed when contaminant levels are elevated. Examining terns at hatching as well as at an older stage from other sites, both clean and contaminated, would help to clarify whether some ovotestes presence is normal at hatch and gonadal differentiation is affected by contaminants, as well as the possibility that all ovotestes are normal.

Ovotestes related to contaminants other than determined here:

Another explanation for the high number of abnormalities at both sites but lower contaminant levels and EROD activities at Nauset is that the abnormalities are related to contaminants, but not a contaminant represented by EROD activities in livers or total PCBs in yolk sacs. This possibility is relevant if ovotestes are caused entirely by contaminants or if a background level of ovotestes is normal, but is increased by contaminant exposure. The contaminants could be particular PCB congeners or other organic contaminants that do not vary with the levels of total PCBs. Ovotestes also could be related to metabolites of PCBs or of other organic contaminants, and these likely would not be found in the yolk sac. A metabolite would be very difficult to correlate since even levels that might be found in blood or liver would likely not be representative of levels throughout development. The contaminant also could be unrelated to halogenated organic contaminants, and instead, other contaminants such as organo-mercury may be important. Finally, even if the gonadal effect is related to contaminants that were measured, it could be highly dependent on the

time of exposure during development. Exposure during a critical developmental window may not be correlated well with the levels of contaminants in the yolk sac or with EROD activities. The importance of exposure during a critical period could apply to any contaminant that may be causing the development of ovotestes.

Summary:

The Nauset and Bird Island site comparisons do not confirm or disprove the hypothesis that incidence of ovotestes in male common tern embryos is related to contaminant exposure. It is difficult to interpret these results, in part, because Nauset may not represent a suitable clean site. Although EROD activities and total PCB levels were significantly lower at Nauset than at Bird Island, some individuals at Nauset showed high contaminant levels. Observations of deformities and necropsy data at the two sites did not further elucidate differences in the health of the two populations. There were no differences in gross measurements between Nauset and Bird Island other than decreased relative spleen weight at Bird Island; a possible indicator of stressed immune systems. Crossed beaks were observed at both sites, which could suggest that exposure to an agent affecting development may not be exclusive to Bird Island. It is also difficult to compare the reproductive success of the breeding populations at Nauset and Bird Island. Due to owl predation, Nauset has consistently lower reproductive success than Bird Island terns (Table 5), and therefore no useful comparisons can be made. The lack of differences in health-related measurements of birds from the two sites is also consistent with the occurrence of ovotestes. Although the incidence of ovotestes presence was lower at Nauset than at Bird Island, this difference was not significant. Furthermore, the incidence of ovotestes at both Nauset and Bird Island was very high (60%, 78%). As discussed, several possibilities are raised. The ovotestes could be related to contaminants or metabolites that were not examined, or dependent on exposure during a critical period of development. Additionally,

Table 5. Reproductive success data for common terns breeding at Bird Island and Nauset, Massachusetts in 1994-1996.

	Year 1994-1996	Bird Island	Nauset
nests		2,000[a]	1,000-2,000
Productivity (chicks fledged per nest)	1994 1995 1996	1.2 1.4-1.5 0.9-1.0[b]	near zero moderate very low
Productivity limited by:		food availability (no significant predation)	owl predation (not limited by food supply)

[a] Source of data: I.T.C. Nisbet, unpublished data.

[b] Productivity would have been ~1.1 except for flooding losses during tropical storm Bertha.

the presence of ovotestes could also be normal in common tern embryos, or normal at a background level which could mask any effect related to contaminants.

Further examination of the possible relationship between contaminants and the incidence of ovotestes among male common tern is necessary. Terns should be examined on an individual basis for relationships between contaminants and the presence of ovotestes because of the high variation in contaminant levels among individuals. Contaminants need to be examined more specifically by looking at individual PCB congeners, organic pesticides, mercury levels, and dioxin-like compounds. These issues will be addressed in Chapter Three. It is also critical to establish whether the incidence of ovotestes in male common terns is normal. This can be addressed by examining terns from additional sites, particularly cleaner sites. Examination of terns at both hatching and a later stage of development also would help answer this question; this will be addressed in Chapter 4.

CHAPTER 3: RELATIONSHIP OF CHEMICAL CONTAMINANTS TO THE PRESENCE OF OVOTESTES IN COMMON TERN EMBRYOS COLLECTED IN 1994.

Chapter 3: Relationship of chemical contaminants to the presence of ovotestes in common tern embryos collected in 1994.

Introduction

Chemical contaminants, particularly chlorinated hydrocarbon pesticides and aryl-hydrocarbon receptor (AhR) agonists such as coplanar PCBs and dioxins, have had dramatic effects on the reproduction and development of colonial waterbird populations (Peakall 1970; Fry and Toone 1981; Gilbertson, Kubiak et al. 1991). Terns breeding at Bird Island historically have had high PCB contaminant levels (Nisbet and Reynolds 1984), and therefore the relationships of specific chemical contaminants to the presence of ovotestes and other characteristics in 1994 common tern embryos were examined. Several different classes of contaminants were selected, including AhR agonists measured as dioxin equivalents (TCDD-EQs), polychlorinated-biphenyls (PCBs), chlorinated hydrocarbon pesticides, and mercury. These contaminant classes were selected because they are known to affect reproduction and development in colonial waterbird populations (Peakall 1970; Fry and Toone 1981; Gilbertson, Kubiak et al. 1991), and also because they are major contaminants in the Bird Island tern population (Nisbet and Reynolds 1984; Burger, Nisbet et al. 1992) .

AhR agonists, or dioxin-like compounds, include non-ortho and mono-ortho substituted PCBs, PCDDs, and PCDFs. In this study, AhR agonist activity of yolk sac contaminants was determined by measuring bioassay-derived dioxin-equivalents (TCDD-EQs). Exposure to AhR agonists has been shown to cause increased embryo mortality and developmental abnormalities among colonial waterbirds including terns, gulls, and cormorants (Gilbertson 1983; Gilbertson, Kubiak et al. 1991; Sanderson, Norstrom et al.

1994). In addition, dioxin-like compounds have anti-estrogenic properties, alter testosterone levels, and may result in developmental and reproductive abnormalities (Gilbertson 1983; Gilbertson, Kubiak et al. 1991; Sanderson, Norstrom et al. 1994). The areas near Bird Island and Nauset are historically low in PCDDs, but high levels of mono- and non-ortho substituted PCBs are associated with the high PCB contaminant levels near Bird Island (Lake, McKinney et al. 1995).

In addition to the AhR agonist activity of certain PCB congeners, PCBs can also disrupt the endocrine system. Individual PCB congeners and Aroclor mixtures have been shown to have reproductive and endocrine disrupting effects (Birnbaum 1994), including estrogenic and antiestrogenic properties (Bitman and Cecil 1970; Bulger and Kupfer 1985; Jansen, Cooke et al. 1993). Hydroxy metabolites of the lower chlorinated PCBs may bind to the estrogen receptor (Korach, Sarver et al. 1987), and also have been shown to alter sexual determination in turtles (Bergeron, Crews et al. 1994). There are very high levels of PCBs, including lower chlorinated PCBs, in New Bedford Harbor near Bird Island (Lake, McKinney et al. 1995), increasing the probability that lower-chlorinated hydroxy-PCBs and other PCB congeners could be affecting the health of terns breeding on Bird Island.

Chlorinated hydrocarbon pesticides include the dichlorodiphenylethanes (DDT, DDD, Dicofol, Methoxychlor), the chlorinated cyclodienes (aldrin, dieldrin, endrin, heptachlor, heptachlor epoxide, chlordane, endosulfan, mirex, chlordecone), and the chlorinated benzenes and cyclohexanes (hexachlorobenzene (HCB), hexachlorocyclohexane (HCH), lindane) (Matsumura 1975; Amdur, Doull et al. 1991). Many of these chlorinated hydrocarbon pesticides affect reproductive and endocrine systems (Colborn, vom Saal et al. 1993), exhibit estrogenic properties and/or binding to the estrogen receptor (Bitman and Cecil 1970; Bulger, Mucitelli et al. 1978; Bulger and Kupfer 1985; Reel and Lamb 1985; McLachlan 1993) and also may act as anti-androgens (Kelce, Stone et al. 1995; Kelce, Lambright et al. 1997). High levels of DDT have been a

particular problem for colonial water birds, beginning with severe eggshell thinning that resulted in dramatic decreases in embryo survival (Peakall 1970; Giesy, Ludwig et al. 1994). DDT exposure during development also was related to the presence of severe ovotestes in California herring gulls, and likely resulted in the observed abnormal breeding behavior and lowered reproduction (Fry and Toone 1981; Fry, Toone et al. 1987). Low levels of pesticides including DDT, dieldrin, endrin, heptachlor epoxide, HCB, alpha-chlordane, and oxychlordane, previously have been detected in Bird Island tern eggs (Nisbet and Reynolds 1984).

Like the PCBs, PCDD/Fs, and chlorinated hydrocarbon pesticides described above, methylmercury can bioaccumulate to high levels in fish, resulting in high methylmercury exposure of piscivorous birds such as terns (Scheuhammer 1987). Laying female birds deposit methylmercury in the albumen of their eggs, resulting in exposure of the developing embryo (Scheuhammer 1991). Most of the effects from methylmercury exposure are neurological, but at lower levels, reproductive effects such as decreased egg production, decreased hatchability, and increased hatching mortality occur (Heinz 1974; Heinz 1976; Finley and Stendell 1978; Scheuhammer 1987; Scheuhammer 1991). Although specific hormone-like effects of methylmercury have not been shown, organo-tin (another organo-metal) alters invertebrate reproductive tract development and function resulting in masculinization of females (Gibbs, Spencer et al. 1991). Terns at Bird Island have had elevated levels of total mercury previously (Burger, Nisbet et al. 1992), suggesting that methylmercury exposure of embryos could be important; it is highly unlikely that inorganic mercury would be deposited in the eggs.

This chapter examines chemical contaminants in the 1994 common tern embryos, including PCBs, chlorinated hydrocarbon pesticides, mercury, and AhR agonists. Contaminant levels were examined in a subset of nineteen male common tern embryos with complete gonad histology and two female embryos with crossed beaks. Yolk sacs from

these birds were used as a measure of embryo contaminant exposure, and extracted for persistent organic contaminants. Chemical measurements on yolk sac extracts were performed for a suite of PCB congeners and chlorinated hydrocarbon pesticides. The yolk sac extracts also were used to measure bioassay derived dioxin equivalents (TCDD-EQs), using a chick embryo hepatocyte bioassay. Muscle tissue from the embryos was used for total mercury measurements, likely mainly methylmercury. Site differences in contaminant levels, relationships among the contaminants to the presence of ovotestes in the male common terns, and relationships of contaminants to other embryo characteristics including EROD activity and deformities were examined.

Methods

Extract Preparation and Chemical Analysis. Extraction of persistent organic contaminants from egg homogenates (1995 Bird Island eggs- Chapter 4, 1996 Bird Island egg pools-Chs 3,4) and yolk sacs (1994 Bird Island, Nauset, 1996 Bird Island- Ch 2,3,4,5, 1996 Great Lakes sites- Ch 2, Table 4) was carried out according to the method of Kennedy et. al., 1996, with minor modifications (Norstrom, Simon et al. 1986; Norstrom, Simon et al. 1990; Kennedy, Lorenzen et al. 1996). Final extracts contained at least all PCDDs, PCDFs, PCBs, structurally related nonpolar HAHs (e.g. polybrominated biphenyls, chloronaphthalenes, and diphenyl ethers), and chlorinated hydrocarbon pesticides (e.g. DDE, Dieldrin, and Mirex). This method is described in the flow chart in Figure 1. Briefly, 5 grams of egg homogenate or entire yolk sacs were dried with sodium sulfate, extracted with dichloromethane (DCM)/hexane (1:1), and cleaned up by gel permeation chromatography (GPC) to remove lipids. The lipid fraction from GPC was collected and

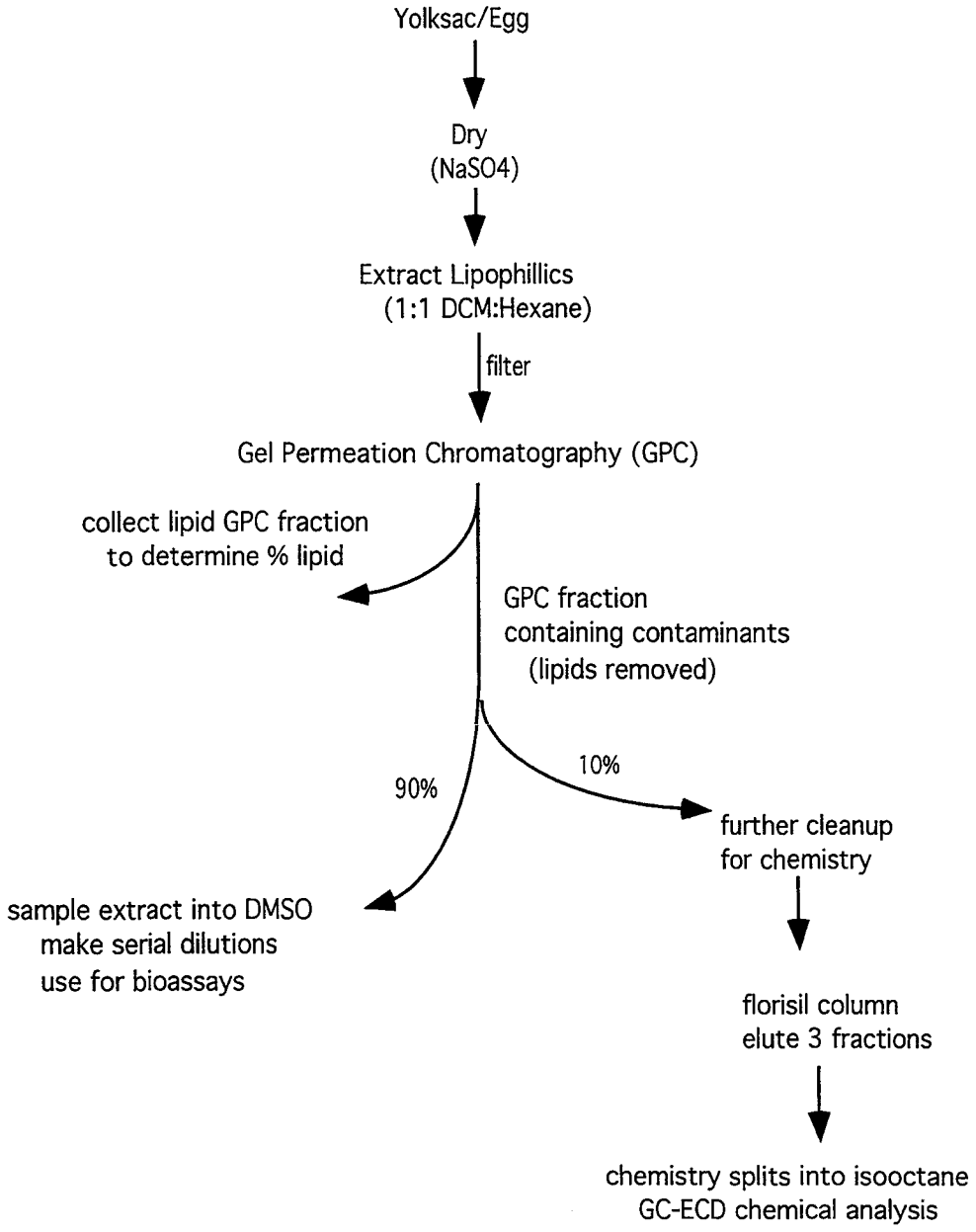


Figure 1. Flow chart for extract preparation method. Lipophillic organic contaminants were extracted from egg and yolksac samples for chemical analysis and use in bioassays.

Table 1. Summary of total peak areas and percentages of PCBs eluting from the GPC during described collection intervals.

sample	total peak area (all peaks)	% total area
GPC collection minute:		
minute 29	47224	0.04
minute 30	52916	0.04
minute 31	104876	0.09
minute 32	667935	0.55
minute 33	3598879	2.98
minute 34	11528720	9.55
minute 35	29803940	24.70
minute 36	35286390	29.24
minutes 37-61	39588900	32.80
sum total area minutes 29-61	120679780	100.00
Aroclor1260 standard	120736500	

used to determine the percent lipid in the sample. The contaminant containing fraction collected from the GPC was split; 10% of the sample was used for chemical analysis and 90% for bioassays. The 90% portion of the split was transferred quantitatively into 200 µl of DMSO using a rotary evaporator and water bath heated to 25° C. The DMSO solutions were vortexed, and serial dilutions in DMSO were prepared. The 10% split for chemical analysis was further cleaned on a florisil column according to the standard procedures of pesticide and PCB trace contamination determination at the National Wildlife Research Center (NWRC) (Won 1992). The sample was eluted from the florisil column in three fractions: fraction 1 was eluted with hexane, fraction 2 with 15% DCM in hexane, and fraction 3 with DCM/hexane (1:1). These three fractions were used to measure a suite of PCB congeners and organic pesticides by gas chromatography electron capture detection (GC-ECD), as carried out by Henry Won at NWRC, Hull, Quebec. Samples were injected into an HP 5890 GC #1 using an HP 7673A auto injector. Throughout the method, all glassware was rinsed with acetone and hexane three times prior to use, to remove any contaminants.

When necessary, a rotary evaporator was used to reduce solvent volumes. The method was tested with PCB 12, and only minimal sample losses occurred by using rotary evaporation. Nitrogen gas was not used to evaporate samples, since this would have resulted in loss of lower (mono, di, tri) chlorinated PCBs.

The GPC dump and collection times were tested with Aroclor 1260 in order to be certain that the maximum amount of lipid was removed from the sample (dump time, the first 30 minutes) without loss of the chemical contaminants (collect time, the second 30 minutes). The results of this study are given in Table 1, and show that the PCBs do not elute significantly from the GPC before minute 33. The dump time used to remove lipids was 30 minutes, well before the chemicals elute. The lack of lipids in the collected sample after GPC allowed transfer of the 90% split into DMSO without further cleanup. This also

allowed retention of any potential hydroxylated metabolites and other compounds present in the sample that normally would be removed by cleanup on an alumina or florisil column.

Recovery studies were also carried out to test the method prior to extraction of yolk sacs or eggs, and appropriate controls were used throughout all extractions. Recovery studies used homogenized chicken egg samples spiked with 1,2,5,10,15, or 25 ppm Aroclor 1248. These samples were extracted as described above, and analyzed in the TCDD-EQ CEH bioassay (described below) along with 1,2,5,10,15 or 25 ppm Aroclor 1248 controls which had been placed directly into DMSO for use in the bioassay. Bioassay results were highly correlated between extracted and control Aroclor samples ($r2 = 0.86$), and recoveries were between 70-90% (Data not shown).

Control samples were used throughout the extractions of yolk sacs and eggs to monitor recovery. Controls included blank eggs, solvent blanks, spiked eggs, and spiked solvents, all of which were extracted. In addition, an unextracted spiked solvent control was used for comparison with extracted spiked eggs and spiked solvent controls. Recoveries were based on the results of chemical analysis (data not shown). Two solvent blanks and one blank egg showed no detectable contaminants; a second blank egg contained a total PCB level of 0.05 ppm. Results from four spiked eggs and one spiked solvent control showed recoveries from 89.5 to 103.7%. These results indicate the extraction method had good recoveries and did not result in contamination of the samples.

TCDD-EQ CEH bioassay. Primary hepatocyte cultures were prepared from 19-day-old chicken embryos in 48-well plates as described previously (Kennedy, Lorenzen et al. 1993). At 24 hours, duplicate plates were dosed with each egg or yolk sac extract dilution series. All yolk sac extracts were compared in a single cell culture, while all egg extracts were compared in a second cell culture. For each cell culture, triplicate 48-well plates were dosed (3 wells/dose) with TCDD (range of doses 0.0001 to 3 nM). After dosing, cells

were incubated for another 24 hours, at which time the medium was removed, the cells were rinsed, and plates were frozen on dry ice before transferring to a -80° C freezer. After thawing, EROD and protein assays were carried out in the cell culture plates as described previously (Kennedy, Jones et al. 1995), and the reaction products (resorufin and fluorescamine-protein adducts) were measured with a fluorescence plate reader (Cytofluor 2300, Millipore Ltd.). EROD dose-response curves were fitted empirically to a modified Gaussian curve as described previously (Kennedy, Lorenzen et al. 1996), and the maximal EROD activity and the EC_{50} were determined for each dose-response curve. Bioassay derived TCDD-EQs then were calculated as described previously (Kennedy, Lorenzen et al. 1996) according to the following equation:

TCDD-EQbio (ng/g lipid) = (TCDD EC50 (ng/ml of medium)/extract EC50) x
(volume of medium (ml)/volume of DMSO (µl)) x
(volume of extract stock (µl)/mass of lipid in sample (g)).

Hg analysis: Total mercury (Hg) was analyzed in the laboratory of Dr. Tony Scheuhammer at NWRC Hull, Quebec according to the Metals Toxicology Laboratory standard operating procedure of May 1995. Two ethanol preserved samples for each embryo in a subset of 1994 embryos were analyzed, one of leg muscle and the other of leg skin and feathers. It was determined that Hg had not leached out of the embryo samples into the ethanol. Briefly, the samples were freeze dried, digested with nitric acid, further digested with sulfuric acid and hydrochloric acid, and then brought to volume with potassium dichromate solution, dilute hydrochloric acid, and octanol. Hg was determined on these samples by cold vapor technique atomic absorption spectrophotometry using tin chloride in hydrochloric acid as the reductant on a Perkin Elmer 3030B atomic absorption spectrophotometer equipped with a Varian vapor generator accessory (VGA-76), a Varian autosampler (PSC-55), and a Perkin Elmer electrodeless discharge lamp.

Immunoblotting: Immunoblots were carried out on chick embryo hepatocytes dosed with three yolk sac extracts. 48-well plates were dosed, rinsed, and frozen as described above. Frozen hepatocyte cultures were then solubilized (Hahn, Woodward et al. 1996), and wells from each dose were pooled on ice in sample treatment buffer (0.25 M Tris-HCl, pH 6.8, 40% (v/v) glycerol, 4% sodium dodecyl sulfate, 0.008% bromophenol blue, and 5% (v/v) beta- mercaptoethanol) to a final concentration of 1.5 ug total protein/ul. The samples were boiled for 5 minutes to ensure complete solubilization and to inactivate proteases. Then 15 ug total cell protein was separated on sodium dodecyl sulfate polyacrylamide gels and electrophoretically transferred to Rad-Free (Biorad) membranes. Aliquots of the same preparation of BNF induced scup liver microsomes were used as standards for all blots. Immunodetection of cytochrome P450 1A was performed with MAb 1-12-3, and immonoreactive proteins were detected by chemiluminescence (Lorenzen, Shutt et al. 1997).

1996 egg and yolk sac samples: Eggs and yolk sacs from Bird Island embryos and from five Great Lakes sites (described in Table 4, Chapter 2) collected during the 1996 breeding season were extracted as above, except that 50% of the sample was split for chemical analysis and 50% was quantitatively put into DMSO. The split for chemical analysis was used to determine PCDD/PCDFs and coplanar PCBs 37, 81, 77, 126, 169, and 189 in addition to the suite of PCBs and organic pesticides described above. This chemical analysis was carried out at NWRC, Hull, Quebec according to standard methods for determination of PCDD, PCDF and non-ortho PCB contaminants by high resolution GC/high resolution MS system (Norstrom, Simon et al. 1986; Norstrom, Simon et al. 1990; Won 1992; Ford, Muir et al. 1993).

For Bird Island samples, calculated dioxin equivalents (TCDD-EQcalc) were determined by multiplying the concentration of each congener by the relative potency (RP) of that congener to 2,3,7,8-TCDD to give the dioxin equivalents for that congener (TCDD-EQcongener), and then summing all the TCDD-EQcongeners for that sample:

TCDD-EQcalc = $\sum$ (RPcongener x concentration of congener (ng/g lipid))

The relative potencies used are further described in the text.

Statistics: Analyses of Variance (ANOVAs) were carried out to test differences in samples. Two-way ANOVAs were carried out to examine the effects of site ((Bird Island early, Bird Island late, Nauset,) and/or (Bird Island, Nauset)), gonad (presence or absence of ovotestes), and the interaction between site and gonad on log transformed data (contaminant concentrations and EROD activities). If the main effect(s) or the interaction term of the model had a p value > 0.2, it was removed from subsequent analysis. Significance levels were $p \leq 0.05$.

Correlations were tested using Pearson product moment correlation coefficients, r, and Bartletts Chi-square statistic probabilities, p. Significance levels were $p \leq 0.05$.

To analyze the PCB congener patterns among samples, principal component analysis (PCA) was carried out (Joliffe 1986). PCB congeners in each yolk sac sample were categorized by the number of chlorines (3 - 9). Each chlorine category was expressed as a proportion of the total PCBs in the corresponding sample. PCA was conducted on individual yolk sac PCB data.

Results

Chemical Analysis:

All PCB congeners, chlorinated hydrocarbon pesticides, and TCDD-EQs are reported per gram lipid. These contaminant levels were normalized to lipid weight because lipid is the reservoir for these extracted chemical contaminants, and the percent lipid in the yolk sacs was variable, ranging from 17% to 34% lipid. All sites showed a similar range of the lipid content of yolk sacs, and as confirmed by ANOVA, no statistically significant difference occurred among sites in the percent lipid of the samples ($p = 0.39$).

PCB contaminant levels varied widely at both Bird Island, and Nauset. At Bird Island total PCBs (defined as the sum of all PCB congeners measured including PCB 31, 28, 29, 52, 49, 44, 42, 64, 74, 70, 66, 60, 101, 99, 97, 87, 84, 77, 110, 151, 149, 118, 146, 153, 105, 141, 137, 138, 158, 129, 182, 183, 128, 185, 174, 171, 200, 172, 180, 170, 201, 203, 195, 194, 206) ranged from 17.2 to 663.4 ug/g lipid, while at Nauset total PCBs ranged from 8.4 to 178.4 ug/g lipid. Among sites, total PCBs were not significantly different between Bird Island early and late, but when Bird Island early and late were combined into one Bird Island site, total PCBs were significantly higher than at Nauset (Table 2). The two females with crossed beaks had total PCB levels of 83.9 (Bird Island) and 14.4 (Nauset) ug/g lipid, which were intermediate and low levels relative to other samples from Bird Island and Nauset. All individual PCB congeners were highly correlated with total PCBs (Pearson product correlation coefficients, $r > 0.85$) except for PCB 128 and 185, of which most samples had levels below the detection limit, and some of the highest chlorinated congeners such as 206 and 194, which also had very low levels. Levels of PCB congener 29, previously suggested for further examination as a possible important congener in relation to ovotestes development (Nisbet, Fry et al. 1996), were below the detection limit (0.00005 ppm) in all samples.

Table 2. Site comparison of contaminant data collected from 1994 common tern embryos. Mean (standard error), (range).

Contaminant	Bird Island n = 14 [c]	Nauset n = 8
sum PCB (ug/g lipid)	114.59 (45.00) A (17.16 - 663.43)	35.36 (20.60) B (8.38 - 178.41)
TCDD-EQbio (ng/g lipid)	39.74 (13.62) A (7.05 - 174.07)	12.36 (6.52) B (4.48 - 57.78)
sum p,p'-DDTs (ug/g lipid)	3.67 (0.45) A (0.91 - 6.79)	3.33 (0.62) A (1.78 - 6.83)
trans-nonachlor (ug/g lipid)	0.73 (0.32) A (0.07 - 4.63)	0.28 (0.14) A (0.02 - 1.25)
Mirex (ug/g lipid)	0.24 (0.07) A (0.05 - 0.94)	0.25 (0.08) A (0.0001 - 0.62)
total Hg (ug/g dry wt.)	1.46 (0.14)[d] A (0.97 - 2.76)	1.01 (0.11)[e] B (0.76 - 1.53)

A,B Within rows, means with the same letter are not significantlly different at $p \leq 0.05$.

[c] Samples from Bird Island early and late collections were combined. There were no significant differences between any contaminant levels in Bird Island early and Bird Island late at $p \leq 0.05$.

[d] n = 13

e n = 7

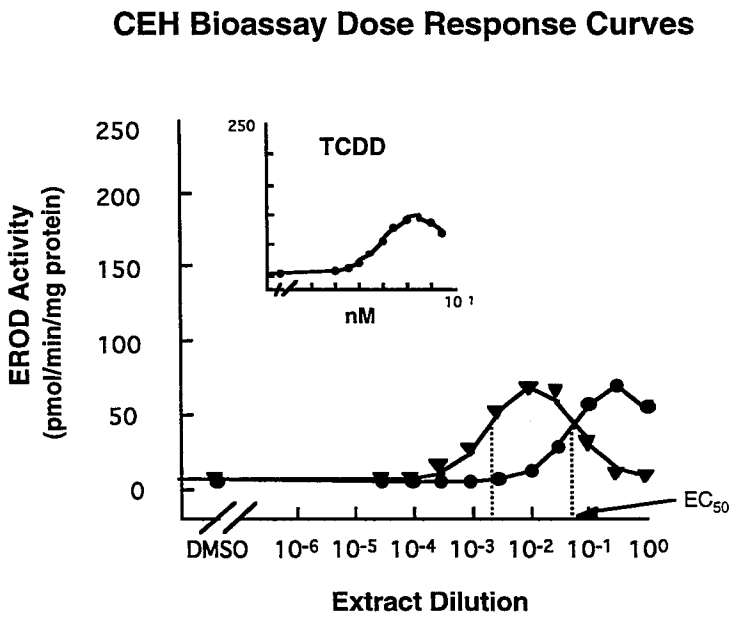


Figure 2. Chick embryo hepatocyte (CEH) bioassay dose-response curves from two common tern embryo yolk sac extracts collected in 1994, and TCDD. The response measured is EROD activity. EC50s from yolk sac extract dose response curves are compared to EC50s from TCDD in order to calculate TCDD-EQs. Yolk sac extract dose response curves are typical for all yolk sac and egg extracts examined.

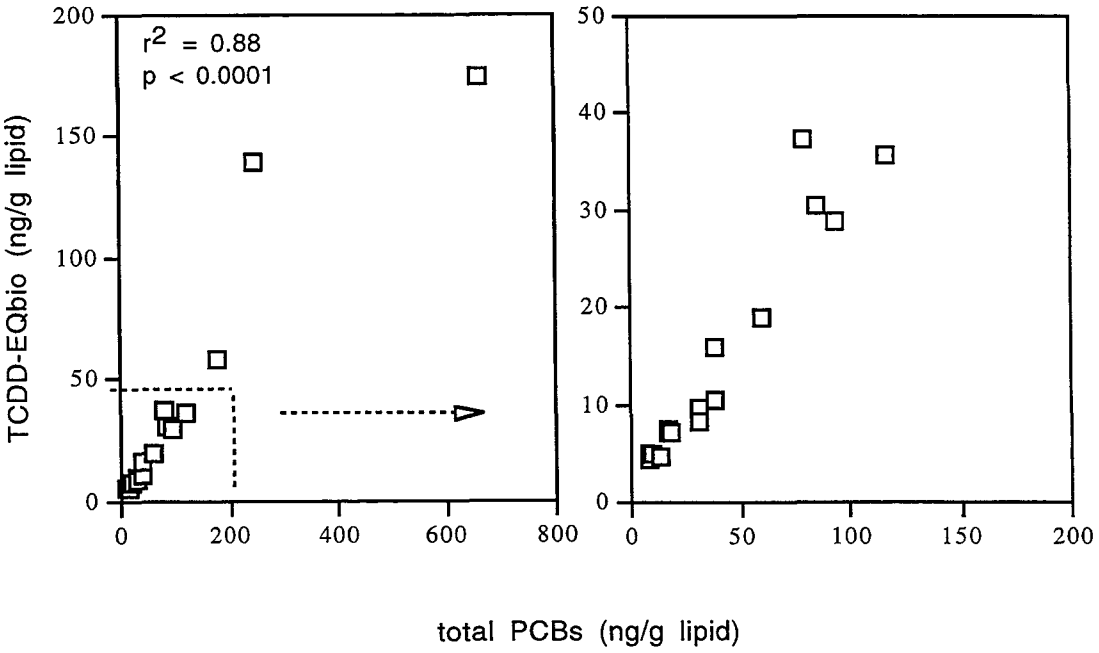


Figure 3. Bioassay-derived TCDD-EQs versus total PCBs in pipping common tern embryo yolk sacs collected from Bird Island and Nauset in 1994.

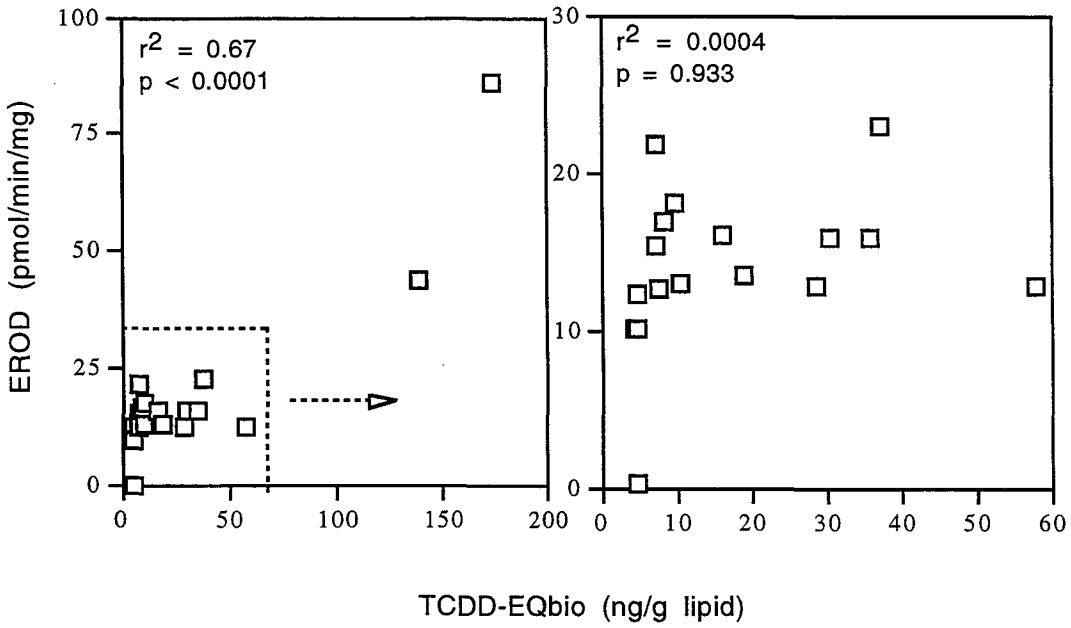


Figure 4. Hepatic EROD activity versus bioassay-derived TCDD-EQs in yolk sacs from pipping common tern embryos collected from Bird Island and Nauset in 1994.

