Université d'Ottawa • University of Ottawa

ENDOCRINE DISRUPTING EFFECTS OF ENVIRONMENTAL CONTAMINANTS IN HERRING GULL EMBRYOS AND CULTURED AVIAN HEPATOCYTES

ANGELA LORENZEN

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
in partial fulfillment of the requirements for the
Ph.D. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
l'École des études supérieures et de la recherche
Université d'Ottawa
en vue de l'obtention du doctorat
L'Institut de biologie d'Ottawa-Carleton

July 2001

ABSTRACT

The effects of non-polar environmental contaminants on components of the hypothalamo-pituitary adrenal (HPA) axis and the hypothalamo-pituitary-ovary (HPO) axis were examined in herring gull (*Larus argentatus*) embryos and cultured avian hepatocytes. In the HPA axis studies, regression analysis of herring gull embryo yolk sac organochlorine residues against basal plasma corticosterone concentrations indicated statistically significant inverse relationships for polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDDs/PCDFs), total polychlorinated biphenyls (PCBs), non-*ortho* PCBs, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs). The activities of two intermediary metabolic enzymes were inversely correlated with PCDDs/PCDFs. In a second study, high incidences of early onset of hatching were observed for herring gull eggs collected from some Great Lakes sites. Embryos from Scotch Bonnet Island were observed to have higher than average renal PEPCK activities and lower than average corticosterone stress responses. Regression analysis of embryo yolk sac organochlorine residues from three Great Lakes sites indicated a statistically significant positive relationship for basal plasma corticosterone concentrations and chlorinated hydrocarbons (CHCs), including organochlorine pesticides.

HPO axis studies involved development of a reverse-transcription-polymerase chain reaction (RT-PCR) bioassay to semi-quantitatively measure mRNA for the estrogen-inducible egg yolk precursor protein, vitellogenin (VTG), in avian embryo hepatocyte cultures. Short regions of VTG, β-actin and albumin cDNAs were cloned and sequenced for several species of birds. Hepatocyte

cultures were prepared from both chicken and herring gull embryos and treated with the estradiol analogue, moxestrol, or the organochlorine insecticide, *o,p'*-DDT. Herring gull embryo hepatocyte cultures responded with VTG mRNA induction at 1 nM moxestrol, compared with 10 nM for chicken embryo hepatocyte cultures. Both herring gull and chicken embryo hepatocyte cultures responded with significant VTG mRNA induction when treated with 10000 nM *o,p'*-DDT. In chicken embryo hepatocyte cultures, 4-*tert*-octylphenol (OP) was determined to be very weakly estrogenic, whereas TCDD, benzo[k]fluoranthene (B[k]F), three halogenated dimethyl bipyrroles (HDBPs) and an extract prepared from a common tern (*Sterna hirundo*) egg were determined to be anti-estrogenic. These results indicate that the semi-quantitative avian RT-PCR VTG mRNA bioassay may be useful for predicting whether wild birds may be sensitive (or exposed) to estrogenic or anti-estrogenic environmental contaminants.

RÉSUMÉ

Les effets des contaminants environnementaux sur des composantes de l'axe hypothalamo-pituitaire-surrénal (HPS) et de l'axe hypothalamo-pituitaire-ovaire (HPO) d'embryons de Goéland argenté (*Larus argentatus*) et de cultures d'hépatocytes aviaires ont été examinés. Pour les études de l'axe HPS, l'analyse de régression entre les concentrations résiduelles d'organochloré du jaune d'oeuf d'embryons de Goéland argenté et les concentrations basales de corticostérone plasmique, ont démontré des relations inverses significatives pour les dibenzodioxines/dibenzofuranes polychlorés (PCDD/PCDF), les biphényles

polychlorés (BPC), les BPC non-*ortho*, et le 2,3,7,8-tétrachlorodibenzo-*p*-dioxine (TCDD) en équivalents toxiques (TEQs). Les activités de deux enzymes du métabolisme intermédiaire étaient inversement corrélées aux PCDD/PCDF. Dans le cadre d'une deuxième étude, on a observé une augmentation de la fréquence d'éclosion hâtive d'oeufs de Goéland argenté, recueillis dans certaines régions des Grands Lacs. On a observé chez les embryons de l'île Scotch Bonnet une activité de la PEPCK rénale supérieure à la moyenne, et des réactions au stress de la corticostérone inférieures à la moyenne. Des analyses de régression avec les concentrations résiduelles d'organochloré du jaune d'oeuf, provenant de trois sites des Grands Lacs, ont démontré une relation positive statistiquement significative entre les concentrations basales de corticostérone plasmique et les concentrations d'hydrocarbures chlorés, incluant des pesticides organochlorés.

Les études de l'axe HPO ont exigé le développement d'un essai biologique de technique de transcription inverse suivie de réaction en chaîne de la polymérase (RT-PCR), pour mesurer l'induction semi-quantitative d'ARNm pour la vitellogénine (VTG) – protéine précurseur du jaune d'oeuf qui est susceptible aux oestrogènes – chez des cultures de cellules hépatiques d'embryons d'oiseaux. De courtes sections d'ADNc de VTG, de ß-actine et d'albumine ont été clonées et les séquences ont été déterminées pour plusieurs espèces d'oiseaux. Des cultures de cellules hépatiques provenant d'embryons de poulet et d'embryons de Goéland argenté ont été traitées avec un produit analogue de l'estradiol, le moxestrol, ou de l'insecticide organochloré – *o,p'*-DDT. Les cultures de cellules hépatiques du Goéland argenté ont répondu avec l'induction d'ARNm de VTG à 1 nM moxestrol, comparativement à

10 nM pour les cultures de cellules hépatiques du poulet. Les deux cultures ont répondu avec l'induction significative d'ARNm de VTG lors du traitement avec 10000 nM de *o,p'*-DDT. Le 4-*tert*-octylphénol ne s'est avéré que très faiblement oestrogénique sur des cultures de cellules hépatiques d'embryons de poulet, tandis que le TCDD, le benzo[k]fluoranthène, trois diméthyl bipyrroles halogénés (HDBPs) et un extrait provenant d'oeufs de la Sterne pierregarin (*Sterna hirundo*) ont eu des effets anti-oestrogéniques. Les résultats indiquent que l'essai biologique pourrait être utile pour prédire si des oiseaux sauvages sont vulnérables (ou sont exposés) aux contaminants environnementaux oestrogéniques ou anti-oestrogéniques.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
Ah	aryl hydrocarbon
AHH	arylhydrocarbon hydroxylase
ALAT	alanine aminotransferase
APND	aminopyrine-*N*-demethylase
ASPAT	aspartate aminotransferase
B[k]F	benzo[k]fluoranthene
BPA	bisphenol A
Br4	1,1'-dimethyl-5,5'-dichloro-3,3',4,4'-tetrabromo-2,2'-bipyrrole
Br5	1,1'-dimethyl-5-chloro-3,3',4,4',5-pentabromo-2,2'-bipyrrole
Br6	1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole
cDNA	complementary DNA
CHCs	chlorinated hydrocarbons including organochlorine pesticides
CRH	corticotropin releasing hormone
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
EC50	concentration to produce a half-maximal response
ERE	estrogen-response-element
EROD	ethoxyresorufin-O-deethylase
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
HAH	halogenated aromatic hydrocarbon
HCB	hexachlorobenzene
HCP	highly carboxylated porphyrin
HDBP	halogenated dimethyl bipyrrole
HPA	hypothalamo-pituitary-adrenal
HPO	hypothalamo-pituitary-ovary
IEF	induction equivalency factor
LH	luteinizing hormone

MDBP	methylated DNA binding protein
ME	malic enzyme
OP	4-*tert*-octylphenol
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
PCDDs/PCDFs	polychlorinated dibenzodioxins & polychlorinated dibenzofurans
PEPCK	phosphoenolpyruvate carboxykinase
RT-PCR	reverse transcription-polymerase chain reaction
STP	sewage treatment plant
TCA	tricarboxylic acid
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEQ	2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent
VHDL	very high density lipoprotein
VLDL	very low density lipoprotein
VTG	vitellogenin

ACKNOWLEDGEMENTS

First I would like to thank Dr. Tom Moon for agreeing to supervise this project even when I was not on campus, for always being available for discussion and advice, and allowing me to work independently. I also thank him for valuable advice on matters outside this thesis.

I would also like to thank my advisory committee, Dr. Bill Casley, Dr. Laird Shutt, Dr. Sean Kennedy, Dr. Jim Fenwick and Dr. Ken Storey for helpful advice concerning the thesis. Furthermore, I am grateful to Dr. Sean Kennedy for supporting my enrollment in the Ph.D. program, to Dr. Laird Shutt and Kim Williams for assistance both in the field and laboratory, to Glen Fox for his continued interest in this thesis and, in particular, Dr. Bill Casley for taking the time (and having the patience) to teach me the techniques and theory required to develop the semi-quantitative RT-PCR bioassay.

Research in this thesis was largely funded by the Wildlife Toxicology Program of the Canadian Wildlife Service and I thank Keith Marshall for allowing me to conduct this work.

Finally, I would like to thank Hans for his patience and support.

This thesis is dedicated to the memory of my father, Carl Lorenzen (1936-1997).

CHAPTER 1. INTRODUCTION

1.A. RATIONALE, OBJECTIVES AND GENERAL INTRODUCTION

1.A.1. Rationale

The endocrine system is extremely sensitive to even small perturbations of hormone levels. Thus assays and measurements based on endocrine endpoints have the potential for detecting subtle, yet significant, effects due to chronic exposure to low levels of contaminants. The current lack of data on the effects of endocrine disrupting chemicals on herring gulls (*Larus argentatus*) is partially due to the limited number of techniques developed or optimized for assessing the impact of these chemicals in birds. Since recent surveys indicate significant declines in certain Great Lakes herring gull populations (R. Morris *et al.*, manuscript in preparation). Further efforts are required, therefore, to develop and apply techniques to determine whether current levels of complex mixtures of environmental contaminants are adversely affecting the health of this species. Furthermore, development and application of these endpoints may provide important insight into potential adverse health effects that may be associated with industrial contamination of the Great Lakes ecosystem.

1.A.2. Objectives

The potential endocrine-disrupting effects of environmental contaminants in birds were investigated by measuring endpoints related to two endocrine pathways: the hypothalamo-pituitary-adrenal (HPA) axis and the hypothalamo-pituitary-ovary (HPO) axis. The specific experimental objectives were 1) to develop and apply

sensitive methods for the evaluation of the endocrine-disrupting effects of organochlorine contaminants in herring gull embryos, and 2) to develop bioassay methods that can be used to assess the potential estrogenicity and/or anti-estrogenicity of individual or complex mixtures of environmental contaminants in avian embryo hepatocyte cultures.

1.A.3. Overview of Endocrine Effects of Contaminants in Wildlife

Over the past 30 years a number of incidences of endocrine disruption in wildlife exposed to industrial and agricultural chemicals have been reported. These effects are observed in species as diverse as gastropods, fish, alligators, birds, seals and other mammals (Sonstegard and Leatherland, 1976; Fry and Toone, 1981; Bergman and Olsson, 1985; Bryan *et al.*, 1986; Moccia *et al.*, 1986; McMaster *et al.*, 1992, 1996; Guillette *et al.*, 1994, 1996; Nisbet *et al.*, 1996). The nature of the endocrine effects are equally diverse. Reproductive failure and behavioural abnormalities related to reproduction are among the most commonly reported endocrine-related effects of contaminant exposure. Depending on the species and contaminant, these effects have been documented as development of male secondary sex characteristics in females (Bryan *et al.*, 1986), abnormal steroid hormone concentrations (McMaster *et al.*, 1992, 1996; Guillette *et al.*, 1994, 1996), developmental abnormalities of gonads (Guillette *et al.*, 1994, 1996), and feminization of embryonic reproductive tracts (Fry and Toone, 1981; Nisbet *et al.*, 1996). In addition, other endocrine-related effects have been reported including

thyroid and adrenocortical hyperplasia (Sonstegard and Leatherland, 1976; Bergman and Olsson, 1985; Moccia *et al.*, 1986).

In the most contaminated areas of the Great Lakes basin, effects including embryo lethality, failure to thrive ("wasting"), deformities, changes in biochemical, immunological and reproductive parameters (eg. hatching success, number of young fledged and length of egg incubation) continue to be reported for fish-eating birds and their embryos (Gilbertson *et al.*, 1991; Harris *et al.*, 1993; Ludwig *et al.*, 1993; Yamashita *et al.*, 1993; Grasman *et al.*, 1996). Although the incidences of these biological endpoints are highly variable from site to site, a combination of chemical residue data and epidemiological criteria support an important etiologic role for non-*ortho* substituted polychlorinated biphenyls (PCBs) and other "dioxin-like" organochlorines in some locations and species (see references cited above). Since fish-eating birds occupy high trophic levels in aquatic-based food chains, they are at risk from exposure to persistent lipophilic contaminants, several of which have been shown to exhibit reproductive or adrenotoxic effects in fish (Mac and Edsall, 1991).

1.A.4. Estrogenic Effects of Environmental Contaminants

Recent studies have indicated that wildlife exposed to high concentrations of some environmental contaminants appear to be experiencing a feminizing influence. For example, alligators in a highly contaminated lake in Florida showed reduced hatchability, increased mortality, high estrogen:testosterone ratios and feminized internal organs in males (Guillette *et al.*, 1994). In fish, exposure to detergent

breakdown products, such as nonylphenols, has resulted in the estrogen receptor-mediated production of the egg yolk protein, vitellogenin, in males (Harries *et al.*, 1997). Based on these and other findings, certain chemicals are grouped together and referred to as "environmental estrogens".

Non-chlorinated compounds, or by-products, that are currently being produced and are suspected, or known, to have estrogenic or anti-estrogenic properties include polycyclic aromatic hydrocarbons (PAHs), ethinylestradiol, bisphenol A, alkylphenols, alkylphenol ethoxylates, and other plasticizers and detergents (Soto *et al.*, 1995, Schlenk *et al.*, 1997; Larsson *et al.*, 1999; Nicolas, 1999). In fish, PAHs have been shown to reduce circulating vitellogenin and steroid hormone levels, exhibit estrogenic and anti-estrogenic effects, retard oocyte maturation, and reduce reproductive success (Nicolas, 1999). Regulated (but persistent) organochlorine contaminants including PCBs, dichlorodiphenyltrichloroethane (DDT), Kepone and dieldrin also have estrogenic or anti-estrogenic properties (Jansen *et al.*, 1993; Soto *et al.*, 1995; Schlenk *et al.*, 1997). High environmental concentrations of the chlorinated insecticide, DDT, are associated with eggshell thinning in birds (Ratcliffe, 1967), female-female pairing of herring gulls (Fry *et al.*, 1987), and feminized reproductive tracts in herring gulls (Fry and Toone, 1981). Similarly, common tern embryos exposed to high concentrations of industrial organochlorine contaminants showed a significant degree of feminization (Nisbet *et al.*, 1996). The organochlorine pesticide, Kepone (chlordecone) also affects avian reproduction directly by interfering with normal egg production and indirectly by altering hormone secretion (Rattner *et al.*, 1984).

1.A.5. The Herring Gull as a Monitor of Environmental Contamination

The herring gull is a common avian species that breeds and resides year-round on all five of the Great Lakes. Since herring gulls live in colonies, population studies are possible. Furthermore, the colonies are located not only on the Great Lakes, but also in other regions of Canada and across the world. This allows selection of study sites which encompass a gradient of contamination levels both within and outside of Canada. Although the herring gull is omnivorous and an opportunistic feeder, under normal circumstances more than 70% of its diet consists of fish, placing it at a high trophic level (Ryckman *et al.*, 1997). As a consequence, PCB and dichlorodiphenyldichloroethylene (DDE, a metabolite of the insecticide DDT) concentrations in herring gull eggs are estimated to be 25 million times higher than in the surrounding water and about 50 times greater than in the fish it consumes (Norstrom *et al.*, 1978). Because of these characteristics, the herring gull is a particularly useful indicator of contamination of the Great Lakes ecosystem (Ryckman *et al.*, 1997).

During the early to mid 1970's, exposure to toxic industrial chemicals resulted in significant impairment of herring gull reproduction at many Great Lakes sites, particularly those in Lake Ontario (reviewed in Hebert *et al.*, 1999). Poor reproductive success was indicated by decreases in the number of chicks fledged per nest, reduced eggshell thickness, incidences of nest and egg disappearance, early embryonic mortality, poor hatching success and chick death (Gilbertson, 1974;

Gilbertson and Hale, 1974a, 1974b). Although DDE residues were associated with herring gull eggshell thinning (reviewed in Gilbertson, 1974), other contaminants such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), PCBs and hexachlorobenzene (HCB) were also present at high concentrations in Great Lakes biota. Even when HCB levels were dramatically reduced by the mid 1970's and eggshell thickness had increased, reproductive impairment of the herring gulls continued (Gilman *et al.*, 1977). Thus it was strongly suspected that PCDDs, PCDFs and "dioxin-like" PCBs were the major contaminants resulting in the observed reproductive problems (reviewed in Hebert *et al.*, 1999).

As a result of regulations implemented in the late 1960's and early 1970's restricting the use and production of persistent organochlorine chemicals, levels of DDE, PCBs, PCDFs, PCDDs and other contaminants decreased dramatically. With the possible exception of PCBs (that achieved a steady state concentration in the early to mid 1980's), levels of most organochlorine contaminants measured in herring gull eggs from the Great Lakes basin continue to decline (reviewed in Hebert *et al.*, 1999). Coincidentally, the breeding success of herring gulls and other species of colonial waterbirds on the Great Lakes has improved (reviewed in Hebert *et al.*, 1999). However, as mentioned previously, several toxicological effects continue to be reported for fish-eating birds and their embryos in the most contaminated areas of the Great Lakes basin. For example, increased levels of highly carboxylated porphyrins (HCPs) reflecting altered heme biosynthesis were measured in livers of adult herring gulls collected from 1980 to 1985 (Fox *et al.*, 1988). The likely cause of the elevated HCPs was later shown to be PCBs (Kennedy *et al.*, 1998). Another

study conducted from 1992 to 1994 showed a strong exposure-response relationship between organochlorines and suppressed T-cell-mediated immunity and reduced plasma retinol (vitamin A) in both herring gull and Caspian tern *(Sterna caspia*) chicks (Grasman *et al.*, 1996). Thus, despite the fact that environmental organochlorine concentrations are 10-20 times lower than they were in the 1970's, it appears that even these levels are sufficiently high to cause measurable biological effects that may impair the health of fish-eating birds of the Great Lakes.