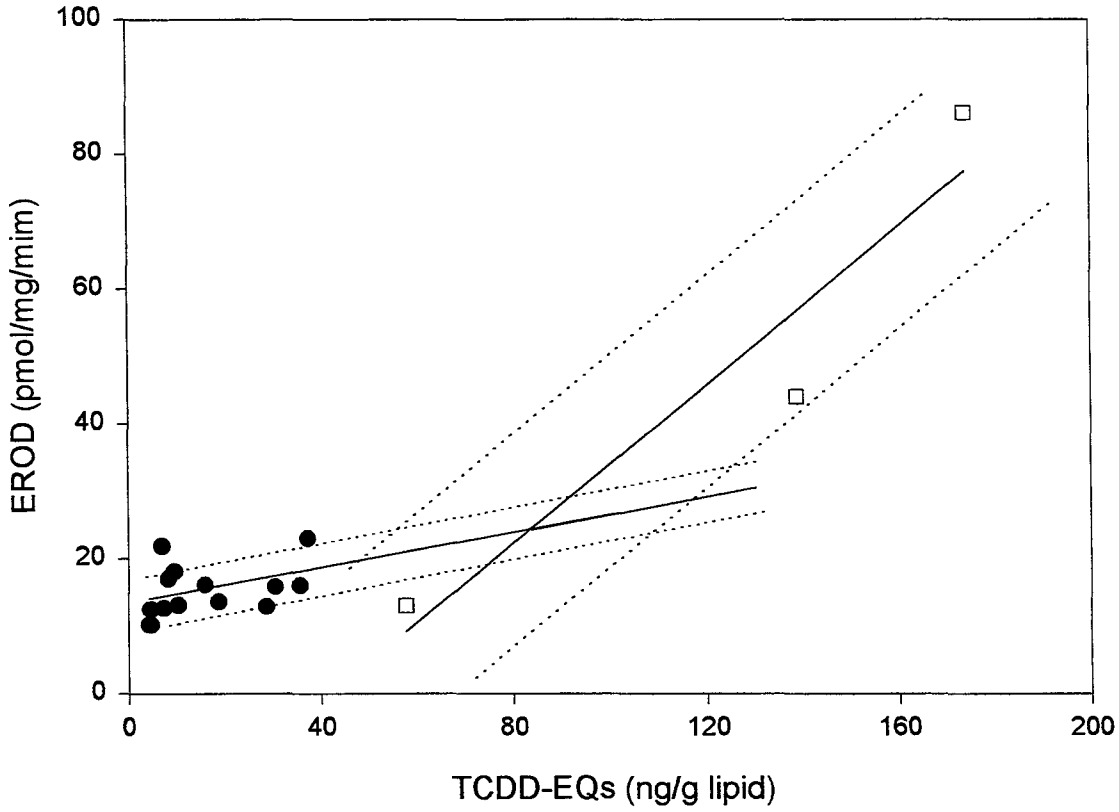


Figure 5. Regressions of hepatic EROD activity and bioassay-derived TCDD-EQs from yolk sacs of pipping common tern embryos collected from Bird Island and Nauset, 1994. The dotted lines indicate the standard error of each regression estimate (3.7 and 15.7 for the regression lines on the left and right respectively). The lines intersect at the TCDD-EQ value 82.5.

The chlorinated hydrocarbon pesticides that were analyzed showed low levels at both Nauset and Bird Island sites (Table 2). For most pesticides, samples had levels below 0.5 ug/g lipid. The exceptions were trans-nonachlor, mirex, and p,p'-DDE. Mirex levels were less than 1 ug/g lipid, and trans-nonachlor levels were also below 1 ug/g lipid except for 3 samples with levels of 1.2, 1.6, and 4.6 ug/g lipid. The only pesticide with sample levels commonly above 2 ug/g lipid was p,p'-DDE, with levels ranging from 0.9 - 6.7 ug/g lipid. p,p'-DDE, p,p'-DDT, and p,p'-DDD were summed to give total p,p'-DDTs, the primary component of which is p,p'-DDE (sum range 0.9-6.8 ug/g lipid). Total p,p'-DDTs were not significantly different among any of the sites, Bird Island early, late, Bird Island combined, or Nauset. Levels of o,p'-DDTs were not measured here, but have been measured at Bird Island in previous years at levels that were extremely low or non-detectable (Nisbet and Reynolds 1984).

The results of the chemical analysis for all PCB congeners and chlorinated hydrocarbon pesticides are listed in appendix B.

TCDD-EQs:

Typical EROD dose response curves from two yolk sac extracts and TCDD are shown in Figure 2. The maximums of all the yolk sac extract dose-response curves were within 49 - 96% of the maximum of the TCDD dose-response curve. Immunoblotting with antibody to CYP1A showed that levels of CYP1A protein followed the EROD dose-response levels, increasing up to the maximum EROD response and then decreasing (data not shown).

TCDD-EQs ranged from 7.0 - 174.1 ng/g lipid at Bird Island, and from 4.5 - 57.8 at Nauset (Table 2). There were no significant difference between Bird Island early and late TCDD-EQs, so data from Bird Island early and late were combined. With Bird Island as one site, TCDD-EQs were significantly higher at Bird Island than at Nauset. TCDD-

EQs of the two females with crossed beaks were 29.7 (Bird Island) and 5.3 (Nauset) ng/g lipid, which were intermediate and low TCDD-EQs relative to other samples at Bird Island and Nauset.

TCDD-EQs were highly correlated with total PCBs, ($r^2 = 0.88$, $p < 0.0001$) (Figure 3). TCDD-EQs also were correlated with EROD activity measured in the livers of the corresponding embryos ($r^2 = 0.67$, $p < 0.0001$). However, upon removing the two highest EROD activity values, the correlation becomes highly non-significant ($r^2 = 0.0004$, $p = 0.933$) (Figure 4). Because of the biphasic nature of the EROD versus TCDD-EQ relationship, two regression lines were fitted to the EROD data (Figure 5).

Mercury (total Hg):

There was no difference in mercury levels determined in muscle compared to those determined in the feather/skin samples. Because the feather/skin samples were less homogeneous, the muscle values for mercury were used. Total mercury values in these samples were all very low, ranging from 0.97 - 2.76 ug/g dry weight at Bird Island and from 0.76 - 1.53 ug/g dry weight at Nauset (Table 2). Only one embryo had a mercury level above 2.0 ug/g in these samples, and that is the 2.76 value at Bird Island. There was no significant difference in the mercury levels among BIE, BIL, and Nauset, but when Bird Island data were combined, mercury levels were significantly higher than those at Nauset. The Hg levels are correlated with the total PCB contaminant levels ($r^2 = 0.61$, $p = 0.0001$); however this correlation is driven mainly by the single value above 2.0 ug/g dry weight, and removing it results in the correlation becoming non-significant ($r^2 = 0.18$, $p = 0.09$).

Relationship of contaminants to the presence of ovotestes:

The contaminant data was classed by site (either Bird Island or Nauset) with ovotestes being either present (severity 2-4) or absent (severity 1), and graphical results are shown in Figures 6-9. Two-way ANOVAs showed no significant differences in contaminant levels between embryos with and without ovotestes for any of the four contaminants at either site. There were significant differences in contaminant levels between sites, with Bird Island having higher total PCBs, TCDD-EQs, and Hg than Nauset, as was previously summarized in Table 2.

Contaminant data for total PCBs, TCDD-EQs, total p,p'-DDTs, and total mercury were plotted against the ovotestis severity index (1 absence - 4 intersex) to look for a possible dose-response relationship. As can be seen in Figure 10, there is no clear dose-response relationship between any of these contaminants and severity of the ovotestes in the embryos. Lower levels of contaminants are observed in birds with all levels of ovotestes severity, from 1(absent) to 4 (intersex). The birds with the highest levels of PCBs and TCDD-EQs had ovotestes, with severity ranging from 2-4.

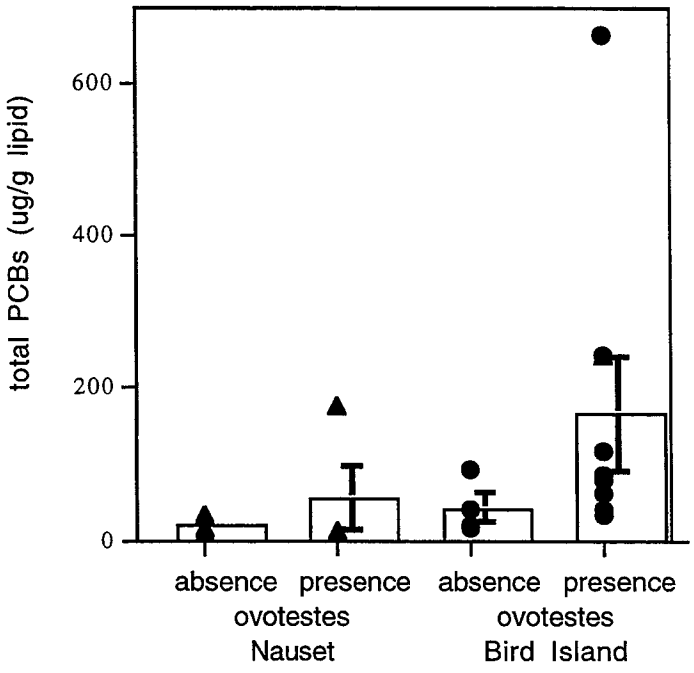


Figure 6. Comparison of ovotestes presence with levels of total PCBs in yolk sacs of pipping common tern embryos collected at Nauset and Bird Island in 1994. There were no significant differences in total PCB levels between embryos with and without ovotestes at either site. The Bird Island site had significantly higher total PCB levels than Nauset.

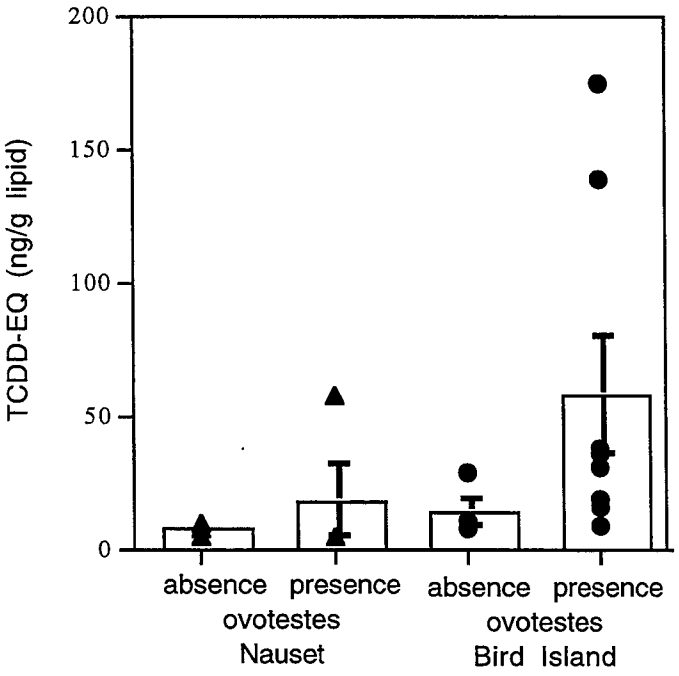


Figure 7. Comparison of ovotestes presence with levels of bioassay-derived TCDD-EQs in yolk sacs of pipping common tern embryos collected from Nauset and Bird Island in 1994. There were no significant differences in TCDD-EQs between embryos with and without ovotestes at either site. The Bird Island site had significantly higher TCDD-EQls than Nauset.

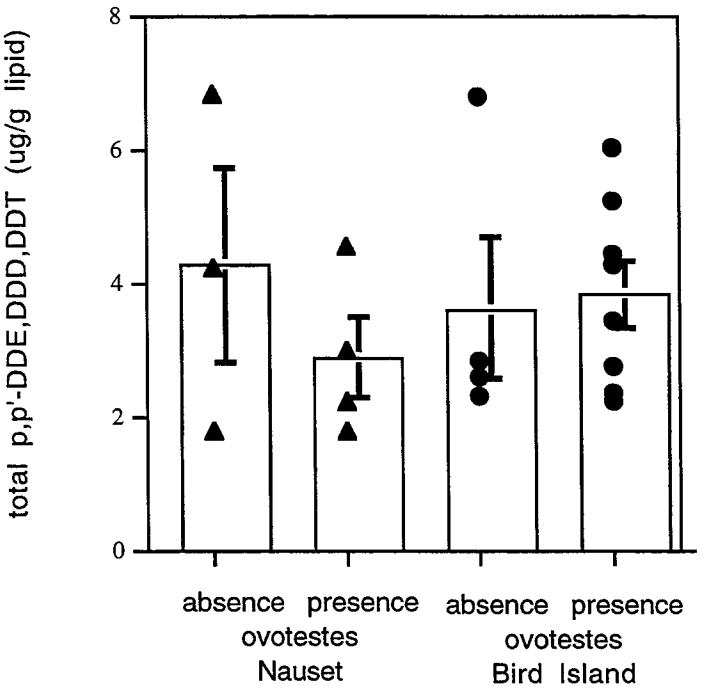


Figure 8. Comparison of ovotestes presence with levels of total p,p'-DDE,DDD,DDT in yolk sacs from pipping common tern embryos collected at Nauset and Bird Island in 1994. There were no significant differences in total p,p'-DDTs between embryos with and without ovotestes at either site, or between sites.

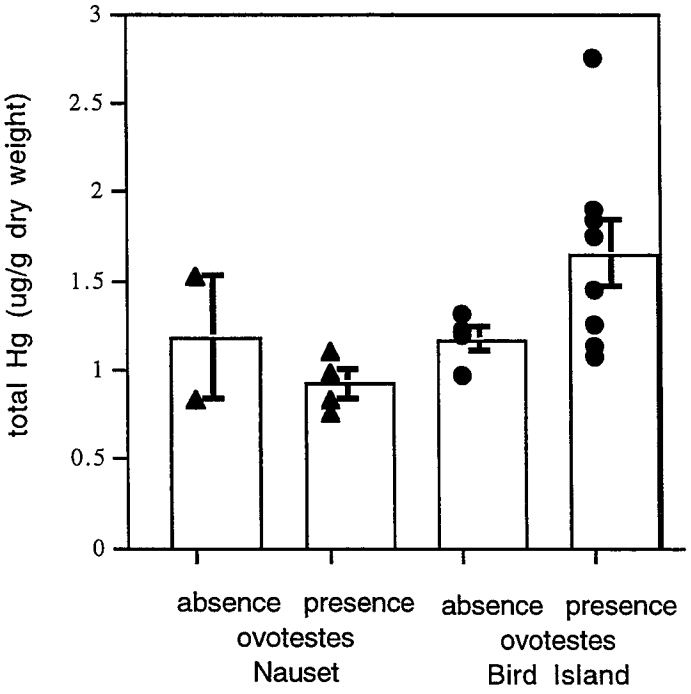


Figure 9. Comparison of ovotestes presence with Hg levels in muscle tissue of pipping common tern embryos collected at Nauset and Bird Island in 1994. There were no significant differences in Hg levels between embryos with and without ovotestes at either site. The Bird Island site had significantly higher Hg levels than Nauset.

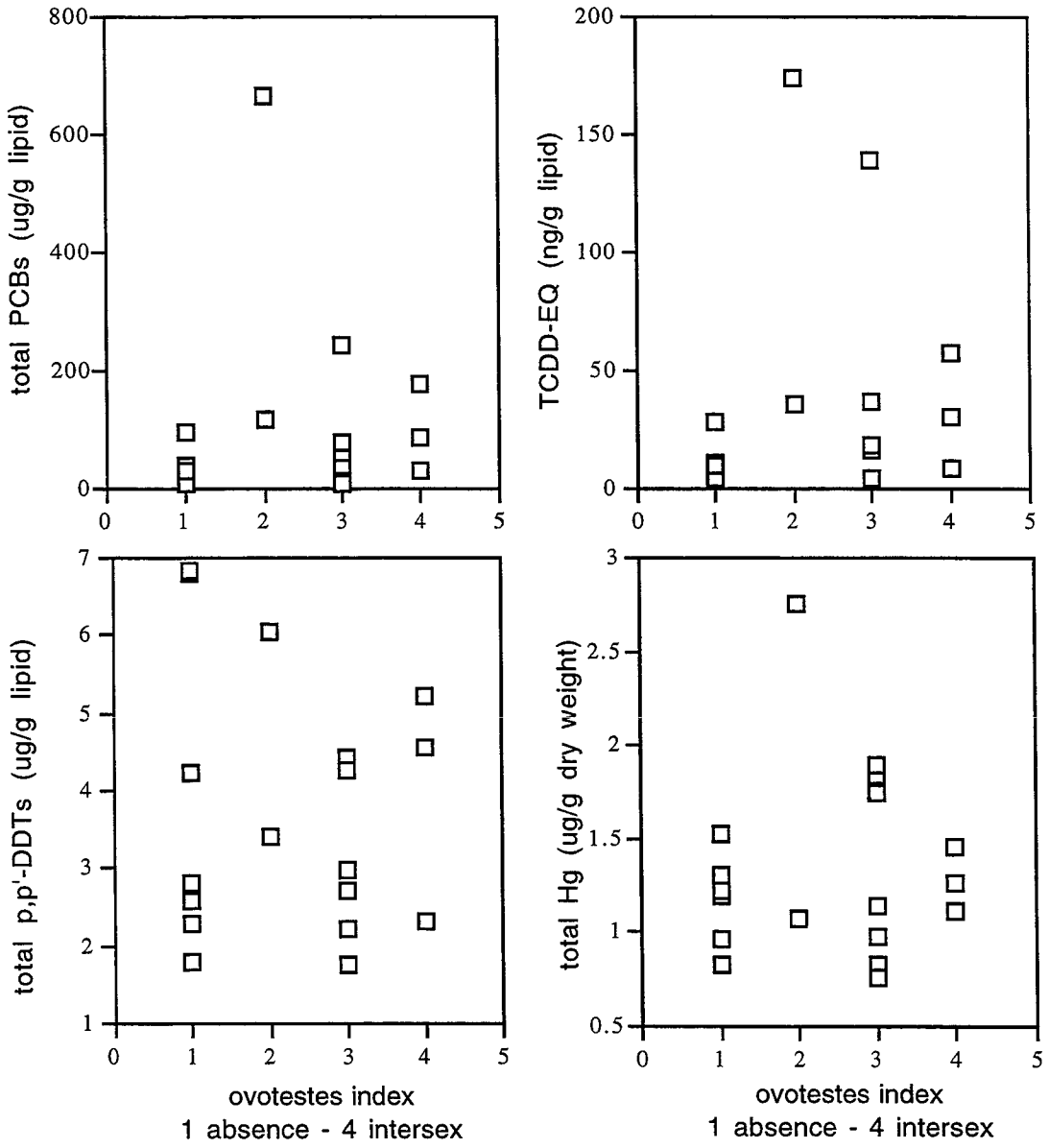


Figure 10. Contaminants (total PCBs, bioassay-derived TCDD-EQs, total p,p'-DDTs, total Hg) versus ovotestes severity index (1=absence to 4=intersex). Contaminants were measured in yolk sac or muscle (Hg) tissues of pipping common tern embryos collected in 1994 from Bird Island and Nauset.

Discussion

Because of the known effects of chemical contamination on developing wildlife, a suite of chemicals was measured in tern embryos. The goal of these analyses was to determine any relationship between the chemical contaminants and the presence of ovotestes, deformities, and other characteristics in the tern embryos.

Chemical Contamination:

PCBs: Unlike the consistently low levels of chlorinated hydrocarbon pesticides and total mercury, PCB levels were quite variable and were extremely high in some samples. High total PCB levels in terns at Bird Island exceeded those measured in terns at five Great Lakes sites, including the contaminated location of Hamilton Harbor where the highest total PCB level measured was 137.2 ug/g lipid (Table 4, Chapter 2). Compared to contaminated sites in the Netherlands, the highest levels of PCB 118 in Bird Island terns were greater, but the highest levels of PCB 153 at Bird Island were lower than those observed in the Netherlands by approximately four fold (Table 4, Chapter 2) (Bosveld, Gradener et al. 1995). This suggests that PCB congener patterns in the Netherlands sites may be quite different from those at Bird Island.

Total PCB levels were particularly variable at Bird Island, where levels ranged from 17.2 to 663.4 ug/g lipid (Table 2, Figure 6), suggesting that some birds are feeding in contaminated sites while others are feeding in cleaner sites. At Nauset, most embryos had contaminant levels under 20 ug/g lipid, but two samples had higher levels of 31.5 ug/g and 178 ug/g lipid. This indicates that terns at Nauset are not feeding only in clean areas, and may have been feeding in more contaminated sites, possibly including areas near Bird Island, prior to arriving at the Nauset breeding grounds. On a site basis, Bird Island is more contaminated than Nauset, but because of the high variability in PCB levels among

individuals the relationship of contaminants to ovotestes presence was examined on an individual basis rather than a site basis alone.

TCDD-EQs: The TCDD-EQs were highly correlated to total PCB levels (Figure 3), and therefore showed the same pattern of variability among samples as the PCBs. To investigate the major contributors to the bioassay derived TCDD-EQs, TCDD-EQcalc values were determined using yolk sac and egg samples collected in 1996 from Bird Island. The 1996 Bird Island samples could be more accurately used to determine the major congeners contributing to TCDD-EQcalc because PCDD, PCDF, and coplanar PCB congeners were measured in these samples, but not in the 1994 Bird Island samples.

The relative potencies used to calculate the contribution of each congener to the total TCDD-EQcalc are shown in Table 3. Four different sets of relative potencies were tested, three of which were determined using the same chick embryo hepatocyte (CEH) bioassay utilized here. CEH bioassay relative potencies are referred to as EC50 EST , EC50 TAP, and 10% TAP potencies. The EC50 EST and EC50 TAP potencies are both calculated by comparing the EC50s of the congeners to the EC50s of 2,3,7,8-TCDD, but the EC50 TAP potencies do not include values for all the PCB congeners (Kennedy, Lorenzen et al. 1996). The 10% TAP relative potencies are calculated by determining the concentration of congener required to produce a response equivalent to 2,3,7,8-TCDD at 10% of its maximal activity (Kennedy, Lorenzen et al. 1996). Also included are WHO (World Health Organization) relative potency values which take into consideration relative potencies determined by different methods. The WHO relative potencies also included values for 14 additional PCDD/F congeners and PCB 189, which combined contributed less than 1% of the total TCDD-EQcalc in tern samples (not shown).

Table 3. Relative potency of selected PCDD/F and PCB congeners compared to 2,3,7,8-TCDD.

Congener	% max. activity EST[a]	RP EC50 EST[b]	RP EC50 TAP[c]	RP 10% TAP[d]	RP WHO[e]
2,3,7,8-TCDD	100	1	1	1	1
1,2,3,7,8-PeCDD	100	1.1			1
2,3,7,8-TCDF	100	1.1	1.1	1	1
3,4,4',5-TCB (81)	100	0.2	0.2	0.2	0.1
3,3',4,4'-TCB (77)	70	0.03	0.03	0.02	0.05
3,3',4,4',5-PeCB (126)	80	0.3	0.3	0.3	0.1
3,3',4,4',5,5'-HxCB (169)	60	0.02	0.02	0.005	0.001
2,3,3',4,4'-PeCB (105)	10	0.005	0.005	0.00004	0.0001
2,3',4,4',5-PeCB (118)	30	0.001	0.0008	0.00007	0.00001
3,4,4'-TriCB (37)	10	0.0004			
2,3',4,4'-TCB (66)	10	0.002			
2,3',4',5-TCB (70)	10	0.0004			
2,3,3',4',6-PeCB (110)	5	0.00005			
2,2',3,3',4,4',-HxCB (128)	10	0.001			
2,2',3,4,4',5'-HxCB (138)	10	0.001			
2,2',3,3',4,4',5-HpCB (170)	10	0.0002			
2,2',3,4,4',5,5'-HpCB (180)	10	0.0002			
2,2',3,3',4,4',5,5'-OCB(194)	5	0.00005			

[a] Percent maximal EROD activity relative to maximal activity elicited by 2,3,7,8-TCDD in the chick embryo hepatocyte (CEH) bioassay. Values as listed in Kennedy et.al., Environmental Science and Technology (EST), 1996.

[b] Relative potency (RP) values as listed in Kennedy et.al., EST, 1996, using the CEH bioassay. Relative potencies were calculated by comparing the EC50s of the congeners to the EC50s of 2,3,7,8-TCDD.

[c] RP values as listed in Kennedy et.al., Toxicology and Applied Pharmacology (TAP), 1996, using the CEH bioassay. Relative potencies were calculated by comparing the EC50s of congeners to the EC50s of 2,3,7,8-TCDD. These relative potencies are essentially the same as the EC50 EST values, but values for the lower set of PCB congeners were not used.

[d] RP values as listed in Kennedy et. al., TAP, 1996, using the CEH bioassay. Relative potencies were calculated by determining the concentration of congener required to produce a response equivalent to 2,3,7,8-TCDD at 10% of its maximal activity.

[e] RP values for birds as listed by the World Health Organization (WHO), 1997. Values take into consideration relative potencies determined by different methods.

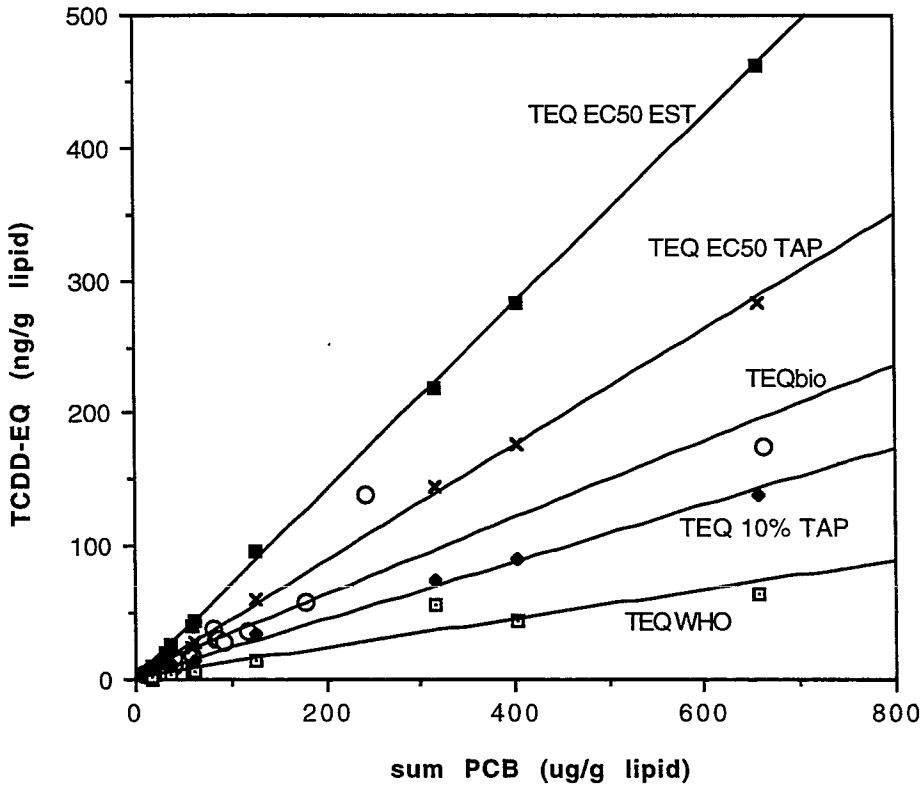


Figure 11. Comparison between bioassay-derived and calculated ΣTCDD-EQ, where calculated ΣTCDD-EQ are determined using the relative potencies described in Table 1.

Table 4. Comparison of the percent contribution of selected congeners to the ΣTCDD-TEQs for common tern embryo samples collected in 1994 (Bird Island and Nauset, MA) and 1996 (Bird Island, MA).

	1996	1994
ΣPCB ug/g lipid	172.4 ± 217.1	85.8 ± 142.2
ΣTCDD-EQcalc ng/g lipid	38.2 ± 46.8	-
ΣTCDD-EQbio ng/g lipid	-	29.8 ± 43.6
	% contribution [a]	estimated % contribution [b]
PCB 126	81.7 ± 7.0	N.D.[c]
PCB 77	8.2 ± 4.3	N.D.
PCB 118	4.6 ± 0.6	N.D.
PCB 81	3.8 ± 3.0	2.5 ± 0.8
2,3,7,8-TCDF	1.3 ± 0.9	N.D.
PCB 105	0.4 ± 0.04	0.2 ± 0.06
PCB 169	0.06 ± 0.04	N.D.
2,3,7,8-TCDD	0.05 ± 0.07	N.D.

[a] % contribution is (TCDD-EQcalc congener/ΣTCDD-EQcalc)x100 where TCDD-EQcalc is determined using the 10%TAP relative potencies as described in Table 1.

[b] estimated % contribution is (TCDD-EQcalc congener/ΣTCDD-EQbio)x100 where TCDD-EQcalc is calculated as described above, and ΣTCDD-EQbio is determined using the CEH bioassay as described in the methods.

[c] N.D.: This congener was not specifically measured in 1994 samples.

Table 5. Comparison of the percent contribution of selected congeners to the calculated TCDD-EQs in Bird Island tern samples using relative potencies for chicken and tern.

Congener	RP CEH[a] chicken	RP TEH[a] tern	% contribution[b] chicken	% contribution[b] tern
PCB 126	0.2	0.03	93.43	93.06
PCB 77	0.007	0.0003	5.07	1.44
2,3,7,8-TCDF	1	0.4	1.35	3.58
PCB 169	0.01	0.02	0.13	1.73
2,3,7,8-TCDD	1	1	0.03	0.19
			100.00	100.00

[a] Relative potencies (RPs) of selected congeners to 2,3,7,8-TCDD for both chicken and tern as listed in Lorenzen et. al. (1997) Arch. Environ. Contam. Toxicol. 32: 126-134. Chicken RPs were determined using a chick embryo hepatocyte (CEH) bioassay, while tern RPs were determined using a tern embryo hepatocyte (TEH) bioassay. RPs were calculated by comparing EC50s from the EROD dose response curves of the congeners to the EC50s of 2,3,7,8-TCDD.

[b] Average concentrations of the specific congener (ng/g lipid) in 1996 Bird Island yolk sac and egg samples were used to calculate the estimated % contribution.

The comparison between the bioassay derived TCDD-EQs (1994 samples) and the TCDD-EQcalcs derived from the four sets of relative potencies (1996 samples) is shown in Figure 11. When the TCDD-EQs are normalized to total PCBs, the TCDD-EQcalcs determined using the 10% TAP relative potencies have values closest to the bioassay derived TCDD-EQs, but slightly lower. The TCDD-EQcalcs determined using the EC50 EST and EC50 TAP relative potencies are much higher then the bioassay derived TCDD-EQs. The EC50 EST and EC50 TAP relative potencies do not consider the differences in maximal EROD response compared to TCDD (efficacy) for the different congeners. If all the PCB congeners were included, the TCDD-EQcalcs determined from the EC50 TAP relative potencies would be similar to the higher values determined using the EC50 EST values. The TCDD-EQcalcs determined using the 10% TAP relative potencies compare an identical absolute EROD activity response level rather than a relative response level (50% of the maximum) as in the EC50 methods, and appear to best approximate the bioassay derived TCDD-EQs. The 10% TAP TCDD-EQcalcs may be lower than the bioassay derived TCDD-EQs because congeners for which no relative potency is determined are not included in the TCDD-EQcalcs, but may be contributing to the bioassay derived TCDD-EQs. In addition, the bioassay derived TCDD-EQs may be slightly overestimated because they were calculated by comparing EC50s of the extracts to that of TCDD, which does not consider differences in efficacy of the extracts. Because maximal responses of most of the extracts were between 60-100% of the maximal TCDD response, calculating the bioassay derived TCDD-EQs using the 10% method is not likely to significantly change the TCDD-EQ values (Kennedy, Lorenzen et al. 1996). These data suggest that when calculating TCDD-EQcalcs from individual congeners that have differing efficacies, relative potencies determined by an absolute response level (10% method) rather than a relative response level (EC50 method) may give more accurate estimations of TCDD-EQs.

Table 4 shows the major congeners contributing to TCDD-EQs in Bird Island 1994 and 1996 samples. The contributions of different congeners were estimated using the 10% TAP relative potencies, since these TCDD-EQcalcs were most similar to the bioassay derived TCDD-EQs. The major contributors to the TCDD-EQcalcs determined in the 1996 samples are PCB 126 (81.7 %), PCB 77 (8.2 %), PCB 118 (4.6 %), PCB 81 (3.8 %), 2,3,7,8-TCDF (1.3 %), and PCB 105, PCB 169, and 2,3,7,8-TCDD (less than 1 % combined). The estimated contribution of the PCB congeners 118 and 105 measured in the 1994 samples is 2.5 % and 0.2 % respectively, similar to the contribution of those congeners in 1996. Likely, PCBs, mainly PCB 126, are the major contributors to the TCDD-EQs in 1994 as well as in 1996. The levels of PCDD/Fs measured in 1996 Bird Island samples were higher than samples collected from contaminated sites in the Great Lakes (Table 4, Chapter 2), due largely to high levels of 2,3,7,8-TCDF; TCDD levels at Bird Island were lower than the reference sites in the Great Lakes. Even with higher levels of PCDFs at Bird Island than other areas, PCBs are much more important in contributing to TCDD-EQs.

In terns, the relative contributions of the different PCB and PCDD/F congeners to total TCDD-EQs may differ because the relative potencies of congeners is different between terns and chickens. Lorenzen et. al. compared the relative potencies of selected congeners (2,3,7,8-TCDD/F, PCBs 77, 126, 169) between tern and chicken using a chick or tern embryo hepatocyte bioassay for EROD induction (Lorenzen, Shutt et al. 1997). Relative potencies determined for tern and chick by Lorenzen et al. (Table 5) were used here to compare how the percent contribution of specific congeners to total TCDD-EQs in Bird Island samples may change when determined specifically for terns (Table 5). While PCB 126 remains the greatest contributor to TCDD-EQs at 93% in both chickens and terns, contributions from other congeners change. In terns, the contribution from PCB 77 becomes less important; this is because tern hepatocytes were unresponsive to EROD

induction by PCB 77 (Lorenzen, Shutt et al. 1997). The contribution of TCDF, PCB 169, and TCDD become more important since terns were responsive to these congeners, although at levels lower than those in chickens. Although the relative potencies of congeners to 2,3,7,8-TCDD differ between chick and tern, this comparison suggests PCB 126 would also be the major contributor to TCDD-EQs in terns at Bird Island; however, TCDF may be a more important contributor to TCDD-EQs in terns than is determined using chick derived relative potencies.

EROD activity: EROD activity levels were measured in common tern embryo livers as a biomarker of exposure to AhR agonists. Figure 4 shows the correlation of hepatic EROD activity levels with the bioassay derived TCDD-EQs, a measure of the cumulative AhR agonist activity in these samples and thus of exposure of the tern embryos to these compounds. There appears to be a threshold level of TCDD-EQs above which EROD activity is elevated. Separate regressions to the high EROD values and the lower EROD values were used to model the threshold level response as shown in Figure 5. The regression lines intersect at TCDD-EQ of 82.5 ng/g lipid ± standard error of regression estimates. This suggests that TCDD-EQs must be between 56.3 - 108.7 ng/g lipid in the yolk sacs before hepatic EROD activities in the tern embryo will be elevated. This threshold of TCDD-EQs above which tern hepatic EROD activity is elevated also may indicate the threshold above which AhR induced toxic effects occur in the common tern embryo.

These results also suggest that EROD activity in common tern embryo livers is not as sensitive a biomarker of exposure to AhR agonists as the chick embryo hepatocyte bioassay used here to measure TCDD-EQs. This is not surprising since tern hepatocyte cultures were shown to be 80-fold less sensitive than chick hepatocyte cultures to EROD induction by TCDD (Lorenzen, Shutt et al. 1997).

Crossed-beak embryos: Two female tern embryos, one from Bird Island and the other from Nauset, had crossed beaks. Deformities in terns, including crossed beaks, have been associated with elevated PCBs and TCDD-EQs (Ludwig, Kurita-Matsuba et al. 1996); therefore the relationship of crossed beaks to contaminants was examined. Both their total PCBs and TCDD-EQs were intermediate (Bird Island embryo 83.9ug/g lipid, 29.7 ng/g lipid respectively) and low (Nauset embryo 14.4 ug/g lipid, 5.3 ng/g lipid) relative to other samples from Bird Island and Nauset, and pesticide and mercury levels in these embryos were similar to all other samples. The EROD activities in livers from these embryos were not elevated relative to other samples at Bird Island and Nauset (Bird Island embryo 9.8, and Nauset embryo 10.4 pmol/min/mg). Thus, the incidence of crossed beaks among common terns studied here is not clearly associated with elevated levels of any contaminants measured in the embryos.

Relationship of contaminants to the presence of ovotestes:

To examine the relationship between ovotestes and contaminants, ovotestes were classified as either present (severity 2-4) or absent (severity 1) and compared by site (Figures 6-9). For all four contaminants, 2-way ANOVA showed no significant interaction between site and ovotestes, indicating that the relationship of contaminant levels to ovotestes presence is the same at the two sites. Embryos with ovotestes presence showed no significant difference in contaminant levels from embryos without ovotestes. Although the PCB and TCDD-EQ means suggest a difference in contaminant levels of terns with ovotestes and those without, this difference was not significant. This was because the variance of contaminant levels within the ovotestes present/absent groups was greater than the variance between these groups, and may be influenced by the small sample sizes.

In an attempt to examine the effect of sample size on the statistical results, the same analysis was carried out with hepatic EROD data from tern embryos using both a sample

size of 19 (the same samples used in the contaminant data) and a sample size of 33 (the complete set of samples for which male gonadal histology was determined) (Fig. 12). Similar to the total PCB and TCDD-EQ data, Bird Island EROD activity levels were significantly higher than those at Nauset for both EROD sample sizes. Results of 2-way ANOVA were the same for both EROD sample sizes, showing that site ovotestes interactions and differences in ovotestes presence and absence were not significant, even with a larger sample size. However, EROD has been shown to be an insensitive indicator of contaminant exposure; therefore, larger samples sizes of more sensitive contaminant measures, such as TCDD-EQs determined in the CEH bioassay, may still show significant differences.

The data were also examined for the presence of a dose-response relationship between contaminant levels and the presence of ovotestes. Total PCB, TCDD-EQ, total p,p'-DDT, and total mercury contaminant levels were plotted against the ovotestes severity index in corresponding embryos (Fig. 10). A similar range of total p,p'-DDT contaminant levels (0.9-6.8 ug/g lipid) was found for all classifications of ovotestes (severity 1-4), indicating no dose-response relationship. For total PCB, TCDD-EQ, and Hg, birds with low contaminant levels were present in all classifications of ovotestes (severity 1-absent to 4-intersex), suggesting no clear dose-response relationships. However, samples with the highest contaminant levels of total PCBs, TCDD-EQs, and Hg always had ovotestes present (severity 2-4). This may indicate that total PCBs higher than 100 ug/g lipid and TCDD-EQs higher than 30 ng/g lipid are threshold levels above which the formation of ovotestes in the embryo is likely to occur. Although this relationship holds true for Hg present in the samples as well, (Hg levels above 1.75 ug/g dry weight have ovotestes present), the low range and levels of Hg (0.76-2.76) suggest it would not be a suitable predictor.

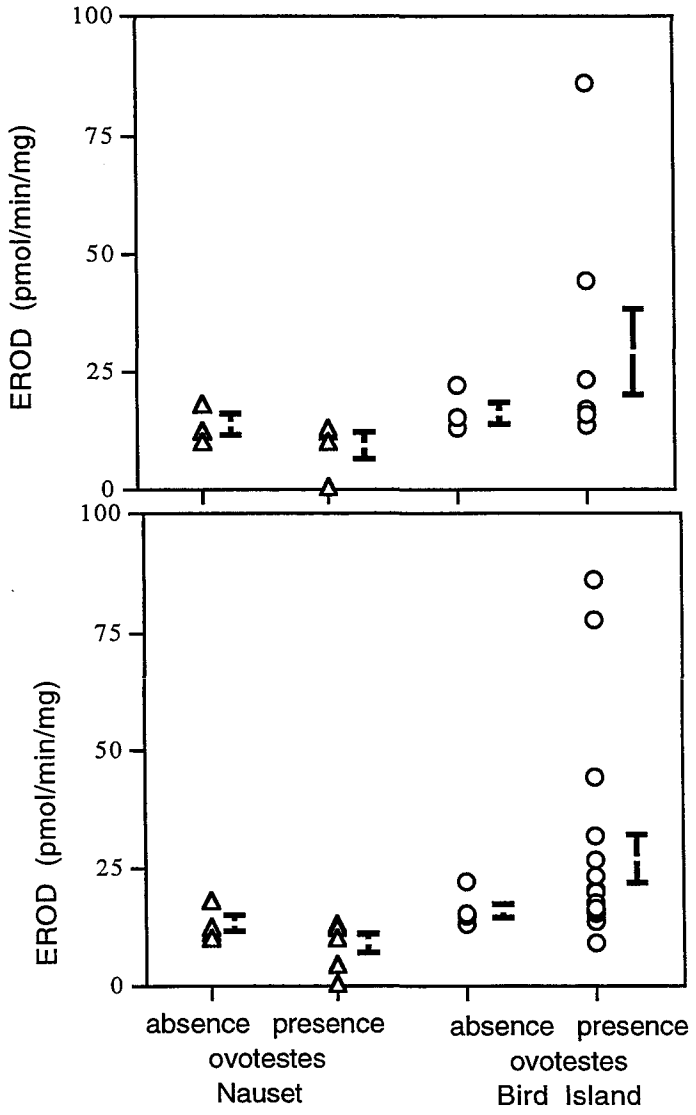


Figure 12. Comparison of ovotestes presence with levels of hepatic EROD activity in common tern embryos at Nauset and Bird Island. Top panel shows EROD activity for a sample size of 19, the same samples as for the contaminant data. Bottom panel shows EROD activity for a sample size of 33, the samples for which complete male embryo gonadal histology were determined. There were no significant differences in EROD activities between embryos with and without ovotestes at either site. The Bird Island site had significantly higher EROD levels than Nauset with the larger sample size.

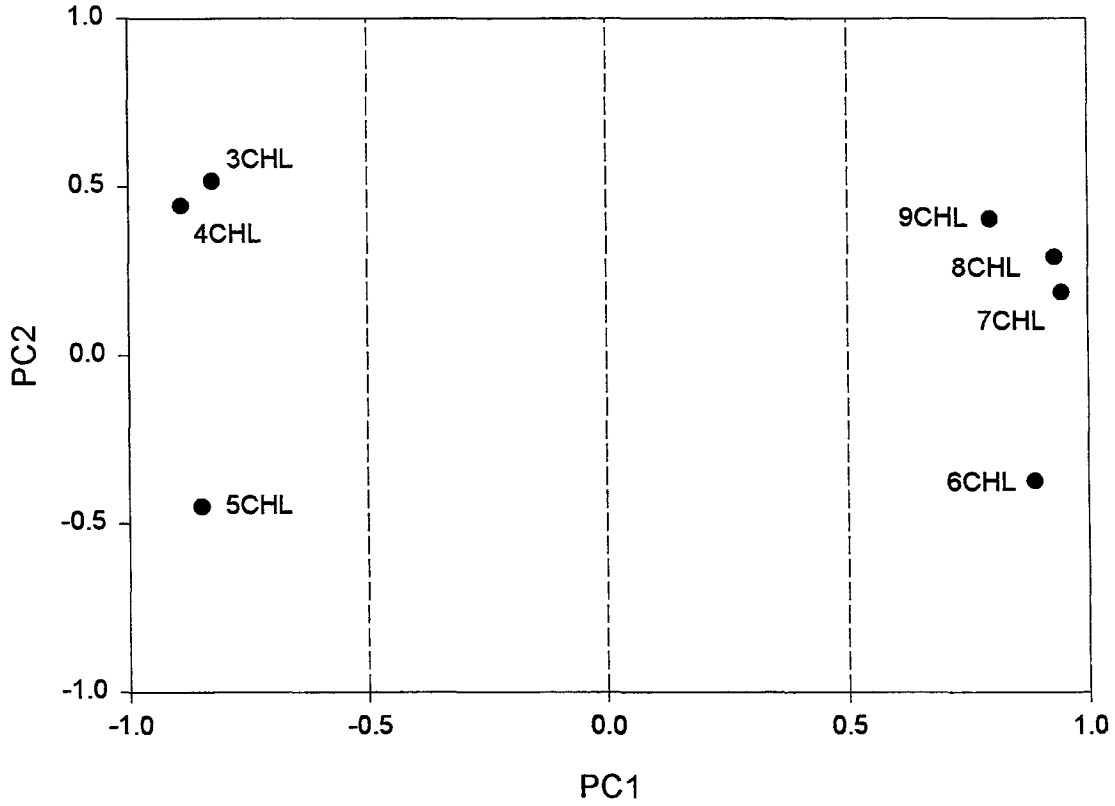


Figure 13. Loadings for the 1st and 2nd principal components. Negative values of PC1 indicate elevated levels of PCBs 3,4 and 5 while positive scores signify a predominance of 6,7,8 and 9 chlorinated PCBs. Similarly, negative and positive PC2 scores descriminate 5 and 6 chlorinated from 3,4 and 9 chlorinated PCBs. PC1 and 2 accounted for 77% and 16% of the total variation in the data respectively. PCs 3-7 explained a total of 7% of the variation in the data and were excluded from the analysis.

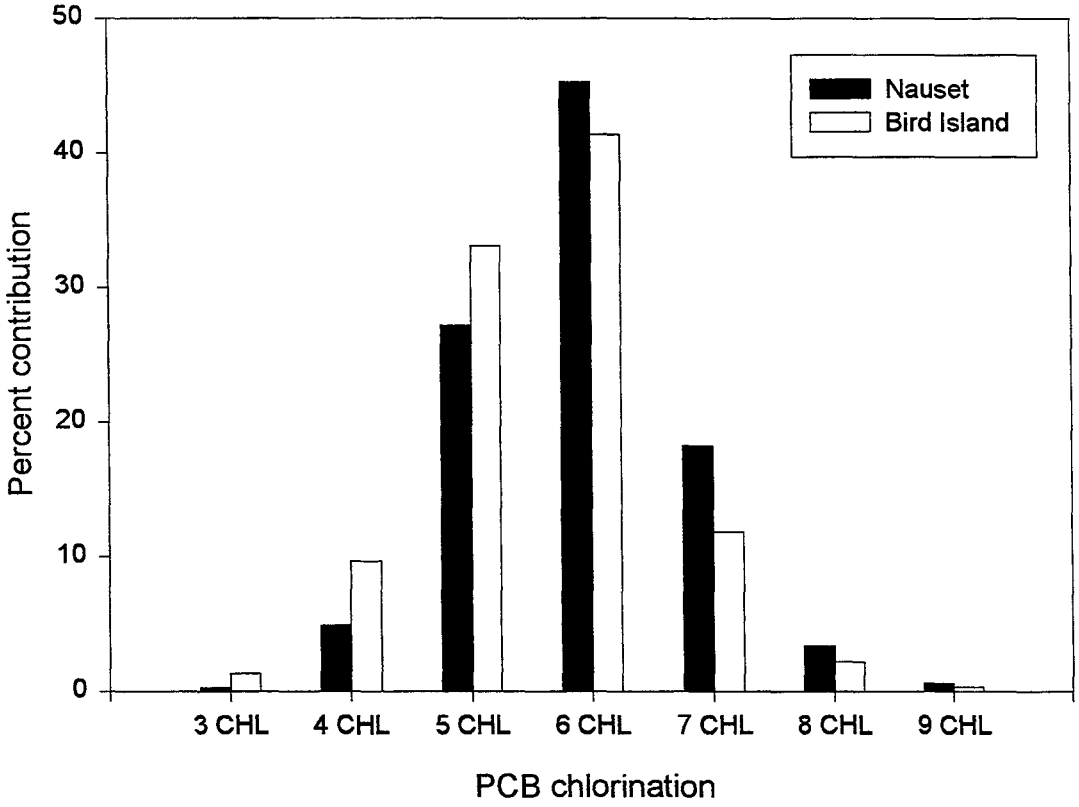


Figure 14. Differences in the proportions of PCB congeners, as classified by the number of chlorines, in common tern embryo yolk sacs collected at Bird Island and Nauset. Yolk sacs from embryos collected at Bird Island have higher proportions of lower chlorinated PCBs than those collected at Nauset.

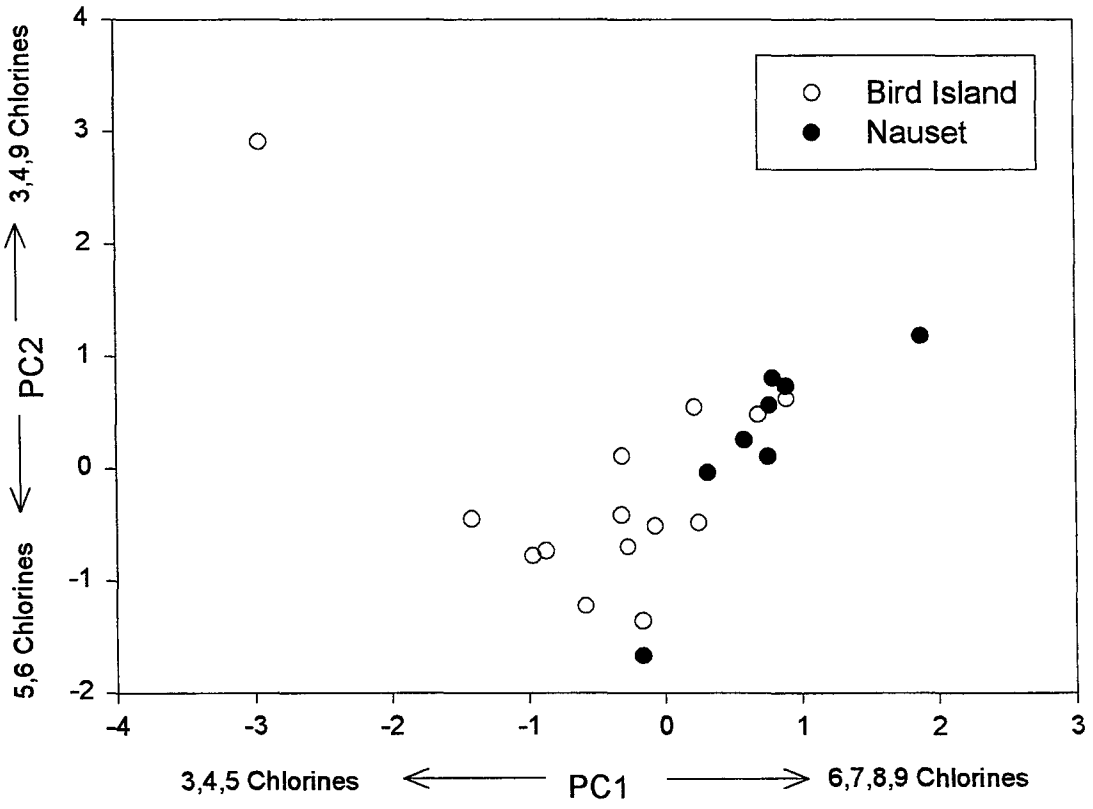


Figure 15. Principal component analysis of PCBs in common tern embryo yolk sacs categorized by site. Tern embryo yolk sacs collected from Bird Island tend to contain a higher proportion of lower chlorinated PCBs than those collected at Nauset.

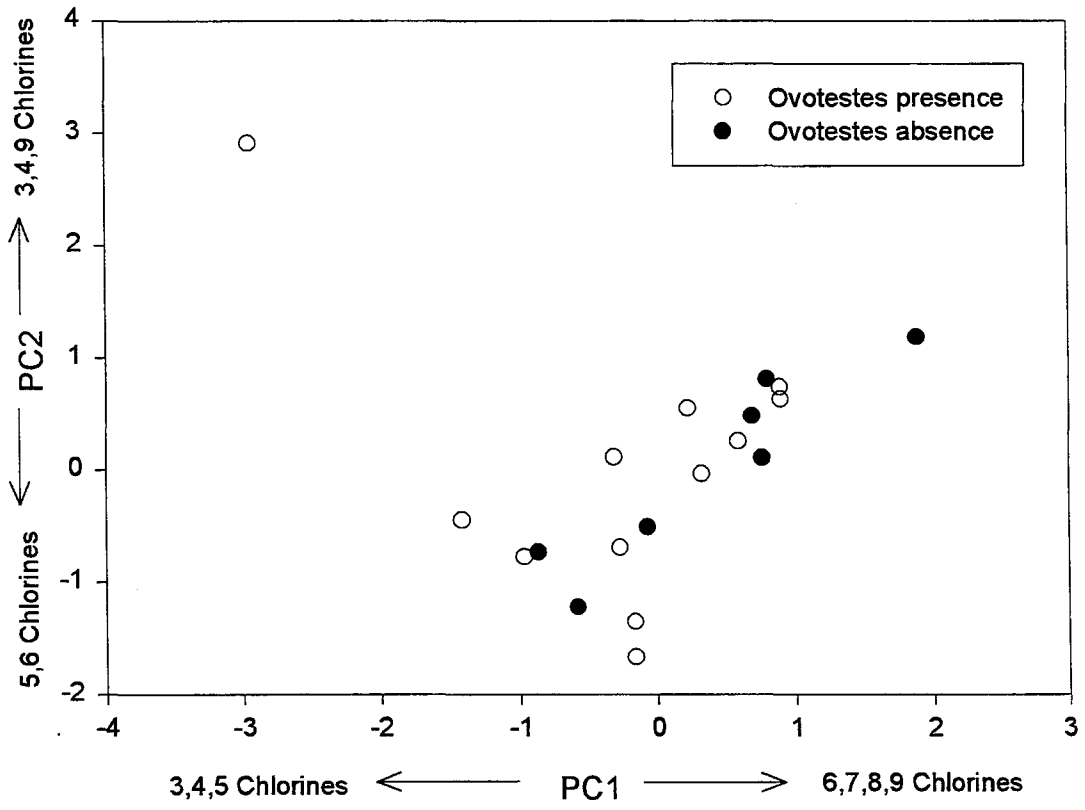


Figure 16. Principal component analysis of PCBs in common tern embryo yolk sacs categorized according to ovotestes presence or absence. The distribution of PCB congener patterns in yolk sacs from embryos with ovotestes present is not distinct from those where ovotestes were absent.

Although there are no statistically significant differences in contaminant levels of embryos with and without ovotestes at the two sites, it is true that samples with the highest levels of PCBs and TCDD-EQs have ovotestes present, but samples with intermediate and low levels of PCBs and TCDD-EQs may or may not have ovotestes present. Principal component analysis (PCA) was carried out with PCB congener data in order to examine the possibility that PCB congener patterns may be different in samples with ovotestes compared to those without ovotestes, even though total PCB levels are not different. PCB congeners in each yolk sac sample were categorized by the number of chlorines (3 - 9), and each chlorine category was expressed as a proportion of the total PCBs in the corresponding sample. The analysis yielded 7 principal components. Since only the first two factors had eigenvalues greater than 1, the remaining 5 were exclued from further analysis. The first principal component (PC1), which accounted for 77% of the variability in the data, descriminated between lower and higher chlorinated PCBs. PC2, which accounted for only 16% of the variation in the data, is more difficult to interpret. It appears that high values of PC2 represent PCB congeners that are not usually found in high proportions (3,4, and 9 chlorines), while low PC2 values represent more prevalent congeners (Figure 13). PCA was also performed with PCBs classified by individual congeners, and results were similar to those described below for PCBs classified by isomers (data not shown).

The PCA results revealed interesting differences between the Nauset and Bird Island sites. Tern embryo yolk sacs collected at Bird Island had a higher proportion of lower chlorinated PCBs (3, 4 and 5 chlorines) than those collected at Nauset (Figures 14 and 15). Nauset samples were distinguished from Bird Island samples by higher proportions of 6 and 7 chlorinated PCBs (Figures 14 and 15). The prevalence of lower chlorinated PCBs in Bird Island samples likely reflects the contamination of tern prey with lower chlorinated PCBs originating from New Bedford Harbor (Lake, McKinney et al.

1995). The most highly contaminated sample from Bird Island (663.43 ng/g lipid total PCBs) showed a much higher proportion of 4 and 5 chlorinated congeners than other samples (Figure 15, upper left data point); this sample contained 31% of 4 chlorinated PCBs versus a mean of 8% for all samples. This may indicate that the laying female tern had been feeding near highly contaminated areas within New Bedford Harbor.

Although the PCA results showed clear differences in tern yolk sac PCB patterns between Bird Island and Nauset, they revealed no differences between terns with ovotestes presence or absence. The distribution of PCB patterns in terns without ovotestes overlaps those with ovotestes present (Figure 16). Hydroxylated metabolites of lower chlorinated PCBs have been shown to be estrogenic (Korach, Sarver et al. 1987), and have been suggested as a possible agent affecting common tern gonadal development (Nisbet, Fry et al. 1996). However, based on the results of PCA there is no association between elevated concentrations of lower chlorinated PCBs and the presence of ovotestes.

Summary of the relationship of contaminants to ovotestes presence:

Although the presence of ovotestes and behavioral abnormalities in California herring gulls was associated with high levels of DDT (Fry and Toone 1981), it seems unlikely that the low DDT levels in Massachusetts common terns are related to the presence of ovotestes. Although only p,p'-DDT, -DDE, and -DDD congeners were measured, the o,p'-DDT/E/D congeners have been measured in previous years at levels that were non-detectable or extremely low (Nisbet and Reynolds 1984; Nisbet, Fry et al. 1996). The only DDT congener of significant levels was p,p'-DDE, and it is the primary component (over 95%) of total p,p'-DDTs which ranged from 0.9-6.8 ug/g lipid in tern yolk sacs. As determined in herring gull egg injection studies (Fry, Toone et al. 1987), the lowest concentrations of DDT required to cause significant development of ovotestes were 20 ppm wet weight p,p'-DDE, 50 ppm p,p'-DDE/T (4:1), and 2 ppm o,p'-DDT, and in

addition one male showed ovotestes development with exposure to 5 ppm p,p'-DDE/T (4:1). These concentrations are estimated as 10 times higher when converted to ppm lipid weight of eggs, and thus are all much (7-30 fold) greater than any concentrations of DDT congeners measured in Bird Island and Nauset tern embryos. Unless terns are much more sensitive to ovotestes development induced by DDT congeners than herring gulls, it is unlikely that DDT is related to tern ovotestes development. However, potent anti-androgen activity of p,p'-DDE and p,p'-DDT has been shown at *in vitro* concentrations of 65 ppb (approximately 0.6 ug/g lipid) (Kelce, Stone et al. 1995), suggesting even very low levels of p,p'-DDE could result in alteration of endocrine function. Furthermore, the percentage of embryos with ovotestes at both Bird Island and Nauset were high and very similar (89% BIE, 71% BIL, and 60%N), suggesting the cause could be a contaminant present in similar levels at both sites. Therefore, the possibility that p,p'-DDE levels may be affecting the presence of ovotestes cannot be completely eliminated, although based on injection study results by Fry this seems highly unlikely.

Like p,p'-DDE, the levels of other chlorinated hydrocarbon pesticides were of similar ranges throughout all the embryos sampled, and levels were likely too low to cause developmental or endocrine effects. Estrogenic potencies of chlorinated hydrocarbon pesticides, like o,p'-DDT, are generally 1/1000th that of estradiol or less (Bulger and Kupfer 1985). Considering the low levels of almost all the other chlorinated hydrocarbon pesticides measured (10-1000 times lower than p,p'-DDE), and their weak estrogenic potencies, it is unlikely that the levels of these compounds in tern yolk sacs were of any toxicological significance.

Although embryos at Bird Island had statistically higher mercury levels than Nauset (means, BI = 1.49 ± 0.14, N = 1.01± 0.12 ug/g dry weight), mercury levels in these embryo samples at both sites were low with a low range (0.76-2.76 ug/g dry weight). Mercury levels measured in common tern eggs from colonies in the Great Lakes and

Germany were unrelated to hatching failure and abnormalities, and were similar to or higher than mercury levels in Bird Island and Nauset common tern embryos (Becker, Schuhmann et al. 1993; Hoffman, Smith et al. 1993). Reproductive effects resulting from mercury exposure generally occur at concentrations approximately one fifth of those causing acute mercury toxicity (Scheuhammer 1991). Using this information and data from Finley et. al., 1978 (Finley and Stendell 1978), the estimated concentration of mercury that could cause reproductive effects in an embryo is 3.1-5.2 ug/g dry weight in muscle. This level is higher than levels found in Bird Island and Nauset embryos, but only by a small margin (2-5 times higher than the means at both sites), suggesting that mercury could affect embryo development. Although no histological analysis was performed, mercury exposure was shown to delay testicular development and increase ovarian development in young quail at levels of 2 ppm in the diet (Hill and Soares Jr. 1984), suggesting the possibility that mercury could be involved in altering testicular development in common terns. As discussed in relation to p,p'-DDE, the similarity of ovotestes incidence at both sites may implicate a contaminant present in similar levels at both sites. Mercury levels were only slightly higher at Bird Island, and although the range of levels was low, terns with levels above 1.75 ug/g dry weight could be predicted to have ovotestes present. It is possible that mercury contamination could be related to ovotestes development, although the lack of direct evidence for effects at these mercury levels suggests it is unlikely.

Because of their prevalence in samples, PCBs and TCDD-EQs are the contaminants most likely to affect reproductive development of common tern embryos. The lipid weight levels of PCBs and TCDD-EQs in some common tern embryo yolk sacs measured here are greater than levels measured in tern eggs in other areas where effects such as reduced hatchability, reduced body weight, increased incubation time, and congenital abnormalities have been observed (Kubiak, Harris et al. 1989; Hoffman, Smith et al. 1993; Ludwig,

Kurita-Matsuba et al. 1996). The levels of total PCBs in some tern samples are also at or above levels of Aroclor mixtures shown to cause estrogenic effects in mice, although this is a different species and direct comparisons may not be appropriate (Jansen, Cooke et al. 1993). The relationship of ovotestes presence to PCB and TCDD-EQ contaminant levels suggests a possible contaminant level threshold above which the formation of ovotestes in tern embryos is likely to occur. At intermediate and lower levels of these contaminants, ovotestes may or may not be present, and no relationship between contaminant levels and ovotestes presence is apparent. In addition, no relationship between embryos with and without ovotestes presence was indicated by PCB congener patterns. One explanation for these results is that a normal background incidence of ovotestes exists, and highly elevated PCB/TCDD-EQ contaminant levels increase this incidence, possibly by delaying development or by altering the endocrine system. Alternatively, PCBs/TCDD-EQs could be responsible for all cases of ovotestes but timing of exposure, individual sensitivity, or activation of metabolites determine whether the contaminant exposure results in ovotestes formation.

In addition to the relationship between ovotestes in terns and contaminants measured in this study, there are several other considerations. It is possible that additional contaminants not measured here could be related to ovotestes development. It is also possible that the presence of ovotestes in common terns at hatching is normal and unrelated to contaminants, and this will be discussed further in the following chapter. One reason it is difficult to assess this possibility is because Nauset is not a good control site. Although the mean PCB levels were lower in Nauset tern yolk sacs than in Bird Island samples, some Nauset birds showed contaminant levels intermediate to those at Bird Island. This suggests that a better control site is needed for evaluation of whether ovotestes presence at hatching in terns is normal.

CHAPTER 4: PERSISTENCE OF OVOTESTES IN COMMON TERN PREFLEDGLINGS COLLECTED AT BIRD ISLAND IN 1995

Chapter 4: Persistence of Ovotestes in Common Tern Prefledglings Collected at Bird Island in 1995

Introduction

Examining the persistence of ovotestes in common terns may help address whether their presence affects reproductive capabilities, is related to contaminants, or is normal. The prevalence of testes with cortical areas containing ovarian primordial germ cells (ovotestes) was high in pipping male common tern embryos collected at Bird Island in 1993 and 1994. Between 70% to 89% of the embryos showed some ovotestes development (Chapter 2). However, it is not known whether these ovotestes persist and affect reproductive performance. Although three tern embryos had significant ovotesticular development, none were so severe as to appear ovarian under macroscopic examination nor did any of the males show development of oviducts. Furthermore, the incidence of ovotestes at Bird Island was only slightly higher than at Nauset (60 %) in 1994, which is a less contaminated site. This lack of ovotestes severity and similarity of ovotestes incidence at the two colonies raise important issues. The presence of ovotestes may be abnormal and/or contaminant-induced, but the persistence and severity of ovotestes development still may not be great enough to affect tern reproductive capability. Alternatively, ovotestes may be present normally in terns at hatching. Determining if the ovotestes observed in pipping embryos persist may indicate whether the development of these tissues is related to lowered tern reproductive success.

It has been suggested that feminization of male birds may affect their reproductive capabilities. Fry suggested that the presence of contaminant induced ovotestes may be correlated with decreased reproduction in California Western gull (*Larus occidentalis*)

populations, as well as in Great Lakes Herring gull (*Larus argentatus*) populations, and that terns may be similarly affected (Fry and Toone 1981; Fry, Toone et al. 1987). Evidence for this began with the observation that lowered reproductive success in populations of Western gulls in California was correlated with high levels of organochlorine pesticides (DDT), a sex ratio skewed in favor of females, female-female pairing, and the increased incidence of supernormal clutches (Fry and Toone 1981; Fry, Toone et al. 1987). A female-biased sex ratio also can result from bird population expansion (Greenwood 1980); however, the Western gull population had been declining from 1960-1978, and had an unusually high excess of females (Hunt, Wingfield et al. 1980; Fry, Toone et al. 1987). Furthermore, the incidence of supernormal clutches tended by female pairs approached 15% on the contaminated Santa Barbara Island population (Hunt, Wingfield et al. 1980), much higher than observed in other gull populations (Hunt and Hunt 1977; Shugart 1980; Nisbet and Drury 1984). Western gull and California gull (*Larus californicus*) eggs injected with amounts of DDT comparable to levels found in eggs at Californian gull colonies resulted in the development of ovotestes and oviducts in male gulls, and indicated that these gulls were more sensitive to feminization by estrogenic compounds (10-50 times) than chickens, quails, and finches (Fry, Toone et al. 1987). In addition to feminization of the male reproductive system, other egg injection studies have shown that exposure to estrogen and testosterone during development may also result in demasculinization and feminization of male brain development and permanent suppression of male sexual and copulatory behavior (Domm 1939; Domm 1940; Domm and Davis 1941; Adkins 1975; Adkins 1976; Adkins 1978; Adkins 1979). It was suggested that contaminant induced feminization of male reproductive tracts and behaviors in California gull populations, and similarly in Great Lakes populations, may result in observed female-biased sex ratios because of differential male mortality or male self-exclusion from the breeding colony,

consequently reducing the reproductive success of the gull population (Fry and Toone 1981; Fry, Toone et al. 1987; Fry 1995).

Similar to the Californian gull population, the presence of ovotestes in terns may reduce reproductive success at Bird Island, in which case it might be expected that ovotestes observed in the tern embryos may persist into the prefledgling stage. Persistence of ovotestes has been observed in some studies where reproductive success was affected. Chickens dosed with estrogen during incubation showed abnormal copulatory behavior and infertile copulations at two years of age, and also possessed ovotestes when examined (Domm 1939; Domm 1940; Domm and Davis 1941). In another study, feminized chickens showed reversion of secondary sex characteristics back to the male form, but ovotestes were still present when examined at nine months (Snedecor 1949). Other studies with chickens suggest that male ovotestes may begin reversion to testes shortly after hatching, with complete reversion to testes occurring anywhere from a few weeks to nine months posthatch (Snedecor 1949; Pincus and Erickson 1962; Taber 1964; Haffen, Scheib et al. 1975; Scheib 1983). In other bird species, estrogen-induced ovotestes tend to persist longer than in chickens. These include quail, dove, herring gull, and turkey, although ovotestes in quail and doves eventually tend to revert back to testes (Riddle and Dunham 1942; Boss and Witschi 1947; Taber 1964; Haffen, Scheib et al. 1975). Evidence from a variety of hormone dosed bird species suggests that induced ovotestes may persist until the prefledgling stage, although reversion back to testes may occur earlier as indicated by some individuals in most species examined.

In some birds, ovotestes may be present normally at hatching. Ovotestes found in birds at hatch often do not persist for more than a few days to weeks, but in some instances they may persist for many months. In hatching male ring doves, testes may exhibit traces of ovarian cortex; this ovarian tissue normally does not persist past four days posthatch, and only rarely persists beyond two weeks posthatch (Riddle and Dunham 1942). Male

duck embryos also show a cortical area on the testes, which often persists until hatching and then becomes less prominent by day two posthatch, but remnants of the cortical tissue may persist until approximately 30 days posthatch (Lewis 1946). Hawks have very persistent traces of cortical tissue on their testes, which may still be observed four months after hatching (Stanley 1937). Quail testes also may show areas of cortical tissue that persist and proliferate until hatching, and may remain visible in early posthatching. Groups of meiotic primordial germ cells are present in these persistent cortical areas of the testes, and are beginning maturation comparable to oocytes in the cortical layer of the ovary at the same age (Haffen, Scheib et al. 1975). In contrast, many other birds- including chickens, gulls, and pigeons- normally do not exhibit cortical tissue on the testes when examined at hatch (Lahr and Riddle 1945; Boss and Witschi 1947; Romanoff 1960; Fry, Toone et al. 1987). If the presence of ovarian cortical tissue on the testes of male common terns at hatching is normal, it might not persist into the prefledgling stage, or might regress significantly in many male terns.