1.A.6. Sensitivity of the Herring Gull to Organochlorine Contaminants

Results from both field and laboratory studies often are interpreted to suggest that the herring gull may be relatively insensitive to the toxic and biochemical effects of organochlorine contaminants compared with other species of birds (Mineau *et al.*, 1984; Kennedy *et al.*, 1996a; Hebert *et al.*, 1999). For example, severe eggshell thinning was observed in pelican eggs at DDT concentrations that were approximately 5-times lower than those shown to produce similar effects in herring gull eggs (reviewed in Fry and Toone, 1981; Fry *et al.*, 1987). Egg injection studies have also shown neither mortality nor deformities in herring gull embryos exposed *in ovo* to 1000 μg/kg 3,3',4,4'-tetrachlorobiphenyl (PCB 77; IUPAC nomenclature) (Brunstrom, 1988). In contrast, embryos of various breeds of chickens responded with a mortality rate of 70-100% after exposure to doses of PCB 77 as low as 20 μg/kg (Brunstrom, 1988). However, embryos of other species, such as Mallard and domestic ducks, domestic goose, pheasant, goldeneye and blackheaded gull, were also much less sensitive to *in ovo* PCB 77 exposure than chickens. Similar to the

herring gull, embryos of these species, with the exception of the pheasant, did not show significant mortality or any deformities at PCB 77 doses as high as 100 μg/kg (Mallard duck), 1000 μg/kg (domestic goose, goldeneye, blackheaded gull) or 5000 μg/kg (domestic duck) (Brunstrom and Reutergardh, 1986; Brunstrom, 1988). Pheasant embryos exhibited 100% mortality at a dose of 100 μg/kg PCB 77 (Brunstrom and Reutergardh, 1986).

Biochemical effects associated with contaminant exposure have also been measured in herring gulls to assess the sensitivity of this species to these compounds. This work has primarily involved measurement of the induction of mixed function oxidases, particularly cytochrome P4501A (CYP1A). Many of the toxic and biochemical effects of halogenated aromatic hydrocarbons (HAHs), such as PCDDs, PCDFs, and PCBs, are thought to be mediated by a common mechanism involving binding of the HAH (ligand) to an intracellular receptor protein (Ah receptor). The receptor-ligand complex then stimulates the expression of specific genes, including those of the *CYP1A* subfamily. *CYP1A* expression, or "induction", is typically measured enzymatically as either ethoxyresorufin-*O*-deethylase (EROD) or arylhydrocarbon hydroxylase (AHH) activities. Studies conducted by Ellenton *et al.* (1985) indicated that the AHH activities of microsomal preparations from 25 day old herring gull embryos collected in 1981 were significantly higher at two Great Lakes colonies compared with an Atlantic coast colony. Furthermore, there was a significant positive correlation between site mean AHH activities and mean 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) concentrations determined in pooled egg homogenates from each site. In contrast, a subsequent

study conducted in 1982, reported EROD activities in microsomal preparations from 25 day old herring gull embryos to be much lower than that previously reported for AHH activities (Boersma *et al.*, 1986). There were no significant correlations between any of the contaminants measured, including TCDD, and activities of this enzyme (Boersma *et al.*, 1986). As different techniques were used to prepare the microsomes in these two studies it is difficult to assess whether EROD activity is a suitable endpoint for measurement of herring gull embryo CYP1A induction. Interestingly, results of a study on herring gull chick liver samples collected from the Atlantic coast indicated that while AHH and aminopyrine-*N*-demethylase (APND, an enzymatic assay for other mixed function oxidases) activities correlated with each other and cytochrome P450 concentrations, no correlations were found for EROD activities (Peakall *et al.*, 1986). These investigators suggested that EROD activity may be influenced by factors other than those that regulate AHH or APND activities. More recently, attempts to correlate EROD activities and CYP1A protein (as determined by western blot) with total PCB and organochlorine pesticide concentrations in herring gull liver samples did not reveal any significant relationships (Kennedy *et al.*, manuscript in preparation). Based on these results, the investigators suggested that herring gulls may only show EROD/CYP1A induction when exposed to very potent Ah receptor agonists such as TCDD or 3,3',3,4',5-pentachlorobiphenyl (PCB 126, IUPAC nomenclature).

Despite the possibility that EROD activity may be a poor indicator of CYP1A induction in herring gull embryos, *in vitro* studies were conducted with avian embryo hepatocyte cultures using this endpoint. Results of these studies indicated that

herring gull embryo hepatocyte cultures were approximately 50 times less sensitive than chicken embryo hepatocyte cultures to TCDD (Kennedy *et al.*, 1996a). Of all species tested (four breeds of chicken, pheasant, turkey, three breeds of domestic ducks, herring gull), the rank order of sensitivity to EROD induction was chicken > pheasant > turkey ~ duck ~ herring gull (Kennedy *et al.*, 1996a). As discussed previously, this ranking is very similar to the incidences of mortality observed after *in ovo* exposure of avian embryos to PCB 77 (Brunstrom and Reutergardh, 1986; Brunstrom, 1988). Several PCBs were also tested in hepatocyte cultures prepared from herring gull embryos and embryos of other species. The concentrations at which half-maximal EROD induction (EC50) was observed for these compounds were more variable among species, but chicken embryo hepatocyte cultures were always at least 10-fold more sensitive to EROD induction than the other species tested. Overall these results and the results obtained from *in ovo* studies suggest that although chicken embryos are exquisitely sensitive to HAHs, it is more difficult to conclusively ascertain the relative sensitivities of other species of birds using mortality or CYP1A induction (measured as EROD activity) as endpoints.

Much less is known regarding the relative sensitivities of species of birds to the physiological effects of environmental contaminants. Only effects on the thyroid gland and the reproductive system have been studied in any detail in the herring gull. Histopathological analyses of adult herring gull thyroids collected between 1974 and 1983 indicated that the majority of gulls from the Great Lakes sites suffered from goitre (Moccia *et al.*, 1986). The anatomical characteristics of the thyroids from the Great Lakes gulls were similar to those observed in humans with iodine

deficiency and included increased mass, reduced follicle size and hyperplastic epithelial components. However, based on temporal and spatial differences for sample collection time and site, and the observation of thyrotoxic effects on rodents fed similarly affected fish, these investigators concluded that HAHs were the probable cause of the altered thyroid histology (Moccia *et al.*, 1986). More recent studies have shown that the relative thyroid mass has decreased over time and as of 1991 were only slightly enlarged (Fox *et al.*, 1998). This observation is consistent with the decline in organochlorine residues documented over this period of time.

Studies on the effects of estrogen and estrogenic environmental contaminants on the herring gull reproductive system have provided interesting insight into the potential sensitivity of this species to these compounds. Early work showed that *in ovo* and post-hatching exposure of herring gulls to the synthetic estrogen, stilbestrol, resulted in partial feminization of males (Boss, 1943). These effects, persisted for 2 years or more and included alteration of the shape and structure of the left gonad to form an ovotestis characterized by an ovarian-like cortex over a testicular medulla. Further studies demonstrated that stilbestrol injected into incubating eggs interfered with the normal development of the oviducts of both sexes and treatment of the embryos prior to gonad determination and differentiation was required for these changes to occur (Boss and Witschi, 1947). These effects were observed at very low doses of stilbestrol (2.5 μg/egg, or approximately 25 ppb) (Boss and Witschi, 1947). More recently, exposure of California and western gull embryos to estradiol also resulted in feminization of male embryos and abnormal development of the right oviduct in females (Fry and Toone,

1981, Fry *et al.*, 1987). Estradiol at the lowest concentration injected (0.5 ppm, or approximately 50 μg/egg) caused extensive feminization of gull females (Fry and Toone, 1981, Fry *et al.*, 1987). In comparison, similar effects are observed in chicken and duck embryos exposed to typically 10-50-fold higher concentrations of either stilbestrol or estradiol (reviewed in Romanoff, 1960; Fry *et al.*, 1987). However, due to differences in dosage units and route of exposure, it is difficult to draw any firm conclusions from these data regarding the apparent enhanced sensitivity of herring gulls to estrogens.

Besides estrogens, such as stilbestrol and estradiol, contaminants known to be estrogenic in mammals were also studied in herring gulls and other birds. The most estrogenic DDT isomer (*o,p'*-DDT) and another organochlorine pesticide, methoxychlor, produced feminizing effects at doses as low as 2-5 ppm in male and 20 ppm in female herring gull embryos. Furthermore, the levels of DDT that caused feminization in this study were of the same magnitude as those found in seabird eggs in California in the late 1960's and 1970's and may explain the highly skewed sex ratio and reduced number of male gulls breeding in this area in the females (Fry and Toone, 1981, Fry *et al.*, 1987). Studies have also assessed the effects of commercial mixtures of DDT on the urogenital tract of chickens and quail (Lutz-Ostertag and David, 1973). Morphological and histological abnormalities of the gonads were observed in both species. However, similar to the studies with estradiol and stilbestrol, different exposure routes (egg dipped in aqueous solution *vs* egg injection) and formulations of DDT used make it difficult to compare the sensitivities of these species with that of the herring gull for this estrogenic contaminant. Thus

further studies are required to elucidate the sensitivity of the herring gull to the feminizing effects of estrogen and organochlorine contaminants such as *o,p'*-DDT.

1.B. THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN BIRDS AND BIRD EMBRYOS

1.B.1. Introduction

The hypothalamo-pituitary-adrenal (HPA) axis regulates and integrates many physiological functions in response to environmental stressors (e.g. temperature, predation) and in the maintenance of internal homeostasis. Activation of the HPA axis during a "stress response" results in the sequential release of various hormones (Fig. 1.B.1). Corticotropin releasing hormone (CRH), is released from neurosecretory cells of the hypothalamus and acts on the anterior pituitary to produce adrenocorticotropic hormone (ACTH). Subsequently, ACTH stimulates the secretion of corticosteroids (including glucocorticoids and mineralcorticoids) from the adrenal cortex. HPA axis activation resulting in corticosteroid production initiates several important physiological changes in birds including effects on intermediary metabolism, electrolyte balance, social and reproductive behaviour and permissive actions on other hormones. As well, high concentrations of corticosteroids can influence growth, immune function and inflammatory responses. Although chronic activation of the HPA axis is detrimental to reproductive success and survival, short term activation of this system enables birds and other vertebrates to respond and adapt to stressors (reviewed in Wingfield, 1994; Carsia and Harvey, 2000).

1.B.2. Embryology and Anatomy of the Avian Adrenal Gland

The origin of the avian adrenal gland is well characterized only for the domestic chicken (*Gallus domesticus*) (reviewed in Freeman and Vince, 1974a). In

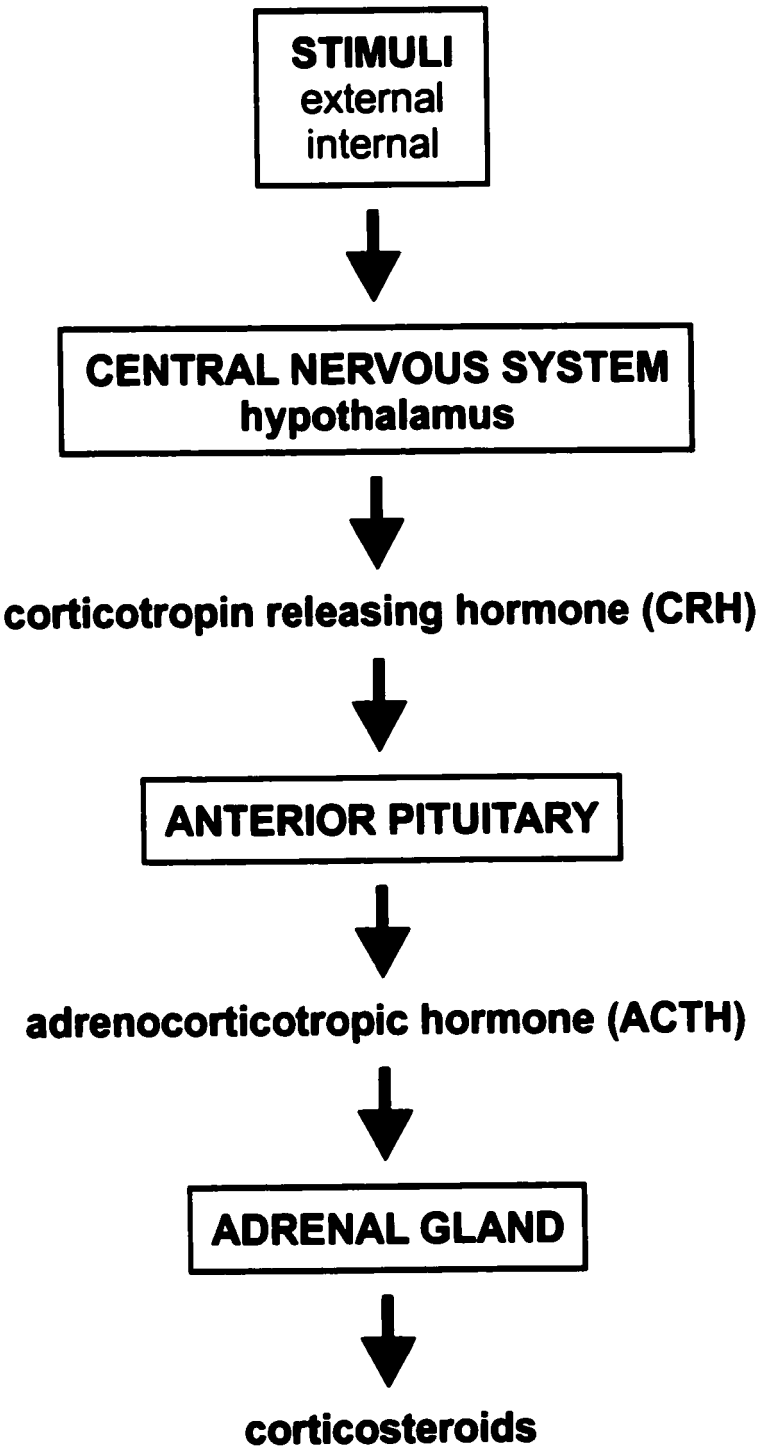


Figure 1.B.1 Activation of the HPA axis.

this species, primordial adrenal steroidogenic cells arise from the mesoderm medial to the mesonephros on day 4 of incubation. These cells develop quickly and by day 7 they are arranged in characteristic cords. The chromaffin cells (that synthesize and secrete the catecholamines epinepherine and norepinephrine) begin to associate with the steroidogenic cells (that synthesize and secrete corticosteroids) between days 4 and 5. By day 10 of incubation the adrenal glands become encapsulated (Freeman and Vince, 1974a).

Avian adrenal glands are small, usually paired structures lying in the thoracoabdominal cavity close to the lungs, gonads and kidneys. Although two distinct glands occur in the domestic chicken and duck, the glands may lie close or be fused together in other species. Furthermore, for species such as the herring gull, fusion of the two separate glands occurs in some individuals, but not in others (Hodges, 1981). Unlike mammals, the adrenal gland of birds is not divided into distinct cortical and medullary regions. Instead, the cortical or steroidogenic tissue and the medullary or chromaffin tissue are mixed together throughout the gland. The proportions of steroidogenic and medullary tissues and their spatial interrelationships differ greatly between species and between individuals of the same species (Hodges, 1981).

1.B.3. Functional Development of the HPA Axis

The functional development of the HPA axis has been thoroughly characterized in terms of 1) biochemical and ultrastructural studies; 2) *in vitro* experiments; 3) the onset and pattern of glucocorticoid secretion; 4) the ability of the

avian embryo to respond to stressful stimuli; and 5) experiments involving hypophysectomy, adrenal grafting, and/or administration of adrenal extracts.

Early observations on the functional development of the adrenal gland were made by Straznicky and colleagues (1966). These investigators noted biochemical and ultrastructural changes in the steroidogenic cells of the chicken embryo adrenal gland at days 14 to 15 of incubation. At this time, the specific enzymatic activities of Δ^5-3-keto-isomerase and Δ^5-3β-hydroxysteroid dehydrogenase gradually increase. These enzymes play important roles in the conversion of pregnenolone to progesterone and adrenal androgen production. Furthermore, just prior to the onset of enzyme activation, the mitochondria of the steroidogenic cells acquire tubules and vesicles and become round or oval shaped while the smooth endoplasmic reticulum becomes dilated. Based on decapitation studies that resulted in suppression of the ultrastructural changes and enzymatic activities of the adrenal steroidogenic cells, it was concluded that the onset of these two endpoints is due to ACTH production by the pituitary. Furthermore, other *in vitro* studies by Pedernera (1971) indicated that the chicken embryo adrenal gland could respond to ACTH as early as day 5 of incubation, but hormonal secretion by the adrenal gland in the absence of ACTH could only be detected after day 8 of incubation.

Corticosteroid levels in the developing chicken embryo were first determined by Woods and colleagues (1971). After day 14.5, hypophysectomized embryos showed significant reductions in the concentrations of adrenal corticosteroids and their metabolites. This effect was abolished when pituitaries were transplanted onto the chorioallantoic membrane. These results supported earlier findings that the

pituitary-adrenal axis became functional in the chicken embryo at days 14-15 of incubation. Other studies by Wise and Frye (1973) showed that corticosterone was present in chicken embryo plasma as early as day 10 and increased steadily through day 20 of incubation. However, the concentration of plasma corticosterone in 20 day embryos was significantly higher than that determined in newly hatched or 1 week post-hatch chicks.

Experiments involving hypophysectomy, pituitary grafts and physical stressors were conducted to determine the functional state of the HPA axis at various stages of incubation. Wise and Frye (1973) concluded that adrenal function was controlled at 3 different levels in the chicken embryo, each level being subject to control by the next higher level. First the adrenal itself was capable of autonomous (basal) secretions of corticosteroids that was not reduced in hypophysectomized embryos. This basal secretion persisted throughout the incubation period. Secondly, after day 14, pituitary control was imposed upon the adrenal and was required for the progressive rise in plasma corticosterone that occurred after day 14 of incubation. Thirdly, the hypothalamic level of control, that was necessary for the response to stress, became functional between days 14 and 16 of incubation. Overall, this work established that the chicken embryo has a typical HPA axis that is qualitatively similar to other vertebrates and that becomes functional several days before hatching.

1.B.4. Developmental Profiles of Avian Embryo Corticosteroids

Corticosterone is the predominant steroid hormone found in the blood and in

the adrenal gland of the various adult birds species studied (reviewed in Kalliecharan and Hall, 1974). Aldosterone, cortisol and cortisone have also been found, but in reduced amounts (reviewed in Kalliecharan and Hall, 1974). Unlike the hatched chick, neither corticosterone, cortisol nor cortisone predominates during embryonic development (Kalliecharan and Hall, 1974, 1976). Only late in development and post-hatching did corticosterone predominate, suggesting a switch over to the adult pattern at hatch. During embryonic development, synthesis of progesterone, corticosterone, cortisol and cortisone, occurred as early as day 9 of incubation. Furthermore the changes in concentration of each of the four steroids in the plasma reflected prior fluctuations in the adrenal glands. These data suggest that during this period the adrenal steroids are being rapidly synthesized, and support previous studies showing that pituitary control of the adrenal gland may begin during this time period (Wise and Frye, 1973; Kalliecharan and Hall, 1974). More recently it has been shown that radioimmunoassay (RIA) procedures can be used to determine concentrations of corticosterone in chicken embryo plasma (Scott *et al.*, 1981). Results of these studies demonstrated a similar pattern of corticosterone secretion as shown previously using fluorometric (Woods *et al.*, 1971) or competitive protein binding assays (Wise and Frye, 1973; Kalliecharan and Hall, 1974, 1976). The structures and biosynthetic pathways for corticosterone, cortisol and cortisone are illustrated in Figure 1.B.2.