In order to examine the persistence of ovotesticular tissue in common terns, the gonads of prefledglings (approximately 21 days old) were examined histologically. Contaminants were measured in a subset of previously collected eggs from the same nests as an indication of embryonic contaminant exposure, since there is a strong correlation between contaminant concentrations in paired eggs and embryos from the same nests (Custer, Pendleton et al. 1990; Custer, Custer et al. 1997). Female prefledglings also were examined for possible abnormalities in ovarian development.

Methods

Collection: All prefledgling tern and egg samples were collected from Bird Island, MA in the 1995 breeding season. Ten prefledglings (8 males and 2 females) were collected at approximately 21 days of age on July 1 (4 birds) and July 6 (6 birds). An egg from the same nest as these 10 prefledglings had been collected previously and the contents stored frozen in acid washed glass jars. Because we wanted to collect only males, these prefledglings were sexed prior to collection using a PCR-based method with blood in feather tips, as described elsewhere (Sabo, Kesseli et al. 1994). The sex marker is located on the female W chromosome, and a male is indicated by a negative PCR result. In the two cases where a female was collected, the negative PCR result was apparently indicative of an unsuccessful PCR experiment rather than the presence of a male.

An additional 18 prefledglings killed by an owl (11 males and 7 females), were collected on July 4 and necropsied within 24 hours of death. These birds were approximately 14-21 days old. Nine adult male terns that died of natural causes throughout the 1995 breeding season also were collected.

Necropsy: The 10 prefledglings were weighed and decapitated. Blood was collected, centrifuged to separate plasma from red blood cells, and stored frozen at -80° C. After removal of the gall bladder, the liver was removed and weighed. A small slice of liver was placed into 70% neutral buffered formalin (NBF), approximately 1 gram was frozen at -80°C in acid washed jars for chemical analysis, and the remainder was frozen in liquid nitrogen. The pluck (trachea, esophagus, stomach, intestinal tract, and attached organs/glands) was removed and placed into NBF; the canoe (back with gonads, kidneys, cloaca), lungs, and head were also were placed in NBF. Within several hours of collection, slices were cut from tissues in NBF and placed into histology cassettes for

further fixation in NBF. Organs processed included thymus, thyroid, spleen, kidney, adrenal, trachea, heart, liver, lung, intestine, pancreas, and gonad. Fixed tissues were embedded in paraffin blocks within 24 hours.

The additional prefledgling and adult terns found dead were necropsied within 24 hours of death. Gonads were fixed in NBF and embedded in paraffin blocks. The time between fixation and embedding was within 24 hours for owl killed prefledglings, and between one and three months for adults.

Gonadal histology: Paraffin embedded gonads were sectioned (3-5 um thickness) and stained with hematoxylin and eosin. A minimum of 20 sections through the left gonad were examined for the presence of ovarian tissue in testes of males, and for the presence of double follicles in ovaries of females. Gonad sections were examined for the presence of other abnormalities as well.

Egg extraction, contaminant analyses: Eggs were homogenized and 5 gram aliquots were extracted as described in Chapter 3. The chemical analysis, and TCDD-EQ bioassay was carried out on egg extracts also as described in Chapter 3. No mercury analysis was carried out on these samples.

Results

Necropsy

No gross abnormalities were observed in male or female prefledglings. No males showed development of oviducts, and no testicular abnormalities were evident by macroscopic examination.

Gonad Histology

The prefledgling and adult gonadal histology results are described in Table 1 and Figures 1-3. The ovaries of all of the female prefledglings examined appeared normal (Table 1). Figure 1 shows a typical section through the ovary of a common tern prefledgling showing the ovarian follicles within the developed ovarian cortex. Double follicles may be observed in hormone injected females (Fry, personal communication); however, none were observed among common tern prefledglings examined here.

Among male prefledglings, most testes were normal and all lacked ovarian cortical tissue (Table 1). A typical normal testis is shown in Figure 1, with the tunica albuginea encapsulating the testis and organized seminiferous tubules within which the primordial germ cells are located. The seminiferous tubules are well organized with a distinctive lining of Sertoli cells, which are the elongated irregular cells with basal nuclei and cytoplasm extending into the lumen. Numerous primordial germ cells are within the seminiferous tubules, distinguished by their large nuclei.

Several irregularities were observed among the male testes. At least five males showed small nodules of tissue within the tunica albuginea; these appeared to be isolated seminiferous tubules or seminiferous tubules extending into the tunica albuginea. These intracapsular nodules appeared testicular because of their organization with Sertoli cells lining the base of the nodule. Some nodules contained primordial germ cells while others did not. The majority of these nodules appeared quiescent, and were small, extending through only 3-15 sections. Figure 2 shows typical examples of testes with intracapsular nodules. One male showed an abnormal, undeveloped seminiferous tubule which appeared to be a rest of cells (grouping of similar cells), likely Sertoli cells. This tubule contained no primordial germ cells and showed no organization or development of a lumen. The male that contained this abnormal tubule also showed an area of extracapsular cells, which may have been a cortical remnant, but which contained no primordial germ cells. One male

testis showed disorganized seminiferous tubules that contained very few primordial germ cells, and also showed primordial germ cells located outside the seminiferous tubules. Although this testis appeared slightly disorganized, there was no evidence of an ovarian cortical area.

In summary, among the 19 male prefledglings examined, one showed disorganized tubules with extra-tubular primordial germ cells, and one showed a slightly thickened extracapsular area and an abnormal seminiferous tubule consisting of a rest of cells. At least five showed intracapsular nodules of testicular tissue but otherwise appeared normal, and the remainder appeared completely normal. No male prefledglings showed any development of ovarian cortex containing primordial germ cells.

As expected, the adult male testes were much more developed than testes of the prefledglings, showing a dramatically increased area of seminiferous tubules which contain developing spermatogonia, spermatocytes and spermatids (Figure 3d). The basal lining of Sertoli cells and lumen of the seminiferous tubules were also distinct in the adults. The edges of adult testes were often slightly irregular or lobed rather then completely rounded, possibly due to enlargement during the breeding season or handling during dissection, fixation, or embedding (Figure 3a). However, the tunica albuginea (epithelial capsule) completely surrounded the testes, and the lobes showed no indication of containing ovarian tissue (Figure 3b). Rather, the lobules consisted of seminiferous tubules containing developing spermatids. In one testes, a seminiferous tubule along the testis edge appeared to be surrounded by tunica albuginea, but this tubule also appeared to be functioning normally and contained spermatids (Figure 3c).

Contaminant Data

Gonadal histology from 10 prefledglings and paired chemical analysis results from previously-collected same-nest eggs are shown in Table 2. The contaminants were

normalized to lipid weights to compare levels in eggs and yolk sacs. The average total PCB levels were 75.13 ± 52.44 (SE), ranging from 14.35 to 546.58 ng/g lipid. However, only one bird showed the very high total PCB level of 546.58 ng/g lipid, while the range of the remaining samples was 14.35 to 37.49 ng/g lipid. Similarly, bioassay derived TCDD-EQs ranged from 3.73 to 16.93 ng/g lipid except for one sample at 114.07 ng/g lipid (the same sample which had the highest PCB levels). The average TCDD-EQs were 18.57 ± 10.68 (SE). Total PCBs and TCDD-EQs were significantly correlated, both including the highest value (r^2= 0.99, p = 0.009) and excluding the highest value (r2 = 0.82, p = 0.02) (Figure 4).

Pesticide contaminant levels were low for all pesticides measured, with most values from nondetectable to less than 1 ug/g lipid. Total p,p'-DDT showed the highest mean pesticide levels (0.83 ± 0.13 ug/g lipid), with over 95% of the total from p,p'-DDE (Table 2). Levels of mirex (0.055 ± 0.012 ug/g lipid) and t-nonachlor (0.45 ± 0.31 ug/g lipid) are also given in Table 2, and are representative of (or higher than) the remaining pesticide levels.

There was no apparent relationship between egg contaminant data and prefledgling gonadal histology. The two females showed normal gonadal histology, and the eight males showed no development of ovotesticular tissue. Also among males, the presence of intracapsular nodules and the abnormal seminiferous tubule consisting of a rest of cells did not appear related to contaminant levels in the corresponding eggs. Only one egg sample had highly elevated total PCBs and TCDD-EQs, and this egg was from the same nest as a normal male prefledgling.

Complete chemical data for all PCB congeners and pesticides analyzed in the 10 egg samples can be found in Appendix C.

Table 1. Gonadal Histology from common tern prefledglings and adults collected from Bird Island in 1995.

Samples	Normal Gonads	Feminized Gonads	Gonads with other irregularities
prefledglings- female n = 8	8	0	0
prefledglings- male n = 19	10	0	9[a]
Adults- male n = 9	8	0	1[b]

a Irregularities in the 10 male prefledglings included:
8 testes with intracapusular (within the tunica albuginea) nodules of testicular tissue; most very small, and 1 consisting of a single primordial germ cell (PGC);
1 testis with disorganized seminiferous tubules containing low numbers of PGCs, and PGCs located outside of the seminiferous tubules but within the medullary area of the testis;
1 testis with a group of similar cells, or rest of cells, in place of a normal seminiferous tubule, a small extracapsular group of cells, as well as small intracapsular nodules (included in the 8 testes with nodules as well).
b One adult testis contained a functioning seminiferous tubule completely surrounded by tunica albuginea.

Figure 1. Normal common tern prefledgling gonads, ovary of female and testis of male

1a. Normal ovary of female. Oocytes can be seen within ovarian follicles. 100x.

1b. Normal testis of male. Epithelial capsule (tunica albuginea) surrounds the testis. Seminiferous tubules are located throughout the medullary area, and primordial germ cells are located within the seminiferous tubules. 100x.

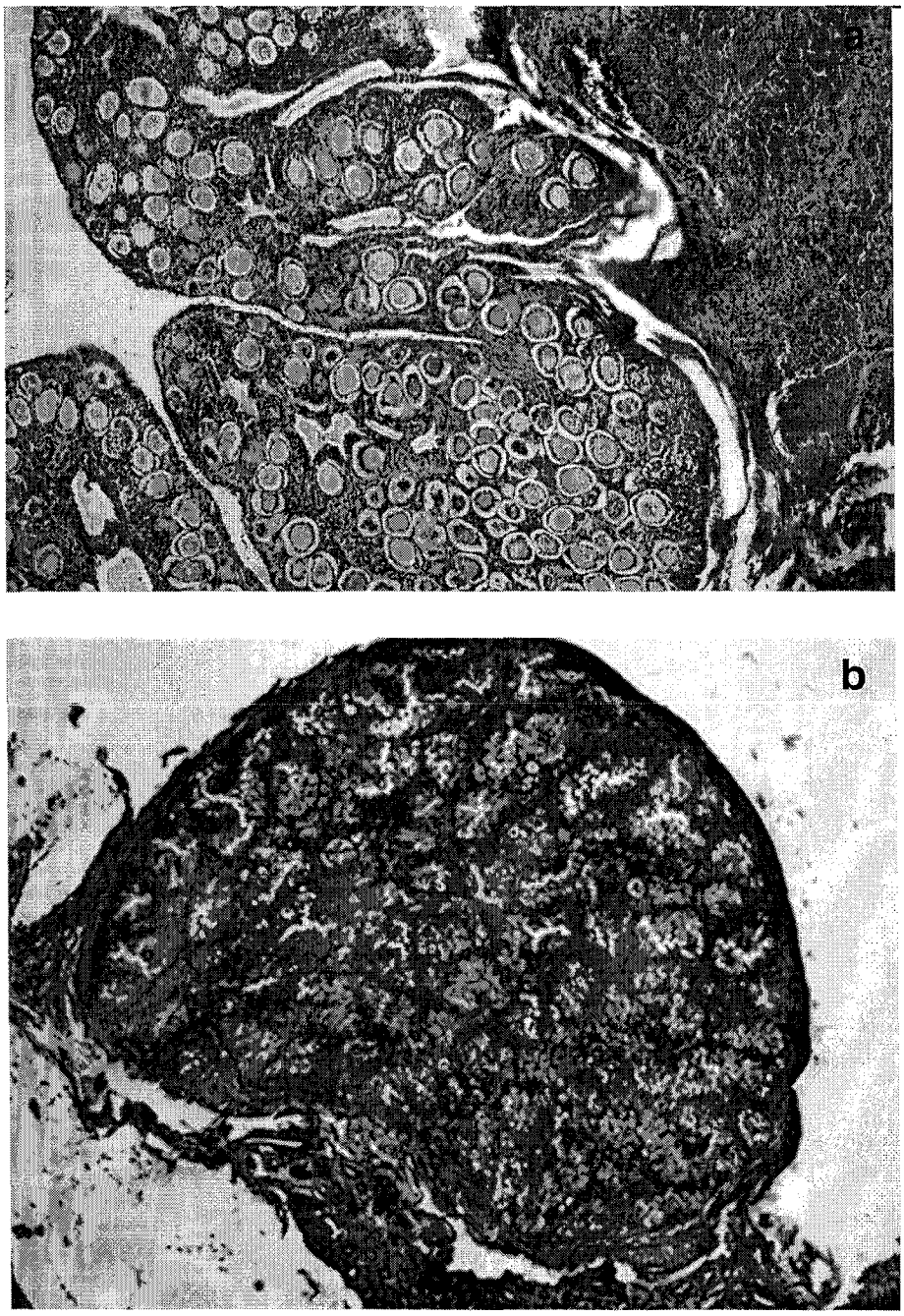

a
b

Figure 2. Examples of intracapsular testicular nodules (arrowheads) in male prefledgling common terns.

2a. Testis with intracapsular nodule of a large size. 100x.

2b. Same testis with large intracapsular nodule, organization appears like that of a seminiferous tubule. 200x.

2c. Testis with intermediate size intracapsular nodule. 200x.

2d. Testis with large intracapsular nodule. 200x.

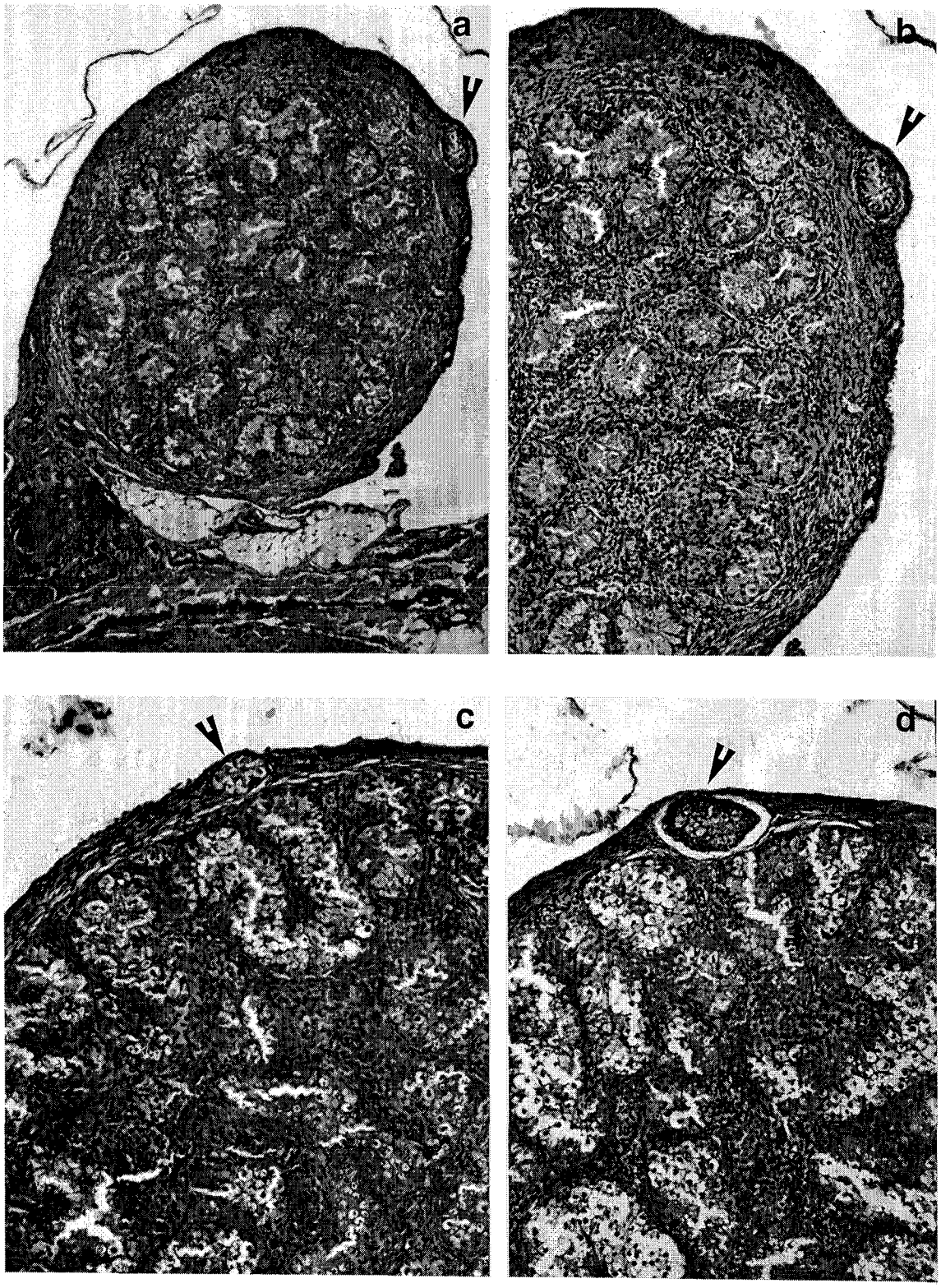

a
b
c
d

Figure 3. Adult common tern testes.

3a. Normal adult male testis, showing lobules. 25x.

3b. Same adult testis, enlargement of one lobule (arrowhead). Lobule appears to contain seminiferous tubules like that of the main testicular area. 100x.

3c. Same adult testis, enlargement of isolated seminiferous tubule surrounded by epithelial capsule (arrowhead). 200x.

3d. Enlargement of testis showing seminiferous tubules (st) containing developing spermatogonia, spermatocytes, and spermatids. The basal lining of Sertoli cells and lumen of the seminiferous tubules are distinct in the adult testis. The epithelial capsule (ec) surrounding the testis is also shown. 200x.

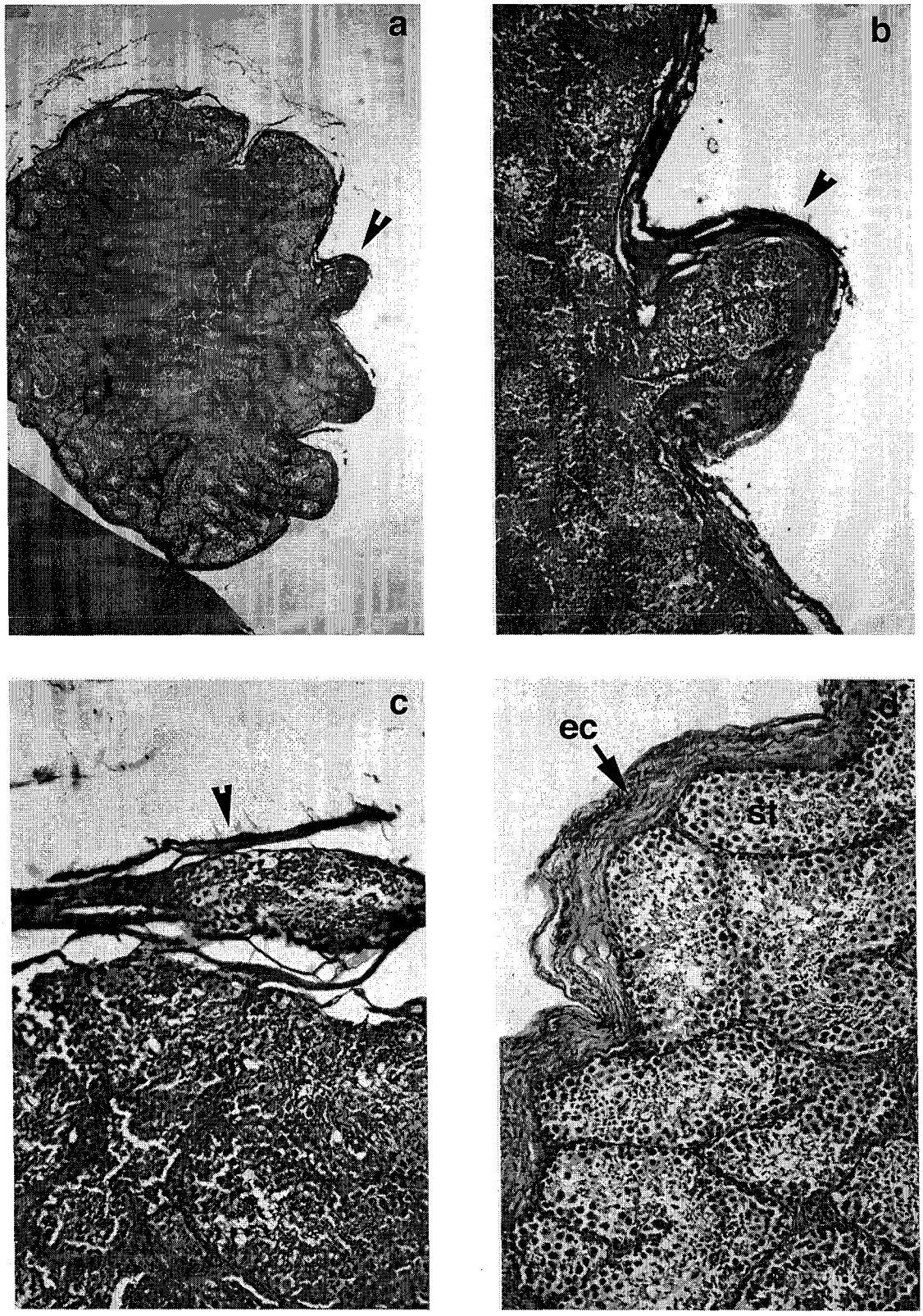

a
b
c
d
ec
st

Table 2. Summary egg chemistry data and gonadal histology of paired pre-fledglings collected from Bird Island, 1995.

Egg number	Total PCBs ug/g lipid	Bioassay-derived TCDD-EQs ng/g lipid	Total p,p'-DDTs ug/g lipid	Mirex ug/g lipid	t-Nonachlor ug/g lipid	Sex of paired pre-fledgling	Gonadal histology
257	24.42	6.36	1.25	0.07	0.28	M	R,I(2),E[a]
527	18.24	7.30	0.56	0.06	0.09	M	I(1)
530	30.28	9.15	1.62	0.03	0.25	M	I(3)
531	16.67	7.26	0.51	0.03	0.07	M	N
674	546.58	114.07	0.73	0.00	3.24	M	N
765	15.02	3.73	0.35	0.11	0.05	M	I(4)
855	37.49	16.93	1.16	0.05	0.26	F	N
938	14.35	3.80	0.37	0.12	0.04	F	N
952	19.62	6.82	1.08	0.03	0.08	M	I(2)
1024	28.65	10.31	0.70	0.06	0.18	M	I(3)
Mean	75.13	18.57	0.83	0.06	0.45		
± s.e.	± 52.44	± 10.68	± 0.13	± 0.012	± 0.31		

[a] Letters represent classification of gonadal histology as follows:
N=normal
I=Intracapsular nodules of testicular tissue. Number indicates size of nodule;
(1)=1 PGC
(2)=nodules contain few cells, and do not persist through more than 3 sections
(3)=nodules contain more cells than (2) and persist through 3-7 sections
(4)=nodules contain many cells, organization is like that of a seminiferous tubule (only much smaller) and persists through many sections
R=rest of cells in place of a normal seminiferous tubule
E=extracapsular cells

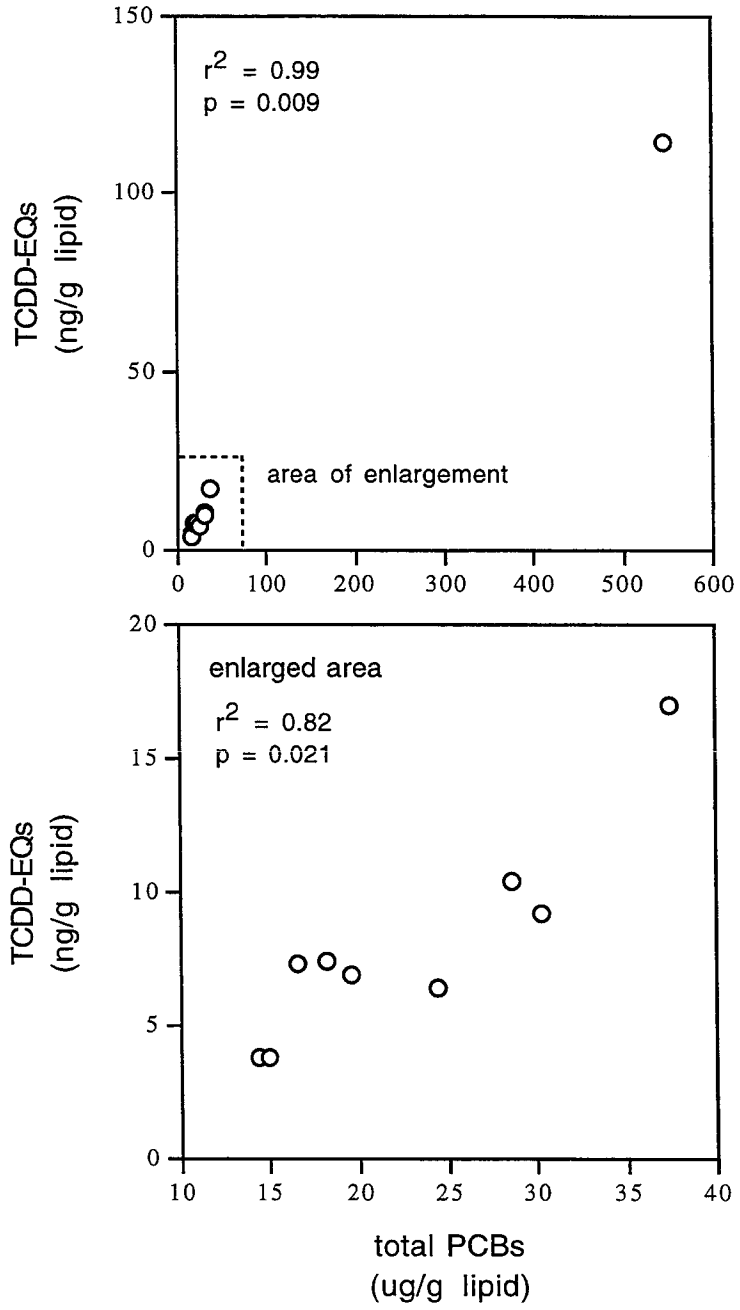


Fig. 4. Chick embryo hepatocyte (CEH) bioassay derived TCDD-EQs versus total PCBs. Samples are egg extracts from common tern eggs collected in 1995 from Bird Island.

Table 3. Summary chemistry data for tissues collected at Bird Island. 1994-1996.

Year of collection	Tissue (n)	Total PCBs ug/g lipid	TCDD-Eqs ng/g lipid	Total p,p'-DDTs ug/g lipid	Mirex ug/g lipid	t-Nonachlor ug/g lipid
1994-early	Yolk sacs (8)	133.15 ± 76.5[a] (31.56-663.43)	38.37 ± 19.66[b] (8.34-174.07)	3.42 ± 0.61 (.91-6.04)	0.28 ± 0.12 (.051-.94)	0.88 ± 0.54 (.148-4.63)
1994-late	Yolk sacs (6)	89.84 ± 33.91 (17.16-244.61)	41.57 ± 20.07[b] (7.05-138.57)	3.98 ± 0.73 (2.22-6.79)	0.18 ± 0.06 (0.06-0.37)	0.55 ± 0.23 (0.07-1.61)
1995	Eggs (10)	75.13 ± 52.44 (14.35-682.92)	18.57 ± 10.68[b] (3.78-114.07)	0.83 ± 0.13 (0.35-1.62)	0.06 ± 0.01 (.0006-.12)	0.45 ± 0.31 (.04-4.02)
1996	Yolk sacs (8)	208.95 ± 81.32 (16.5-658.74)	46.34 ± 17.42[c] (2.37-138.05)	3.27 ± 0.49 (1.23-5.19)	0.13 ± 0.03 (0-0.30)	1.2 ± 0.47 (.18-3.98)
1996	Egg pool[d] (2)	26.04 ± 10.5 (15.5-36.6)	5.45 ± 2.55[c] (2.91-8.0)	1.25 ± 0.22 (1.03-1.47)	0.06 ± 0.003 (0.06-0.07)	0.14 ± 0.07 (0.07-0.21)

[a] mean ± s.e., range in parentheses

[b] TCDD-EQs were derived using the chick embryo hepatocyte (CEH) bioassay

[c] TCDD-EQs were calculated from PCB and TCDD congener data

[d] Five eggs per pool

Table 4. Comparison of PCB, DDT, and Hg contaminant levels in common tern eggs and hepatic AHH activity in pipping common tern embryos from Bird Island, the Great Lakes, and Germany.

SITE	AHH activity[a] pmol/min/mg	total PCB[b] ug/g wet wt	total DDT[b] ug/g wet wt	Hg[b] ug/g wet wt
Bird Island, 1995 10 eggs	N.A.[c]	6.5 (1.3-45.9)	0.08[d] (0.03-0.20)	N.A.
Bird Island, 1996 2 pools of 5 eggs/pool	N.A.	1.3 (0.7-1.9)	0.06[d] (0.05-0.08)	N.A.
Great Lakes[1]				
Cut River, 1984 Non-industrialized site, Lake Michigan	22 ± 3	6.7 (4.8-10)	2.9[e] (1.6-5.0)	0.3 (0.2-0.4)
Cut River , 1985	10 ± 1	4.7 (2.5-8.0)	1.7[e] (1.0-2.4)	0.3 (0.2-0.5)
Lime Island, 1984 Non-industrialized site, Lake Huron	25 ± 4	3.9 (2.7-7.3)	1.8[e] (1.3-2.3)	0.3 (0.2-0.5)
Saginaw Bay, 1984 Industrialized site, Lake Huron	39 ± 4	10.9 (5.4-24)	1.7[e] (0.6-3.4)	0.3 (0.1-0.5)
Saginaw Bay, 1985	33 ± 4	8.5 (4.3-13)	1.6[e] (0.80-2.3)	0.3 (0.19-0.48)
Green Bay, 1985 Industrialized site, Lake Michigan	23 ± 3	10.0 (5.4-22)	1.7[e] (0.9-3.7)	0.8 (0.4-1.2)
Germany[2]				
Augustgroden, 1988 Southern North Sea coast	N.A.	3.8 (2.2-8.4)	2.4 (1.5-4.3)	0.9 (0.5-1.4)
Hullen, 1988 Highly contaminated site at the Elbe estuary	N.A.	5.2 (2.3-10.3)	7.9 (3.5-16.5)	6.2 (0.8-11.3)

[a] Aryl hydrocarbon hydroxylase, mean ± s.e., [b] Mean (range), [c] N.A. - not available,
[d] p,p'-DDE represents >95% of reported values
[e] Values reported represent p,p'-DDE only.
[1] From Hoffman, et. al. (1993) Environ. Tox. Chem. 12: 1095-1103.
[2] From Becker, et. al. (1993) Environ. Poll. 79: 207-213.

Discussion

Contaminant Data:

Contaminant levels in tern eggs collected in 1995 from Bird Island were similar to those in tern embryo yolk sacs collected in 1994 (Table 3). In both 1995 eggs and 1994 yolk sacs, most samples had intermediate total PCB levels and a few showed extremely high total PCB levels, while pesticides were fairly low and similar among all the samples. This pattern of contaminant levels continues to hold true for yolk sacs and eggs collected in 1996 (Table 3).

Contaminants in the 1995 eggs are compared to other sites in the Great Lakes and Germany in Table 4. Total PCB levels in 1995 Bird Island eggs are higher than those of both reference and highly contaminated sites in Germany, but are lower than contaminated Saginaw Bay and Green Bay sites in the Great Lakes. Although the means of total PCB levels in Bird Island eggs are lower than those at contaminated Great Lakes sites, the range of contaminant levels at Bird Island exceeds those of any other sites. This further supports the idea that individual terns at Bird Island may feed in areas highly contaminated with PCBs, accumulating exceedingly high levels of PCBs which are deposited into eggs, while other Bird Island terns may feed in relatively clean sites. Unlike total PCB levels, pesticide levels in Bird Island eggs are low compared to other sites in the Great Lakes and in Germany (Table 4), including reference sites in these areas.

There was a high correlation between PCBs and TCDD-EQs (Figure 3), as was observed in embryo yolk sac data (Chapter 3). The contaminant pattern in eggs is very similar to that of yolk sacs collected in 1994 and yolk sacs and eggs collected in 1996. As described in Chapter 3, the major contributors to TCDD-EQs are likely PCBs, primarily PCB 126.

Relationship of Gonadal Histology to Contaminants

Among the abnormalities observed histologically in the testes of prefledglings, there appeared to be no relationship to contaminants, nor any indication that these abnormalities would affect reproductive ability. As can be seen in Table 2, the presence of intracapsular nodules and the abnormal seminiferous tubule found among eight male prefledglings appears unrelated to contaminant levels. Furthermore, the prefledgling from the same nest as the egg with the highest contaminant levels shows no testicular abnormalities. The intracapsular nodules of seminiferous tubules/testicular tissue found in some prefledglings appear to be either quiescent or a typical seminiferous tubule, and of little consequence to testicular function. One of the adults examined contained a seminiferous tubule surrounded by tunica albuginea which appeared very similar to the intracapsular nodules found in the prefledglings. This could suggest that the nodules in the prefledglings may be misplaced seminiferous tubules, which in some cases can continue to develop into a functioning seminiferous tubule. None of the prefledgling abnormalities would be likely to affect the reproductive function of the testis. In addition, adults examined showed no indication of abnormal development, although this may be expected since they were found at the breeding grounds.

None of the abnormalities in the common tern prefledglings appeared ovarian. There is clearly no development of an ovarian cortical area similar to that found in female prefledglings of the same age. This may be expected since among hormonally dosed birds with ovotestes, the ovarian areas do not consistently continue to develop into oocytes that contain yolk granules and that are located in follicles. However, this can be observed in some cases (Domm 1939; Domm 1940; Riddle and Dunham 1942; Snedecor 1949; Taber 1964; Scheib 1983). The further development of ovarian areas also might be less likely to occur because the exposure to hormone-like contaminants may decrease after the embryo has hatched and resorbed the yolk sac, resulting in reduced stimulation of the ovarian

cortical areas. While contaminants in the yolk sac are from the laying female's contaminant burden, which is accumulated over time from numerous prey items, after hatching, contaminant exposure will be from single prey items.

It is possible that some of the abnormalities observed in the male prefledglings might be indicative of regressing ovarian cortical areas found in the embryos. For example, the observed quiescent intracapsular nodules in several males and the single intracapsular primordial germ cell could indicate regressing cortical areas of development. Among male hawks, areas identified as regressing cortical areas on the testes looked similar to the intracapsular nodules in the common terns (Stanley 1937). In addition, cortical areas formed in estrogen dosed gulls were described as transforming into testicular nodules that rose like blisters on the surface of an otherwise normal testis, and microscopically were almost completely enclosed in capsules formed by the fibrous tunica albuginea, but with narrow tubular connections with the main part of the testes (Boss and Witschi 1947). Although the intracapsular areas on the common tern testes could not be seen macroscopically, their description is similar to that of testicular nodules in the gull that were thought to be regressing cortical areas. The sample with an extracapsular area of thickened cells also could represent a regressing cortical area, although it is common to see varying cortical thicknesses among normal males at hatching; therefore regression of this area may not be a good indication of feminization when primordial germ cells are lacking (Fry, Toone et al. 1987). Additional time points between the hatching and prefledgling stage might clarify whether these features are related to regression of ovarian cortical areas. Nevertheless, it is clear from the prefledgling observations that the ovarian tissue seen in hatchling male tern embryos does not persist unchanged, or continue to develop in a typical ovarian manner in the prefledglings.

Since no definitively ovotesticular tissue was apparent, there appeared to be no relationship between contaminants and ovotestes development in common tern

prefledglings. The most highly contaminated sample, if following the pattern of the common tern embryos in 1994, would be expected to show development of ovotestes, but this is not the case. Regardless of the contaminant levels, no ovotestes were found in any common tern prefledglings. No ovotestes were found among the eight males with contaminant data from matched eggs, or in the other 11 male prefledglings examined without contaminant data. Since 75-80% of the pipping embryos showed ovotestes development of some severity (Chapter 2), this data suggests that the ovotestes in common tern embryos do not persist to the prefledgling stage, and therefore are unlikely to affect reproduction.

Indications from lack of ovotestes persistence:

The lack of persistence of ovotestes suggests that the effects of chemical alteration of testicular development do not permanently affect the gonad, or that the presence of ovotestes in common terns at hatching could be normal. Since only common tern embryos were collected in 1994, and only prefledglings in 1995, another possibility is that no ovotestes were present in 1995 embryos. However, since the embryos consistently showed similar incidences of ovotesticular development in 1993 and 1994, and the contaminant data is similar from 1993, 1994, 1995, and 1996 eggs and yolk sacs, this possibility is highly unlikely.

Ovotestes presence may be related to contaminants:

If the development of ovotestes in common tern embryos is related to contaminants but regresses by prefledgling age, it is possible that the bird health or reproduction could be affected in ways other than testicular function. If contaminants are acting like hormones to

cause the embryonic ovotestis development, then they also could affect other hormonally controlled developmental processes. This could include alteration of sexually dimorphic brain differentiation and result in alteration of masculine reproductive behaviors (Domm and Davis 1941; Adkins 1975; Adkins 1978; Adkins 1979) and in lowered copulatory success, or altered parental behavior affecting hatchability. Even if contaminants are acting as hormones, ovotestes might be expected to regress after hatching because of decreased hormone-like stimulation since the embryo is no longer exposed to concentrated egg yolk contaminants. The embryo also may be more sensitive to hormonal effects than the prefledglings so that embryonic ovotesticular tissue may regress or fail to be maintained in the prefledglings.

Contaminants also could be related to the development of ovotestes in common tern embryos by causing delayed development. It is known that during avian testes development some degree of ovarian cortical development often occurs, which normally regresses before hatching. This cortex has been observed in many developing bird species, including terns (*Sterna paradisea*) as described by Hoffman in 1892 (as cited in Swift 1916). The presence of dioxin-like compounds has been associated with delayed hatching and development in some bird populations. Therefore, these contaminants could lead to delayed gonadal development as well, resulting in delayed regression of ovotesticular tissue. If contaminant exposure results in delayed gonadal development, the ovotestes presence potentially could be used as an indicator of generalized developmental delay and associated effects, rather than a direct hormonal effect that would alter testicular function.

If contaminants are related to ovotestes presence in common terns, it is also possible that roseate terns may be more severely affected. The endangered roseate terns on Bird Island are showing declines, a female biased sex ratio, and much higher incidences of female-female pairing and supernormal clutches than common terns on Bird Island. Thus, even though the ovotesticular development may not indicate or cause dramatic effects in

common tern health and reproductive success, roseate terns could be a more sensitive species.

Ovotestes presence may be normal:

It is also possible that contaminants are not related to incidence of ovotestes. Instead, ovotestes could be normally present at hatching in common tern embryos. This is supported by the lack of oviduct development observed in any male common tern embryos or prefledglings. In embryos from eggs dosed with estrogenic contaminants or hormones, some oviduct development generally is observed, particularly when ovotestes of intermediate severity are present (Kozelka and Gallagher 1934; Willier, Gallagher et al. 1937; Gaarenstroom 1939; Snedecor 1949; Pincus and Erickson 1962). In addition, the incidence of ovotesticular development changes from very high (70-80%) in pipping embryos to none in prefledglings in a rapid time span of approximately three weeks. This is a similar time frame for regression of ovotestes in most other bird species in which ovotesticular tissue is normally observed at hatching, including doves, ducks, and quails (Riddle and Dunham 1942; Lewis 1946; Haffen, Scheib et al. 1975). In male ring doves examined at hatching, 76% (13/17) of birds showed traces of ovarian cortex on the left testes, which rarely persisted beyond two weeks posthatch, and usually was gone within four days posthatch (Riddle and Dunham 1942); this example of normal development in ring doves has similar percentages of ovotestes and time periods for their disappearance as those observed in common terns. Also, as previously mentioned, during embryonic development avian male testes often possess areas of ovarian cortical development which persist for a short time. This was first observed in 1886 in chicken (Laulanie 1886), and was also observed in terns (*Sterna paradisea*) by Hoffman in 1892 (as cited in Swift 1916). It is not unreasonable that remnants of this cortical tissue still may be observed at the time

of hatching, as has been seen in some other species as discussed (Stanley 1937; Riddle and Dunham 1942; Lewis 1946; Haffen, Scheib et al. 1975).

In order to directly address the possibility that ovotestes in common terns are normal, another clean site in New Brunswick, Canada, will be examined. This site has very low contaminant levels in tern eggs, with total PCB levels at 1 ppm wet weight or less. If pipping common tern embryos from this site show the presence of ovarian cortex at a similar frequency and to a similar degree as those from Bird Island and Nauset, it will strongly suggest that ovotesticular tissue in common tern males at hatch is normal. Alternatively, if common terns from the Canadian clean site show no development of ovotesticular tissue at hatching, the common terns at Bird Island could be abnormal and the presence of ovotesticular tissue could be related to contaminant exposure.

In summary, the presence of ovotestes in male common tern testes does not appear to persist to the prefledgling stage (approximately 3 weeks of age), and testes and seminiferous tubules appear to be normal. This suggests that reduced functioning of the testes is not a cause of impaired reproduction in common terns. From the data available, it is not possible to determine whether ovotestes presence in hatching common terns is related to contaminant exposure or whether it is normally present. If contaminants do cause ovotestes development in tern hatchlings, other adverse effects could occur in the terns even though the ovotestes appear to regress. A contaminant acting hormonally to cause ovotestes development may alter other hormonally controlled processes. Behavior, regulation of hormonal cycles, functioning of the reproductive system, and other aspects of the endocrine system could be permanently affected. A contaminant also could be acting to delay development in the embryonic bird, resulting in prolonged persistence of an ovarian-like cortical area that appears on the male testis normally during development. Contaminants that cause delayed development also could cause other adverse effects in the terns. If contaminants are causing ovotesticular development by either of these

mechanisms, it is possible that roseate terns may be much more sensitive to effects. Alternatively, contaminants may be unrelated to ovotestes development, but rather the persistent cortical remnants found on the testes of male common terns at hatching may be normal. Further investigation, particularly examination of common terns at an additional clean site, should distinguish between these possibilities.

CHAPTER 5: ESTROGENIC BIOASSAY USING COMMON TERN YOLK SAC EXTRACTS

Chapter 5: Estrogenic bioassay using common tern yolk sac extracts

Introduction

Estrogen receptor binding was examined as a possible mechanism by which contaminants could be causing feminization in common tern embryos. Injection studies have shown that embryonic exposure to estrogens can result in feminization of the male gonads (development of ovotestes) in all avian species examined (Gaarenstroom 1937; Willier, Gallagher et al. 1937; Domm 1939; Riddle and Dunham 1942; Lahr and Riddle 1945; Lewis 1946; Boss and Witschi 1947; Romanoff 1960; Pincus and Erickson 1962; Taber 1964; Haffen, Scheib et al. 1975; Scheib 1983). Furthermore, recent studies have shown many environmental contaminants to have estrogenic or anti-estrogeninc activity, including PCBs, phthalates, alkyl-phenols, and many organochlorine pesticides including o,p'-DDT, methoxychlor, and chlordecone among others (Korach, Sarver et al. 1987; Soto, Lin et al. 1992; Jansen, Cooke et al. 1993; Klotz, Beckman et al. 1996; Coldham, Dave et al. 1997; Harris, Henttu et al. 1997) . Many of the contaminants with estrogenic activity were able to bind to the estrogen receptor. Therefore, we wanted to examine the estrogenic potency of contaminants in common tern yolk sac extracts by determining their ability to bind to tern estrogen receptor.

The ability of tern yolk sac extracts to displace radiolabled estradiol from estrogen binding sites was examined. Tern hepatic cytosol was used as a source of tern estrogen receptor. In addition, chicken ovary or oviduct cytosol was used as another avian source of estrogen receptor.

Methods

Cytosols: Tern hepatic cytosols and chicken ovarian and oviduct cytosols were made by differential centrifugation according to (Hahn, Poland et al. 1994). Powdered tern liver, chicken ovary or oviduct were homogenized with 5 volumes of cold 25mM MOPS buffer, pH 7.5 at 20°C, containing 1mM EDTA, 5mM EGTA, 0.02% NaN_3, 20mM Na_2MoO_4 , and 10% (v:v) glycerol, and made 1mM in dithiothreitol (15mg/100ml) on the day of use and 0.1 % (v:v) of 0.1M PMSF (phenylmethylsulfonyl fluoride) in isopropanol just prior to homogenization. In addition, the following protease inhibitors were present: 20uM TLCK (Tosyl-L-lysine chloromethyl ketone), 5 ug/ml leupeptin, 100 ug/ml aprotinin, and 7 ug/ml pepstatin A. Tissues were homogenized on ice with a Teflon-glass homogenizer (10 passes), and centrifuged for 10 minutes at 750g and 10 minutes at 12,000g. The supernate was then centrifuged at 100,000g for 70 minutes. The upper lipid layer was removed. Then the supernate fraction (cytosol) was removed and frozen in liquid nitrogen until use. Tern hepatic cytosols had been made in 1991 from common terns collected at Bird Island. Chicken (*Gallus gallus*) tissues were a kind gift from Dr. John Teal just prior to this study, and were collected fresh, frozen immediately in liquid nitrogen, powdered, and stored in liquid nitrogen until cytosols were prepared.

Estrogen receptor binding assay: Estrogen receptor binding was measured in either tern hepatic cytosols, or chicken ovary or oviduct cytosols as described by Leake and Habib (Leake and Habib 1987) and Janz and Bellward (Janz and Bellward 1996). The [2,4,6,7,16,17-^{3}H]estradiol (141 Ci/mmol; greater than 97% radiochemical purity) was purchased from DuPont NEN. Duplicate 150 ul volumes of cytosol were diluted in 25mM MOPS buffer (as above, but with only DTT added) to give a protein concentration of 1 mg/ml, and were added to glass test tubes, on ice. A 50 ul volume of each working

solution of [^{3}H]estradiol was added to give a concentration between 0.01-12 nM (concentrations vary with experiment) to determine total binding (TB) in a volume of 200 ul. Identical duplicate tubes except for the presence of 1000-fold molar excess of diethylstilbestrol (DES) or estradiol (fold excess and DES or estradiol vary with experiment) were used to determine nonspecific binding (NSB). Tubes were vortex-mixed and incubated for 18 hours at 4°C. After incubation, a 5 ul aliquot was removed and added to 5 ml scintillation cocktail to determine total counts. To remove unbound estradiol, 200 ul of Dextran-coated charcoal (DCC; 0.5% w/v activated charcoal and 0.05% w/v Dextran T-70) were added to the incubation mixtures, and tubes were vortex-mixed and allowed to sit for 15 minutes at 4°C. The DCC was pelleted by centrifugation at 1000g and 4°C for 10 minutes. Then 200ul of supernatant were added to a 5 ml volume of scintillation cocktail. Radioactivity was measured using a Beckman LS 5000TD scintillation counter. Specific binding was defined as the difference between total binding (incubations containing [^{3}H]estradiol) and nonspecific binding (incubations containing [^{3}H]estradiol plus a 1000-fold excess of estradiol or DES).

A preliminary experiment was conducted to examine the effect of varying cytosolic protein concentrations on specific binding at 0.1, 0.5 and 1.5 nM [^{3}H]estradiol concentrations. Specific binding was linear for each radioligand concentration at the cytosolic protein concentrations tested (1, 2, 4 mg/ml) (data not shown).

The concentration of dextran coated charcoal used to remove unbound ligand was tested to assure that DCC was able to remove all the free ligand. DCC at 0.25, 0.5, 1.0, 2.5, and 5 % activated charcoal were tested. Incubations were carried out with 1nM and 3nM [^{3}H]estradiol plus or minus 1000-fold excess estradiol both with and without cytosolic protein. Concentrations of DCC at 0.5 % activated charcoal and above resulted in removal of free ligand (data not shown). The full binding curve experiments used 1% DCC while remaining experiments used 0.5 % DCC.

The concentrations of excess DES and estradiol for use in nonspecific binding tubes were tested. DES was tested at 100-fold, 200-fold, and 1000-fold excess in tern hepatic cytosol. Estradiol was tested at 100-fold, 200-fold, 500-fold, 1,000-fold, 2,000-fold, and 10,000-fold excess in tern hepatic and chicken ovary cytosol. DES and estradiol were compared at 250-fold excess in hepatic tern and chicken ovary and oviduct cytosol. A 1,000-fold excess concentration of either competitor was optimal for determination of nonspecific binding.

For the common tern yolk sac extract experiment, duplicate total binding tubes contained tern hepatic cytosol (1mg/ml), 1 nM [3H]estradiol and one of the following: buffer alone, 5 or 10 ul DMSO, or 5ul of tern yolk sac extracts in DMSO (see Chapter 3 for extract methods). Duplicate nonspecific binding tubes contained tern hepatic cytosol (1mg/ml), 1 nM [^{3}H]estradiol, either buffer alone or 5 or 10 ul DMSO, and 1000-fold excess DES (1uM). For all tubes, total incubation volumes were 200 ul, and the DMSO at both 2.5% and 5% had no effect on specific binding results.

Full binding curves were determined using tern hepatic cyotsols and chicken ovary cytosols. Cytosols were incubated at 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.5, 10.0, 12.5, and 17.5 nM [^{3}H]estradiol. Nonspecific binding tubes were incubated with a 1000-fold excess of estradiol.

Results

Specific binding of [^{3}H]estradiol in tern hepatic cytosol and chicken ovary cytosol with differing amounts of excess estradiol as the cold competitor is shown in Figure 1. Results show that 1000-fold excess estradiol is the optimal concentration for determining nonspecific binding, although 200-1000 fold excess estradiol is adequate.

The ability of common tern yolk sac extracts to compete with [^{3}H]estradiol for binding sites was determined in tern hepatic cytosol. Figure 2 shows total binding when different competitors are added to the incubation. All of the extracts displaced more [^{3}H]estradiol than either DES or estradiol.

The full binding curves for [^{3}H]estradiol in chicken ovary cytosol and tern hepatic cytosol are shown in Figures 3 and 4. The specific binding curve did not reach a clear maximum for [^{3}H]estradiol binding at 12-15 nM [^{3}H]estradiol in either chicken or tern cytosols. Binding data were analyzed using double reciprocal plots and Scatchard plots in both chicken and tern cytosols (Figures 5-8). From these plots, the estimated Kd for [^{3}H]estradiol binding in chicken cytosol is 9 - 11.1 nM, and the total concentration of binding sites is 298-358 fmol/mg. In tern hepatic cytosol, the Kd is 5.28 and the total concentration is 344 fmol/mg as determined from the double reciprocal plot; the Scatchard plot indicates binding is very low affinity and non-saturable, and a single point suggests the possibility of a biphasic binding curve and thus two types of binding sites.

Specific binding of [^{3}H]estradiol in chicken ovary cytosol and tern hepatic cytosol with either DES or estradiol as the cold competitor is shown in Figure 9. In both tern and chicken cytosols, DES was unable to compete well with [^{3}H]estradiol for binding sites, resulting in almost no specific binding observed. Estradiol competed in both tern and chicken cytosols, resulting in higher specific binding.

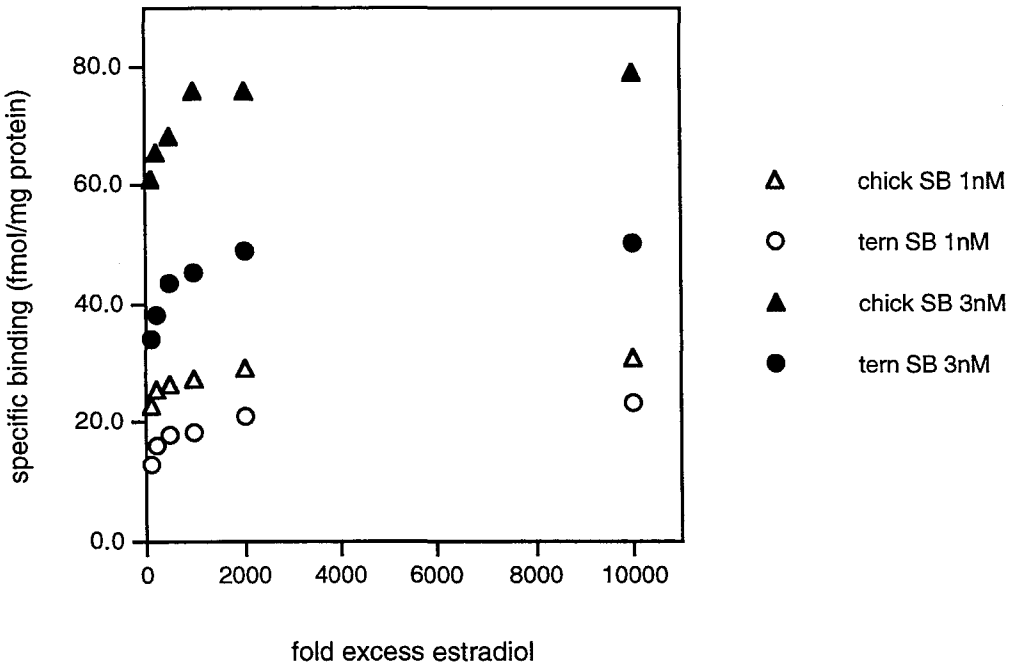


Figure 1. Specific binding (SB) of [H3]estradiol (1nM and 3nM incubations) in tern hepatic cytosol and chicken ovary cytosol with differing amounts of excess estradiol as the cold competitor for determination of nonspecific binding. Amounts of excess estradiol used were 100-fold, 200-fold, 500-fold, 1,000-fold, 2,000-fold, 10,000-fold excess.

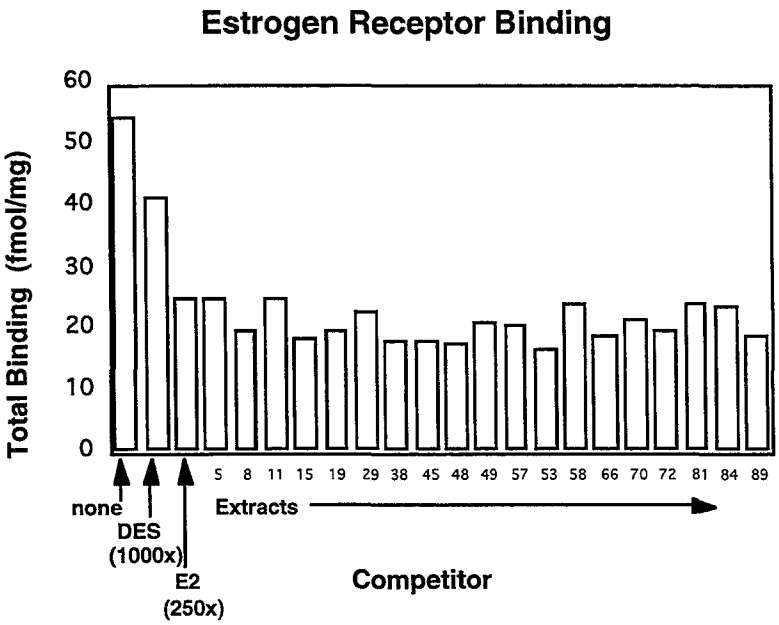


Figure 2. Comparison of total binding of [H^3]estradiol in tern hepatic cytosol with different competitors. Competitors include 1000-fold excess DES, 250-fold excess estradiol, and common tern yolk sac extracts.

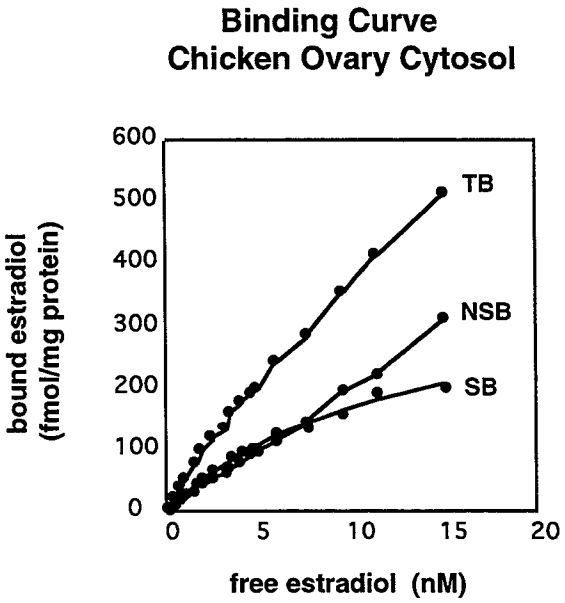


Figure 3. [H^3]Estradiol binding curve in chicken ovary cytosol. TB = total binding, NSB = nonspecific binding, SB = specific binding (SB = TB - NSB).

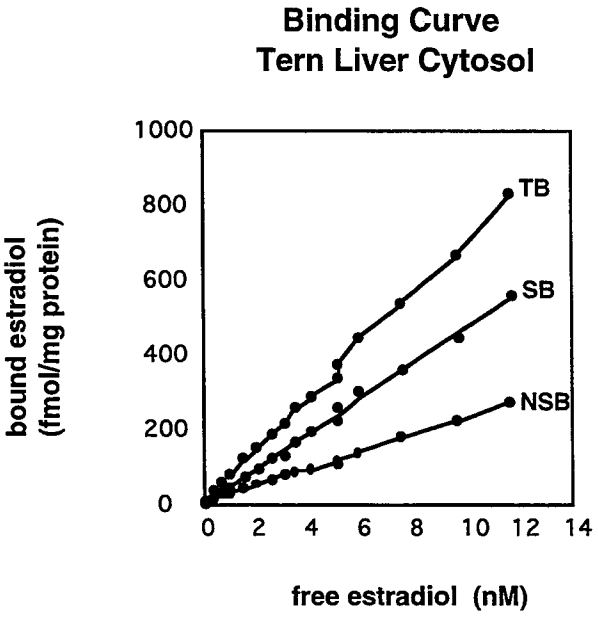


Figure 4. [H^3]Estradiol binding curve in tern hepatic cytosol. TB = total binding, NSB = nonspecific binding, SB = specific binding (SB = TB - NSB).

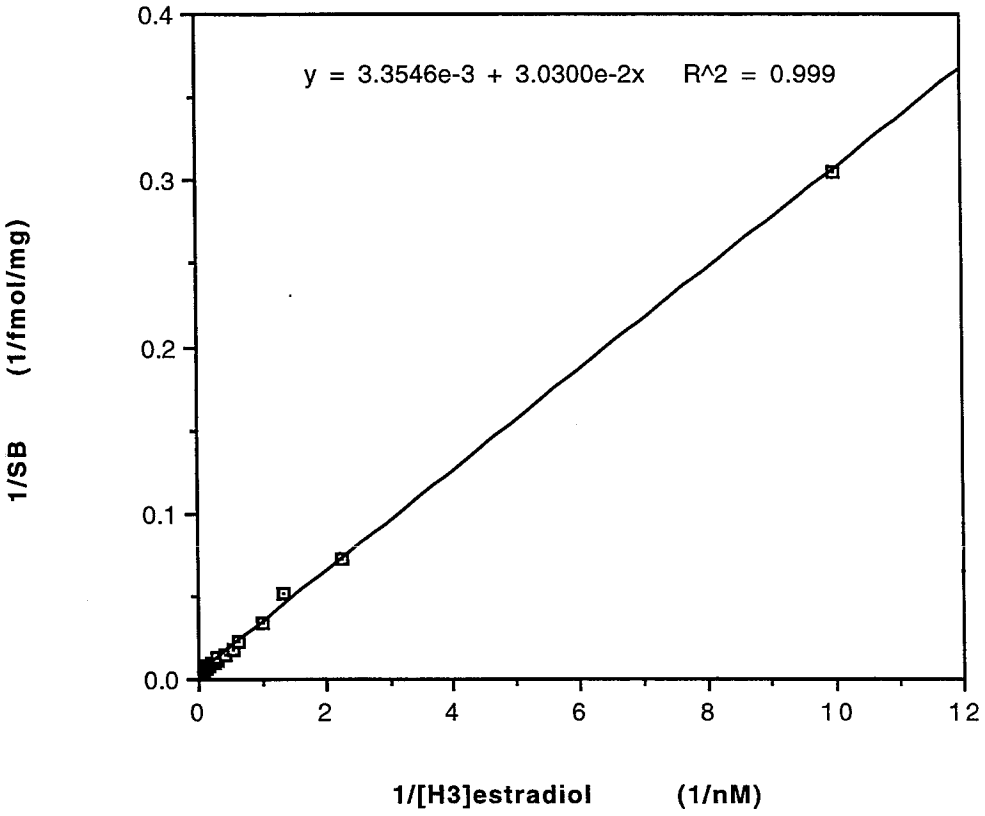


Figure 5. Double reciprocal plot of chicken ovary cytosol [H^3]estradiol binding curve. Calculated from the plot, the Kd = 9 nM and the total concentration of binding sites is 298 fmol/mg.

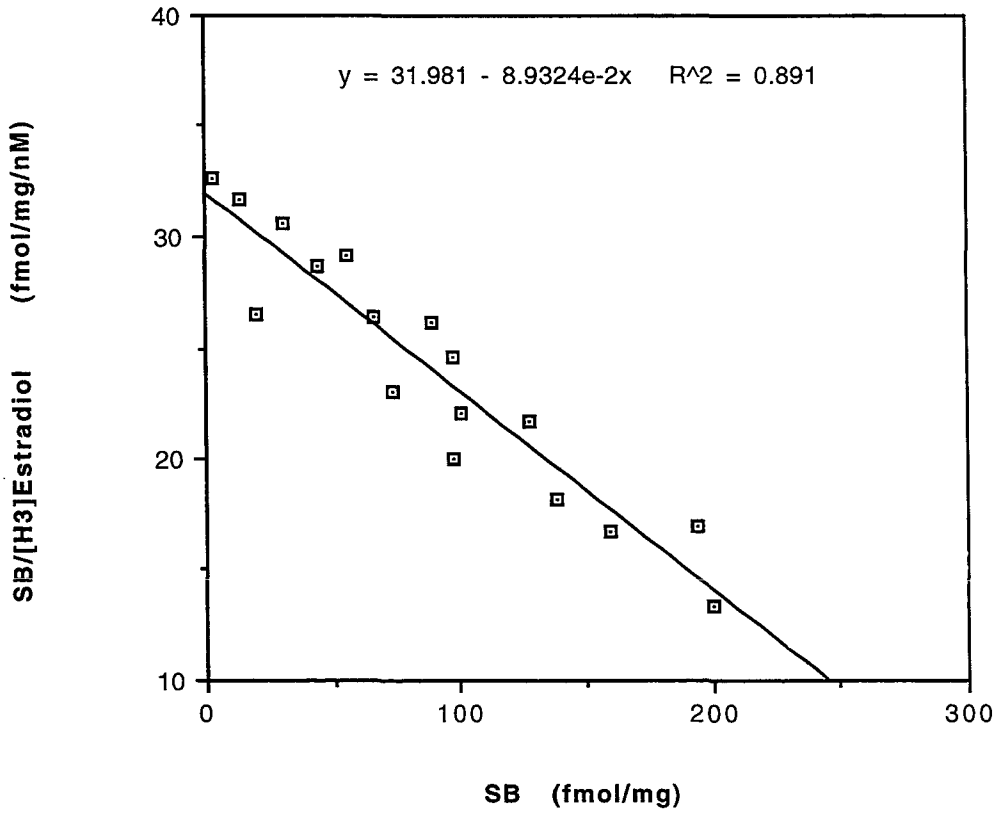


Figure 6. Scatchard plot of chicken ovary cytosol [H^3]estradiol binding curve. Calculated from the plot, the K_d = 11.1 nM and the total concentration of binding sites is 358 fmol/mg.

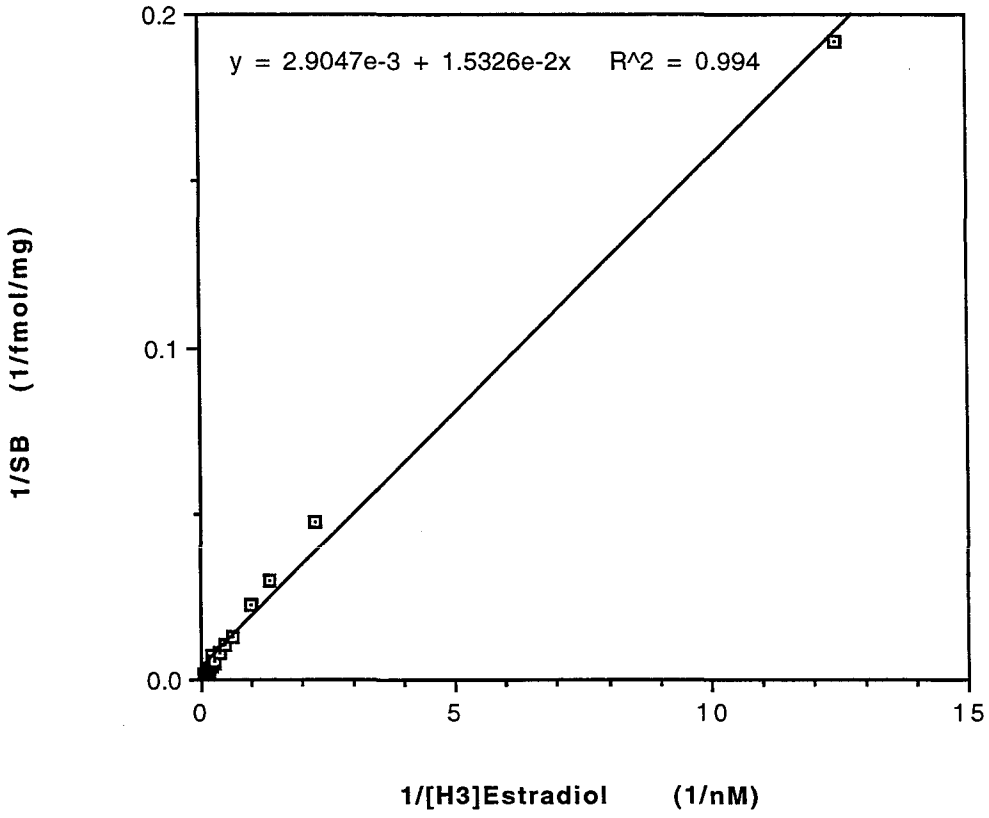


Figure 7. Double reciprocal plot of tern hepatic cytosol [H^3]estradiol binding curve. Calculated from the plot, the Kd = 5.28 nM and the total concentration of binding sites is 344 fmol/mg.

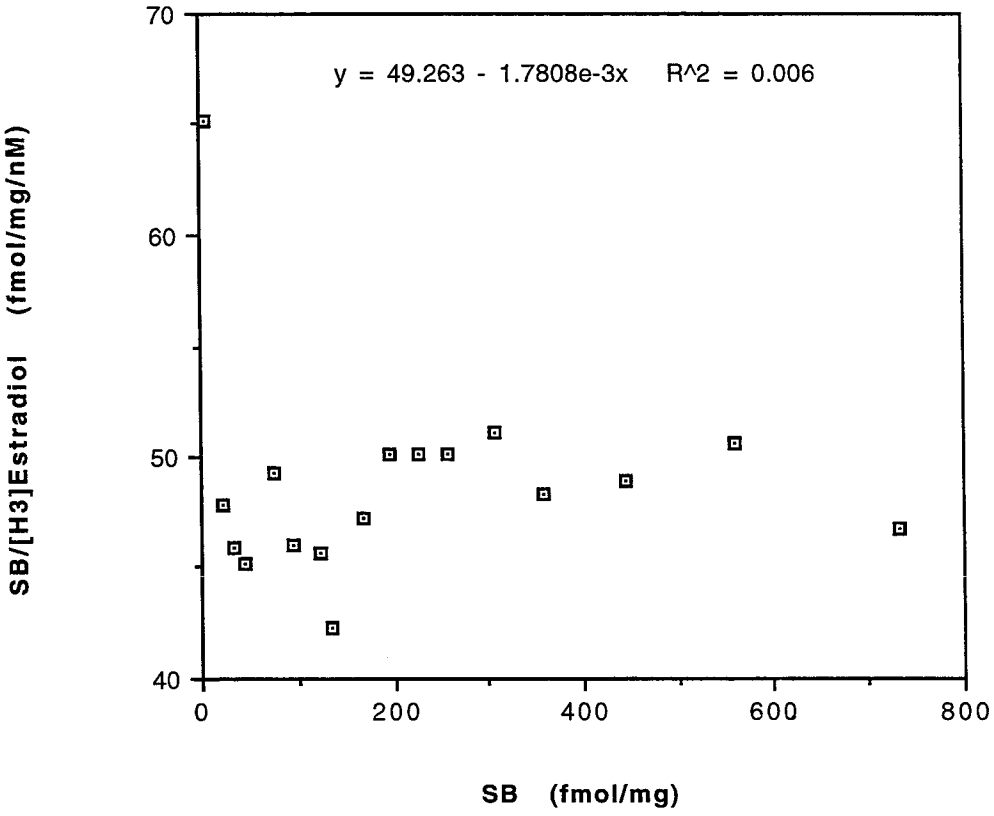


Figure 8. Scatchard plot of tern hepatic cytosol [H^3]estradiol binding curve.