Corticosteroid binding globulin (CBG or transcortin) has also been characterized in chicken embryo plasma during development. Siegel and Gould (1976) noted that the CBG binding capacity for corticosterone was negligible before

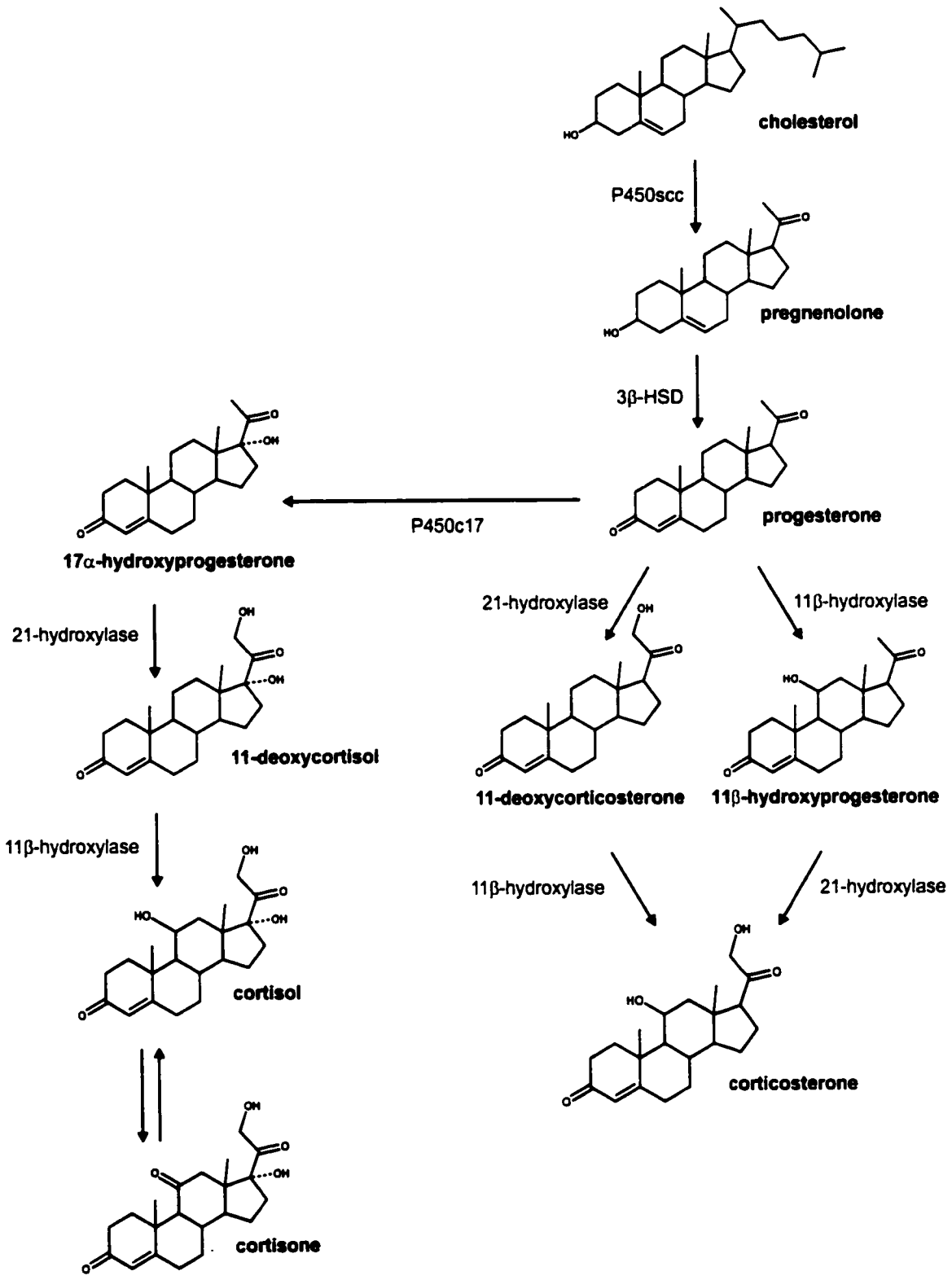


Figure 1.B.2 Biosynthetic pathways for corticosterone, cortisol and cortisone. P450c17 = 17α-hydroxylase/C_{17-20} lyase; 3β-HSB = 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase. Modified from Harvey *et al.*, 1986.

day 10, but rose rapidly between days 10 and 12 of incubation. The binding capacity of CBG for corticosterone remained high until day 16 of incubation and then declined before hatch. Plasma corticosterone concentrations were also measured in the same study and showed a pattern similar to that obtained by Kalliecharan and Hall (1974). This pattern of CBG binding activity was later confirmed for corticosterone and other C_{21} steroids in a study by Martin *et al.* (1977). Based on these data, both groups of investigators concluded that since the unbound fraction of corticosteroids is believed to represent the biologically active component, then an essential role for these hormones late in embryonic development was likely when CBG binding activity was low.

1.B.5. Functions of Corticosteroids in the Avian Embryo

Adrenal corticosteroids play key roles in regulation of water and mineral balance, carbohydrate, fat and protein metabolism, and stress tolerance in adult birds and mammals. Although little is known regarding the function of corticosteroids in the avian embryo, these hormones appear to have physiological actions, particularly during the second half of incubation. Gradual increases in hormone levels for an extended period of time are necessary for the differentiation of the duodenal epithelium and the abrupt increase in alkaline phosphatase levels in the chicken embryo (Moog and Richardson, 1955; Moog, 1961). Hypophysectomy studies with chicken embryos showed that alkaline phosphatase activity was essential for early ontogenic processes of bone differentiation and skeletal development (McWhinnie and Thommes, 1973). Results with low concentrations of

exogenously administered hormones suggested that corticosterone triggered the synthesis of surfactant phospholipid and influenced the growth and hydration of the chicken embryonic lung, presumably in preparation for hatching and exposure to a gaseous environment (Hylka and Doneen, 1983). Adrenal corticosteroids may also be involved in the maturation of the exocrine pancreas and the gonads, cholesterol metabolism, and the stimulation of Na^+/K^+-ATPase and adrenal phenylethanolamine-N-methyltransferase (PNMT) activities (reviewed in Freeman and Vince, 1974a).

Corticosteroids in the avian embryo may also be involved in water and mineral balance and embryonic kidney function. Hypophysectomy of chicken embryos resulted in a greater allantoic fluid volumes during days 15 to 17 of incubation than those characteristic of the intact embryo (Woods *et al.*, 1971). The amniotic fluid volumes were partially restored when pituitaries were transplanted into hypophysectomized embryos. In other studies, type II glucocorticoid receptors peaked at day 15 of incubation in chicken embryonic kidney (Beaudry *et al.*, 1983; Bellabarba *et al.*, 1983).

The *in ovo* administration of pharmacological concentrations of corticosteroids at various stages of incubation has also been used to characterize the functions of these adrenal hormones in avian embryo development. When day 8 to 10 chicken embryos were treated with large doses of corticosteroids, embryonic growth was retarded (Karnofsky *et al.*, 1951; Moog and Richardson, 1955), but administration of the same dose to day 16 embryos resulted in slight weight increases (Moog and Richardson, 1955). Cortisone acetate was also found to

markedly inhibit the growth of chicken embryos prior to day 8 of incubation (Karnofsky *et al.*, 1951). High concentrations of corticosterone administered to chicken embryos resulted in condensation of yolk (Wishart *et al.*, 1977), delayed hatching and decreased hatchability (Brooks and Ungar, 1967). In contrast, hatchability of turkey embryos increased when high circulating concentrations of corticosterone (540 ng/embryo) were present just prior to hatching (Wentworth and Hussein, 1985). Based on these results, it is apparent that the avian embryo is sensitive to exogenous corticosteroid administration. However, the large doses of corticosteroids employed in these experiments renders the physiological significance of these observations obscure.

1.B.6. Environmental and Genetic Influences on HPA Axis Activation

The physiological significance of corticosteroids in the avian embryo has also been characterized by the ability of the embryo to respond to stress with activation of the HPA axis. Avian embryos are well-suited for these studies. First, there is no interfering connection of the embryo to a maternal organism. Second, after day 15 of incubation, chicken embryos have a sufficiently mature HPA axis for studying its reactivity towards stressors *in ovo*, thus avoiding the influence of many complicating external environmental factors.

The effect of temperature change on the HPA axis in developing chicken embryos has been examined. Studies by Jacobs (1996) indicated that continued exposure of 18 or 19 day old embryos to cold temperatures (22 or 12°C) did not elevate plasma corticosterone concentrations. In contrast, embryos incubated for

17, 18, 20 or 21 days showed a significant response to continuous heating (43°C) as indicated by elevated plasma corticosterone levels. In a separate experiment, cooling of embryos to 12°C then re-warming at 38°C (normal incubation temperature) resulted in a rapid peak in corticosterone levels within 30 min. Other studies by Al-hassani (1993) indicated that chicken embryos exposed to 10°C for 30 min on day 16 of incubation had increased hatchability, decreased mortality, and decreased liver glycogen levels compared to controls. Although corticosteroid levels were not measured in this study, it can be hypothesized that the presence of a functional HPA axis triggered the observed physiological responses.

Avrutina *et al.* (1984) investigated genotypic influences on the HPA axis using embryos of two strains of chickens selected for either high or low reactivity towards a cold stress. Results of these studies indicated that the highly reactive strain responded with maximal synthesis and secretion of glucocorticoids 30 min after ACTH injection, whereas in the low reactivity strain, peak levels were reached 1 h after ACTH injection. Furthermore, exposure of 18 day old embryos from each of these strains to a heat stress (3 h at 41-41.5°C) led to a greater increase in the glucocorticoid content in the adrenals of 18 day old embryos from the highly reactive strain in comparison to the low reactivity strain. Similar studies conducted by Decuypere *et al.* (1989) showed that 30 min after ACTH treatment, corticosterone concentrations were nearly twice as high in 18 day old embryos from a broiler strain of chickens compared with 18 day old embryos from a layer strain of chickens. Overall, these studies established the important role of genotype in influencing the functional capacity of the HPA axis in the developing avian embryo.

1.B.7. Corticosteroid Pharmacology

The basic parameters of adrenal steroidogenic function in avian embryos were assessed in one study. Carsia *et al.* (1987) evaluated basal and maximally stimulated production of corticosterone, cortisol and aldosterone and the effects of steroidogenic agents in enriched adrenal steroidogenic cell populations prepared from 18, 19, 20 or 21 day chicken embryos. Basal corticosteroid production by isolated adrenal steroidogenic cells was predominated by corticosterone for all ages of embryos studied. On average corticosterone concentrations were 8- and 48-times greater than basal aldosterone and cortisol production, respectively. Similarly, corticosterone was the predominant corticosteroid produced by isolated cells stimulated for 2 h by ACTH, such that corticosterone concentrations were on average 52- and 115-times greater than maximal aldosterone and cortisol production, respectively. Based on these data it was concluded that since aldosterone predominated over cortisol, the 21-hydroxylase pathway predominated over the 17α-hydroxylase pathway in the chicken embryo (see Fig. 1.B.2).

The responses of isolated adrenal steroidogenic cells from 18 and 19 day chicken embryos were determined in the same study for a wide range of concentrations of ACTH, 8-Br-cyclic AMP (8-Br-cAMP) and the steroid precursor, 25-hydroxycholesterol (Carsia *et al.*, 1987). For 18 day embryos, maximal corticosterone production after ACTH and 8-Br-cAMP treatment were both ~70-fold higher than basal corticosterone levels. Treatment of cells with 25-hydroxycholesterol resulted in a maximal corticosterone concentration that was ~50-fold greater than basal concentrations. For adrenal steroidogenic cells prepared

from 19 day embryos a similar pattern was observed, but all concentrations of corticosterone, including basal concentrations, were significantly higher. In terms of potency of the three steroidogenic agents tested, ACTH elicited half-maximal (EC50) values (18 day, 0.01 nM; 19 day, 0.0065 nM) which were much less than those of either 8-Br-cAMP (~180,000 nM) or 25-hydroxycholesterol (~3,000 nM) in cells prepared from either 18 or 19 day embryos. Statistically significant age-dependent changes in potency were only observed for ACTH. For this compound, adrenal steroidogenic cells prepared from 18 day embryos were less sensitive than cells prepared from 19 day embryos. Based on these data it was concluded that the differences in steroidogenic capacity between 18 and 19 days of development appeared to be due to differences in the amount of steroidogenic enzymes within the cells.

1.B.8. Adrenal Corticosteroid Regulation of Intermediary Metabolism

HPA axis activation, with subsequent adrenal steroid hormone release, affects the activities of various key intermediary metabolic enzymes in mammals. However, much less is known regarding the effects of corticosteroids on avian intermediary metabolism. Since the mammalian literature is more complete, general aspects of corticosteroid regulation of intermediary metabolism will first be considered for this class of animals. Subsequently, comparisons will be made to birds where sufficient data exist. The major metabolic pathways thought to be regulated by corticosteroids include glycolysis, gluconeogenesis and lipogenesis (Fig. 1.B.3). In mammals, corticosteroid effects on intermediary metabolism are

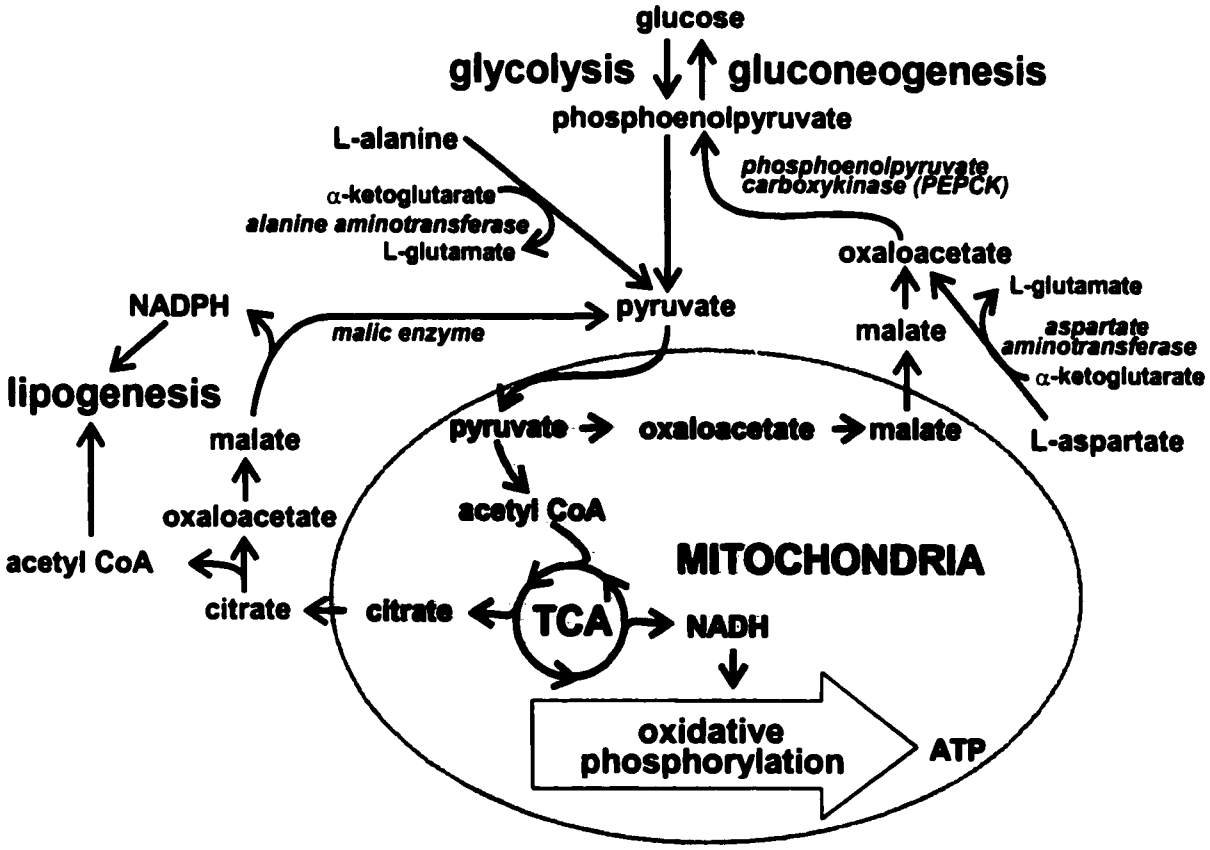


Figure 1.B.3. Simplified view of metabolic pathways and enzymes measured in this study.

essentially antagonistic to the effects of insulin. Stimulation of most, if not all, of the enzymes involved in gluconeogenesis involves increased hepatic synthesis of enzyme proteins (reviewed in Pilkis and El-Maghrabi, 1988; Gumbleton and Nicholls, 1996). However, because enzyme induction generally requires a time period in the order of hours, it cannot account for the earliest effect of corticosteroids on gluconeogenesis. Recent studies have indicated that corticosteroids also interact with other regulatory factors which influence the transcription of gluconeogenic enzymes (reviewed in Gumbleton and Nicholls, 1996).

In addition to effects on specific intermediary metabolic enzymes, corticosteroids regulate carbohydrate metabolism by many other mechanisms (reviewed in McMahon *et al.*, 1988). Corticosteroids enhance gluconeogenesis by inhibiting glucose uptake and utilization by peripheral tissues and have permissive effects on the actions of other gluconeogenic hormones, such as glucagon and epinephrine (Exton, 1979; Fain, 1979; reviewed in Goldberg *et al.*, 1980; Malbon *et al.*, 1988). Glucocorticoids also promote mobilization of amino acids from muscle protein, possibly by inhibiting the stimulatory effect of insulin on protein synthesis (Goldberg *et al.*, 1980). Inhibition of peripheral glucose uptake and glycerol production by corticosteroids results in increased lipolysis (due to a permissive effect on growth hormone) and elevated plasma free fatty acid levels (Fain, 1979). These effects are further potentiated by the permissive actions of corticosteroids on catecholamine-mediated lipolytic pathways (Birnbaum and Goodman, 1977). However, corticosteroid-induced lipolysis is generally counteracted by the lipogenic

actions of insulin; the net result being fat re-distribution as opposed to loss of adipose tissue (Fain, 1979). Corticosteroid stimulation of gluconeogenesis also results in increased glycogen deposition, hyperglycemia and decreased glucose tolerance. Generally, these effects are also compensated by increased insulin secretion.