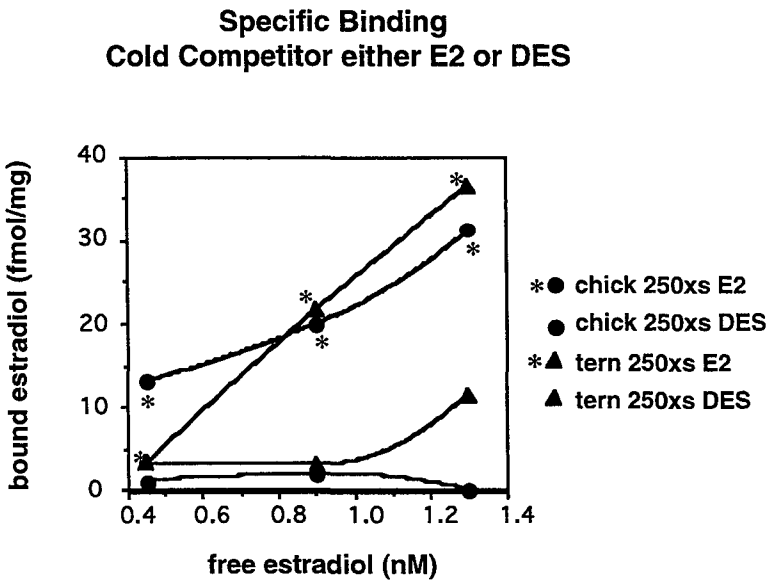


Figure 9. Comparison of specific binding of [H^3]estradiol in chicken ovary cytosol and tern hepatic cytosol when either excess DES or excess estradiol is used to determine nonspecific binding.

Discussion

Surprisingly, the yolk sac extracts were able to displace either more or an equivalent amount of radiolabled estradiol than either excess DES or excess estradiol (Figure 2). Specific binding is total binding (no competitor) minus nonspecific binding (binding with either excess estradiol or DES as competitor). Thus, both estradiol and DES should maximally displace radiolabled estrogen from specific binding sites. However, the extracts were better or equivalently able to displace radiolabled estradiol from binding sites than the natural or synthetic hormone, indicating that either the extracts were extremely estrogenic or the assay was not measuring binding to the estrogen receptor alone. Further investigation with full binding curves and estradiol and DES competitors suggest that binding is not specific to the estrogen receptor.

Binding curves for both chicken ovary and tern hepatic cytosol (Figures 3 and 4) indicate that specific binding continues to increase without reaching a maximum even with radiolabled estradiol concentrations nearing 12 nM. Results at the highest [^{3}H]estradiol incubation concentration (15 nM) in chicken ovary cytosol suggest the binding curve may be approaching a maximum (Figure 3). Literature binding curves for estradiol to avian estrogen receptor suggest that a maximum should be reached by 1 - 3 nM estradiol (Lazier and Haggarty 1979; Janz and Bellward 1996; Stevens 1996). Furthermore, the present results show the Kd values determined from double reciprocal and Scatchard plots of binding data are between 9 and 11.5 nM for chicken ovary cytosol and are either 5.3 or too high to be determined for tern hepatic cytosol. Literature values for avian estrogen receptor Kds are usually from 0.1 to 1 nM, and only seldom are values above 2.0 nM reported (Lazier and Haggarty 1979; MacLaughlin, Hutson et al. 1983; Janz and Bellward 1996; Stevens 1996). The unusually high Kd values and the lack of a maximum observed in the binding curves suggest that binding may not be specific for the estrogen receptor.

Further support that these cytosolic incubations are not measuring specific estrogen receptor binding alone is shown by specific binding curves comparing estradiol and DES as cold competitors (Figure 9). For both tern and chicken cytosol, almost no specific binding was observed with DES as cold competitor, yet specific binding was observed with estradiol as cold competitor. DES is routinely used as a cold competitor in estrogen binding studies, including studies in chicken, great blue heron, and pigeon (Lazier and Haggarty 1979; Janz and Bellward 1996). Furthermore, high affinity binding of DES to estrogen binding sites often is used as a check that binding is specific for the estrogen receptor, and is used across vetebrate species including mammals, birds, turtles, alligators, sting rays, and fish (Lazier and Haggarty 1979; Lazier, Lonergan et al. 1985; Giannoukos and Callard 1996; Yamamoto, Suzuki et al. 1996; Vonier, Guillette Jr. et al. 1997). All previous work indicates that DES should bind to the estrogen receptor of chicken with an affinity equivalent to estradiol. There have been no previous studies in vertebrates where DES does not bind to the estrogen receptor with high affinity. Thus, the possibility that DES is unable to bind to tern estrogen receptor is extremely unlikely. Finally, while both estrogen and DES should bind similarly to an estrogen receptor, it is known that estrogen has a much greater affinity for binding to steroid binding plasma proteins than does DES, which has an extremely low affinity for these proteins (MacLaughlin, Hutson et al. 1983).

Further support that binding is not specific for the estrogen receptor is given in Table 1, which compares the Kds and concentrations for estrogen binding sites from literature with those determined in this study. The Kd values for the binding observed in tern hepatic cytosol and chicken ovary cytosol are most similar to those observed for plasma binding proteins, with both Kd values and binding site concentrations being much larger than values for estrogen receptors. Also, DES bound with very low affinity in tern and chicken cytosols, similar to binding studies with plasma proteins. In the case of true estrogen receptors, DES bound only with high affinity.

Table 1. Comparison of estradiol and DES binding affinities to estrogen binding sites in the tissues described. Binding sites are estrogen receptors, plasma proteins, and undetermined estrogen binding sites. Values listed are for estradiol binding; DES binding is high affinity unless otherwise noted.

Tissue source	Kd (nM)	Binding site concentrations (fmol/mg protein)
common tern hepatic cytosol[a]	5.3 - low affinity DES very low affinity	345 - ∞
chicken ovary cytosol[a]	9 - 11.1 DES very low affinity	295 - 360
Estrogen Receptors		
chicken oviduct purified receptor[b]	1) 0.06 high affinity form 2) 0.8 phosphorylated lower affinity form	not available
chicken oviduct cytosol[c]	1) 0.12 2) lower affinity form	21 -65
Great blue heron hepatic cytosol[d]	0.06 - 0.14	33 - 53 (one at 133)
Chicken hepatic cytosol[d]	0.16 - 0.46	17 - 50
pigeon hepatic cytosol[d]	0.09 - 1.25	25 - 98
cockerel hepatic cytosol[e]	0.4 - 2.6	20 - 33
salmon hepatic cytosol[f]	2.9	60
Plasma proteins		
Human pregnancy plasma (has SHBG and αFP)[g]	1) 2 2) lower affinity form DES very low affinity	3
rat pregnancy plasma[g]	2.5 DES very low affinity	4.5
salmon plasma[f]	13 DES very low affinity	16.8 nM in plasma

[a] this study
[b] Stevens (1996)
[c] Maclaughlin, Hutson, and Donahoe (1983)
[d] Janz and Bellward (1996)
[e] Lazier and Haggarty (1979)
[f] Lazier, Lonergan, and Mommsen (1985)
[g] Sheehan and Young (1979)

Although studies of avian estrogen receptor have been succesful with simple hepatic cytosol preparations (Janz and Bellward 1996), other studies with cytosol preparations suggest that it is not unusual for the presence of other hormone binding proteins to mask estrogen receptor binding, or for estrogen receptor binding to be too low for detection. A non-receptor steroid binding protein was found in chicken hepatic cytosol; removal of this protein was required before estrogen receptor binding in preparations could be detected (Lazier and Haggarty 1979; Lazier, Lonergan et al. 1985). In experiments examining unfractionated chicken hepatic cytosol for high-affinity [^{3}H]estradiol binding, copious quatities of bound hormone were detected, but less than 5% suppression of total binding by inclusion of 100-1000-fold excess of unlabelled estradiol or DES in the assay mixture was found (Lazier and Haggarty 1979). Salmon liver cytosol preparations showed either very low or nondetectable levels of estrogen receptor binding, but did show lower affinity binding, the source of which could have been liver cytosol or contamination from plasma (Lazier, Lonergan et al. 1985); salmon plasma did show binding of estradiol to a non-receptor hormone binding protein, which also did not bind DES (Lazier, Lonergan et al. 1985). Furthermore, cytosols from oviduct of alligator and turtle showed [^{3}H]estradiol binding to non-receptor hormone binding proteins, with no detectable binding to a hormone receptor. The protein was shown to be a non-receptor protein by the equal ability of estradiol and testosterone to displace [^{3}H]estradiol from the binding proteins, the inability of DES to displace [^{3}H]estradiol, and by DNA-cellulose purification of cytosols which showed minimal binding in the estrogen receptor fraction and significant binding in the non-estrogen-receptor fraction (Crain, Noriega et al. 1997).

It is likely that the tern and chicken cytosol preparations may contain very low levels of estrogen receptor, which are masked by other binding proteins. The [^{3}H]estradiol binding results in tern liver cytosol were similar to the results observed in chicken and salmon liver cytosol preparations where high affinity estrogen receptor binding was

nondetectable. The greatest specific binding was observed in chicken ovary cytosol, and this preparation also contained the most blood in the preparation. Thus, binding might be greatest due to the increased presence of plasma binding proteins. Specific binding was much lower in chicken oviduct cytosol preparations, which contained little blood (data not shown). Results from tern and chicken cytosolic preparations were consistent with many other studies; cytosol preparations of tissues from chickens, salmon, alligator, and turtle all proved to have large amounts of non-receptor hormone binding proteins present, while high affinity receptor binding was undetectable without further purification (Lazier and Haggarty 1979; Lazier, Lonergan et al. 1985; Crain, Noriega et al. 1997; Vonier, Guillette Jr. et al. 1997).

Similar results to those with tern extracts were observed in studies looking at contaminant binding to hormone receptors and hormone binding proteins. Studies of [^{3}H]5α-DHT binding were carried out with prostate cytosol, which is known to contain a steroid binding protein other than an androgen receptor that is fairly nonspecific and not readily saturable. Incubations with environmental contaminants such as DDTs were able to inhibit [^{3}H]5α-DHT binding to a greater extent than cold 5α-DHT. Sucrose gradient studies showed that while both DDTs and 5α-DHT reduced binding to androgen receptor, the DDTs were better able to reduce binding to the additional unknown binding protein (Danzo 1997). A similar protein that binds contaminants with a higher affinity than estradiol could be present in avian cytosols.

Although no conclusions about the ability of common tern yolk sac extracts to bind to the estrogen receptor can be made with certainty, extracts were able to displace radiolabled estradiol from other binding sites, likely from a hormone binding plasma or cytosolic protein. Hormone binding proteins are important for regulation of metabolism, clearance, and bioavailability of steroid hormones, and interaction with these proteins by contaminants in the extracts may affect estrogen signaling (Crain, Noriega et al. 1997;

Danzo 1997). Since extracts may be able to displace estradiol from non-receptor hormone binding proteins, they may be able to displace estradiol from estrogen receptors as well. Previous studies have shown that contaminants interacted with both receptor and nonreceptor hormone binding proteins to different degrees (Crain, Noriega et al. 1997; Danzo 1997). The preliminary results with tern yolk sac extracts suggesting their ability to displace estradiol from binding sites are intriguing. They indicate the need for further research to determine specifically the hormone binding proteins and hormone receptors with which extracts can interact, and if indeed, environmental contaminants in the extracts result in the interaction. Further research should include use of pure estrogen receptor preparations to determine whether these extracts are able to displace estradiol from the estrogen receptor, and use extracts from "clean" eggs as a control for endogenous compounds.

CHAPTER 6: SUMMARY AND CONCLUSIONS

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Terns breeding at Bird Island, Massachusetts historically have had high levels of contaminants in their eggs and tissues, most significantly PCBs (Nisbet and Reynolds 1984; Burger, Nisbet et al. 1992). This occurs mainly because the feeding grounds of some Bird Island terns are at or near New Bedford Harbor, a highly contaminated Superfund site with high levels of PCBs and heavy metals, including unusually high levels of lower chlorinated PCBs (Weaver 1984; Pruell, Norwood et al. 1990; Lake, McKinney et al. 1995). Roseate terns breeding at Bird Island were observed to have a female-biased sex ratio and to show female-female pairing and supernormal clutches. Similar observations among gull populations in the 1970s were suggested to be related to feminization of the reproductive tract of male gulls resulting from highly elevated levels of DDT and other organochlorine contaminants (Fry and Toone 1981; Fry, Toone et al. 1987). In 1993, a limited sampling of common tern embryos from Bird Island (sampled as a surrogate for endangered roseate terns) revealed feminization of testes in 73% (11/15) of males, consisting of ovarian-like cortical areas containing primordial germ cells located on the testis (ovotestes). The severity of ovotestes was compared with the levels of selected PCB congeners in the 1993 embryo livers and no clear relationship was found, although further investigation of PCBs was suggested (Nisbet, Fry et al. 1996). The high percentage of ovotestes along with high PCB levels in Bird Island terns prompted further study to examine the relationship of contaminants to ovotestes presence in common terns, as described in this thesis. A more extensive sampling of pipping common tern embryos was examined and compared with tern embryos collected from Nauset, a control site on outer Cape Cod. Contaminants in embryo yolk sacs, including chemical analysis of a suite of PCB congeners and chlorinated pesticides, bioassay-derived TCDD-EQs, estrogenic

potencies, and mercury (in embryo muscle tissue) were compared between sites and were examined for their relationship to the presence of ovotestes. In addition, prefledgling common terns were studied to determine the persistence of ovotestes.

The major findings of this study were:

1. Total PCBs were significantly higher in Bird Island tern embryos (mean 114, range 17-663 ug/g lipid) than in Nauset tern embryos (mean 35, range 8-178 ug/g lipid); but were highly variable at both sites. The need for a better reference site is indicated.
2. Levels of chlorinated pesticides and mercury were low and similar at both sites.
3. Principal Component Analysis showed clear site distinctions in PCB isomer patterns, with Bird Island having relatively higher levels of lower-chlorinated PCBs.
4. Total PCBs were highly correlated with TCDD-EQs, and PCB 126 was determined to be the major contributor (over 80%) to the TCDD-EQs.
5. Tern hepatic EROD activity was relatively insensitive to induction; only when TCDD-EQs were above 82 ± 26 ng/g lipid were EROD activities elevated.
6. Spleen weights of terns at Bird Island were significantly lower than those at Nauset.
7. Two female terns with crossed beaks were found, one from Bird Island and one from Nauset. Both of these terns had relatively low contaminant levels.
8. The percentage of male tern embryos with ovotestes at Nauset (60%) and Bird Island (78%) was high and not significantly different; ovotestes in terns from both sites ranged in severity from absent (1) to intersex (4).
9. There was no significant relationship between ovotestes severity and any of the contaminants measured.
10. The data did suggest a contaminant level threshold of 100 ug/g lipid total PCBs and 30 ng/g lipid TCDD-EQs above which the formation of ovotestes in tern embryos is more likely to occur.

11. Principal Component Analysis of PCB isomer patterns showed no difference in PCB patterns of common terns with and without ovotestes.

12. Examination of common tern prefledglings indicated that ovotestes do not persist to the prefledgling stage (approximately 3 weeks of age).

13. The results support more than one possibility for the relationship between contaminants and ovotestes:

a) Contaminants could be related to ovotestes, either completely or by increasing the incidence of ovotestes above a normal background incidence. If contaminants are related to ovotestes, mechanisms could include: 1) altering hormonal control of testicular development; or 2) delaying development which could result in the persistence of ovarian cortical areas normally present during avian embryonic gonadal differentiation.

b) The presence of ovotestes development in hatching male common tern embryos could be normal and unrelated to contaminants. This possibility is very important to consider since the normal presence of persistent ovarian cortical tissue in testes has been observed in several avian species at hatching.

14. It is critical to determine whether ovotestes are normally present at hatching in common terns before final conclusions can be made. In order to establish this, studies with common terns from a pristine site are currently underway.

Discussion:

Comparison of the relationships between contaminants and health of common terns at the Bird Island and Nauset sites provided several interesting results. Of the chemicals measured here, the major contaminants in both Bird Island and Nauset terns were PCBs, while levels of organochlorine pesticides were lower than in many Great Lakes sites, and

mercury as measured in embryo muscle tissue was below levels known to be of toxicological significance. Tern embryo yolk sacs and tern eggs from Bird Island had PCB levels ranging from those near low values at Nauset to levels greater than those observed in terns from any Great Lakes sites. This shows that contaminant levels vary widely in Bird Island terns, and thus the risk of contaminant related effects may vary widely, depending on contaminant levels of prey in feeding grounds. Results from Principal Component Analysis (PCA) on PCB isomer patterns at Bird Island and Nauset clearly showed different isomer patterns at the two sites, with Bird Island terns having relatively more lower-chlorinated PCBs. The Bird Island embryo with the highest PCB levels observed also showed much greater relative levels of 4-chlorinated PCBs (31%) than the average among all samples (8%). This may indicate that the breeding female had been utilizing feeding grounds within New Bedford Harbor, resulting in the extremely elevated PCB levels with unusually high relative amounts of lower chlorinated PCBs. Also of importance, terns at Nauset did not have consistently low contaminant levels. Although most birds showed fairly low PCB levels, several Nauset birds had PCB levels typical of Bird Island terns, suggesting that Nauset terns had been feeding at a more contaminated area prior to arriving at their nesting grounds. Thus, although average contaminant levels at Nauset are lower than at Bird Island, Nauset is not a satisfactory control site.

Measures of exposure to AhR agonists revealed several relationships. The chick embryo hepatocyte (CEH) bioassay-derived TCDD-EQs were highly correlated with total PCBs, with PCB 126 likely being the major contributor (over 80%) to the TCDD-EQs for both chickens and terns. CEH bioassay-derived TCDD-EQs were more sensitive than tern hepatic EROD as an indicator of exposure to AhR agonists. This may be expected since tern hepatocytes showed 80-fold lower sensitivity to EROD induction by 2,3,7,8-TCDD than chicken hepatocytes (Lorenzen, Shutt et al. 1997). There appeared to be a threshold level of 82.5 ± 26.2 ng/g lipid TCDD-EQs above which tern hepatic EROD activities were

induced, and below which activities were all within a similar range. In contrast, TCDD-EQ concentrations down to 3.5 ng/g lipid were determined in the CEH bioassay.

The necropsy data revealed few differences in the overall health of the common tern populations at Bird Island and Nauset. Two female tern embryos with crossed beaks were found, one at Bird Island and one at Nauset. Although it has been suggested that crossed beaks are related to exposure to PCBs, and particularly TCDD-EQs (Ludwig, Kurita-Matsuba et al. 1996), the results here do not support that hypothesis since the terns with crossed beaks had relatively low levels of PCBs, TCDD-EQs and other contaminants. Lower spleen weights and relative spleen weights at Bird Island compared to Nauset were the only significant differences in necropsy data. This may be interesting to pursue further since it could be related to suppression of the immune system by high levels of PCBs/TCDD-EQs, which were elevated at Bird Island compared to Nauset.

Results from these studies did not clearly elucidate the relationship between contaminants and the presence of ovotestes in male common terns, but rather raised several possibilities. These include the possibility that 1) occurence of ovotestes is related to contaminants, either those measured here or others; 2) there is a normal background incidence of ovotestes which is increased by contaminants; or 3) the presence of ovotestes at hatching in male common terns is completely normal and unrelated to contaminants.

There is some support for the idea that the occurence of ovotestes could be related to contaminants. Although there is no clear dose-response relationship between contaminant levels and severity of ovotestes, there appears to be a threshold level of total PCBs (100 ug/g lipid), TCDD-EQs (30 ng/g lipid), and EROD activity (25 pmol/min/mg) above which ovotestes incidence is likely to occur. Below this level, there is no apparent relationship between the presence of ovotestes and contaminant levels or EROD activities. Furthermore, PCA analysis revealed no relationship between PCB isomer patterns and ovotestes presence. Although there was no clear dose-response relationship, nor any

relationship between PCB congener patterns and the presence of ovotestes it is possible that other factors influence whether ovotestes develop below the threshold levels. For example, the timing of exposure as well as the sensitivity of individuals may play a role in determining whether ovotestes develop. Furthermore, a metabolite of a contaminant measured here could be involved, in which case correlation to the parent compound may not be clear.

These results could also support the possibility that ovotestes presence is normal at a certain background level, but that this incidence is increased upon exposure to contaminants. As just described, while there is no clear dose-response relationship between any contaminants and severity of ovotestes, there does appear to be a threshold level response for PCBs, TCDD-EQs and EROD activity above which the presence of ovotestes is more likely to occur. The more extensive set of gonad histology and EROD activity data suggests a background incidence of ovotestes. The incidence of ovotestes is 78% at Bird Island and 60% at Nauset, but after removing samples with an EROD activity above 25 pmol/min/mg, the ovotestes incidence becomes closer at the two sites: 56% at Bird Island and 60% at Nauset. Thus, a background level of ovotestes presence could be 55-60%, and this incidence may be increased by high levels of contaminants. However, as discussed, Nauset is not a "clean" site and therefore, gonad histology in terns from an uncontaminanted site needs to be examined before a background incidence of ovotestes can be assessed.

The lack of an obvious dose -response relationship between ovotestes development and contaminants also could occur if the development of ovotestes was related to a contaminant not measured here. In a recent study of New Bedford Harbor sediment contaminants, compounds including bis(2-ethylhexyl) phthalate and pyrazole were measured in addition to PCBs and PAHs (Ho, McKinney et al. 1997). Although, the phthalate ester found in New Bedford Harbor sediments was not found to be estrogenic

(Harris, Henttu et al. 1997), some phthalate esters have been shown to be estrogenic *in vitro*, with potencies 1 x 10^6 times less than estradiol or lower (Harris, Henttu et al. 1997). Possibly, other compounds such as alkyl phenols may be present at both Nauset and Bird Island feeding grounds and may lead to development of ovotestes in common tern embryos.

If the presence of ovotestes is either elevated by or completely related to contaminants, at least two mechanisms of contaminant action are possible. One mechanism is that contaminants could alter hormonal control of gonadal differentiation. This could occur at many levels, including mimicking of steroid hormones. Although no conclusions can be made without further studies, estrogenic bioassays suggested common tern yolk sac extracts may be able to displace estradiol from binding sites. A second mechanism is that contaminants could affect the incidence of ovotestes by slowing embryonic development and prolonging the normal developmental stage in which testes of male birds may posesses ovarian cortical-like areas. Contaminants, including PCBs and TCDD-EQs, have been shown to lengthen the incubation period, suggesting that development is slowed when contaminant levels are elevated (Kubiak, Harris et al. 1989; Murk, Boudewijn et al. 1996).

In addition, if contaminants are related to ovotestes development, roseate terns may be more sensitive, and thus, more greatly affected. They may be more sensitive to additional effects, such as behavioral alterations or other hormonally controlled processes, that might occur along with ovotestes development. A higher sensitivity of roseate terns to contaminant related effects might explain why abnormal reproductive behavior is observed in roseate terns but not often in common terns.

In addition to the possibility that ovotestes are related to contaminants, another possible explanation of ovotestes also is supported: that the presence of ovotestes in common tern embryos is normal. During differentiation of the gonad into a testis, there is a time period where a potential ovarian cortex exists; this tissue may show areas of ovarian

cortical development and contain primordial germ cells. The presence of this cortical area during development of the testes was first observed in the chicken in 1886 (Laulanie 1886), and also observed in a species of tern in 1892 (as cited in Swift 1916). In chickens and many other species this cortical area normally has regressed completely by the time of hatching (Romanoff 1960); however other birds including quail, doves, pigeons, ducks, and hawks are known to have late persisting areas of ovarian-like cortical tissue that still may be present at hatching (Stanley 1937; Riddle and Dunham 1942; Lahr and Riddle 1945; Lewis 1946; Romanoff 1960; Haffen, Scheib et al. 1975). These testicular cortical areas have been observed to contain primordial germ cells developing in the same manner as ovarian primordial germ cells of the same age (Haffen, Scheib et al. 1975). Observations of the normal presence of persistent cortical tissue in the testes of many types of birds suggest that concern about feminizing effects of contaminants on bird populations as measured by ovotestes development in hatching birds may be exaggerated. Furthermore, it is necessary to clearly establish whether persistent testicular cortical areas are common in the bird species of concern. Before strong conclusions can be made about terns from Bird Island, this must be established.

In addition to the historical incidences of ovotesticular tissue normally present at hatching, several results from this study support the idea that ovotestes presence may be unrelated to contaminants. The percentage of ovotestes in Nauset terns (60%) was high and not significantly different from that in Bird Island terns (78%), even though levels of PCBs, TCDD-EQs, and EROD activities in Nauset terns were significantly lower than at Bird Island. Furthermore, examination of the PCB congener patterns in common tern embryo yolk sacs by PCA determined that isomer patterns of terns without ovotestes completely overlapped those of terns with ovotestes. Levels of other contaminants including pesticides and mercury were likely too low at either Bird Island or Nauset to be of any toxicological significance. In addition, the persistence of ovotestes was examined in

common tern prefledglings from Bird Island, and no tissue that appeared ovarian was found. In other species where ovotestes presence at hatching is normal, the same pattern of a high percentage of birds with ovotestes presence at hatching followed by complete disappearance of ovotestes within a few days to weeks posthatch also is observed. In doves, 76% of birds possessed ovotestes at hatch, which normally disappeared by 4 days posthatch, and rarely persisted past 2 weeks posthatch (Riddle and Dunham 1942). In addition, the presence of oviducts was not observed in any of the feminized common tern embryos or prefledglings. Hormonal injection studies have indicated that oviduct development is usually observed along with intermediate development of ovotestes (Willier, Gallagher et al. 1937; Pincus and Erickson 1962), and thus we may have expected to see some oviduct development. While some tern embryos possessed extensive cortical areas, it certainly is possible that this could be normal development; extensive cortical development has been observed normally in some bird species, particularly hawks (Stanley 1937).

Future Studies:

Before conclusions can be made concerning the relationship of contaminants to ovotestes, it is critical to establish whether the presence of ovotestes at hatching in common terns is normal. This will be accomplished by examining common tern embryo gonads from a clean site in New Brunswick, Canada that consistently has shown low contaminant levels, including total PCB levels of less than 1.5 ppm wet weight in eggs and embryo yolk sacs (compared to a range of 2.0 - 44.5 ppm wet wet in Nauset tern embryo yolk sacs). If embryos from this site have a high percentage of ovotestes presence at hatching, it will strongly suggest that this may be normal in common terns. Conversely, if no ovotestes are found in these embryos it will suggest that their presence at hatching is not normal. Studies

at several other Canadian sites with varying contaminant levels and types of contamination also will help to answer this question. These studies are underway. In addition, sampling common tern embryos at different developmental stages prior to hatching and posthatching may help clarify normal testicular development in terns.

Whether or not ovotestes are normal, the highly elevated contaminant levels in many common terns and the abnormal reproductive behavior in roseate terns suggest that it may be of interest to continue examining the health of these terns in relation to contaminant exposure on an individual basis. Alteration of hormonal systems as well as suppression of immune systems may be important, as indicated by differences in spleen weights between Bird Island and Nauset colonies. Histological examination of spleens, thymus, and thyroid may be revealing. Hormone levels were determined for all 1994 common tern embryos collected, and will be examined. In addition, blood from prefledglings may be used to look for the presence of vitellogenin or zona radiata (eggshell) proteins as indicators of exposure to estrogenic compounds in prefledglings.

On a mechanistic level, the types of contaminants and their activities need to be further examined. The estrogenic bioassay utilized here did not give clear results, but did suggest that compounds in the extracts may be able to compete with estrogen for binding sites. Continuing this study with improved estrogen receptor, either purified or isolated from an MCF7 human breast cancer cell line may provide intriguing results. Similarly, bioassays may be used to determine other activities of the common tern extracts, such as their ability to act as anti-androgens, androgens, or thyroid hormones. *In vitro* bioassays for vitellogenin induction in chick embryo hepatocytes also may be utilized. In addition, the affects of metabolites might be addressed by incubating pure compounds or extracts with tern hepatic microsomes and then using the resulting metabolites in bioassays. Injection studies using tern eggs and either pure compounds, metabolites, or extracts also may be used to study effects.

Multiple common tern sites with varying contaminant compositions and levels will also be compared. Common tern embryos have been collected from five sites in Canada that range from very clean to highly contaminated. It will be interesting to compare various aspects of embryo health and contaminant types among these sites and Bird Island and Nauset samples.

Finally, because roseate terns show much greater population declines and abnormal reproductive behavior than common terns, it may be important to examine roseate terns that die of natural causes.

In summary, this thesis examines the relationship between contaminants and the presence of ovotestes, as well as other health related effects, in Bird Island and Nauset common terns. Before conclusions about the relationship of contaminants to the presence of ovotestes can be determined, examination of tern embryos from an uncontaminated site is necessary. This work is underway. The highly elevated contaminant levels and unusual PCB patterns in some Bird Island terns, as well as the abnormal reproductive behavior in roseate terns, suggest that further studies should be pursued.

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APPENDICES

Appendix A: Collection, necropsy and biochemistry data for pipping common tern embryos collected in 1994 from Bird Island and Nauset

Appendix B: Levels of Contaminants measured inthe yolk sacs of pipping common tern embryos collected in 1994 from Bird Island and Nauset

Appendix C: Contaminant concentrations measured in common tern eggs collected from Bird Island in 1995

Appendix A. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection site	Dissection date	Developmental stage	Sex	Gonadal score	Egg mass(g)	Egg length (mm)	Egg Width (mm)	Body mass(g)	Yolk mass(g)
94-301	Bird Island, MA	15-Jun-94	pipping	m	N.A.[1]	16.45	N.A.	N.A.	14.14	1.06
94-302	Bird Island, MA	15-Jun-94	pipping, seal open	f	N.A.	15.73	41.7	30.29	N.A.	0.66
94-303	Bird Island, MA	15-Jun-94	pipping, seal open	f	N.A.	16.94	41.6	30	14.14	1.8
94-304	Bird Island, MA	15-Jun-94	pipping	f	N.A.	16.85	42.52	29.87	14.63	1.28
94-305	Bird Island, MA	15-Jun-94	pipping	m	2	17.05	43.38	30	14.18	1.36
94-306	Bird Island, MA	15-Jun-94	pipping, yolk sac extended	m	3	18.33	40.78	31.59	14.92	2.05
94-307	Bird Island, MA	15-Jun-94	starred	f	N.A.	N.A.	N.A.	N.A.	14.91	1.37
94-308	Bird Island, MA	15-Jun-94	starred	m	3	16.83	41.62	30.55	14.02	1.62
94-309	Bird Island, MA	15-Jun-94	starred	f	N.A.	16.61	41.41	30.34	14.37	1.3
94-310	Bird Island, MA	15-Jun-94	starred	m	2	17.5	40.36	30.2	14.98	1.63
94-311	Bird Island, MA	15-Jun-94	pipping	m	2	17.51	40.6	30.84	14.94	1.27
94-312	Bird Island, MA	15-Jun-94	pipping (very small hole)	f	N.A.	14.95	40.77	28.61	12.31	1.84
94-313	Bird Island, MA	15-Jun-94	pipping	f	N.A.	19.77	43.57	32.17	16.61	1.73
94-314	Bird Island, MA	15-Jun-94	pipping	f	N.A.	17.96	43.93	30.71	14.81	2.57
94-315	Bird Island, MA	15-Jun-94	pipping	m	4	17.83	41.52	31.3	15.62	1.39
94-316	Bird Island, MA	15-Jun-94	pipping (very small hole)	f	N.A.	14	40.99	28.24	11.7	0.59
94-317	Bird Island, MA	15-Jun-94	pipping	f	N.A.	15.31	43.5	30.1	13.6	0.75
94-318	Bird Island, MA	15-Jun-94	pipping	m	N.A.	16.76	42.64	30.73	14.86	1.07
94-319	Bird Island, MA	15-Jun-94	pipping	m	1	15.97	40.95	30.23	13.74	1.27
94-320	Bird Island, MA	15-Jun-94	pipping	f	N.A.	17.02	42.99	30.11	14.29	1.62
94-321	Bird Island, MA	16-Jun-94	pipping	f	N.A.	17.55	41.98	30.55	14.88	1.71
94-322	Bird Island, MA	16-Jun-94	pipping	m	N.A.	16.73	42.51	29.94	14.64	0.98
94-323	Bird Island, MA	16-Jun-94	pipping	f	N.A.	16.9	42.57	30.62	14.47	0.86
94-324	Bird Island, MA	16-Jun-94	pipping	m	2	17.44	43.06	29.93	15.06	1.55
94-325	Bird Island, MA	16-Jun-94	pipping	f	N.A.	14.79	42.1	28.88	12.63	0.67
94-326	Bird Island, MA	16-Jun-94	pipping	f	N.A.	15.52	41.35	30.14	13.4	0.98
94-327	Bird Island, MA	16-Jun-94	pipping	f	N.A.	16.46	41.35	29.49	13.97	1.35
94-328	Bird Island, MA	16-Jun-94	pipping	f	N.A.	14.85	39.04	28.46	12.45	2.02
94-329	Bird Island, MA	16-Jun-94	pipping	m	3	17.58	42.48	30.84	15.18	1.57
94-330	Bird Island, MA	16-Jun-94	N.A.	f	N.A.	17.91	43.37	30.88	15.09	0.86