The effects of corticosteroid deficiency on intermediary metabolism have also been characterized. Generally, in fed adrenalectomized animals, basal hepatic gluconeogenesis is not impaired, although there is a decreased response to glucagon or catecholamines, and an increased sensitivity to insulin (Exton, 1979; Gumbleton and Nicholls, 1996). However, in fasted or diabetic animals adrenalectomy results in decreased gluconeogenesis that is reversed by cortisol or corticosterone treatment (Exton, 1979). These data support corticosteroids being required for the gluconeogenic response to fasting and in insulin deficiency, but not for basal gluconeogenesis in the fed state (Exton, 1979).

Corticosteroids in birds have the same general metabolic roles as in mammals. In particular, corticosterone plays an important role in carbohydrate and lipid metabolism (reviewed in Hodges, 1981). For example, corticosterone in birds increases gluconeogenesis by stimulating protein catabolism. Long-term corticosteroid treatment of chickens results in hepatic glycogen deposition and an insulin-resistant hyperglycemia that persists for as long as hormone treatment is continued (Stamler *et al.*, 1954; Greenman and Zarrow, 1961; reviewed in Hazelwood, 1986). As in mammals, adrenalectomy results in hypoglycemia under conditions of fasting. However, unlike mammals where lipolysis is stimulated by

corticosteroids, fat *deposition* is favoured in corticosteroid-treated birds. This is evident as both an increase in the synthesis of fatty acids and an increase in the ratio of saturated to unsaturated fatty acids (Nagra and Meyer, 1963). These data imply that, in birds, the potent lipogenic effects of insulin dominate the lipolytic effect of corticosteroids typically observed in mammals.

1.B.9. Review of Selected Metabolic Enzymes

A number of metabolic enzymes are influenced by corticosteroids. Those measured in the present study were phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (ALAT), aspartate aminotransferase (ASPAT) and malic enzyme (ME). Fig. 1.B.3. illustrates that PEPCK is a key gluconeogenic enzyme and is involved in the production of glucose from non-carbohydrate precursors. The aminotransferases, ALAT and ASPAT, are supporting enzymes of gluconeogenesis and supply carbon skeletons from the amino acids alanine and aspartate for use in this pathway. Malic enzyme has a function that is distinct from gluconeogenesis, supplying reducing equivalents in the form of NADPH for lipogenesis or fatty acid synthesis. Fatty acid synthesis is required for several processes during cell membrane synthesis and energy storage. The following text provides an overview of each of these enzymes in birds and how corticosteroids may regulate their activities.

1) Phosphoenolpyruvate Carboxykinase (PEPCK)

In birds and mammals, PEPCK is a key regulatory gluconeogenic enzyme catalyzing the conversion of oxaloacetate to phosphoenolpyruvate (PEP):

$$\text{oxaloacetate} + \text{GTP} \leftrightarrow \text{PEP} + \text{GDP} + CO_2$$

Approximately 70% of gluconeogenesis in the domestic fowl occurs in the liver and the remainder occurs in the kidney (Tinker *et al.*, 1986). The efficacy of various metabolites as gluconeogenic precursors in the avian liver is: lactate ~ glycerol > pyruvate > alanine > asparate > serine (Langslow, 1978). Although lactate is the best substrate for gluconeogenesis in avian liver, pyruvate is the preferred substrate in mammalian liver. This difference is thought to be due to a limited supply of NADH which is required for the reduction of 1,3-diphosphoglycerate to triose phosphates in avian liver cytosol (Langslow, 1978; Watford, 1985; Stevens, 1996). Thus, as shown in Fig. 1.B.4, the probable mechanism for preferentially using lactate for gluconeogenesis in avian liver is as follows:

1) cytosolic lactate is oxidized to pyruvate, generating NADH for the reduction of 1,3- diphosphoglycerate;
2) pyruvate enters the mitochondrial matrix where both pyruate carboxylase and PEPCK are present; and,
3) the PEP formed is transported out of the mitochondria and then converted to glucose in the cytosol.

Pyruvate, or alanine which can be transaminated to pyruvate (see below), could also be converted to PEP by the same route, but use of these substrates would not generate the cytosolic NADH required for gluconeogenesis (Stevens, 1996).

Avian hepatic PEPCK is present almost exclusively in the mitochondria, whereas in most laboratory rodents it is present primarily in the cytosol (Söling *et al.*, 1973; Watford, 1985). Furthermore, it has been demonstrated immunochemically that most PEPCK activity present in the cytosolic fraction of adult chicken liver may

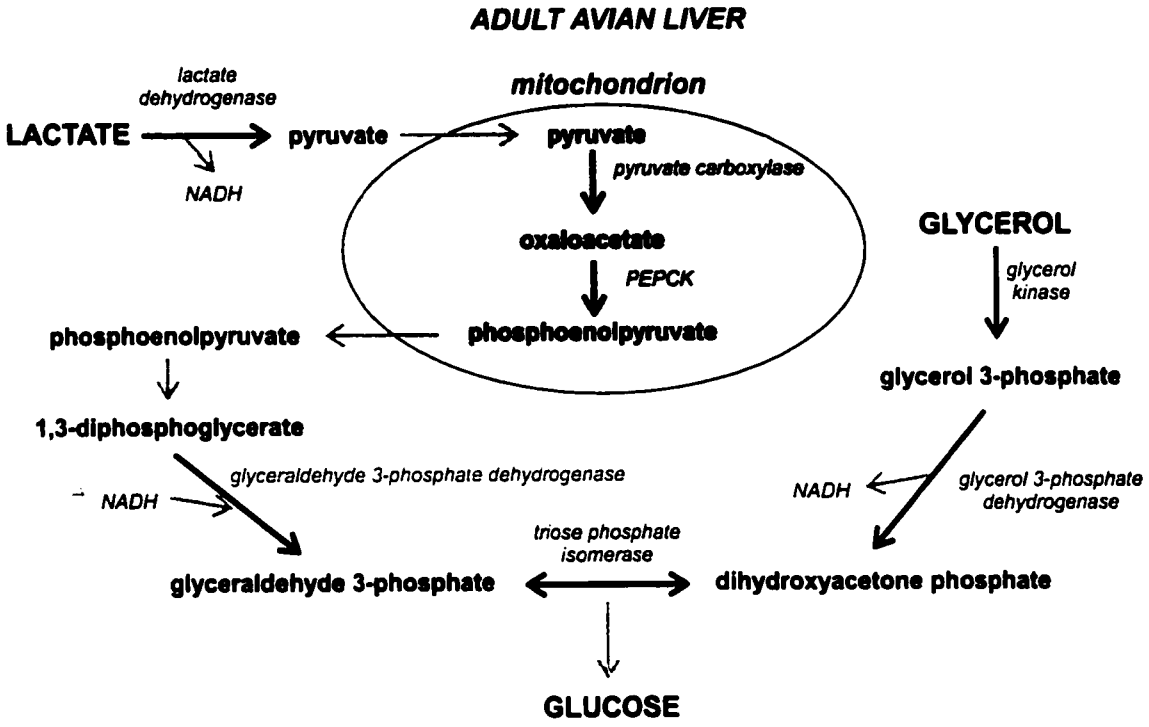


Figure 1.B.4. Preferred substrates and gluconeogenic pathways in adult avian liver.

actually be the mitochondrial form of the enzyme (Watford *et al.*, 1981). In contrast to adult avian liver, avian kidney has two distinct PEPCK isozymes - mitochondrial and cytosolic - such that between 20-50% of kidney PEPCK activity is present in the cytosol (Shen and Mistry, 1979; Watford *et al.*, 1981). Similar to mammalian liver, pyruvate is a better gluconeogenic substrate than lactate for avian kidney cytosolic PEPCK (Fig 1.B.5). The two avian kidney PEPCK isozymes have been purified and their complementary DNAs (cDNAs) sequenced (Weldon *et al.*, 1990). Since only ~60% cDNA homology was detected, it was concluded that these two isozymes are products of distinct genes. Furthermore, it has been determined that whereas the mitochondrial isoenzyme is constitutive, the cytosolic form adapted in response to dietary or hormonal control, thereby enabling pyruvate and amino acids to be converted to glucose (Watford, 1985).

Although hepatic PEPCK is located primarily (>90%) in mitochondria of adult birds (Söling *et al.*, 1973; Watford, 1985), both mitochondrial and cytosolic forms are present in the liver of the developing avian embryo (Felicioli *et al.*, 1967; Hamada and Matsumoto, 1984; Savon *et al.*, 1993). Based on a study by Savon *et al.* (1993) hepatic mitochondrial PEPCK mRNA was present at high levels throughout development of the avian embryo. In the same study, hepatic cytosolic PEPCK was also expressed throughout the embryonic period, but mRNA levels gradually decreased as development proceeded and became negligible at the time of hatching. In contrast, both Felicioli *et al.* (1967) and Hamada and Matsumoto (1984) observed that hepatic cytosolic PEPCK activity increased just prior to hatching and did not decrease until after hatching. In the carbohydrate limiting environment of the

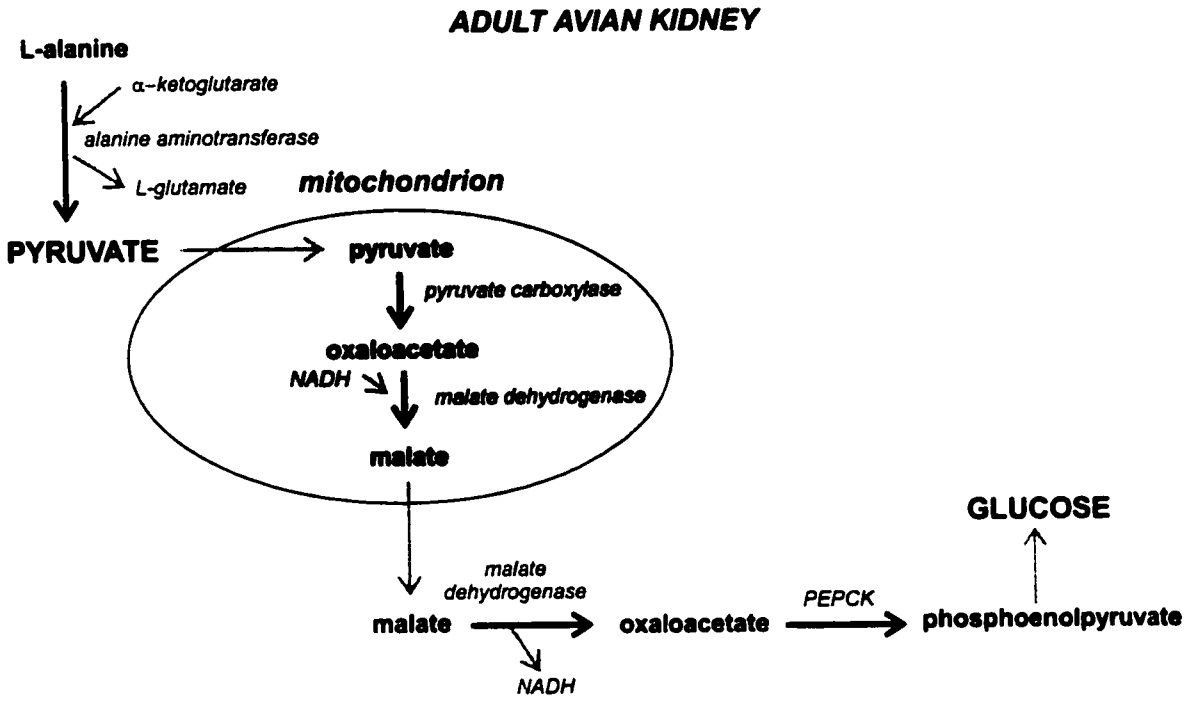


Figure 1.B.5. Adult avian kidney gluconeogenic pathway involving cytosolic PEPCK.

egg, the avian embryo synthesizes glucose from glycerol and amino acids during development. Furthermore, studies have shown that the incorporation of ^{14}C amino acids and $^{14}CO_2$ into glucose by liver slices from embryonic chickens is temporally associated with the activity of the gluconeogenic enzymes, pyruvate carboxylase, fructose 1,6-diphosphatase, glucose 6-phosphatase and PEPCK (Nelson *et al.*, 1966; Yarnell *et al.*, 1966). Thus, it has been suggested that the role of the cytosolic form of hepatic PEPCK in the avian embryo is necessary for maximal glucose production from amino acids (Savon *et al.*, 1993).

Both cytosolic and mitochondrial PEPCK activities have been demonstrated in chicken embryo kidney preparations as in embryonic liver (Shen and Mistry, 1979). In this study, the activities of mitochondrial and cytosolic PEPCK were low and increased from 8 days before hatching until the time of hatching. After hatching, mitochondrial PEPCK activity continued to increase for 8 days and then returned to a value similar to that observed near the time of hatching. Cytosolic PEPCK activity decreased slightly at hatching to a value similar to that observed in 30-58 day chickens.

In all animals studied to date, the mitochondrial form of PEPCK is constitutive while the cytosolic form is subject to long-term, or adaptive, regulation (Watford, 1989). Although, cytosolic PEPCK expression is modified by many hormones, dietary factors, and metabolic acidosis (reviewed in Watford, 1985, 1989; Pilkis and El-Maghrabi, 1988) only the regulatory effects involving adrenal corticosteroids will be considered here. In isolated chicken kidney tubules, cytosolic PEPCK mRNA was increased after incubation with the potent synthetic glucocorticoid, dexamethasone

(Watford, 1989). Similarly, in other studies using an avian hepatoma cell line (LMH), that expresses both the cytosolic and mitochondrial forms of PEPCK, dexamethasone treatment resulted in a three-fold increase in cytosolic PEPCK mRNA (Savon *et al.*, 1993). As expected, the expression of the mitochondrial PEPCK form was completely unresponsive to glucocorticoid treatment.

Experiments on the *in vivo* developmental regulation of basal and corticosteroid-inducible hepatic cytosolic PEPCK gene expression have also been conducted with chicken embryos. McCaffrey and Hamilton (1994) determined that embryo hepatic cytosolic PEPCK mRNA was constitutively expressed only between days 12 and 16 of incubation and was transiently inducible by dexamethasone. Induction by dexamethasone progressively diminished between days 12 and 15 of incubation, such that by day 16 expression of chicken embryo hepatic cytosolic PEPCK mRNA was no longer responsive to dexamethasone. Similar studies have not been conducted for avian embryo kidney cytosolic PEPCK.

Based on the results of these studies, cytosolic PEPCK expression in early embryonic and adult birds is sensitive to glucocorticoids. However, the mechanism of action by which glucocorticoids regulate this expression is complex. Increased mRNA levels after dexamethasone treatment of isolated kidney tubules, LMH cells or chicken embryos during days 12 to 15 of incubation indicate that glucocorticoid receptor-mediated pathways are likely involved (Watford, 1989; Savon *et al.*, 1993; McCaffrey and Hamilton, 1994). However, recent mammalian studies indicate that glucocorticoids may also stabilize PEPCK mRNA by inducing the synthesis of another "stabilizing" protein that binds to the 3'- end of the message (Gumbleton

and Nicholls, 1996). Thus, corticosteroids probably also regulate PEPCK activity by post-transcriptional mechanisms in birds.

2) Alanine Aminotransferase (ALAT)

All aminotransferases involve the transfer of the α-amino group from L-amino acids to the α-carbon atom of α-ketoglutarate. The products are the α-keto acid of the reacting L-amino acid plus L-glutamate. In the case of ALAT the reaction is

L-alanine + α-ketoglutarate ↔ pyruvate + L-glutamate

The pyruvate produced can be directly utilized for gluconeogenesis, while L-glutamate can be re-oxidized to α-ketoglutarate and ammonia by glutamate dehydrogenase. The α-ketoglutarate formed by this reaction can be utilized in the tricarboxylic acid (TCA) cycle and the ammonia can be excreted (Griminger and Scanes, 1986).

Essentially no cytosolic ALAT activity was observed in chicken liver, unlike the rat liver where approximately 85% of ALAT activity is located in the cytosol and the remainder is primarily mitochondrial (DeRosa and Swick, 1975). Similarly, in the same study, negligible ALAT activity was present in chicken kidney cytosol, whereas in the rat, essentially all kidney ALAT activity was confined to the cytosolic fraction. It was concluded that high levels of ALAT activity were associated with the subcellular fraction having high levels of PEPCK activity (see above). In the rat, where hepatic PEPCK activity is primarily cytosolic, hepatic ALAT activity was also primarily cytosolic, and in the chicken, where hepatic PEPCK activity is primarily mitochondrial, ALAT activity was also primarily mitochondrial. The explanation for this possible co-localization was based on the interconversions required to enter the

gluconeogenic pathway by pyruvate (DeRosa and Swick, 1975). In species such as the rat, where hepatic PEPCK is primarily cytosolic, oxaloacetate formed in the mitochondria must first be converted to malate or aspartate in order to cross the mitochondrial membrane (see Fig. 1.B.3.). This transfer is coordinated by the malate-aspartate shuttle that involves aspartate aminotransferase (see next section). Thus, ALAT may ultimately regulate the supply of oxaloacetate to PEPCK in the cytosol. On the other hand, when PEPCK is located primarily in the mitochondrial compartment, as in chicken liver (see Fig. 1.B.4.), phosphoenolpyruvate is freely diffusible across the mitochondrial membrane and no transamination reactions are involved when lactate is the precursor. Thus in this situation, ALAT activity is simply associated with intramitochondrial transamination of alanine to pyruvate to be used as a substrate by pyruvate carboxylase to produce oxlaloacetate which is subsequently converted to oxaloacetate by PEPCK. Although the apparent localization of ALAT in the chicken liver mitochondria can be logically related to its co-localization with other enzymes in the gluconeogenic pathway (DeRosa and Swick, 1975), these investigators failed to explain why chicken kidney ALAT, that was also determined to be essentially mitochondrial, is not co-localized with the relatively high concentration of cytosolic PEPCK in the avian kidney. In fact, it has been reported in another study that ALAT is present in both the cytosolic and mitochondrial fractions of chicken liver in approximately a 2:1 ratio (Sarkar, 1974). Thus, despite the use of markers or controls to ensure their preparations were either purely mitochondrial or purely cytosolic in both the study by Sarkar (1974) and the study by DeRosa and Swick (1975), further experiments are required to define the

subcellular distribution of ALAT in avian liver and kidney and its physiological function.

3) Aspartate Aminotransferase (ASPAT)

ASPAT catalyzes the reaction

L-aspartate + α-ketoglutarate ↔ oxaloacetate + L-glutamate

The oxaloacetate produced can be directly utilized in the TCA cycle. As mentioned briefly, both ASPAT and ALAT may play roles in the movement of malate across mitochondrial membranes in tissues, such as rat liver and adult avian kidney, where PEPCK is found in the cytosol (see Fig. 1.B.2. and above). It is uncertain whether the malate-asparate shuttle is also important for avian liver gluconeogenesis. A study by Söling *et al.* (1973) indicated that, since aminooxyacetate (an inhibitor of ASPAT) did not decrease hepatic gluconeogenesis from lactate, the translocator role of ASPAT was probably not important in avian liver. However, a more recent study by Ochs and Harris (1980), indicated that aminooxyacetate treatment *did* block hepatic lactate gluconeogenesis when pyruvate concentrations were lower and the incubation period was longer than that used by Söling and colleagues (1973).