[1] N.A. - not available

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Yolk-free body mass (g)	Liver mass(g)	Liver frozen for usomes (g)	Liver fixed (g)	Liver frozen for chem. (g)	Spleen mass(g)	Upper beak length(mm)	Lower beak length(mm)	Fluore-escence
94-301	Bird Island, MA	13.08	0.36	0.24	0.12	N.A.	5.11	12.01	12.01	none
94-302	Bird Island, MA	N.A.	0.23	0.15	0.08	N.A.	6.49	10.67	10.2	none
94-303	Bird Island, MA	12.34	0.33	0.25	0.08	N.A.	8.01	10.43	10.43	none
94-304	Bird Island, MA	13.35	0.37	0.22	0.15	N.A.	4.01	9.46	9.46	none
94-305	Bird Island, MA	12.82	0.32	0.16	0.16	N.A.	5.86	10.49	10.49	none
94-306	Bird Island, MA	12.87	0.23	0.15	0.08	N.A.	3.35	9.6	9.6	none
94-307	Bird Island, MA	13.54	0.35	0.20	0.15	N.A.	5.23	10.11	10.11	none
94-308	Bird Island, MA	12.4	0.4	0.30	0.10	N.A.	9.69	9.26	9.26	none
94-309	Bird Island, MA	13.07	0.35	0.20	0.15	N.A.	5.9	9.13	9.13	none
94-310	Bird Island, MA	13.35	0.37	0.21	0.16	N.A.	5.85	9.18	9.18	none
94-311	Bird Island, MA	13.67	0.38	0.28	0.10	N.A.	4.84	N.A.	N.A.	none
94-312	Bird Island, MA	10.47	0.21	0.13	0.08	N.A.	5.7	8.97	8.97	none
94-313	Bird Island, MA	14.88	0.46	0.27	0.19	N.A.	5.47	10.81	9.69	none
94-314	Bird Island, MA	12.24	0.27	0.18	0.09	N.A.	4.8	9.5	9.5	N.A.
94-315	Bird Island, MA	14.23	0.35	0.25	0.10	N.A.	6.74	10.77	10.77	none
94-316	Bird Island, MA	11.11	0.26	0.17	0.09	N.A.	4.37	9.57	9.57	none
94-317	Bird Island, MA	12.85	0.37	0.14	0.23	N.A.	5.9	8.97	8.4	none
94-318	Bird Island, MA	13.79	0.42	0.24	0.18	N.A.	5.56	10.17	10.17	none
94-319	Bird Island, MA	12.47	0.3	0.22	0.08	N.A.	8.82	9.78	8.96	N.A.
94-320	Bird Island, MA	12.67	0.34	0.19	0.15	N.A.	4.18	9.35	9.35	none
94-321	Bird Island, MA	13.17	0.35	0.20	0.15	N.A.	4.3	10.09	10.09	none
94-322	Bird Island, MA	13.66	0.34	0.20	0.14	N.A.	N.A.	10.34	10.34	none
94-323	Bird Island, MA	13.61	0.41	0.24	0.17	N.A.	6.02	9.87	9.87	N.A.
94-324	Bird Island, MA	13.51	0.31	0.21	0.10	N.A.	5.9	9.53	9.53	none
94-325	Bird Island, MA	11.96	0.35	0.21	0.14	N.A.	5.14	9.19	9.19	none
94-326	Bird Island, MA	12.42	0.31	0.22	0.09	N.A.	4.38	9.61	9.61	none
94-327	Bird Island, MA	12.62	0.4	0.24	0.16	N.A.	3.52	9.45	see notes	none
94-328	Bird Island, MA	10.43	0.34	0.15	0.19	N.A.	4.29	8.46	8.46	none
94-329	Bird Island, MA	13.61	0.35	0.22	0.13	N.A.	4.89	10.25	10.25	none
94-330	Bird Island, MA	14.23	0.44	0.28	0.16	N.A.	4.57	9.98	9.98	none

[1] N.A. - not available

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection site	Microsomal protein conc. mg/ml	Microsomal yield mg prot./g	EROD pmol/min/mg	PROD	Comments
94-301	Bird Island, MA	10.44	21.75	14.76	1.44	left eye orbit slightly depressed
94-302	Bird Island, MA	8.30	27.67	7.22	0.79	left eye orbit slightly behind right
94-303	Bird Island, MA	9.53	19.07	29.01	2.29	
94-304	Bird Island, MA	7.25	16.48	19.49	1.65	
94-305	Bird Island, MA	8.18	25.58	15.99	1.80	
94-306	Bird Island, MA	3.95	13.17	20.26	2.13	
94-307	Bird Island, MA	6.90	17.26	12.07	1.42	
94-308	Bird Island, MA	11.11	18.51	16.08	1.39	
94-309	Bird Island, MA	5.44	13.60	16.51	2.08	
94-310	Bird Island, MA	5.46	26.02	14.22	0.99	
94-311	Bird Island, MA	5.23	18.68	86.16	2.99	
94-312	Bird Island, MA	1.71	13.12	44.50	5.16	
94-313	Bird Island, MA	3.64	13.47	31.56	2.75	
94-314	Bird Island, MA	2.40	13.32	21.86	2.92	
94-315	Bird Island, MA	2.51	10.03	17.01	2.53	no rightt or left oviduct
94-316	Bird Island, MA	2.07	12.18	11.72	1.77	
94-317	Bird Island, MA	2.23	15.91	10.83	1.82	
94-318	Bird Island, MA	3.33	13.87	14.42	1.51	
94-319	Bird Island, MA	4.50	20.46	13.08	1.75	right testes and mesonepheros displaced
94-320	Bird Island, MA	3.01	15.85	16.90	2.44	
94-321	Bird Island, MA	2.64	13.21	9.49	2.13	
94-322	Bird Island, MA	2.63	13.15	9.09	2.29	
94-323	Bird Island, MA	5.02	20.91	11.75	1.80	
94-324	Bird Island, MA	2.68	12.77	19.69	2.72	
94-325	Bird Island, MA	3.60	17.16	17.21	2.17	
94-326	Bird Island, MA	3.48	15.81	11.38	2.39	
94-327	Bird Island, MA	6.68	13.91	9.80	0.78	cross beak, pigtail right ovary, left orbit recessed
94-328	Bird Island, MA	1.54	10.26	9.76	1.84	slight pericardial and neck edema,slt. beak turn
94-329	Bird Island, MA	4.42	20.11	13.56	1.32	
94-330	Bird Island, MA	5.52	19.73	11.79	1.28	

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Dissection Date	Developmental stage	Sex	Gonadal score	Egg mass(g)	Egg Length (mm)	Egg Width (mm)	Body mass(g)	Yolk mass(g)
94-331	Bird Island, MA	16-Jun-94	pipping	m	N.A.	13.29	38.22	27.52	10.81	1.4
94-332	Nauset, MA	7-Jul-94	hatched	f	N.A.	1.59 shell	N.A.	N.A.	12.76	0.4
94-333	Nauset, MA	7-Jul-94	hatched	f	N.A.	N.A.	N.A.	N.A.	12.82	0.63
94-334	Nauset, MA	7-Jul-94	hatched	m	1	2.06 shell	N.A.	N.A.	13.64	0.71
94-335	Nauset, MA	7-Jul-94	pipped	f	N.A.	18.17	43	30.9	15.96	1.54
94-336	Nauset, MA	7-Jul-94	dead	dead	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
94-337	Nauset, MA	7-Jul-94	hatching	f	N.A.	19.08	45.6	30.84	16.87	1.84
94-338	Nauset, MA	7-Jul-94	hatching	m	3	15.6	40.98	30.34	13.23	1.47
94-339	Nauset, MA	7-Jul-94	large pip	f	N.A.	17.39	43.24	30.46	14.94	1.3
94-340	Nauset, MA	7-Jul-94	large pip	f	N.A.	15.94	41.64	29.86	13.87	0.73
94-341	Nauset, MA	7-Jul-94	dead	dead	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
94-342	Nauset, MA	7-Jul-94	small pip	f	N.A.	15.04	40.26	29.5	12.89	0.96
94-343	Nauset, MA	7-Jul-94	small pip	m	3	16.54	39.28	30.68	14.27	1.91
94-344	Nauset, MA	8-Jul-94	hatching	unknown	N.A.	18.36	47.33	29.21	16.26	1.81
94-345	Nauset, MA	8-Jul-94	large pip	m	4	14.45	38.8	29.02	12.65	1.01
94-346	Nauset, MA	8-Jul-94	large pip	m	3	14.92	42.22	28.74	12.72	0.53
94-347	Nauset, MA	8-Jul-94	large pip	f	N.A.	15.99	44.35	29.25	14	0.97
94-348	Nauset, MA	8-Jul-94	large pip>hatching	m	1	17.67	43.87	31.41	15.41	1.46
94-349	Nauset, MA	8-Jul-94	large pip	m	3	13.93	40.04	28.74	11.91	0.88
94-350	Nauset, MA	8-Jul-94	large pip	f	N.A.	17.91	42.53	31.76	15.76	1.04
94-351	Nauset, MA	8-Jul-94	large pip	f	N.A.	14.85	40.94	28.51	12.84	0.74
94-352	Nauset, MA	8-Jul-94	large pip	f	N.A.	16.94	42.63	29.91	14.23	1.41
94-353	Nauset, MA	8-Jul-94	large pip	m	1	13.87	40.99	28.15	11.85	0.9
94-354	Nauset, MA	8-Jul-94	large pip	f	N.A.	16.13	41.45	29.47	13.58	1.63
94-355	Nauset, MA	8-Jul-94	large pip	f	N.A.	20.7	49.17	30.34	17.24	1.59
94-356	Nauset, MA	8-Jul-94	large pip	f	N.A.	16.23	41.77	29.31	14	1.18
94-357	Nauset, MA	8-Jul-94	large pip	m	1	16.95	42.13	29.86	14.85	1.24
94-358	Nauset, MA	8-Jul-94	large pip	m	3	19.96	42.3	31.73	16.41	2.7
94-359	Nauset, MA	8-Jul-94	large pip	unknown	N.A.	16.36	40.15	30.15	13.89	1.36
94-360	Nauset, MA	8-Jul-94	small pip	f	N.A.	17.81	43.88	30.64	15.3	1.47

[1] N.A. - not available

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Yolk-free body mass (g)	Liver mass(g)	Liver frozen for usomes (g)	Liver fixed (g)	Liver frozen for chem. (g)	Spleen mass(g)	Upper beak length(mm)	Lower beak length(mm)	Fluore-escence
94-331	Bird Island, MA	9.41	0.2	0.11	0.09	N.A.	2.8	9.06	9.06	none
94-332	Nauset, MA	12.36	0.33	0.22	0.11	N.A.	8.66	8.35	N.A.	none
94-333	Nauset, MA	12.19	0.28	0.13	0.10	0.05	7.75	8.34	N.A.	none
94-334	Nauset, MA	12.93	0.33	0.12	0.08	0.13	5.06	9.37	N.A.	none
94-335	Nauset, MA	14.42	0.38	0.16	0.12	0.10	9.56	11.5	N.A.	none
94-336	Nauset, MA	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
94-337	Nauset, MA	15.03	0.47	0.12	0.16	0.19	7.88	10.8	see notes	none
94-338	Nauset, MA	11.76	0.28	0.11	0.09	0.08	9.21	10	N.A.	none
94-339	Nauset, MA	13.64	0.37	0.10	0.12	0.15	5.5	10.75	N.A.	none
94-340	Nauset, MA	13.14	0.32	0.12	0.11	0.09	4.21	9.5	N.A.	none
94-341	Nauset, MA	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
94-342	Nauset, MA	11.93	0.41	0.13	0.13	0.15	4.42	10.42	N.A.	none
94-343	Nauset, MA	12.36	0.28	0.08	0.11	0.09	5.2	10.04	N.A.	none
94-344	Nauset, MA	14.45	0.39	0.30	0.09	N.A.	6.03*	10.14	N.A.	none
94-345	Nauset, MA	11.64	0.33	0.21	0.12	N.A.	4.29	9.61	N.A.	none
94-346	Nauset, MA	12.19	0.28	0.16	0.12	N.A.	5.42	10.7	N.A.	none
94-347	Nauset, MA	13.03	0.4	0.25	0.15	N.A.	9.3	10.51	N.A.	none
94-348	Nauset, MA	13.95	0.38	0.25	0.13	N.A.	7.04	9.96	N.A.	none
94-349	Nauset, MA	11.03	0.31	0.18	0.13	N.A.	4.59	10.01	N.A.	none
94-350	Nauset, MA	14.72	0.39	0.26	0.13	N.A.	9.57	10.04	N.A.	none
94-351	Nauset, MA	12.1	0.34	0.20	0.14	N.A.	7.82	9.44	N.A.	none
94-352	Nauset, MA	12.82	0.37	0.21	0.16	N.A.	4.17	10.21	N.A.	none
94-353	Nauset, MA	10.95	0.33	0.24	0.09	N.A.	13.47	9.99	N.A.	none
94-354	Nauset, MA	11.95	0.28	0.19	0.09	N.A.	6.61	10.1	N.A.	none
94-355	Nauset, MA	15.65	0.43	0.26	0.17	N.A.	6.43	10.64	N.A.	none
94-356	Nauset, MA	12.82	0.28	0.20	0.08	N.A.	4.98	9.63	N.A.	none
94-357	Nauset, MA	13.61	0.36	0.20	0.16	N.A.	5.94	10.32	N.A.	none
94-358	Nauset, MA	13.71	0.3	0.19	0.11	N.A.	3.23	10.21	N.A.	none
94-359	Nauset, MA	12.53	0.32	0.19	0.13	N.A.	4.28	9.77	N.A.	none
94-360	Nauset, MA	13.83	0.42	0.28	0.14	N.A.	7.74	10.54	N.A.	none

[1] N.A. - not available

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Microsomal protein conc. mg/ml	Microsomal yield mg prot./g	EROD pmol/min/mg	PROD	Comments
94-331	Bird Island, MA	1.18	10.69	50.51	5.70	right gonad missing, left gonad bilobed or sectioned
94-332	Nauset, MA	3.26	14.81	8.55	1.30	
94-333	Nauset, MA	1.96	15.11	9.17	1.55	
94-334	Nauset, MA	1.09	9.05	11.61	2.66	
94-335	Nauset, MA	1.38	8.63	16.52	2.66	
94-336	Nauset, MA	N.A.	N.A.	N.A.	N.A.	
94-337	Nauset, MA	1.51	12.55	10.38	1.56	upper mandible twisted to left,low mandible straight
94-338	Nauset, MA	1.08	9.82	0.54	1.12	
94-339	Nauset, MA	0.90	9.04	7.71	2.69	
94-340	Nauset, MA	1.37	11.44	15.73	2.53	
94-341	Nauset, MA	N.A.	N.A.	N.A.	N.A.	
94-342	Nauset, MA	1.65	12.72	12.81	3.41	
94-343	Nauset, MA	1.34	16.76	12.64	2.94	
94-344	Nauset, MA	4.75	15.82	6.95	1.19	spleen only 1/2-1/3 whole
94-345	Nauset, MA	3.50	16.67	13.03	2.11	
94-346	Nauset, MA	2.39	14.94	4.84	1.33	
94-347	Nauset, MA	3.65	14.62	9.17	1.87	
94-348	Nauset, MA	3.83	15.32	12.73	1.82	
94-349	Nauset, MA	2.75	15.27	12.45	2.57	
94-350	Nauset, MA	5.05	19.42	10.90	1.91	
94-351	Nauset, MA	3.11	15.55	12.51	1.94	
94-352	Nauset, MA	3.12	14.85	9.09	2.00	
94-353	Nauset, MA	4.57	19.04	10.22	1.83	
94-354	Nauset, MA	2.79	14.70	8.49	1.62	
94-355	Nauset, MA	4.00	15.39	9.82	1.88	
94-356	Nauset, MA	2.89	14.44	7.40	1.50	
94-357	Nauset, MA	3.08	15.42	18.11	2.58	
94-358	Nauset, MA	2.47	13.02	10.25	2.01	
94-359	Nauset, MA	2.38	12.55	13.82	2.23	
94-360	Nauset, MA	3.34	11.93	12.55	1.97	

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Dissection Date	Developmental stage	Sex	Gonadal score	Egg mass(g)	Egg Length (mm)	Egg Width (mm)	Body mass(g)	Yolk mass(g)
94-361	Nauset, MA	8-Jul-94	small pip	f	N.A.	16.22	40.76	29.98	13.78	0.88
94-362	Nauset, MA	8-Jul-94	starred	f	N.A.	18.36	43.61	30.77	15.6	1.24
94-363	Nauset, MA	8-Jul-94	starred	unknown	N.A.	15.55	40.15	30.01	13.08	0.88
94-364	Bird Island, MA	12-Jul-95	hatched	m	2	N.A.	N.A.	N.A.	15.37	1.09
94-365	Bird Island, MA	12-Jul-95	hatching	m	N.A.	14.96	N.A.	N.A.	13.11	0.71
94-366	Bird Island, MA	12-Jul-95	pipping	m	4	16.78	39.86	31	14.82	0.68
94-367	Bird Island, MA	12-Jul-95	pipping	m	2	14.52	40.2	28.66	12.51	0.68
94-368	Bird Island, MA	12-Jul-95	pipping	f	N.A.	13.74	39.92	27.84	12.25	0.63
94-369	Bird Island, MA	12-Jul-95	pipping	f	N.A.	17.97	41.91	31	15.62	1.18
94-370	Bird Island, MA	12-Jul-95	starred	m	1	16.92	41.66	30.01	14.62	1.06
94-371	Bird Island, MA	12-Jul-95	starred	f	N.A.	15.34	41.93	29.13	12.7	2.34
94-372	Bird Island, MA	13-Jul-95	hatched	m	1	N.A.	N.A.	N.A.	11.81	0.73
94-373	Bird Island, MA	13-Jul-95	hatched	f	N.A.	12.03	N.A.	N.A.	12.03	0.43
94-374	Bird Island, MA	13-Jul-95	hatching	f	N.A.	18.38	N.A.	N.A.	16.13	1.13
94-375	Ram Island, MA	13-Jul-95	pipping	f	N.A.	15.17	41.03	28.62	13.29	1.8
94-376	Bird Island, MA	15-Jul-95	hatched	m	3	N.A.	N.A.	N.A.	14.78	1.06
94-377	Bird Island, MA	15-Jul-95	pipping	m	3	16.24	42.41	30	14.16	0.92
94-378	Bird Island, MA	15-Jul-95	just pipped	m	3	13.8	40.4	37.5	11.46	0.81
94-379	Bird Island, MA	15-Jul-95	starred	m	3	16.56	41.4	29.4	13.82	1.54
94-380	Bird Island, MA	15-Jul-95	starred	m	3	17.13	39.8	29.8	15.89	2.42
94-381	Bird Island, MA	18-Jul-95	pipping	m	3	18.39	43.53	30.95	15.67	1.02
94-382	Bird Island, MA	18-Jul-95	pipping	f	N.A.	15.83	42.08	29.29	14.07	0.67
94-383	Bird Island, MA	18-Jul-95	pipping	f	N.A.	14.48	41.27	28.4	12.86	0.69
94-384	Bird Island, MA	18-Jul-95	pipping	m	3	16.73	41.58	29.84	27.11	1.6
94-385	Bird Island, MA	18-Jul-95	pipping	m	1	15.66	40.09	29.53	13.65	1.07
94-386	Ram Island, MA	21-Jul-95	hatched	m	N.A.	N.A.	N.A.	N.A.	15.3	1.04
94-387	Ram Island, MA	21-Jul-95	pipped	f	N.A.	14.22	N.A.	N.A.	12	0.95
94-388	Bird Island, MA	21-Jul-95	pipping	f	N.A.	N.A.	41.61	31.21	N.A.	2.41
94-389	Bird Island, MA	21-Jul-95	pipping	m	1	13.82	38.02	28.87	11.88	1.31
94-390	Bird Island, MA	21-Jul-95	just pipped	f	N.A.	16.26	43.11	29.17	13.69	1.95

[1] N.A. - not available

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Yolk-free body mass (g)	Liver mass(g)	Liver frozen for usomes (g)	Liver fixed (g)	Liver frozen for chem. (g)	Spleen mass(g)	Upper beak length(mm)	Lower beak length(mm)	Fluore-escence
94-361	Nauset, MA	12.9	0.28	0.15	0.13	N.A.	5.75	9.95	N.A.	none
94-362	Nauset, MA	14.36	0.41	0.25	0.16	N.A.	7.83	10.66	N.A.	none
94-363	Nauset, MA	12.2	0.31	0.21	0.10	N.A.	7.59	9.94	N.A.	none
94-364	Bird Island, MA	14.28	0.31	0.13	0.08	0.10	5.59	10.83	N.A.	none
94-365	Bird Island, MA	12.4	0.29	0.11	0.13	0.05	6.94	10.8	N.A.	none
94-366	Bird Island, MA	14.14	0.31	0.10	0.11	0.10	5.56	10.59	N.A.	none
94-367	Bird Island, MA	11.83	0.3	0.14	0.10	0.06	N.A.	10.39	N.A.	none
94-368	Bird Island, MA	11.62	0.35	0.09	0.15	0.11	3.51	10.23	N.A.	none
94-369	Bird Island, MA	14.44	0.33	0.12	0.10	0.11	6	9.98	N.A.	none
94-370	Bird Island, MA	13.56	0.33	0.18	0.15	N.A.	9.36	9.51	N.A.	none
94-371	Bird Island, MA	10.36	0.17	0.12	0.05	N.A.	2.28	9.1	N.A.	none
94-372	Bird Island, MA	11.08	0.34	0.22	0.12	N.A.	4.34	10.74	N.A.	none
94-373	Bird Island, MA	11.6	0.34	0.23	0.11	N.A.	6.06	10.65	N.A.	none
94-374	Bird Island, MA	15	0.43	0.28	0.15	N.A.	5.87*	10.43	N.A.	none
94-375	Ram Island, MA	11.49	0.23	0.14	0.09	N.A.	5.81	9.99	N.A.	none
94-376	Bird Island, MA	13.72	0.34	0.20	0.14	N.A.	5.36	10.23	N.A.	none
94-377	Bird Island, MA	13.24	0.33	0.20	0.13	N.A.	4.97	10.44	N.A.	none
94-378	Bird Island, MA	10.65	0.26	0.18	0.08	N.A.	6.07	9.64	N.A.	none
94-379	Bird Island, MA	12.28	0.25	0.20	0.05	N.A.	5.09	10.14	N.A.	none
94-380	Bird Island, MA	13.47	0.19	0.13	0.06	N.A.	3.8	9.94	N.A.	none
94-381	Bird Island, MA	0.4	0.41	0.25	0.16	N.A.	6.87	10.76	N.A.	none
94-382	Bird Island, MA	13.4	0.36	0.19	0.17	N.A.	5.92	10.24	N.A.	none
94-383	Bird Island, MA	12.17	0.36	0.36	0.00	N.A.	6.07	10.17	N.A.	none
94-384	Bird Island, MA	25.51	0.26	0.17	0.09	N.A.	5.76	9.99	N.A.	none
94-385	Bird Island, MA	12.58	0.37	0.19	0.18	N.A.	6.54	10.56	N.A.	none
94-386	Ram Island, MA	14.26	0.43	0.25	0.18	N.A.	5.09*	10.93	N.A.	none
94-387	Ram Island, MA	11.05	0.39	0.22	0.17	N.A.	6.98	9.21	N.A.	none
94-388	Bird Island, MA	N.A.	0.34	0.19	0.15	N.A.	4.54	9.82	N.A.	none
94-389	Bird Island, MA	10.57	0.24	0.14	0.10	N.A.	2.86	9.46	N.A.	none
94-390	Bird Island, MA	11.74	0.28	0.19	0.09	N.A.	3.32	10.01	N.A.	none

[1] N.A. - not available

Appendix A con't. Collection, necropsy and biochemistry data for pipping common tern embryos.

I.D.	Collection Site	Microsomal protein conc. mg/ml	Microsomal yield mg prot./g	EROD pmol/min/mg	PROD	Comments
94-361	Nauset, MA	2.29	15.29	15.47	3.04	
94-362	Nauset, MA	3.68	14.71	12.93	2.15	
94-363	Nauset, MA	3.95	18.80	15.35	2.76	
94-364	Bird Island, MA	2.33	17.94	15.51	2.91	
94-365	Bird Island, MA	1.83	16.67	16.53	3.15	
94-366	Bird Island, MA	1.53	15.32	15.85	3.40	
94-367	Bird Island, MA	2.20	15.73	9.23	2.06	no spleen found
94-368	Bird Island, MA	1.08	12.06	13.35	3.87	
94-369	Bird Island, MA	1.13	9.42	7.77	2.44	
94-370	Bird Island, MA	3.02	16.80	21.89	3.58	
94-371	Bird Island, MA	1.51	12.60	30.80	5.00	starred, yolk sac entirely exterior,some blood included
94-372	Bird Island, MA	2.59	11.79	12.93	1.94	
94-373	Bird Island, MA	4.19	18.22	23.80	2.10	
94-374	Bird Island, MA	4.17	14.90	13.13	1.43	partial spleen weight
94-375	Ram Island, MA	2.03	14.50	18.06	2.33	
94-376	Bird Island, MA	4.76	23.81	17.53	2.33	
94-377	Bird Island, MA	3.77	18.87	31.68	2.73	
94-378	Bird Island, MA	2.60	14.45	16.37	2.04	
94-379	Bird Island, MA	4.18	20.90	77.82	6.14	
94-380	Bird Island, MA	1.62	12.43	26.94	4.31	
94-381	Bird Island, MA	3.34	13.37	22.96	3.64	head fixed
94-382	Bird Island, MA	2.78	14.62	13.57	2.26	head fixed
94-383	Bird Island, MA	5.77	16.01	13.34	2.08	fixed head, no liver fixed
94-384	Bird Island, MA	2.19	12.88	44.06	5.84	head fixed
94-385	Bird Island, MA	3.88	20.44	14.99	2.07	head fixed
94-386	Ram Island, MA	3.92	15.68	10.43	1.37	tip of spleen broken off
94-387	Ram Island, MA	3.04	13.82	49.77	2.57	
94-388	Bird Island, MA	3.42	17.99	9.72	1.69	
94-389	Bird Island, MA	2.65	18.96	15.40	1.80	
94-390	Bird Island, MA	3.42	18.00	26.16	2.90	

Appendix B. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	Total PCBs	TCDD-Eqs ng/g	muscle hg ug/g d.w.	skin hg ug/g d.w.	p,p'-DDE	p,p'-DDD	p,p'-DDT	Total p,p'-DDT's	3-chlorine PCBs	4-chlorine PCBs
94-305	B.I. early	26.00	117.2255	35.5800	1.07	0.99	3.3069	0.0383	0.0562	3.4014	0.1990	6.2145
94-308	B.I. early	23.00	38.9418	15.9400	1.14	2.15	2.6648	0.0209	0.0452	2.7309	0.4088	3.4342
94-311	B.I. early	25.00	663.4348	174.0700	2.76	1.79	5.6145	0.3445	0.0800	6.0390	37.9355	206.9761
94-315	B.I. early	23.00	31.7567	8.3400	1.26	1.46	2.2824	0.0161	0.0321	2.3306	0.5930	2.7303
94-318	B.I. early	32.00	31.5645	13.9100	N.A.	N.A.	0.8573	0.0336	0.0145	0.9055	0.5567	2.2464
94-319	B.I. early	21.00	38.7123	10.4700	1.31	1.28	2.2008	0.0519	0.0362	2.2888	0.3838	2.7627
94-327	B.I. early	19.00	83.8581	29.7400	1.15	1.42	5.1867	0.0543	0.0362	5.2771	0.6019	4.8305
94-329	B.I. early	21.00	59.7034	18.8800	1.84	1.93	4.3843	0.0118	0.0279	4.4239	0.4046	2.6600
94-337	Nauset	19.00	14.3742	5.3100	1.29	1.12	2.1681	0.0439	0.0703	2.2823	0.0461	0.8158
94-338	Nauset	26.00	13.7317	4.6400	0.76	1.05	2.8783	0.0254	0.0620	2.9657	0.0511	0.7949
94-345	Nauset	25.00	178.4077	57.7800	1.11	1.44	4.4773	0.0364	0.0436	4.5573	0.1955	7.6132
94-348	Nauset	18.00	17.1736	7.4900	1.53	1.37	4.1968	0.0020	0.0304	4.2292	0.0696	1.0456
94-349	Nauset	34.00	10.2149	4.8100	0.83	0.76	2.1359	0.0459	0.0503	2.2321	0.0362	0.6183
94-353	Nauset	24.00	8.3833	4.8600	N.A.	N.A.	1.7352	0.0214	0.0243	1.7810	N.D.	N.D.
94-357	Nauset	19.00	31.5971	9.5700	0.83	0.91	6.7182	0.0327	0.0764	6.8273	0.0927	1.4732
94-358	Nauset	17.00	9.0061	4.4800	0.98	0.71	1.7049	0.0173	0.0511	1.7732	0.0511	0.6122
94-366	B.I. late	20.00	85.6854	30.4800	1.45	1.73	5.1762	0.0338	0.0269	5.2369	0.7169	6.4808
94-370	B.I. late	28.00	17.1613	7.0500	1.19	1.43	2.7507	0.0641	0.0162	2.8310	0.0741	1.0555
94-372	B.I. late	31.00	93.6150	28.7600	1.23	N.A.	6.7009	0.0786	0.0141	6.7936	0.7023	7.8205
94-381	B.I. late	28.00	79.7264	37.3200	1.90	2.71	2.1361	0.0668	0.0171	2.2200	0.9654	8.7750
94-384	B.I. late	19.00	244.6131	138.5700	1.75	1.80	4.2034	0.0572	0.0017	4.2624	4.8103	33.5014
94-389	B.I. late	34.00	18.2403	7.2600	0.97	1.98	2.5398	0.0365	0.0079	2.5842	0.1635	1.9544

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	5-chlorine PCBs	6-chlorine PCBs	7-chlorine PCBs	8-chlorine PCBs	9-chlorine PCBs	1-ortho PCBs	2-ortho PCBs	3-ortho PCBs	4-ortho PCBs	PCB 28
94-305	B.I. early	26.00	41.2979	51.4272	15.3255	2.4834	0.2779	26.7759	78.5397	11.2859	0.6241	0.1990
94-308	B.I. early	23.00	11.0145	17.0488	5.6473	1.1345	0.2536	7.2721	27.0779	4.4455	0.1464	0.1894
94-311	B.I. early	25.00	241.3168	143.7632	29.2665	3.7865	0.3903	190.6155	422.6465	49.0258	1.1471	17.2342
94-315	B.I. early	23.00	10.1897	13.7345	3.6239	0.7315	0.1536	7.4645	21.3839	2.7770	0.1312	0.2688
94-318	B.I. early	32.00	9.9700	14.2042	3.8682	0.6400	0.0791	7.4070	21.3152	2.7070	0.1355	0.2685
94-319	B.I. early	21.00	12.0292	17.7965	4.7062	0.8842	0.1496	8.4262	26.3088	3.8150	0.1623	0.1673
94-327	B.I. early	19.00	24.1595	39.9805	11.7552	2.2110	0.3195	17.1133	58.2014	8.1010	0.4424	0.2633
94-329	B.I. early	21.00	15.0029	27.4482	11.4650	2.3539	0.3689	11.0114	42.4032	5.9914	0.2975	0.1932
94-337	Nauset	19.00	3.9529	6.3126	2.5813	0.5629	0.1026	2.4426	9.5042	2.3768	0.0506	0.0461
94-338	Nauset	26.00	3.4123	6.1589	2.7080	0.5117	0.0949	2.3189	9.4223	1.9511	0.0394	0.0511
94-345	Nauset	25.00	62.8786	80.5155	23.6355	3.2514	0.3182	43.2423	120.9636	13.1486	1.0532	0.1955
94-348	Nauset	18.00	4.6652	7.3572	3.1972	0.7156	0.1232	3.1936	11.5400	2.3744	0.0656	0.0696
94-349	Nauset	34.00	2.7948	4.5269	1.8614	0.3152	0.0621	1.7776	6.8217	1.6155	N.D.	0.0362
94-353	Nauset	24.00	1.8771	4.2895	1.7290	0.3605	0.1271	1.0371	6.1243	1.2219	N.D.	N.D.
94-357	Nauset	19.00	8.0814	14.4727	6.2473	1.1027	0.1273	5.1809	22.2868	4.0082	0.1214	0.0927
94-358	Nauset	17.00	2.4859	3.9830	1.6035	0.2368	0.0338	1.7776	6.0786	1.1500	0.0000	0.0511
94-366	B.I. late	20.00	29.2762	37.3100	9.6862	1.8823	0.3331	19.4354	57.9246	7.9700	0.3554	0.7169
94-370	B.I. late	28.00	4.6017	7.6886	3.0279	0.5955	0.1179	3.2579	11.8293	2.0121	0.0621	0.0741
94-372	B.I. late	31.00	33.6373	40.6877	9.0364	1.5264	0.2045	24.8273	61.7782	6.5991	0.4105	0.7023
94-381	B.I. late	28.00	30.8729	30.4107	7.2500	1.2643	0.1882	22.1686	51.0489	6.1943	0.3146	0.9654
94-384	B.I. late	19.00	97.8883	85.6503	19.3607	3.0400	0.3621	70.5214	155.1669	18.0669	0.8579	4.8103
94-389	B.I. late	34.00	7.2207	6.7849	1.7149	0.3416	0.0602	4.5316	11.8714	1.7823	0.0549	0.1635

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	PCB 31	PCB 42	PCB 44	PCB 49	PCB 52	PCB 64	PCB 74	PCB 70	PCB66	PCB60
94-305	B.I. early	26.00	0.0145	0.0869	N.D.	0.4107	0.5531	0.0721	1.4655	0.3993	2.4745	0.7524
94-308	B.I. early	23.00	0.2194	0.1288	N.D.	0.4933	0.7136	0.0706	0.3755	0.4739	0.9000	0.2785
94-311	B.I. early	25.00	20.7013	13.0116	7.1065	41.8987	56.7019	6.1090	16.1303	16.2742	39.1387	10.6052
94-315	B.I. early	23.00	0.3242	N.D.	N.D.	0.6333	N.D.	0.0645	0.4485	0.4318	0.8330	0.3191
94-318	B.I. early	32.00	0.2882	N.D.	N.D.	0.4142	0.0994	0.0579	0.3958	0.3018	0.7130	0.2642
94-319	B.I. early	21.00	0.2165	N.D.	N.D.	0.4950	0.3535	0.0546	0.3777	0.3212	0.7835	0.3773
94-327	B.I. early	19.00	0.3386	N.D.	N.D.	0.8257	0.6076	0.0862	0.8057	0.5590	1.3862	0.5600
94-329	B.I. early	21.00	0.2114	N.D.	N.D.	0.4171	N.D.	0.0496	0.4964	0.4021	0.9404	0.3543
94-337	Nauset	19.00	N.D.	N.D.	N.D.	0.0610	0.1426	N.D.	0.1132	0.0610	0.2410	0.1971
94-338	Nauset	26.00	N.D.	N.D.	N.D.	0.0889	N.D.	0.0180	0.1229	0.1243	0.2471	0.1937
94-345	Nauset	25.00	N.D.	N.D.	N.D.	0.1245	N.D.	0.0573	2.3959	0.2341	3.8495	0.9518
94-348	Nauset	18.00	N.D.	N.D.	N.D.	0.0992	N.D.	0.0204	0.1660	0.1636	0.3188	0.2776
94-349	Nauset	34.00	N.D.	N.D.	N.D.	0.0597	N.D.	N.D.	0.0866	0.0976	0.1872	0.1872
94-353	Nauset	24.00	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
94-357	Nauset	19.00	N.D.	N.D.	N.D.	0.1445	N.D.	0.0323	0.2541	0.1682	0.4936	0.3805
94-358	Nauset	17.00	N.D.	N.D.	N.D.	0.0670	N.D.	0.0119	0.1005	0.1073	0.1881	0.1373
94-366	B.I. late	20.00	N.D.	N.D.	N.D.	0.9900	N.D.	0.1515	1.2746	0.7700	2.5346	0.7600
94-370	B.I. late	28.00	N.D.	N.D.	N.D.	0.0724	N.D.	0.0183	0.1993	0.1476	0.3772	0.2407
94-372	B.I. late	31.00	N.D.	N.D.	N.D.	0.8300	N.D.	0.1327	1.7159	0.8177	3.3864	0.9377
94-381	B.I. late	28.00	N.D.	N.D.	N.D.	1.4007	0.2196	0.1832	1.8346	1.0739	3.1143	0.9486
94-384	B.I. late	19.00	N.D.	0.3090	N.D.	5.0483	1.2676	1.0152	7.2979	2.3214	12.3117	3.9303
94-389	B.I. late	34.00	N.D.	N.D.	N.D.	0.3100	N.D.	0.0426	0.3267	0.3242	0.6809	0.2700