ASPAT activity has been reported to predominate in the cytosolic fraction, with only 6% associated with the mitochondrial fraction in several species of birds (Sarkar, 1974). Furthermore, the activity of chicken hepatic ASPAT was considerably higher than ALAT (Sarkar, 1974). Interestingly, in the same study it was also demonstrated that, unlike the rat and guinea pig, fasted adult chickens were unable to use aspartate for glucose production. The physiological significance

of these findings have not been established.

4) Malic Enzyme (ME)

Malic enzyme provides reducing equivalents in the form of NADPH for the synthesis of *de novo* long-chain fatty acids in both birds and mammals. The main site of lipid biosynthesis in birds is liver, not adipose tissue, such that approximately two thirds of the total lipid biosynthesis in the domestic fowl occurs in the liver (Saadoun and LeClercq, 1983). ME is present in hepatic cytosol and it catalyzes the reaction

$$\text{malate} + NADP^+ \leftrightarrow \text{pyruvate} + CO_2 + NADPH$$

ME in pigeon liver is a tetramer composed of four identical 65 kDa subunits. A detailed study of its kinetics have been made and the cloning and expression of its cDNA carried out (Chou *et al.*, 1994, and references therein). Studies by Goodridge (1968) indicated that, shortly after hatching in chickens, increases in the activity of ME occurred simultaneously with a marked increase in hepatic lipogenesis. The basal activity of ME in pigeon liver homogenate was determined to be about 33-times higher than in rat liver and about 260-times higher than in guinea pig liver (Söling *et al.*, 1973).

Although little information is available on the hormonal, and more specifically, corticosteroid, regulation of ME activity, both nutritional and hormonal factors are generally regarded as the main controls of fatty acid synthesis in birds (Stevens, 1996). The principal hormones that influence lipid biosynthesis in the avian liver are insulin, glucagon, triiodothyronine and glucocorticoids (Stevens, 1996). ME activity was found to be very low in early embryonic ducks, and did not increase until the

newly hatched ducklings were fed (Goodridge *et al.*, 1984). In the same study, starvation of 4 day old ducklings resulted in a pronounced decrease in ME mRNA in the liver. Based on these data it was concluded that, in the duck, ME was regulated by both developmental and nutritional factors and that this regulation occurred primarily at a pretranslational level. Similarly, in another study, feeding of neonatal chickens resulted in increased abundance of mRNAs for ME and fatty acid synthase, while starvation decreased their abundance (Morris *et al.*, 1984).

1.B.10. Toxicology of the Adrenal Gland

The adrenal cortex is a major endocrine target for exogenous substances (Ribelin, 1984). Chemical effects on membrane receptors, cyclic nucleotide levels, protein synthesis and steroid biosynthetic pathways may compromise adrenal and target organ function. Furthermore, the high lipid content of the adrenal cortex is thought to contribute to its susceptibility to contaminants. Since most xenobiotics that enter the body are hydrophobic, the lipid-rich adrenal cortex promotes their deposition and accumulation. In addition, the adrenal has the potential to bioactivate exogenous compounds that are deposited here. It has recently been established that adrenal monooxygenases (P450 family) not only participate in steroid metabolism, but also have the capacity to metabolize xenobiotics, in some cases producing toxic metabolites (Colby and Longhurst, 1996).

Few studies have been conducted to document and characterize the effects of complex mixtures of environmental contaminants on the HPA axis in birds or other species. Mallard ducks fed a petroleum-treated diet showed significantly

higher mortality than untreated controls after exposure to a continuous mild cold stress (Holmes *et al.*, 1979). In the same study, adrenal hypertrophy and lymphoepithelial involution were apparent in all of the birds that died, suggesting that a high level of adrenocortical stimulation preceded death. Other studies by Hontela and colleagues that characterized adrenal and HPA axis function in wild fish and amphibians strongly indicated that HPA axis-related endpoints are sensitive to complex mixtures of environmental contaminants including organochlorines (reviewed in Hontela, 1997). These data support the hypothesis that HPA axis-related endpoints are useful indicators of contaminant exposure and potential toxicity in wild animals. To my knowledge, no prior HPA axis studies have considered the potential toxicological effects of complex mixtures of environmental organochlorine contaminants in birds.

1.C. AVIAN VITELLOGENESIS

1.C.1. Overview

One of the potentially most useful endpoints when evaluating the estrogenicity or anti-estrogenicity of environmental pollutants in wild fish, amphibians, reptiles and birds both *in vivo* and *in vitro* is based on vitellogenin (VTG) induction. VTG is a lipophosphoglycoprotein of the very high density lipoprotein (VHDL) class that is produced after stimulation of hepatocytes of female oviparous vertebrates with estrogen (Ho, 1991). In mature female breeding birds, the process of VTG induction begins with hypothalamic secretion of gonadotropin releasing hormone (GnRH) which stimulates the pituitary to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH, and possibly FSH, are involved in ovarian and follicle development and stimulate steroidogenesis. As a result, plasma estrogen (primarily 17β-estradiol) concentrations increase and specific binding to hepatic estrogen receptors occurs (see Fig. 1.C.1). The receptor-ligand complexes then interact with specific estrogen-response-elements (EREs) associated with VTG genes. Although, the genetic information to produce VTG is also present in male and juvenile birds, physiological levels of estrogen are normally only sufficient to stimulate VTG synthesis in laying female birds. Once synthesized in the hepatocyte, VTG is secreted from the liver and taken up from the blood into developing oocytes by receptor-mediated endocytosis. Within the oocyte the VTG protein is enzymatically cleaved into two major phosphoproteins - phosvitin and lipovitellin. The developing embryo uses these VTG breakdown products as sources

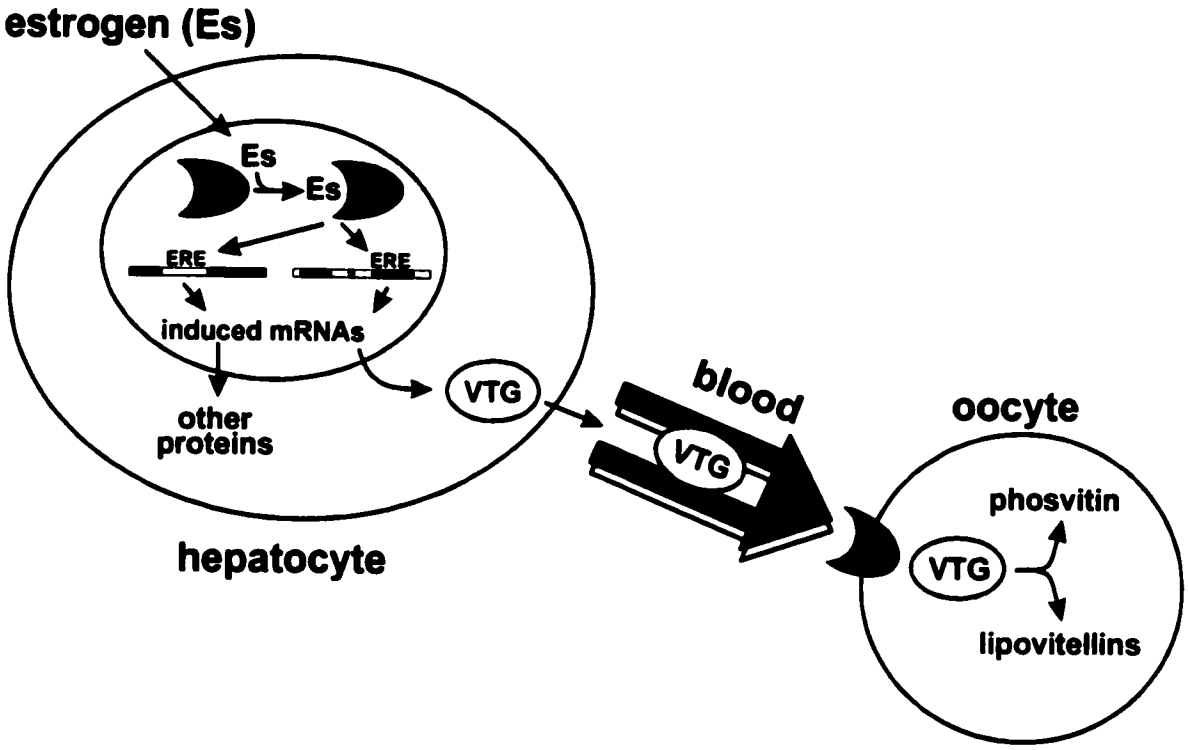


Figure 1.C.1 A simplified view of vitellogenesis. ERE = estrogen response element.

of nutrition during incubation (reviewed in Burley and Vadehra, 1989; Etches, 1996; Stevens, 1996).

1.C.2. Physiology of Yolk Formation and Use by the Developing Embryo

Unlike mammals, the embryonic development of oviparous animals is dependent on energy reserves stored in the form of yolk. Primary oocytes can be identified by day 12 of incubation in female chicken embryos (Burley and Vadehra, 1989). After the chicken hatches, these primary oocytes are transformed into ovarian follicles by the addition of an outer layer of flat follicle cells (presumptive granulosa cells) and connective tissue. The oocytes remain in this state until the deposition of yolk commences prior to egg laying.

The yellow yolk of the egg is composed of tightly packed spheres that contain proteins and lipids. Of the lipid fraction, 95% is composed of very low density lipoproteins (VLDLs) which have molecular weights between 3 and 10 million Da (Etches, 1996). Twelve percent of the yolk protein consists of apoproteins associated with the yolk VLDLs. Phosvitin and lipovitellins, that are derived from the precursor molecule VTG, account for 23% of the yolk protein and most of the remaining 5% of the yolk lipid. As well, phosvitin accounts for approximately 80% of the protein-bound phosphorous within the yolk. The remainder of the protein component consists largely of plasma albumin, plasma glycoprotein and the immunoglobulin subclass IgG (Etches, 1996).

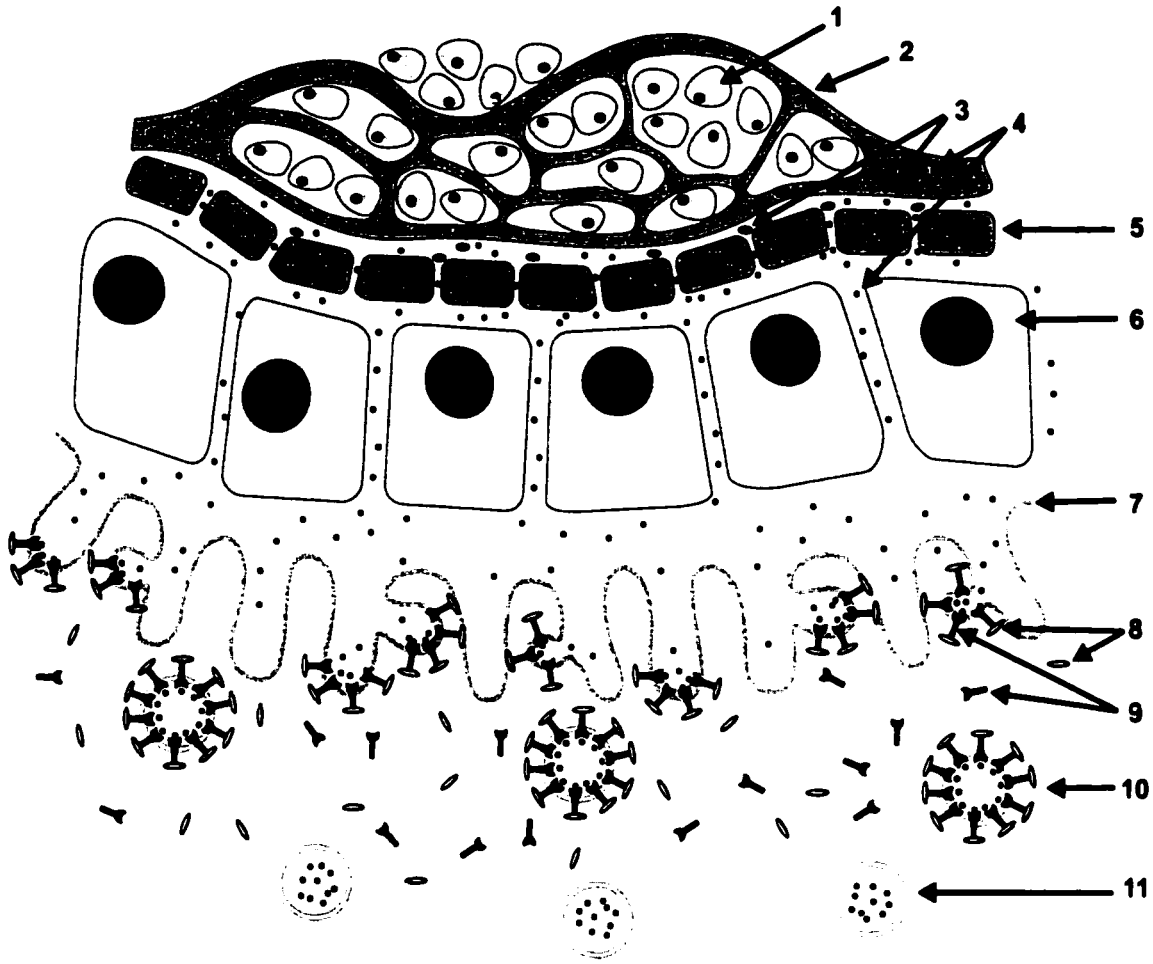
In the hen, the deposition of egg yolk commences approximately 10 days prior to ovulation and is triggered by elevated estrogen concentrations.

Approximately 19 g of egg yolk precursors - primarily VLDLs and VTG - are synthesized by the liver every day (Etches, 1996). In preparation for ovulation, invagination of the oocyte membrane (oolemma) dramatically increases its surface area, and blood capillaries surrounding the follicle acquire "holes" which allow relatively large particles to leave the blood stream. As well, the basal lamina associated with the granulosa cells becomes separated by spaces sufficient to permit passage of VTG and yolk VLDLs to the oolemma. However, the spaces are sufficiently small so that other classes of VLDLs and portomicrons (lipid droplets of dietary origin that have larger diameters than yolk VLDLs and are specific to birds) are excluded (Stevens, 1996). Transfer of yolk VLDLs and VTG across the ooelmma is accomplished by receptor-mediated endocytosis. A 95kDa receptor recognizes and binds both the apoprotein B portion of yolk VLDLs and the lipovitellin portion of VTG. Once sufficient quantities of yolk VLDLs or VTG have bound to receptors to form a coated pit, that section of the oolemma folds in on itself to form a membrane-bound vesicle that moves inside the yolk. Within minutes the yolk VLDLs and VTG are removed from the membrane and the receptors are returned to the oolemma (Etches, 1996; Stevens, 1996). The lysosomal protease, cathepsin D, cleaves apoprotein B into four smaller proteins and VTG into phosvitin and lipovitellin fractions. This enzyme is produced and stored in specialized granules located near the surface of the oocyte. Once the apoproteins are removed from the VLDLs the remaining portions, composed primarily of triacylglycerols and lesser amounts of phospholipids, cholesterol and cholesteryl ester, coalesce to form larger

spheres. Phosvitin and lipovitellin aggregate to form yolk granules (Etches, 1996). The deposition of VTG and VLDL into the yolk is illustrated in Fig. 1.C.2.

Although it is conceivable that yolk lipids and lipoproteins could be transferred across the yolk sac membrane from the yolk to the embryonic circulation without alteration, such a scenario is thought to be unlikely. The VLDLs synthesized by the liver of the laying hen have structural features that serve to promote their uptake by the oocyte. In particular, the presence of estrogen-inducible apoprotein II inhibits breakdown of yolk VLDLs by lipoprotein lipase in the capillaries of the maternal adipose tissue and muscle (Speake *et al.*, 1998) and may have a role in ensuring that the size of the VLDLs is small enough to pass through the basal lamina of the granulosa cells surrounding the oocyte (Stevens, 1996). In contrast, the function of the VLDLs in the embryo, would be to deliver lipid moieties to the embryonic adipose, heart, muscle and other tissues. Experiments have shown that VLDL spherules detected in the exocytotic vesicles were larger than the maternal yolk VLDLs (Speake *et al.*, 1998). These and other data support the notion that yolk lipid is extensively modified during transfer across the yolk sac membrane.

Similarly, yolk proteins may be absorbed by the embryo intact, or the proteins may first be digested into their constituent amino acids prior to absorption. Most of the yolk proteins are located within the yolk granules and within the water-soluble livetin fraction (Juurlink and Gibson, 2000). The yolk granules are composed of 81% lipovitellin, 18% phosvitin and 12% low density lipoprotein (Burley and Vadehra, 1989). Two aspects of whole protein uptake have been demonstrated by Juurlink and Gibson (2000). The first aspect involves coated pinocytotic invaginations and



1. thecal cells
2. blood capillaries
3. large VLDLs and portomicrons
4. VTG and yolk VLDLs
5. basal lamina
6. granulosa cells
7. oolemma
8. clathrin
9. 95 kDa receptor
10. clathrin-coated membrane-bound vesicle
11. membrane-bound vesicle
12. yolk sphere with yolk granules

Figure 1.C.2 Deposition of VTG and VLDL into yolk. The legend indicates the various components involved in yolk deposition as described in section 1.C.2.

vesicles which are indicative of whole protein absorption. The second aspect involves phagocytosis of yolk granules. Furthermore, the vacuoles containing the yolk granules have been shown to contain high acid phosphatase activity that increases throughout the incubation period (Juurlink and Gibson, 2000). Thus, it is likely that most of the yolk proteins are absorbed intact across the yolk sac membrane and the yolk granules are phagocytized and subsequently digested within lysosomes. This mechanism of protein uptake by the avian embryo was further confirmed by the observation that lipovitellin and phosvitin were not detected in the embryonic blood indicating that these molecules were degraded prior to use by the embryo (Burley and Vadehra, 1989).

1.C.3. Avian Estrogen Receptor

Estrogen receptor purified from domestic fowl oviduct has a molecular weight of approximately 66 kDa. The complete cDNA sequence has been determined and it shows a high degree of sequence homology with estrogen receptors from other vertebrates (reviewed in Stevens, 1996). The general mechanism of action of estrogenic substances has recently been reviewed by Zacharewski (1997) and is illustrated in Fig. 1.C.3. Circulating estrogen (primarily 17β-estradiol in birds) passes through the plasma and nuclear membranes of target tissue cells and binds to an estrogen receptor located within the nucleus. Hormone binding results in a conformational change of the receptor that involves dissociation of a heat shock protein (hsp90) and receptor-ligand complex dimerization. These changes enable the homodimer complex to bind to EREs which are located in the regulatory regions

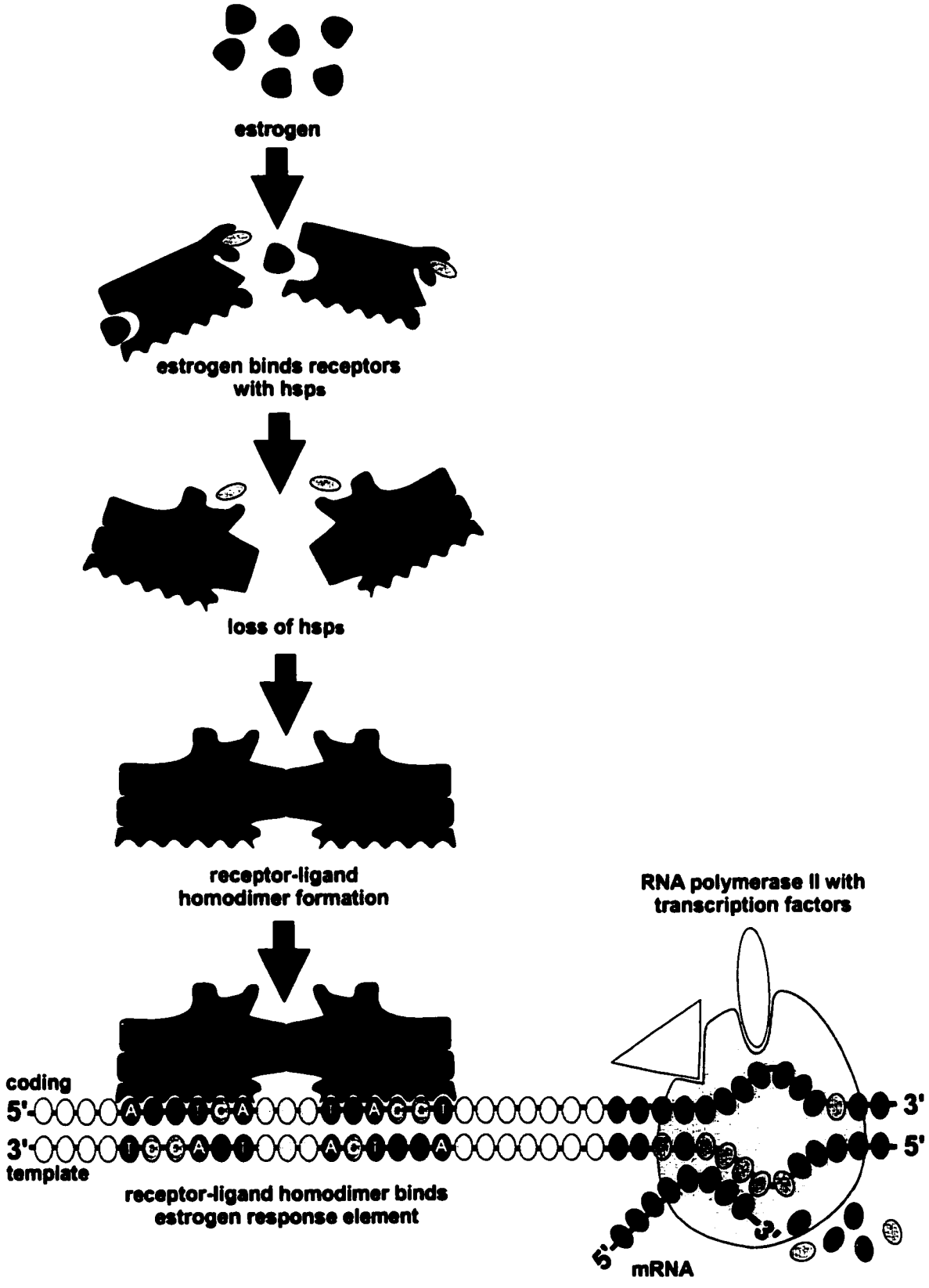


Figure 1.C.3 Estrogen receptor binding and transcription of estrogen-inducible genes. The mechanism of action of estrogen as described in section 1.C.3. is shown. (hsp = heat shock protein)

of estrogen-responsive genes. The EREs are composed of a 15 base pair palindrome consisting of two 6 base pair arms separated by a 3 base pair spacer (nnn) as follows: AGGTCAnnnTGACCT. After the homodimer complex binds to the EREs, transcription factors interact with the promoter region of the gene which results in its increased expression. The mRNA molecules that are synthesized as a result of this induction process are ultimately transcribed and translated into proteins which have many different functions and fates.

Laying hen liver contains approximately 5000 estrogen receptor sites per hepatocyte (Evans *et al.*, 1987). This is approximately half the number observed in chicken oviduct (Evans *et al.*, 1987). Hepatic estrogen receptor synthesis can be stimulated in chicken embryos by injecting embryonated eggs with estrogen (Haché *et al.*, 1987). This process requires approximately 48 h and the embryo must be at least 7-8 days of age at the time of injection (Lazier, 1978; Deeley *et al.*, 1993). The number of estrogen receptors induced in response to estrogen treatment increases during the embryonic period until hatching at which time it reaches 30-40% of induced adult levels (Deeley *et al.*, 1993). Activation of yolk protein genes that are absolutely dependent on estrogen is staggered over a period of at least 4-5 days. Apoprotein II induction can be detected as early as days 7-8, whereas VTG induction becomes apparent between days 10-13 (Deeley *et al.*, 1993). Within this latter time period, it has also been shown that the activation of the various VTG genes shows developmental staging; induction of the VTGII gene is apparent at days 10-11, followed by induction of the minor VTG genes I and III at days 12-13 (Evans *et al.*, 1988; Deeley *et al.*, 1993). Interestingly, the estradiol-induced

expression of the VTG and apoprotein II genes in the chicken becomes diminished in the late embryonic period (approximately 4-5 days before hatching) (Evans *et al.*, 1988). Maximal responses for these genes in chickens were subsequently achieved at distinct ages between 1 and 6 weeks after hatching (Evans *et al.*, 1987). Although, estrogen receptor levels were found to progressively increase to adult levels over this post-hatch period (Evans *et al.*, 1987), the distinct developmental profiles of the respective genes do not correlate well with gross changes in receptor levels (Evans *et al.*, 1988). Thus, it has been proposed that the timing with which the yolk protein genes can be induced reflects the sensitivities of their regulatory regions to activation by estrogen, or it may simply reflect a requirement for transcription factors that only become available at specific developmental stages.

1.C.4. Avian Vitellogenin Genes

Of the three VTG genes known to exist in the domestic chicken, only the gene encoding the major VTGII protein has been thoroughly investigated and completely sequenced (van het Schip *et al.*, 1987). Complementary DNA clones for portions of the minor VTGI and VTGIII genes have also been isolated for this species (Evans *et al.*, 1988). In contrast, there is no literature on the VTG gene(s) in any other avian species The chicken VTGII gene is 20,342 nucleotides in length and contains 35 exons and 34 introns (Fig. 1.C.4; reviewed by Deeley *et al.*, 1993; Stevens 1996). The combined length of the coding sequence is 5787 nucleotides (van het Schip *et al.*, 1987). The phosvitin subunit is derived from the central region of the gene coding for amino acids 1112 to 1328 (van het Schip *et al.*, 1987).

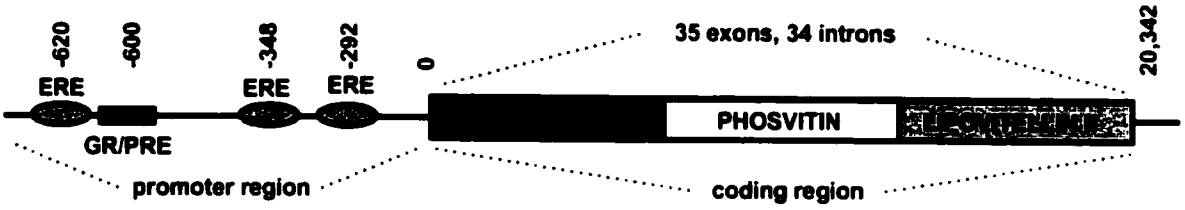


Figure 1.C.4 Simplified view of the chicken VTGII gene. The numbers shown correspond to nucleotide positions with respect to the transcription initiation site (0). ERE = estrogen response element; GR/PRE = progesterone/glucocorticoid response element (after Deeley *et al.*,1993; Stevens, 1996).

Regions on either side of the central phosvitin region encode lipovitellin I (N-terminal) and lipovitellin II (C-terminal). Since amino acid sequences of the lipovitellins have not been determined, the precise regions of the VTGII gene that codes for these subunits are not known. As well, based on the molecular weight of lipovitellin II, it is likely that other polypeptides may originate from the C-terminal sequence of the VTGII gene (van het Schip *et al.*, 1987).

No significant homology was observed when the sequence of the chicken VTGII gene was compared to the gene of the estrogen-inducible yolk VLDL apoprotein II (van het Schip *et al.*, 1987). Comparison of the organization and fine structure of the chicken VTGII gene with that of *Xenopus laevis* has shown that both genes have the same exon-intron organization (Nardelli *et al.*, 1987). However both the nucleotide and amino acid sequences vary considerably between these two species. In particular, the internal region of the VTGII gene that encodes phosvitin is poorly conserved suggesting that this subunit evolved rapidly and/or was not subjected to strong evolutionary constraints (Nardelli *et al.*, 1987). Other regions of the gene appear to be better conserved. It has been speculated that these regions may be involved in critical aspects of vitellogenesis such as secretion of VTG from the liver into the blood, receptor-mediated uptake by the oocyte, or processing and storage in the yolk (Nardelli *et al.*, 1987).

The regulatory regions of the chicken VTGII gene have been thoroughly investigated (reviewed in Deeley *et al.*, 1993; Stevens, 1996). Upon activation of the VTGII gene, nuclease hypersensitive sites that extend for approximately 900 nucleotides upstream from the initiation site become exposed (Deeley *et al.*, 1993).

This observation suggests that the chromatin must undergo reorganization to allow transcription of the VTGII gene. Exposure of the DNase I hypersensitive sites is correlated with demethylation of specific methylated cytosine-guanosine dinucleotides (mCpGs) in the promoter region of the chicken VTGII gene (Jost *et al.*, 1990). Demethylation of mCpGs also occurs in at least one ERE without the appearance of DNase I hypersensitivity sites (Jost *et al.*, 1990). Furthermore, since demethylation in the ERE occurs during the 24h following receptor binding, it is not a prerequisite for the binding of estrogen receptor to this site, nor for gene activation (Deeley *et al.*, 1993). These data suggest that the ERE is always protected by proteins. For example, experiments have shown that the non-histone protein, NHP1, binds with high affinity to the spacer regions of the ERE palindrome and is thought to facilitate the binding of the estrogen receptor-ligand complex to this site (Jost *et al.*, 1990; McEwan *et al.*, 1991).

A perfect ERE (GGTCA*GC*GTGACC), that appears to be the major determinant of estrogen inducibility of the VTGII gene, is located at 620 nucleotides upstream from the transcription initiation site (Fig. 1.C.4; Burch *et al.*, 1988). Two additional EREs are located closer to the initiation site. The first is an imperfect ERE (GGTCA*A*C*A*TAACC) located at nucleotide -348 which acts synergistically with the distal ERE (Burch *et al.*, 1988). The other is a proximal ERE located at 292 nucleotides upstream from the initiation site that does not appear to be functional as determined by *in vitro* transient expression assays (Burch *et al.*, 1988). As well, a glucocorticoid/progesterone response element is located near the distal ERE at nucleotide position -600 (Fig. 1.C.4; Cato *et al.*, 1988). Although *in vitro* experiments

have shown that estrogens and progestins or glucocorticoids bind synergistically to their corresponding response elements, a similar response has not been observed *in vivo* (Cato *et al.*, 1988; Slater *et al.*, 1991; Deeley *et al.*, 1993).

As discussed briefly above, transcription factors also play an important role in chicken VTGII gene expression (reviewed in Deeley *et al.*, 1993; Stevens, 1996). DNA footprinting studies of the EREs indicate that these sites are protected by protein binding in both hen and rooster (McEwan *et al.*, 1991). Sites have been identified in the promoter region of the VTGII gene which bind liver-specific or liver-enriched *trans*-acting factors that are important for both the efficiency and tissue specificity of induction. Other regions within the promoter region of the VTGII gene may be involved with suppression of VTG induction, particularly in non-hepatic tissues of male or immature birds. For example, a protein called the Methylated DNA Binding Protein (MDBP-2) represses the *in vitro* expression of the VTG II gene (Jost *et al.*, 1991). However, in the presence of estradiol, the VTGII gene becomes hypomethylated and the binding of MBDP-2 is down-regulated, allowing expression of the VTG II gene.

Although it has been shown by many researchers that the VTG genes are not expressed in males or immature birds, there is some evidence supporting the contrary view (Shapiro, 1982; Ho, 1991). Some investigators have measured small quantities of VTG protein in the plasma or liver of untreated roosters, quail, and immature chickens. In roosters, low levels of VTG mRNA corresponding to 1-5 molecules per cell and VTG protein corresponding to 4-8 ng/ml have been reported. Similarly, a baseline of VTG synthesis ranging from 0.01-0.07% of total *de novo*

synthesized liver proteins has been reported for control immature male and female chickens (Burch *et al.*, 1988). In comparison the blood of the laying hen contains VTG concentrations of 10-25 mg/ml. Thus it is likely that both male and immature birds have detectable basal VTG mRNA and protein levels.

1.C.5. Avian Vitellogenin Proteins

In the liver of an egg laying hen the levels of mRNA encoding VTGI, VTGII and VTGIII are approximately 11,000, 30,000 and 3000 molecules per cell, or 25%, 68%, and 7% of total VTG mRNA, respectively (Evans *et al.*, 1988). Multiple forms of VTG proteins have been found in all species of birds examined (chicken, quail, duck). However, the number of variants is species-dependent with three forms of VTG described for the chicken and quail, while only two forms are detected in the domestic duck (van den Boogaart *et al.*, 1981).

In preparation for the synthetic effort required to produce VTG, avian hepatocytes undergo several significant changes (Shapiro, 1982; Burley and Vadehra, 1989). This is particularly notable in roosters in which estrogen treatment results in a 3-fold increase in the size of the liver. At the cellular level, there is a doubling of total cellular RNA content. The cytoplasm of hepatic parenchymal cells becomes nearly filled with endoplasmic reticulum and the size of the Golgi apparatus is markedly increased. As well there is an increased capacity for mRNA translation as indicated by an increase in the number of membrane-bound polysomes, particularly those with 30-40 ribosomes. An increase in the activities of

enzymes required for post-translational modification of the nascent VTG protein has also been noted.

After the intron sequences have been spliced from the coding sequences, chicken VTGII mRNA is translated into a 1850 amino acid protein precursor with a molecular weight of 203,047 (van het Schip *et al.*, 1987). As mentioned above, residues 1112 through 1328 represent phosvitin (MW ~30,000) while the N-terminal and C-terminal components represent lipovitellin I (MW ~125,000) and lipovitellin II (MW ~30,000), respectively. Before VTGII is secreted, it is glycosylated and phosphorylated, and calcium ions bind to most of the phosphate groups. Some non-covalently bound lipid is thought to be added prior to secretion and additional lipid may be obtained from the blood during transport to the oocyte. Approximately 80% of the VTG dimer consists of polypeptide and the remainder consists of lipid, covalently bound phosphate, and carbohydrate (Stevens, 1996). The final form of VTGII which is secreted and circulates in the blood is a dimer (~500 KDa). In the dimer formation, it is likely that the monomers lie side by side and are held together by calcium bridges between phosphates (Burley and Vadehra, 1989). Interestingly, it has been shown that a strain of chickens that lays thin-shelled eggs produced VTG that bound less calcium than normal strains (Burley and Vadehra, 1989).

Although there is only one region of the chicken VTGII gene that codes for phosvitin, there may be more than one form of this polypeptide (Burley and Vadehra, 1989). The major form of phosvitin derived from the chicken VTGII gene contains a core region of 99 amino acids, 80 of which are serines, and ~95% of which are phosphorylated (van het Schip *et al.*, 1987). Based on its high level of

phosphorylation, phosvitin is thought to be a source of organic phosphate for the embryo and is also important for metal binding. Approximately 91% of total yolk ferric iron is bound to phosvitin which serves as an iron reservoir for the developing embryo (Burley and Vadehra, 1989). Phosvitin also binds and stores other cations, such as calcium and magnesium, and may act as an antioxidant for yolk lipids (Burley and Vadehra, 1989). The carbohydrate portion of phosvitin is thought to have a role in recognition of membrane receptors and/or transport into the yolk.

Less is known regarding the function of lipovitellins in the egg and developing embryo. The two lipovitellins differ in their amino acid composition and also in the amounts of protein-bound phosphorous and carbohydrate they contain. Several experiments have shown that the proportion of lipovitellins I and II is variable and likely has a genetic basis (Burley and Vadehra, 1989). Despite these differences between the two lipovitellins, both dimerize easily and reversibly and are highly soluble (Burley and Vadehra, 1989).

1.C.6. Vitellogenin Induction Kinetics and "Memory"

After estradiol treatment, chicken VTGII mRNA is detectable as early as 30 min, reaches a maximum in 72 h and decays over the next 7 days with a half-life of 30 h (Deeley *et al.*, 1977). Using RNA excess hybridization analysis, Deeley *et al.* (1977) determined that during this induction process, VTGII mRNA concentrations increase from less than 10 copies to 6000 copies per cell. In comparison, estradiol-treated domestic Pekin duck liver accumulates a maximum of ~18000 VTG mRNA molecules per cell by the fourth day after treatment (van den Boogaart *et al.*, 1981).

In chickens, inhibitors of mRNA transcription such as actinomycin are effective only if given during the first few hours after estradiol treatment. After this point the level of mRNA is sufficient for VTG protein synthesis. In contrast, inhibitors of mRNA translation, such as puromycin, are effective at later time points when the VTG protein is being actively synthesized (Burley and Vadehra, 1989).

A maximal rate of VTG synthesis of 2.1 mg/g liver/h (equivalent to 20% of total protein synthesis) was observed in rooster liver at 4-5 days after estradiol injection (Gruber *et al.*, 1976). Similarly, in domestic ducks, maximal levels of VTG protein were observed 4 days after estradiol treatment and decreased thereafter with an estimated half-life of 10 h (van den Boogaart *et al.*, 1981). In chickens, the half life of VTG has been estimated to be as short as 5-8 h (Bergink *et al.*, 1973; Bergink and Wallace, 1974) to as long as 1 day (Redshaw and Follett, 1976). When estradiol-induced plasma containing ^{32}P-labeled VTG was injected into the blood of estrogenized roosters the half-life of VTG was ~55% longer ($t_{½}$ = 8.4 h) compared with control roosters ($t_{½}$ = 5.2 h) (Bergink *et al.*, 1973; Bergink and Wallace, 1974). These data suggest that estradiol may have a role in stabilizing the VTG protein (Bergink and Wallace, 1974).