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	PCB101	PCB99	PCB97	PCB87	PCB110	PCB151	PCB149	PCB118	PCB146	PCB153
94-305	B.I. early	26.00	5.0676	9.1607	0.4555	1.1493	3.9797	N.D.	2.9445	19.0783	3.7424	21.9107
94-308	B.I. early	23.00	1.9406	2.2206	0.3779	0.3791	1.2609	N.D.	1.1482	4.3085	1.1230	7.1882
94-311	B.I. early	25.00	52.5213	41.3671	13.5058	15.3077	48.0832	2.5342	30.0245	58.2787	8.1497	45.3587
94-315	B.I. early	23.00	1.5282	2.3927	0.0445	0.2985	1.0867	N.D.	0.7197	4.2630	0.5664	6.1082
94-318	B.I. early	32.00	1.1330	2.3891	0.0685	0.3112	0.8927	N.D.	0.6912	4.5930	0.4730	6.3400
94-319	B.I. early	21.00	1.5312	2.7169	0.1535	0.3569	1.0881	N.D.	0.9185	5.4850	1.0988	7.6008
94-327	B.I. early	19.00	2.7405	5.5705	0.1695	0.6957	1.7829	N.D.	1.6295	11.7105	1.8424	17.1948
94-329	B.I. early	21.00	1.4732	3.5329	0.0593	0.3782	1.1457	N.D.	0.8318	7.4654	0.7450	12.6793
94-337	Nauset	19.00	0.6932	0.7797	0.1029	0.1339	0.4590	N.D.	0.6371	1.5558	0.4171	2.7045
94-338	Nauset	26.00	0.5574	0.7094	N.D.	0.1403	0.4254	N.D.	0.3771	1.3429	0.3394	2.7889
94-345	Nauset	25.00	5.0186	15.6836	0.2427	1.2509	5.0673	N.D.	2.4718	31.4405	5.7877	34.8686
94-348	Nauset	18.00	0.7716	0.9588	N.D.	0.1416	0.5952	N.D.	0.4908	1.8900	0.3316	3.3964
94-349	Nauset	34.00	0.5631	0.4921	0.0766	0.1290	0.3514	N.D.	0.4628	1.0352	0.2997	2.0434
94-353	Nauset	24.00	0.2614	0.3905	N.D.	N.D.	0.1881	N.D.	0.2171	0.9233	0.1762	2.1243
94-357	Nauset	19.00	1.2227	1.8086	N.D.	0.2864	0.9718	N.D.	0.6950	3.1745	0.8768	6.3723
94-358	Nauset	17.00	0.4143	0.4865	N.D.	0.1022	0.2897	N.D.	0.2519	1.0197	0.2100	1.8403
94-366	B.I. late	20.00	4.6462	6.9769	0.1392	0.8823	3.2523	N.D.	2.1031	11.8754	1.9023	16.0415
94-370	B.I. late	28.00	0.6714	1.0531	N.D.	0.1203	0.5379	N.D.	0.4183	1.9110	0.3469	3.4714
94-372	B.I. late	31.00	3.7273	8.7018	N.D.	1.0227	2.9182	N.D.	1.6705	15.2509	1.7109	17.2845
94-381	B.I. late	28.00	5.0089	7.0411	0.1614	1.1564	3.2732	N.D.	2.1393	12.4825	1.5979	13.3718
94-384	B.I. late	19.00	18.1290	22.3945	0.5297	3.5572	13.4283	N.D.	6.1538	34.9310	4.9103	32.0448
94-389	B.I. late	34.00	1.5121	1.5995	0.0370	0.2463	1.0595	N.D.	0.6784	2.4330	0.4053	2.9435

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	PCB105	PCB141	PCB137	PCB138	PCB158	PCB129	PCB182	PCB183	PCB128	PCB185
94-305	B.I. early	26.00	2.4069	0.2834	0.8221	20.7890	0.9352	N.D.	3.3045	1.4938	N.D.	N.D.
94-308	B.I. early	23.00	0.5270	0.0821	0.1545	5.7724	0.1970	0.0506	1.2755	0.5742	1.3327	N.D.
94-311	B.I. early	25.00	12.2529	4.2052	1.9342	48.3710	2.2645	0.9213	6.8019	2.7916	N.D.	N.D.
94-315	B.I. early	23.00	0.5761	0.0621	0.1333	4.9806	0.1679	N.D.	0.7245	0.3855	0.9964	N.D.
94-318	B.I. early	32.00	0.5824	0.0582	0.1673	5.1433	0.2264	N.D.	0.7388	0.4224	1.1048	N.D.
94-319	B.I. early	21.00	0.6977	0.1000	0.1623	6.3792	0.2377	N.D.	1.2592	0.4831	1.2992	N.D.
94-327	B.I. early	19.00	1.4900	0.1810	0.4348	14.5371	0.5500	N.D.	2.4505	1.2219	3.6110	N.D.
94-329	B.I. early	21.00	0.9482	0.0782	0.3007	9.9375	0.3704	N.D.	1.3975	1.2221	2.5054	N.D.
94-337	Nauset	19.00	0.2284	0.0497	0.0458	2.3829	0.0755	N.D.	0.7758	0.2710	N.D.	N.D.
94-338	Nauset	26.00	0.2369	0.0474	0.0514	2.4743	0.0803	N.D.	0.6309	0.3089	N.D.	N.D.
94-345	Nauset	25.00	4.1750	0.1768	1.4127	34.1955	1.6023	N.D.	3.8286	2.2186	N.D.	N.D.
94-348	Nauset	18.00	0.3080	N.D.	0.0588	2.9816	0.0980	N.D.	0.7580	0.3600	N.D.	N.D.
94-349	Nauset	34.00	0.1476	N.D.	N.D.	1.6703	0.0507	N.D.	0.5328	0.1866	N.D.	N.D.
94-353	Nauset	24.00	0.1138	N.D.	N.D.	1.3924	0.0529	N.D.	0.3590	0.1919	0.3267	N.D.
94-357	Nauset	19.00	0.6173	0.1164	0.1418	6.0550	0.2155	N.D.	1.5159	0.7264	N.D.	N.D.
94-358	Nauset	17.00	0.1735	N.D.	0.0308	1.5970	0.0530	N.D.	0.4324	0.1795	N.D.	N.D.
94-366	B.I. late	20.00	1.5038	0.2200	0.3315	13.7400	0.4354	N.D.	2.4054	0.9862	2.5362	N.D.
94-370	B.I. late	28.00	0.3079	N.D.	0.0569	2.8203	0.0941	N.D.	0.5979	0.3359	0.4807	N.D.
94-372	B.I. late	31.00	2.0164	0.1827	0.4418	15.8282	0.5441	N.D.	1.8541	0.9927	3.0250	N.D.
94-381	B.I. late	28.00	1.7493	0.2982	0.3421	12.1111	0.4539	0.0964	1.5393	0.7557	N.D.	N.D.
94-384	B.I. late	19.00	4.9186	1.0469	1.0228	32.4414	1.3786	0.7966	5.3159	2.1331	5.8552	N.D.
94-389	B.I. late	34.00	0.3333	0.0749	0.0509	2.5512	0.0807	N.D.	0.4698	0.1805	N.D.	N.D.

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	PCB174	PCB171	PCB200	PCB172	PCB180	PCB170	PCB201	PCB203	PCB195	PCB194
94-305	B.I. early	26.00	0.2448	1.6652	0.6241	0.3003	5.4345	2.8824	0.6659	0.5100	0.1793	0.5041
94-308	B.I. early	23.00	0.0845	0.4252	0.1464	0.0958	2.2145	0.9776	0.2888	0.2988	0.0967	0.3039
94-311	B.I. early	25.00	1.6374	2.9103	1.1471	0.4135	10.1877	4.5239	0.9310	0.7213	0.2832	0.7039
94-315	B.I. early	23.00	0.0533	0.3255	0.1312	0.0452	1.4164	0.6736	0.1779	0.1603	0.0567	0.1855
94-318	B.I. early	32.00	0.0621	0.3682	0.1355	0.0461	1.4997	0.7309	0.1436	0.1539	0.0476	0.1594
94-319	B.I. early	21.00	0.0781	0.4108	0.1623	0.0715	1.6192	0.7842	0.2515	0.1969	0.0673	0.2062
94-327	B.I. early	19.00	0.1386	1.0829	0.4424	0.1767	4.4867	2.1981	0.5833	0.5033	0.1714	0.5105
94-329	B.I. early	21.00	0.0139	0.8518	0.2975	0.1343	5.4075	2.4379	0.4032	0.6932	0.2089	0.7511
94-337	Nauset	19.00	0.0732	0.1435	0.0506	0.0377	0.9100	0.3700	0.1900	0.1365	0.0471	0.1387
94-338	Nauset	26.00	0.0463	0.1589	0.0394	0.0386	1.0611	0.4634	0.1451	0.1454	0.0437	0.1380
94-345	Nauset	25.00	0.0832	2.7677	1.0532	0.4423	9.7323	4.5627	0.5814	0.6700	0.2091	0.7377
94-348	Nauset	18.00	N.D.	0.1896	0.0656	0.0352	1.2956	0.5588	0.2060	0.1824	0.0644	0.1972
94-349	Nauset	34.00	0.0503	0.1083	N.D.	0.0300	0.6683	0.2852	0.1221	0.0907	N.D.	0.1024
94-353	Nauset	24.00	N.D.	0.1043	N.D.	N.D.	0.7524	0.3214	0.1019	0.1205	N.D.	0.1381
94-357	Nauset	19.00	0.0964	0.1550	0.1214	0.1082	2.4655	1.1800	0.3059	0.2877	0.0986	0.2891
94-358	Nauset	17.00	N.D.	0.0916	N.D.	0.0230	0.6000	0.2770	0.0905	0.0703	N.D.	0.0759
94-366	B.I. late	20.00	0.1723	0.8369	0.3554	0.1323	3.4923	1.6608	0.5492	0.4215	0.1623	0.3938
94-370	B.I. late	28.00	N.D.	0.1790	0.0621	0.0283	1.3179	0.5690	0.1434	0.1641	0.0555	0.1703
94-372	B.I. late	31.00	0.1327	0.9686	0.4105	0.1045	3.2786	1.7050	0.3368	0.3332	0.1059	0.3400
94-381	B.I. late	28.00	0.1625	0.7504	0.3146	0.0904	2.6321	1.3196	0.2896	0.2768	0.0925	0.2907
94-384	B.I. late	19.00	0.5200	1.9876	0.8579	0.2759	5.9248	3.2034	0.8021	0.5766	0.2159	0.5876
94-389	B.I. late	34.00	0.0493	0.1316	0.0549	0.0198	0.6021	0.2619	0.1040	0.0786	0.0300	0.0742

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	PCB206	PCB29	PCB84	OCS	T-nonach	p-mirex	Mirex	a-HCH	b-HCH	g-HCH
94-305	B.I. early	26.00	0.2779	N.D.	N.D.	N.D.	0.8121	0.1614	0.9431	0.0100	0.0148	N.D.
94-308	B.I. early	23.00	0.2536	N.D.	0.0541	N.D.	0.4006	0.0503	0.1282	0.0067	0.0106	N.D.
94-311	B.I. early	25.00	0.3903	N.D.	1.4497	0.9848	4.6258	N.D.	0.6155	0.0071	0.0142	N.D.
94-315	B.I. early	23.00	0.1536	N.D.	N.D.	N.D.	0.1479	0.0309	0.0627	0.0055	0.0097	N.D.
94-318	B.I. early	32.00	0.0791	N.D.	N.D.	N.D.	0.1764	N.D.	0.0676	0.0124	0.0155	N.D.
94-319	B.I. early	21.00	0.1496	N.D.	N.D.	N.D.	0.2631	N.D.	0.0512	0.0085	N.D.	N.D.
94-327	B.I. early	19.00	0.3195	N.D.	N.D.	N.D.	0.3586	0.0910	0.1233	0.0086	0.0171	N.D.
94-329	B.I. early	21.00	0.3689	N.D.	N.D.	N.D.	0.2175	0.0871	0.2807	0.0064	0.0150	N.D.
94-337	Nauset	19.00	0.1026	N.D.	0.0654	N.D.	0.3658	0.0535	0.0590	0.0148	0.0171	N.D.
94-338	Nauset	26.00	0.0949	N.D.	N.D.	N.D.	0.1191	N.D.	N.D.	0.0160	0.0160	N.D.
94-345	Nauset	25.00	0.3182	N.D.	N.D.	N.D.	1.2500	0.1345	0.6191	0.0200	0.0273	N.D.
94-348	Nauset	18.00	0.1232	N.D.	N.D.	N.D.	0.0920	0.0740	0.2032	0.0116	0.0132	N.D.
94-349	Nauset	34.00	0.0621	N.D.	N.D.	N.D.	0.2083	0.0679	0.2862	0.0083	0.0110	N.D.
94-353	Nauset	24.00	0.1271	N.D.	N.D.	N.D.	0.0171	N.D.	0.4086	0.0110	N.D.	N.D.
94-357	Nauset	19.00	0.1273	N.D.	N.D.	N.D.	0.1695	N.D.	0.0514	0.0145	0.0282	N.D.
94-358	Nauset	17.00	0.0338	N.D.	N.D.	N.D.	0.0559	0.0819	0.3941	0.0076	0.0162	N.D.
94-366	B.I. late	20.00	0.3331	N.D.	N.D.	N.D.	0.4746	N.D.	0.0892	N.D.	N.D.	N.D.
94-370	B.I. late	28.00	0.1179	N.D.	0.0793	N.D.	0.0738	0.1066	0.3721	N.D.	0.0107	N.D.
94-372	B.I. late	31.00	0.2045	N.D.	N.D.	0.0686	0.5573	N.D.	0.0495	N.D.	0.0182	N.D.
94-381	B.I. late	28.00	0.1882	N.D.	N.D.	0.0932	0.4436	0.0379	0.2107	0.0057	0.0107	N.D.
94-384	B.I. late	19.00	0.3621	N.D.	N.D.	0.3479	1.6072	N.D.	0.3317	N.D.	0.0207	N.D.
94-389	B.I. late	34.00	0.0602	N.D.	N.D.	N.D.	0.1140	0.0333	0.0605	N.D.	N.D.	N.D.

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Note: OCS=octachlorostyrene; T-nonach=Trans nonachlor; p-mirex=photo Mirex; a,b,g HCH=a,b,g Hexachlorocyclohexane

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	HCB	Oxychlor	T-chlor	Cis-chlor	C-nonach	H.E.	Dieldrin	TCPM	1,2,4,5 CB	1,2,3,4 CB
94-305	B.I. early	26.00	0.1762	0.0731	0.0107	0.0307	0.0614	0.0697	0.2066	0.0800	N.D.	N.D.
94-308	B.I. early	23.00	0.2073	0.0918	0.0139	0.0258	0.0724	0.0873	0.2497	0.0448	N.D.	N.D.
94-311	B.I. early	25.00	0.4242	0.2345	0.0129	0.1287	0.2503	0.1110	0.5019	0.1652	N.D.	N.D.
94-315	B.I. early	23.00	0.2079	0.0694	0.0045	N.D.	0.0167	0.0636	0.1315	0.0564	N.D.	N.D.
94-318	B.I. early	32.00	0.0636	0.0564	N.D.	N.D.	0.0242	0.0424	0.1415	0.0324	N.D.	N.D.
94-319	B.I. early	21.00	0.0862	0.0719	0.0054	0.0119	0.0542	0.0512	0.2419	0.0508	N.D.	N.D.
94-327	B.I. early	19.00	0.2233	0.1314	N.D.	N.D.	0.0457	0.1086	0.1814	0.1124	N.D.	N.D.
94-329	B.I. early	21.00	0.2079	0.0918	N.D.	N.D.	0.0200	0.0821	0.1621	0.0714	N.D.	N.D.
94-337	Nauset	19.00	0.1848	0.1552	N.D.	0.0265	0.0861	0.0955	0.1829	0.0294	N.D.	N.D.
94-338	Nauset	26.00	0.1671	0.0711	0.0034	0.0131	0.0340	0.0631	0.1246	0.0449	N.D.	N.D.
94-345	Nauset	25.00	0.2164	0.1623	N.D.	0.0077	0.0786	0.1241	0.2423	0.1295	N.D.	N.D.
94-348	Nauset	18.00	0.1260	0.1152	N.D.	N.D.	0.0320	0.0744	0.1476	0.0520	N.D.	N.D.
94-349	Nauset	34.00	0.1324	0.0628	0.0052	0.0110	0.0576	0.0483	0.0883	0.0514	N.D.	N.D.
94-353	Nauset	24.00	0.0829	0.0495	0.0100	0.0229	0.0214	0.0400	0.0752	0.0343	N.D.	N.D.
94-357	Nauset	19.00	0.2759	0.1732	0.0082	N.D.	0.0500	0.1214	0.1977	0.1041	N.D.	N.D.
94-358	Nauset	17.00	0.1365	0.0678	0.0059	N.D.	0.0195	0.0616	0.0870	0.0197	N.D.	N.D.
94-366	B.I. late	20.00	0.2885	0.1969	0.0108	N.D.	0.0400	0.1377	0.1600	0.0915	N.D.	N.D.
94-370	B.I. late	28.00	0.1145	0.1272	0.0076	N.D.	0.0228	0.0648	0.1303	0.1752	N.D.	N.D.
94-372	B.I. late	31.00	0.2223	0.1600	0.0091	N.D.	0.0214	0.0882	0.1159	0.0832	N.D.	N.D.
94-381	B.I. late	28.00	0.2029	0.1011	0.0111	N.D.	0.0218	0.0689	0.0754	0.0661	0.0127	N.D.
94-384	B.I. late	19.00	0.3683	0.3466	0.0055	N.D.	0.0217	0.1079	0.1141	0.1445	N.D.	N.D.
94-389	B.I. late	34.00	0.0616	0.1081	0.0035	N.D.	0.0202	0.0588	0.1037	0.0667	0.0124	N.D.

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Note: HCB=Hexachlorobenzene; Oxychlor=Oxychlorodane; T-chlor=Trans-chlordane; Cis-chlor=Cis-chlordane; C-nonach=Cis-nonachlor
TCPM=H.E.=Heptachlor Epoxide; Tris(4-chlorophenyl)methanol; 1,2,4,5 CB=1,2,4,5 chlorobenzene; 1,2,3,4 CB=1,2,3,4 chlorobenzene

Appendix B con't. Levels of contaminants measured in the yolk sacs of pipping common tern embryos.
All chemistry values expressed as ug/g lipid weight unless noted otherwise.

I.D.	Site[1]	Lipid content(%)	Penta CB	PCB 1:1
94-305	B.I. early	26.00	N.D.	283.9441
94-308	B.I. early	23.00	N.D.	78.8421
94-311	B.I. early	25.00	N.D.	660.6703
94-315	B.I. early	23.00	N.D.	68.0273
94-318	B.I. early	32.00	N.D.	70.2497
94-319	B.I. early	21.00	N.D.	87.1300
94-327	B.I. early	19.00	N.D.	196.5543
94-329	B.I. early	21.00	N.D.	135.7304
94-337	Nauset	19.00	N.D.	32.5468
94-338	Nauset	26.00	N.D.	33.7949
94-345	Nauset	25.00	N.D.	467.0555
94-348	Nauset	18.00	N.D.	40.7240
94-349	Nauset	34.00	N.D.	22.8141
94-353	Nauset	24.00	N.D.	19.0176
94-357	Nauset	19.00	N.D.	82.7018
94-358	Nauset	17.00	N.D.	21.8130
94-366	B.I. late	20.00	N.D.	187.6662
94-370	B.I. late	28.00	N.D.	38.5214
94-372	B.I. late	31.00	N.D.	216.1877
94-381	B.I. late	28.00	N.D.	165.4179
94-384	B.I. late	19.00	N.D.	443.0976
94-389	B.I. late	34.00	N.D.	34.8449

[1] B.I. early=Bird Is., 15 June, 1994; B.I. late=Bird Is., 21 July, 1995; Nauset=Nauset, 8 July, 1994

Note: Penta CB=pentachlorobenzene; PCB 1:1=estimate based on PCB congener 138.

Appendix C. Contaminant concentrations measured in common tern eggs.
All values expressed as ug/g lipid weight unless noted otherwise.

Egg number	Lipid content (%)	Paired[1] histology #	Total PCBs	TCDD-Eqs ng/g lipid	pp'-DDE	pp'-DDD	pp'-DDT	Total DDTs	3-chlorine PCBs	4-chlorine PCBs	5-chlorine PCBs
257	16.0	H95-050	24.42500	6.36	1.20750	0.03000	0.01625	1.25375	0.37813	3.62500	7.92625
527	8.5	H95-052	18.23765	7.30	0.53294	0.02235	N.D.	0.55529	0.33765	1.72471	5.53765
530	8.9	H95-051	30.27640	9.15	1.57865	0.02247	0.02135	1.62247	0.65056	3.51348	10.51011
531	8.9	H95-053	16.67303	7.26	0.50562	N.D.	N.D.	0.50562	0.08315	1.01236	5.23820
674	8.4	H95-076	546.57500	114.07	0.51667	0.12976	0.08690	0.73333	47.42024	184.67024	175.95357
765	8.4	H95-075	15.02143	3.73	0.32976	0.02024	N.D.	0.35000	0.21548	1.26190	4.36310
855	7.8	H95-073	37.49359	16.93	1.14359	N.D.	0.01667	1.16026	0.51154	2.97949	12.34103
938	8.8	H95-077	14.34659	3.80	0.37045	N.D.	N.D.	0.37045	0.16477	0.55114	3.29432
952	7.6	H95-074	19.61974	6.82	1.03947	0.02368	0.01316	1.07632	0.06974	1.02763	5.66447
1024	8.2	H95-072	28.65366	10.31	0.66463	0.03902	N.D.	0.70366	0.29756	2.15732	8.63537

[1] Histological analyses were conducted on pre-fledged terns taken from the same nests as the corresponding egg.

Egg number	Lipid content (%)	6-chlorine PCBs	7-chlorine PCBs	8-chlorine PCBs	9-chlorine PCBs	1-ortho PCBs	2-ortho PCBs	3-ortho PCBs	4-ortho PCBs	PCB28	PCB31
257	16.0	8.95000	3.04938	0.45188	0.04375	5.19250	15.98063	3.16563	0.08625	0.15625	0.22188
527	8.5	6.87529	3.31647	0.40353	0.04353	3.84941	11.83765	2.48588	0.06471	0.15294	0.18471
530	8.9	11.43820	3.62135	0.49775	0.04382	7.26067	19.29438	3.60337	0.11685	0.26966	0.38090
531	8.9	7.29101	2.64270	0.36517	0.04157	3.49551	11.07865	2.02472	0.07416	0.08315	N.D.
674	8.4	113.65357	22.61071	2.26667	N.D.	175.77262	335.72857	34.27738	0.79643	18.97143	28.44881
765	8.4	5.91905	2.84286	0.31548	0.10238	2.82381	9.64881	2.49286	0.05476	0.08690	0.12976
855	7.8	14.72179	6.02564	0.83077	0.08333	8.55769	24.42821	4.33846	0.16923	0.27692	0.23462
938	8.8	6.49205	3.32386	0.47500	0.04659	2.33068	9.67386	2.29091	0.05114	0.06477	0.10000
952	7.6	8.75132	3.61316	0.44737	0.04605	3.95000	13.04737	2.52368	0.10000	0.06974	N.D.
1024	8.2	12.14268	4.76220	0.59512	0.06463	5.98902	19.00854	3.52073	0.13537	0.13171	0.16585

Appendix C con't. Contaminant concentrations measured in common tern eggs.
All values expressed as ug/g lipid weight unless noted otherwise.

Egg number	Lipid content (%)	PCB42	PCB44	PCB49	PCB52	PCB64	PCB74	PCB70	PCB66	PCB60	PCB101
257	16.0	0.19500	0.17000	0.38000	0.97938	0.06625	0.33438	0.38000	0.89750	0.22250	1.48875
527	8.5	N.D.	N.D.	0.45882	N.D.	0.04471	0.28118	0.26118	0.50824	0.17059	1.00353
530	8.9	0.09213	N.D.	0.65281	0.31461	0.08202	0.50112	0.42921	0.96404	0.47640	1.94382
531	8.9	N.D.	N.D.	0.12809	N.D.	0.02022	0.20112	0.16404	0.38202	0.11685	0.72584
674	8.4	10.65714	5.53333	42.97262	44.87857	5.42857	14.86667	16.39643	33.00952	10.92738	36.86071
765	8.4	N.D.	N.D.	0.28810	0.08452	0.02619	0.20119	0.19524	0.33571	0.13095	0.83929
855	7.8	N.D.	N.D.	0.46538	N.D.	0.07051	0.58718	0.41538	1.11795	0.32308	1.83462
938	8.8	N.D.	N.D.	0.07273	N.D.	0.01250	0.12841	0.08295	0.17386	0.08182	0.41932
952	7.6	N.D.	N.D.	0.15263	N.D.	0.01579	0.21184	0.13553	0.36447	0.14737	0.62237
1024	8.2	0.04024	N.D.	0.40610	0.09634	0.03780	0.38537	0.27927	0.66707	0.24512	1.17439

Egg number	Lipid content (%)	PCB99	PCB97	PCB87	PCB110	PCB151	PCB149	PCB118	PCB146	PCB153	PCB105
257	16.0	1.66563	0.37875	0.25625	1.15813	0.10875	1.00875	2.54813	0.59938	3.49625	0.43188
527	8.5	1.26588	0.06000	0.20118	0.71529	N.D.	0.46588	2.02471	0.32235	3.26824	0.26706
530	8.9	2.44494	0.16854	0.36292	1.35056	N.D.	0.99888	3.67303	0.68539	4.55393	0.56517
531	8.9	1.31236	N.D.	0.14382	0.50787	N.D.	0.36292	2.26517	0.33034	3.55056	0.28315
674	8.4	34.00833	8.50595	9.88452	33.54167	1.80000	18.61905	46.07976	4.61667	37.78452	7.07262
765	8.4	0.91905	0.09048	0.19643	0.57381	N.D.	0.45357	1.55238	0.26548	2.50119	0.19167
855	7.8	3.18333	N.D.	0.35769	1.36410	N.D.	0.74487	4.94103	0.77179	6.69744	0.66154
938	8.8	0.73409	0.04659	0.08068	0.31477	N.D.	0.25568	1.36364	0.31023	2.72841	0.33636
952	7.6	1.43947	N.D.	0.15789	0.42500	N.D.	0.35000	2.68553	0.33816	4.35000	0.33684
1024	8.2	2.21220	0.09024	0.27195	0.77195	N.D.	0.58902	3.66951	0.51098	5.57439	0.44512

Appendix C con't. Contaminant concentrations measured in common tern eggs.
All values expressed as ug/g lipid weight unless noted otherwise.

Egg number	Lipid content (%)	PCB141	PCB137	PCB138	PCB158	PCB129	PCB182	PCB183	PCB128	PCB185	PCB174
257	16.0	0.08563	0.06750	3.24938	0.13813	0.19688	0.94688	0.34125	N.D.	N.D.	0.08688
527	8.5	0.04941	0.05529	2.63412	0.08000	N.D.	0.95294	0.45882	N.D.	N.D.	0.03176
530	8.9	0.10787	0.10449	4.43708	0.22809	0.32247	1.29438	0.45281	N.D.	N.D.	0.09213
531	8.9	0.03933	0.05955	2.86404	0.08315	N.D.	0.73371	0.37191	N.D.	N.D.	N.D.
674	8.4	1.77500	1.49048	36.47738	2.35476	1.23929	6.67738	3.22738	7.49643	N.D.	0.75238
765	8.4	0.04286	0.03929	2.10833	0.14762	0.36071	1.00595	0.44286	N.D.	N.D.	0.07143
855	7.8	0.12692	0.13077	5.92564	0.16154	0.16282	1.63077	0.70385	N.D.	N.D.	0.06282
938	8.8	N.D.	0.32727	2.64091	0.22841	N.D.	0.97159	0.46364	N.D.	N.D.	N.D.
952	7.6	0.03553	0.07632	3.50132	0.10132	N.D.	0.96974	0.51053	N.D.	N.D.	N.D.
1024	8.2	0.05610	0.12317	4.66220	0.25732	0.36951	1.30000	0.60976	N.D.	N.D.	0.04756

Egg number	Lipid content (%)	PCB171	PCB200	PCB172	PCB180	PCB170	PCB201	PCB203	PCB195	PCB194	PCB206
257	16.0	0.35750	0.08625	0.04438	0.86313	0.40938	0.14063	0.09375	0.03813	0.09375	0.04375
527	8.5	0.29529	0.06471	0.03059	1.12353	0.42235	0.10471	0.09882	0.03412	0.10235	0.04353
530	8.9	0.42809	0.11685	0.04270	0.87753	0.43596	0.16067	0.09326	0.04157	0.08539	0.04382
531	8.9	0.30899	0.07416	0.02809	0.81236	0.38876	0.09213	0.08539	0.03034	0.08315	0.04157
674	8.4	2.22738	0.79643	0.24286	6.16905	3.31429	0.49643	0.47738	N.D.	0.49643	N.D.
765	8.4	0.22143	0.05476	0.02500	0.77381	0.30119	0.09762	0.06786	0.02976	0.06548	0.10238
855	7.8	0.64359	0.16923	0.06154	2.14487	0.77821	0.21923	0.19487	0.05641	0.19231	0.08333
938	8.8	0.26136	0.05114	0.03750	1.15568	0.43409	0.12045	0.12727	0.04318	0.13182	0.04659
952	7.6	0.40658	0.10000	0.03026	1.20658	0.48947	0.10526	0.10395	0.03158	0.10658	0.04605
1024	8.2	0.58049	0.13537	0.04756	1.54146	0.63537	0.15122	0.13049	0.04756	0.13049	0.06463

Appendix C con't. Contaminant concentrations measured in common tern eggs.
All values expressed as ug/g lipid weight unless noted otherwise.

Egg number	Lipid content (%)	PCB 29	PCB 84	OCS	T-nonach	p-mirex	Mirex	a-HCH	b-HCH	g-HCH	HCB
257	16.0	N.D.	0.05514	0.01688	0.28188	0.05000	0.07188	N.D.	N.D.	N.D.	0.01813
527	8.5	N.D.	0.08014	N.D.	0.09059	0.07882	0.05882	N.D.	N.D.	N.D.	0.04000
530	8.9	N.D.	0.06284	0.02022	0.24719	N.D.	0.02697	N.D.	N.D.	N.D.	0.03034
531	8.9	N.D.	0.04293	N.D.	0.07416	0.06180	0.02921	N.D.	N.D.	N.D.	0.05393
674	8.4	N.D.	1.27637	0.76071	3.24167	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
765	8.4	N.D.	0.05166	N.D.	0.05238	0.13810	0.11071	N.D.	N.D.	N.D.	0.02738
855	7.8	N.D.	0.06431	0.02436	0.25513	0.09615	0.04744	N.D.	N.D.	N.D.	0.09615
938	8.8	N.D.	0.03878	N.D.	0.03977	0.09773	0.11818	N.D.	N.D.	N.D.	0.02841
952	7.6	N.D.	0.05893	N.D.	0.07632	N.D.	0.03026	N.D.	N.D.	N.D.	0.06447
1024	8.2	N.D.	0.05918	N.D.	0.18171	0.08659	0.05732	N.D.	N.D.	N.D.	0.02317

Note: OCS=octachlorostyrene;T-nonach=Trans nonachlor; p-mirex=photo Mirex; a,b,g HCH=a,b,g Hexachlorocyclohexane; HCB=Hexachlorobenzene

Egg number	Lipid content (%)	Oxychlor	T-chlor	Cis-chlor	C-nonach	H.E.	Dieldrin	TCPM	1,2,4,5 CB	1,2,3,4 CB	Penta CB
257	16.0	0.04250	N.D.	0.02188	0.05500	0.02250	0.11188	0.02750	N.D.	N.D.	N.D.
527	8.5	0.05294	N.D.	0.06000	0.01765	0.02353	0.05765	0.03765	N.D.	N.D.	N.D.
530	8.9	0.05730	N.D.	0.00674	0.03820	0.02697	0.12584	0.10000	N.D.	N.D.	N.D.
531	8.9	0.03596	N.D.	N.D.	0.00787	0.02472	0.05730	N.D.	N.D.	N.D.	N.D.
674	8.4	0.05714	0.11548	0.04762	0.05714	0.01548	0.06548	0.05000	N.D.	N.D.	N.D.
765	8.4	0.02500	N.D.	N.D.	0.01429	0.01786	0.03214	N.D.	N.D.	N.D.	N.D.
855	7.8	0.06026	N.D.	N.D.	0.01154	0.03846	0.07179	0.06538	N.D.	N.D.	N.D.
938	8.8	0.02159	N.D.	N.D.	0.00682	0.01250	0.04205	N.D.	N.D.	N.D.	N.D.
952	7.6	0.03421	N.D.	N.D.	0.01316	0.02632	0.06974	0.73816	N.D.	N.D.	N.D.
1024	8.2	0.03415	N.D.	N.D.	0.01220	0.01220	0.04878	0.13780	N.D.	N.D.	N.D.

Note: Oxychlor=Oxychlordane; T-chlor=Trans-chlordane; Cis-chlor=Cis-chlordane; C-nonach=Cis-nonachlor; H.E.=Heptachlor Epoxide; TCPM=Tris(4-chlorophenyl)methanol; 1,2,4,5 CB=1,2,4,5 chlorobenzene; 1,2,3,4 CB=1,2,3,4 chlorobenzene; Penta CB=pentachlorobenzene

Appendix C con't. Contaminant concentrations measured in common tern eggs.
All values expressed as ug/g lipid weight unless noted otherwise.

Egg number	Lipid content (%)	PCB1:1
257	16.0	44.38313
527	8.5	35.97765
530	8.9	60.61011
531	8.9	39.12135
674	8.4	498.22262
765	8.4	28.80357
855	7.8	80.94231
938	8.8	36.07727
952	7.6	47.81579
1024	8.2	63.67805

Note: PCB 1:1=estimate based on PCB congener 138.