Unlike VTGII mRNA induction, there is a substantial delay or "lag period" between estradiol treatment and the appearance of complete VTGII phospholipoglycoproteins in the blood of the domestic chicken that cannot simply be attributed to the time required for mRNA translation. This delay has been reported to last for 10 (Bergink *et al.*, 1973; Bergink and Wallace, 1974; Jost *et al.*, 1978), 6 (Burley and Vadehra, 1989), or 4-12 (Ho, 1991) hours and may depend on the strain

of chicken studied (Bergink and Wallace, 1974). It has also been shown that initial incorporation of ^{32}P into a phosphoprotein complex occurs about 3-4 h after estradiol injection (Bergink *et al.*, 1973; Bergink and Wallace, 1974). The cause of this lag period remains unclear, but it has been speculated that it may be due to the requirement for up-regulation of enzymes required for post-translational modification of the protein (Bergink *et al.*, 1973).

Early studies showed that after the 10 h lag period, the concentration of VTG in estradiol-treated rooster blood increased slowly for the following 15 h and then increased more rapidly and linearly for several days before declining to basal levels (Bergink *et al.*, 1973; Bergink and Wallace, 1974). In contrast, if the roosters were injected a second time with estradiol once the primary response had subsided, detectable accumulation of VTG started sooner (~ 5 h lag period) and the increase in VTG synthesis after this point immediately became linear and much more rapid than that observed during the primary response (Bergink *et al.*, 1973; Bergink and Wallace, 1974). This phenomenon, termed hepatic "memory", was observed in roosters for at least 50 days after the first hormone injection (Bergink *et al.*, 1973; Bergink and Wallace, 1974).

Similar induction kinetics were observed in studies in which VTGII mRNA concentrations were determined in cockerels or immature chickens after primary and secondary estradiol injections (Deeley *et al.*, 1977; Jost *et al.*, 1978). During the primary response, VTGII mRNA accumulation began after a lag period at a slow rate that then increased to a rapid rate several hours later. Administration of a second estradiol dose several weeks later resulted in an earlier onset of the rapid

accumulation of VTGII. The net result was a maximal VTGII mRNA level which was 1.5 - 2 times higher during the secondary response than during the primary response. More recent studies with chicken embryos, or dispersed hepatocyte suspensions, compared the hepatic memory effect among mRNAs coding for the three VTGs (Evans *et al.*, 1988; Edinger *et al.*, 1997). Similar to VTGII, both VTGI and VTGIII displayed lag times for the onset of rapid accumulation of mRNA after estradiol exposure that were significantly shortened during the secondary response. However, while the lag periods for VTGII and VTGIII were similar, the lag period for VTGI mRNA induction was significantly longer and was the slowest of the egg yolk protein genes to accumulate mRNA in response to estradiol. After a second estradiol treatment, the lag periods for all three VTG mRNAs were significantly shortened and the rates of transcription were increased.

There may also be species differences with respect to the "memory" effect. When ducks were given a second estradiol injection after the initial vitellogenic response had ceased, the rate of accumulation of vitellogenin in the plasma was the same as that observed after primary hormone treatment (van den Boogaart *et al.*, 1981). However, further studies by the same investigators showed that there was a difference in the rate of accumulation of VTG mRNA after primary and secondary stimulation. Eleven hours after secondary estradiol stimulation, the rate of VTG mRNA synthesis in the duck was estimated to be ~1.6 times higher than after primary stimulation (van den Boogaart *et al.*, 1981). In comparison, rates of chicken VTGII mRNA synthesis after a similar time period were ~6 times higher after

secondary stimulation (Jost *et al.*, 1978). Thus it appears that the "memory" effect in the duck is substantially smaller than that observed in the chicken.

Although the mechanism(s) resulting in the different kinetic profiles during primary and secondary estradiol stimulation and the development of hepatic "memory" have not been elucidated, most investigators support the notion that a rearrangement of the structural and promoter regions of the VTG gene is involved (Deeley *et al.*, 1977; Jost *et al.*, 1978; Edinger *et al.*, 1997). Such a change might be expected to stabilize the VTG gene(s) for several months in a conformation that allows the gene to be transcribed more readily during a second estradiol treatment. Evans *et al.* (1987) further suggested that other mechanisms, including changes in synthesis, processing, export or stability of RNA, may also play a role.

1.C.7. Vitellogenin Bioassays for Environmental Contaminants

Since it is difficult to predict the hormonal activity of environmental contaminants based on their chemical structure, the estrogenic potency of a substance, or mixture of substances, must be determined and characterized by biological methods. To this end, both *in vivo* and *in vitro* assays have been developed. *In vivo* estrogenicity bioassays typically involve measurement of effects of test substances on organ weights, cell differentiation and enzyme activities. However, in many cases the utility of *in vivo* bioassays is limited due to their cost, poor sensitivity, and complexity (see Zacharewski, 1997). On the other hand, *in vitro* assays utilize better defined endpoints. Recently, Zacharewski (1997) reviewed

available *in vitro* bioassays for assessing estrogenic substances and these are summarized in Table 1.C.1.

As with the *in vivo* bioassays, there are also important limitations to the use of *in vitro* estrogenicity bioassays (see Zacharewski, 1997). For example, one cannot assume that one *in vitro* bioassay will accurately predict all of the potential *in vivo* responses elicited by an estrogenic compound. Furthermore, *in vitro* systems do not account for bioaccumulation, and metabolism of the test substances is often minimal or non-existent. As well, interactions of test substances with other proteins, such as sex hormone binding proteins, are beyond the scope of *in vitro* bioassays. Finally, most *in vitro* estrogenicity bioassays are based on the assumption that the estrogenic or anti-estrogenic effects of a test substance will be mediated only by direct binding of the substance to the estrogen receptor. Overall, *in vitro* estrogenicity bioassays should only be used as an initial screen in a complete assessment process.

Both *in vivo* and *in vitro* estrogenicity bioassays have been developed that utilize vitellogenin induction as an endpoint. By measuring plasma vitellogenin, investigators in the United Kingdom were able to assess the estrogenic activity of river water by placing caged male trout at various distances downstream from sewage-treatment plant (STP) effluent entry points for a standardized exposure period (Sumpter and Jobling, 1995; Harries *et al.*, 1997). Elevated VTG levels in these fish were tentatively correlated with effluent containing high levels of alkyphenolic chemicals. Similarly, i*n vivo* treatment of both male turtles and frogs

Table 1.C.1 Overview of in vitro estrogenicity bioassays. Based on Zacharewski, 1997.

Bioassay	Endpoint/Examples	Comments
competitive ligand binding	- estrogen receptor - ligand interactions	- does not distinguish agonists from antagonists - binding to estrogen receptor does not infer biological activity - labour intensive
cell proliferation	- cell proliferation in estrogen receptor-positive, estrogen-responsive cell lines - eg. E-screen (Soto *et al.*, 1992; Soto *et al.*, 1995)	- modest responsiveness - long incubation periods - very sensitive
post-confluent cell accumulation and foci formation	- formation of multi-cell clusters (foci) in estrogen-receptor-positive, estrogen-responsive cell lines	- reasonably selective for estrogens - good sensitivity - good responsiveness
induction of protein expression or enzyme activities	- estrogen-inducible proteins - eg. cathepsin D, prolactin, progesterone receptor proteins, sex hormone binding globulin, VTG	- usually labour intensive - require optimization for each species or cell culture system
recombinant receptor/reporter genes	a) endogenous promoter-regulated reporter gene assays - endogenous promoters from estrogen-responsive genes linked to reporter genes encoding luciferase, chloramphenicol acetyltransferase,β-galactosidase or alkaline phosphatase	a) sensitive, stable, easily measured endpoints
	b) response-element-regulated reporter gene assays - reporter genes are regulated by the 13 bp ERE palindrome	b) better selection for estrogen-receptor mediated gene expression - susceptible to induction, synergy or antagonism due to similarity of ERE with other hormone response elements
	c) chimeric receptor/reporter gene assays - chimeric receptor consists of ligand binding domain from an estrogen receptor of interest linked to the DNA binding domain of a yeast transcription factor which is activated by ligand binding. The chimeric receptor is situated next to a yeast-based reporter gene which is regulated by the activated transcription factor. eg. E2 bioassay (Zacharewski, 1997)	c) induction of the reporter gene can only occur through the chimeric estrogen receptor since there are no mammalian transcription factors that can bind the yeast response element and initiate expression of the reporter gene - can determine potential differences in species sensitivity by using chimeric receptors that contain the ligand binding domain for a given species - selective and sensitive - requires specialized equipment and expertise
yeast-based assays	yeast are transformed with estrogen receptor and ERE-regulated reporter genes	- yeast have no endogenous hormone receptors - use chemically-defined growth medium - easy to genetically modify and transform - ligand potencies may not reflect that observed in mammalian systems

with estradiol, diethylstilbestrol (DES) or *o,p'*-DDT resulted in dose-dependent VTG induction (Palmer and Palmer, 1995). More recently, *in vivo* bioassays based on vitellogenin induction in brown trout (Sherry *et al.*, 1999) and fathead minnows (Tyler *et al.*, 1999) exposed to various concentrations of estrogenic compounds also have been developed.

Although data from *in vivo* VTG bioassays more accurately reflect physiological exposures, potencies, and fates of estrogenic compounds, high throughput *in vitro* vitellogenin bioassays have provided much more information regarding identification and relative potencies of estrogenic and anti-estrogenic compounds. For example, induction of vitellogenesis was used as one of the *in vitro* endpoints to test the estrogenicity of specific alkylphenol-polyethoxylates (APEOs) (Jobling and Sumpter, 1993; White *et al.*, 1994). For these experiments, male rainbow trout hepatocytes were prepared and exposed to serial dilutions of each test compound of interest for 48 h. The culture medium was then collected and VTG protein was measured by radioimmunoassay (Sumpter, 1985). Although all the APEOs were substantially (~1000-10,000-times) less estrogenic than estradiol, results from the bioassay indicated that the estrogenicity of these compounds was related to the length of their ethoxy chains and ring substitution patterns. In further experiments, the trout hepatocyte vitellogenin bioassay was used to assess the estrogenicity of other environmental contaminants (Sumpter and Jobling, 1995). Similar to the APEOs, approximately 1000-fold higher concentrations of *o,p'*-DDT, bisphenol A, and Aroclor 1221 were required to induce vitellogenin synthesis compared to estradiol. Furthermore, it was demonstrated that a greater vitellogenin

induction response was observed when hepatocytes were treated with a mixture of five contaminants than when the responses observed for the individual chemicals were summed (Sumpter and Jobling, 1995). Therefore, by using VTG induction as a bioassay endpoint considerable information regarding the estrogenic potencies of various environmental contaminants and contaminant mixtures can be obtained.

The relationship between CYP1A and VTG induction has also been examined using *in vitro* bioassays in primary cultures of rainbow trout hepatocytes (Anderson *et al.*, 1996). Results of these studies indicated that a variety of compounds known to effectively induce CYP1A (e.g., 2,3,4,7,8-PCDF, TCDD, TCDF) inhibited estrogen-induced VTG synthesis at very low concentrations (10^{-12} to 10^{-8} M). In contrast, PCB 114, a weak CYP1A inducer, appeared to potentiate estrogen-induced VTG synthesis. Thus, it was concluded that CYP1A inducing compounds may modulate the estrogen responsiveness of those cell types that contain both Ah and estrogen receptors. These experiments further demonstrate the utility and potential application of *in vitro* VTG bioassays.

Recently, *in vitro* VTG induction-based estrogenicity or anti-estrogenicity bioassays have also been developed for primary hepatocyte cultures prepared from channel catfish (Monteverdi and Di Giulio, 1999) and carp (Smeets *et al.*, 1999a). Data obtained from these systems showed estrogenic potencies for a number of environmental contaminants which were less than or similar to those observed in rainbow trout hepatocytes (Sumpter and Jobling, 1995). As well, TCDD was shown to be a potent anti-estrogen in hepatocyte cultures from all three fish species. Further experiments are required to establish whether there are significant

differences in sensitivity of hepatocyte cultures prepared from different species of fish to estrogen- or contaminant-induced VTG synthesis.

Unlike amphibians and fish, attempts to produce a vitellogenic response *in vitro* in primary avian hepatocyte cultures have not been very successful. Reports that claim successful induction of VTG synthesis *in vitro* in avian systems have required either the use of transfected and transformed cells (Binder *et al.*, 1990), or serum from estradiol-treated birds (Boehm *et al.*, 1988). However, in another study induction of VTG mRNA was detected in primary chicken embryo hepatocyte cultures after treatment with the semi-synthetic estradiol analogue moxestrol (Strijker, 1986). Thus, VTG induction may also be a useful endpoint for estrogenicity or anti-estrogenicity bioassays in avian primary hepatocyte cultures. Therefore one objective of this thesis was to develop an *in vitro* vitellogenin-based bioassay with avian embryo hepatocyte cultures.

CHAPTER 2. RELATIONSHIPS BETWEEN ENVIRONMENTAL ORGANOCHLORINE CONTAMINANT RESIDUES, PLASMA CORTICOSTERONE CONCENTRATIONS, AND INTERMEDIARY METABOLIC ENZYME ACTIVITIES IN GREAT LAKES HERRING GULL EMBRYOS - 1997[1]

2.1. Introduction

The hypothalamo-pituitary-adrenal (HPA) axis is an important system which regulates and integrates many physiological functions to maintain internal homeostasis and in response to environmental stressors. The ontogeny, activation, function and toxicology of the HPA axis in birds and bird embryos was reviewed in Chapter 1.B.

Of particular interest and relevance to the present study are reports describing the reduced abilities of birds to cope with a secondary stress (e.g., cold temperatures) after exposure to the primary stressor, petroleum (Holmes *et al.*, 1978, 1979). Thus, it is conceivable that other environmental contaminants could similarly interfere with the HPA axis to increase the susceptibility of birds to non-anthropogenic environmental stressors. To my knowledge, no prior avian HPA axis studies have considered the potential toxicological effects of complex mixtures of environmental organochlorine contaminants. However, studies by Hontela, Gendron and colleagues (Hontela *et al.*, 1992, 1995, 1997; Gendron *et al.*, 1997; reviewed in Hontela, 1997) which characterized adrenal and HPA axis function in two species of wild freshwater fish and an amphibian (mudpuppy) strongly indicated that HPA axis-

[1] from: Lorenzen, A., Moon, T.W., Kennedy, S.W., and Fox, G.A. (1999). *Environ. Health Persp.* **107**, 179-186.

related endpoints are altered after exposure of these animals to complex mixtures of contaminants. These data further support the idea that HPA axis-related endpoints are useful indicators of contaminant exposure and potential toxicity in wild animals.

2.2. Hypothesis

Current levels of organochlorine contaminants are affecting the hypothalamo-pituitary-adrenal (HPA) axis and associated intermediary metabolic pathways in environmentally-exposed herring gull embryos.

2.3. Objectives

The objectives of this study were to correlate 1) basal plasma corticosterone concentrations and 2) the activities of intermediary metabolic enzymes that are thought to be regulated by corticosterone, with yolk sac organochlorine residues in Great Lakes herring gull embryos collected in 1997.

2.4. Materials and Methods

2.4.1. Egg collection and Incubation

During May and June of 1997, unincubated, fertile herring gull eggs were collected from Kent Island, New Brunswick (near Grand Manan Island in the Bay of Fundy; control site), Middle Island (western basin of Lake Erie), Snake Island (eastern basin of Lake Ontario), Hamilton Harbour (western terminus of Lake Ontario), and Papoose Island (10 km east of Thunder Bay, Ontario in Lake Superior). The eggs were brought to the University of Ottawa (Ottawa, ON, Canada)

and/or the National Wildlife Research Centre (Hull, QC, Canada) where they were artificially incubated for 24 or 26 days at 37.5°C and 60% relative humidity in a Curfew Model RX250 incubator (Althorne, Essex, England) with automatic turning. The normal incubation time for herring gull eggs to hatch is 28 days. All experiments were approved and conducted in compliance with the "Guide to the Care and Use of Experimental Animals" and "Ethics of Animal Experimentation" of the Canadian Council on Animal Care.

2.4.2. Plasma Corticosterone

Blood was collected from individual 24 day herring gull embryos from each site and analyzed for corticosterone based on previously published methods (Decuypere *et al.*, 1989; Rombauts *et al.*, 1994). Briefly, a large blood vessel associated with the shell membrane was located and marked by candling the eggs on day 23 of incubation. A window was cut through the shell of each egg (but not the shell membrane) using a dental drill (Buffalo Dental Mfg. Co. Inc., Syosset, NY,USA) equipped with a small cut-off wheel (Dremel no. 409, Racine, WI, USA) to allow access to the vessel. The window was covered with removable tape and all eggs were returned to the incubator, without turning. On day 24 of incubation, the tape was removed from each egg and the membrane allowed to dry. A drop of mineral oil was applied to the membrane to make it transparent and the selected vessel was cannulated using a 27G needle fitted to 0.30 mm i.d. silastic tubing (Dow Corning, Midland, MI, USA). This tubing was attached to a 3-way stopcock fitted with a heparinized disposable tuberculin syringe. For basal corticosterone analysis, a

single blood sample (100 µl) was taken. All embryos were sacrificed following blood removal by decapitation and yolk sacs were frozen for organochlorine residue analysis. Plasma was separated by centrifuging the blood samples, frozen in liquid nitrogen and stored at -80°C until analyzed for corticosterone using a commercially available ^{125}I radioimmunoassay kit (*Immunchem™ Double Antibody*, ICN, Costa Mesa, CA, USA). Intra- and inter-assay coefficients of variation were reported to be 4.4% - 10.3% and 6.5% - 7.2%, respectively. Cross-reactivity was 0.35% for desoxycorticosterone, 0.1% for testosterone, and < 0.05% for all other steroids tested. Recovery of exogenous corticosterone added to plasma samples ranged from 98.3% to 106.9% and the sensitivity of the assay was at least 25 ng/ml. Plasma dilutions of 1:20 were determined to be optimal for herring gull embryo plasma.

2.4.3. Tissue Preparation

On day 26 of incubation, herring gull embryos from each site were sacrificed by decapitation. The liver was immediately removed, rinsed and homogenized in 3 ml Tris-buffered 0.25 M sucrose (5 mM Tris, pH 7.4) per gram of tissue. Cytosolic fractions were prepared by differential centrifugation. Briefly, the homogenate was centrifuged at 1000g for 10 min at 4°C (Eppendorf, model 5402) and the resulting supernatant was centrifuged at 10,000g for 10 min at 4°C (Beckman, model J2-21M/E). The resulting supernatant was filtered through a 1 µm syringe filter (glass fiber Acrodisc, Gelman Sciences, Ann Arbor, MI, USA). After a final centrifugation of the filtrate at 105,000g for 1 h at 4°C (Beckman, Optima TL), the supernatant

(cytosol) was frozen in aliquots in liquid nitrogen. Cytosol samples were similarly prepared from the kidneys of each embryo. All samples were stored at -80°C until analyzed for protein concentration (BCA, Pierce, Rockford, IL, USA) and enzyme activities. Pools of liver and kidney cytosol prepared from both domestic duck embryos and Kent Island herring gull embryos were used to optimize each assay prior to analysis of individual samples.

2.4.4. Intermediary Metabolic Enzymes

The activities of four intermediary metabolic enzymes were assayed. Previously published methods were optimized and modified to allow measurement of samples in 96-well plates (Corning Costar, Acton MA, USA) using a UV-vis microplate reader (*Spectracount Microplate Photometer*, Canberra-Packard Canada Ltd., Mississauga, ON, Canada). For each enzyme, optimization involved determination of optimal cytosol protein and substrate concentrations which provided a linear response for at least 10 min with absorbances that did not exceed the bounds of the NADH or NADPH standard curves. All concentrations given are based on final reaction volumes and NADH or NADPH standards were included in quadruplicate on each plate. Assay plates for all four enzymes were scanned at 1 min intervals for approximately 10 min and the rate of the reaction for each sample (in quadruplicate) was determined by calculating the change in absorbance at 340 nm over time as compared to the sample blank (in quadruplicate). Enzyme activities are expressed as nmol NAD(H) or NADP(H) oxidized or reduced/min/mg protein.

a) Phosphoenolpyruvate Carboxykinase (PEPCK) - The method described by Petrescu *et al.* (1979) was optimized for herring gull embryo liver and kidney cytosol samples. Kidney or liver cytosol (37.5 µl containing 0.15 mg total protein diluted with TRIS-buffered 0.25 M sucrose as required) was added to 162.5 µl of a mixture of CO_2-saturated $NaHCO_3$ (20 mM), phosphoenolpyruvate (1 mM), NADH (0.1 mM) and malate dehydrogenase (0.5U), all dissolved in TRIS buffer (50 mM, pH 7.4). The plate was shaken on a microplate shaker for 30 sec. The reaction was initiated with 50 µl dGDP (0.2 mM) dissolved in TRIS buffer for a final reaction volume of 250 µl. The absence of $NaHCO_3$ was used as a blank.

b) Malic Enzyme (ME) - The method described by Wise and Ball (1964) was optimized for herring gull embryo liver cytosol samples. Liver cytosol (37.5 µl containing 0.15 mg total protein diluted with Tris-buffered 0.25 M sucrose as required) was added to 162.5 µl of a mixture of $MgCl_2$ (4.5 mM) and NADP (0.2 mM) each dissolved in TRIS buffer (50 mM, pH 7.4). The plate was shaken on a microplate shaker for 30 sec. The reaction was initiated with 50 µl sodium malate (2.5 mM) dissolved in TRIS buffer. Malate was not added to the blank wells.

c) Alanine Aminotransferase (ALAT) - The method described by Segal and Matsuzawa (1970) was optimized for herring gull embryo liver and kidney cytosol samples. Cytosol (50 µl containing 0.1 mg total protein for liver or 0.025 mg total protein for kidney diluted with Tris-buffered 0.25 M sucrose as required) was added to 150 µl of a mixture of α-ketoglutarate (4.1 mM), NADH (liver - 0.17 mM, kidney - 0.34 mM) and lactic dehydrogenase (0.42 U) all dissolved in phosphate buffer (0.1 M, pH 7.3). The plate was shaken on a microplate shaker for 30 sec. The reaction

was initiated with 50 µl L-alanine (100 mM) dissolved in phosphate buffer. Alanine was not added to the blank wells.

d) Aspartate Aminotransferase (ASPAT) - The method described by Segal and Matsuzawa (1970) for ALAT was adjusted and optimized for measuring ASPAT activities in herring gull embryo liver and kidney cytosol samples. Reaction conditions were identical to those described for ALAT except the total protein concentration of herring gull embryo liver and kidney cytosol was 0.01 mg mg/ and 0.42 U malate dehydrogenase replaced lactic dehydrogenase. To start the reaction, 50 µl L-aspartate (100 mM) dissolved in phosphate buffer was added to all but the blank wells.

2.4.5. Chemical Residue Analysis

Twenty-six yolk sac samples, chosen randomly to represent each site and endpoint, were analyzed for PCDDs, PCDFs, non-*ortho* substituted PCBs, organochlorine pesticides and other chlorinated hydrocarbons (CHCs), and total PCBs (sum of 59 congeners). The samples were analyzed by the Laboratory Services Division at the National Wildlife Research Centre (Hull, QC) using standard procedures described in the Canadian Wildlife Service Technical Report Series (Simon and Wakeford, 2000; Won *et al.*, 2001). These methods are based on original publications by Norstrom and Won (1985) and Norstrom and Simon (1991).

2.4.6. Statistical Analysis

All chemical residue and biological measurement data were determined to be normally distributed (Kolmogorov-Smirnov test, $P<0.05$). Contaminant levels and biological measurements were compared among sites with a one-way analysis of variance (ANOVA); significant differences were determined using Tukey's multiple comparison procedure. Since there can be considerable variation in contaminant burdens among individual embryos within a site (e.g. due to differences in diet, over-wintering locations of parent birds etc.) the presumed cause of variation for the biological endpoints was considered to be contaminant exposure and not site. Therefore relationships between contaminant levels and biological measurements were determined using regression analyses and coefficients of determination (R^2). Unless stated otherwise, a value of $P<0.05$ was considered statistically significant in all analyses. The probabilities reported in Table 2.1 do not reflect the experimentwise error rate. Correction can be made using the Bonferroni or Dunn-Sidak methods. All statistical analysis was conducted with Sigmastat statistical software (Jandel Scientific, Chicago, IL, USA).

2.5. Results

2.5.1. Organochlorine Contaminant Levels

The organochlorine residue data for individual yolk sac samples were analyzed to determine means for each major class of contaminants for each site (Fig. 2.1). For all samples combined, the total PCB fraction accounted for greater than 60% of the total residues, while the PCDD/PCDF fraction accounted for less

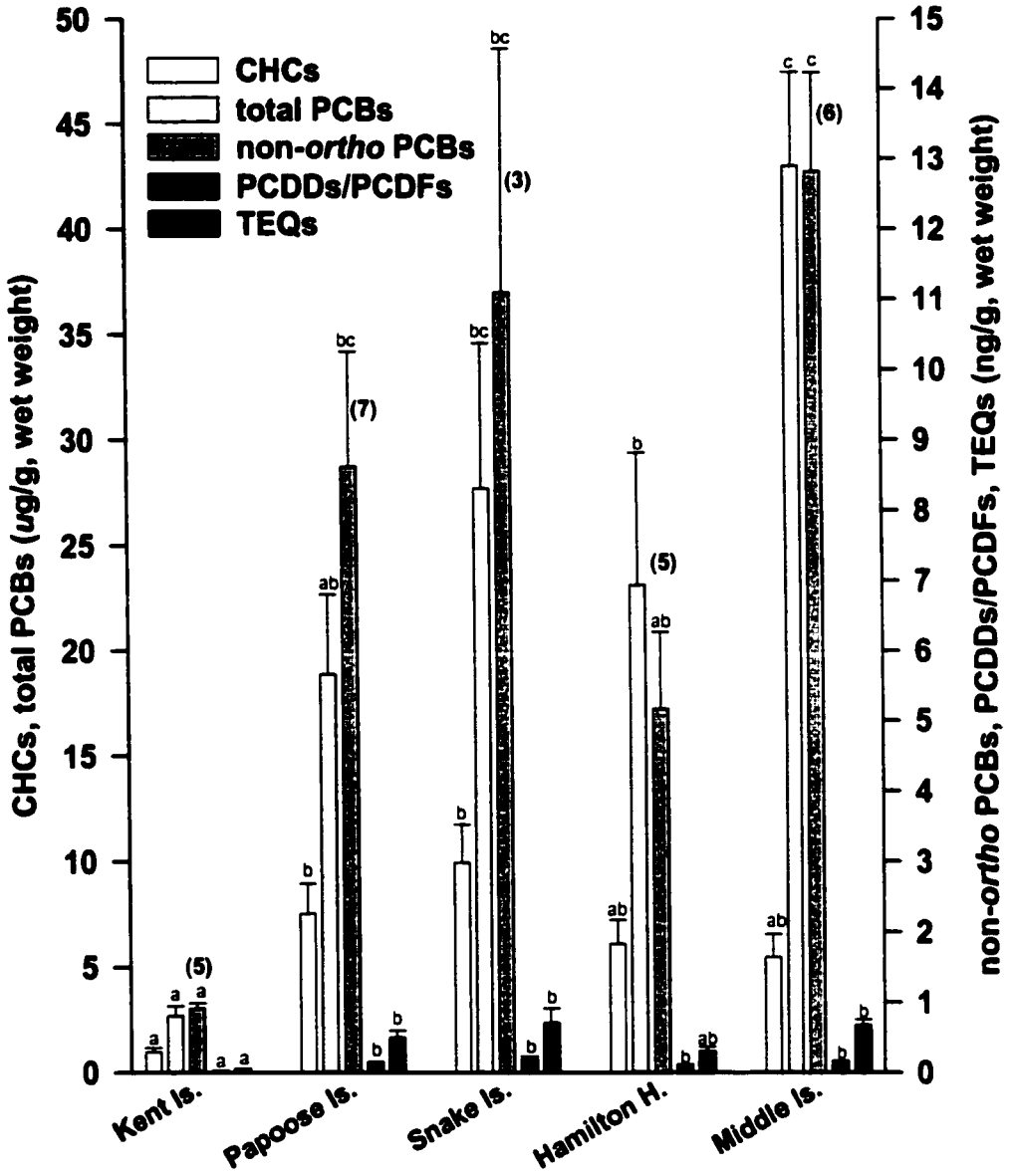


Figure 2.1 Mean organochlorine residues and TEQs in herring gull embryo yolk sacs. Bars for each class of organochlorines, or TEQs, identified with the same letter among sites are not significantly different from each other (ANOVA, Tukey's, P>0.05; (n) = number of yolk sacs; error bars = standard error of mean).

than 1% of the total residues. Of the 59 congeners detected, PCB 153 was the dominant PCB found in the yolk sacs. For the CHCs, *p,p'*-DDE accounted for greater than 75% of this fraction. Similarly, PCB 126 accounted for greater than 75% of the non-*ortho* substituted PCB residues. Samples from the control site (Kent Island) did not always have significantly lower concentrations of the major classes of organochlorine residues than the Great Lakes sites (see results of Tukey test, P>0.05, Fig. 2.1).

Based on a previous study in which the relative cytochrome P4501A (CYP1A) inducing potencies (induction equivalency factors, IEFs) of specific organochlorine contaminants in primary hepatocyte cultures prepared from herring gull embryos were reported (Kennedy *et al.*, 1996a), yolk sac residue data were also expressed as TEQs (Fig. 2.1). Expression of contaminant residue data in this manner allows determination of the potential toxicity of complex environmental mixtures by taking into account the extreme range of potencies of specific isomers and congeners to induce CYP1A, their specific concentrations in a given sample, and interspecies differences in sensitivity to these chemicals (Kennedy *et al.*, 1996b). Similar to the residue concentration data, samples from the control site (Kent Island) did not always have significantly lower TEQs than the Great Lakes sites (see results of Tukey test, *P*>0.05, Fig. 2.1). The low degree of statistically significant differences between the control and test sites can largely be attributed to the high level of individual variation in yolk sac organochlorine residue content. As a result, all biological responses were analyzed by considering mean responses for a given site

and, where data were available, by correlating individual responses with individual organochlorine residue data and TEQs.

2.5.2. *Plasma Corticosterone*

Basal plasma corticosterone concentrations were measured in herring gull embryos from all sites. No statistically significant differences were observed when mean corticosterone concentrations were compared among embryos from different sites (Fig. 2.2, bar graph). However, significant negative correlations were observed when individual yolk sac PCDDs/PCDFs, total PCBs, non-*ortho* PCBs, or TEQs were regressed against basal plasma corticosterone levels for the same individuals (Fig. 2.2, scatter graphs; Table 2.1). There was no significant correlation observed when yolk sac CHC concentrations were regressed against basal plasma corticosterone levels for individual embryos (Table 2.1, $P>0.05$).

2.5.3. *Intermediary Metabolic Enzymes*

The activities of four intermediary metabolic enzymes were measured in herring gull embryo liver or kidney cytosol, or both. Of the enzymes measured only hepatic and renal PEPCK and hepatic ME activities tended to be lower for embryos collected at the Great Lakes sites than at the Atlantic site (Figs. 2.3 and 2.4, bar graphs). However, in many cases, these activities were not statistically different from those measured for embryos collected at Kent Island (see results of Tukey's tests for Figs. 2.3 and 2.4, bar graphs).

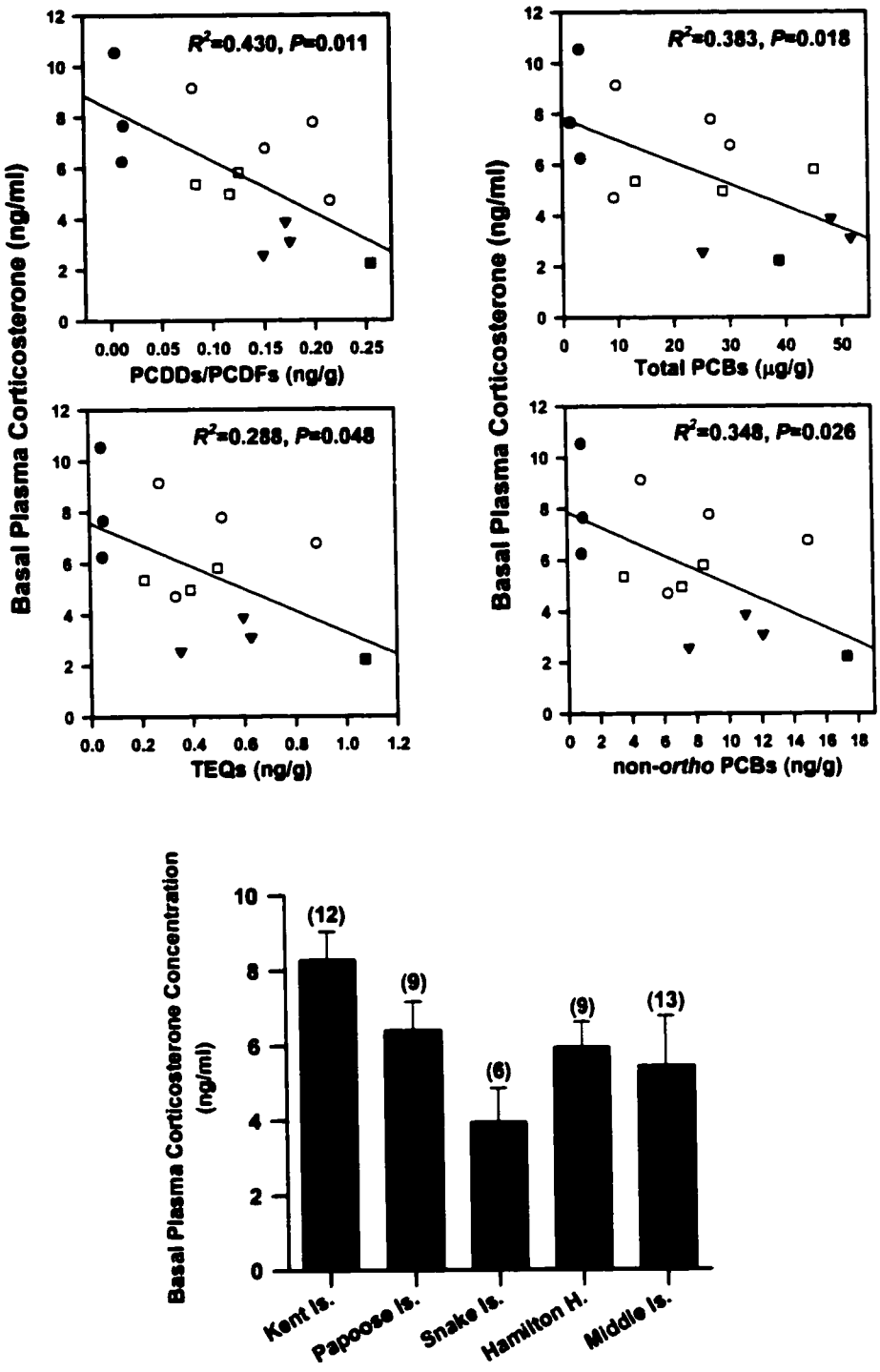


Figure 2.2 Basal plasma corticosterone levels of herring gull embryos. *Bar Graph:* Bars represent mean basal plasma corticosterone concentrations. There are no significant differences among sites (ANOVA, Tukey's, P>0.05; (n); error bars = standard error of mean). *Scatter Graphs:* Linear regression of individual basal plasma corticosterone data for each class of organochlorines, or TEQs, where a significant correlation (P<0.05) was obtained are shown. Symbols represent each site as follows:

● Kent Is., ○ Papoose Is., ■ Snake Is., □ Hamilton H., ▼ Middle Is.

Table 2.1 Coefficients of Determination (R^2). Individual biological measurements for each endpoint were linearly regressed against TEQs, or the concentrations of each of the 4 major classes of organochlorines, determined for the individual yolk sacs. The significance of the correlation was $P<0.05$, unless specifically indicated.

Endpoint	(n)	TEQs	total PCBs	non-ortho PCBs	CHCs	PCDDs/ PCDFs
basal corticosterone	14	0.288 P=0.048	0.383 P=0.018	0.348 P=0.026	0.0851	0.430 P=0.011
PEPCK - liver	12	0.075	0.144	0.0692	0.0262	0.225
PEPCK - kidney	12	0.140	0.177	0.129	0.0933	0.312 P=0.059
malic enzyme	12	0.00655	0.0436	0.00363	0.000459	0.357 P=0.040
ALAT - liver	12	0.203	0.0530	0.187	0.168	0.00323
ALAT - kidney	12	0.000134	0.00913	0.0000209	0.126	0.128
ASPAT - liver	12	0.0662	0.0234	0.0602	0.0543	0.0129
ASPAT - kidney	12	0.101	0.131	0.141	0.00123	0.0130

Correlation coefficients for regression analysis of individual yolk sac organochlorine residue data, or TEQs, against PEPCK or ME activities are shown in Table 2.1. Significant correlations were observed for regression of individual yolk sac PCDD/PCDF concentrations and ME activities for the same individuals (Fig. 2.4, scatter graph; Table 2.1). Regression of individual yolk sac PCDD/PCDF

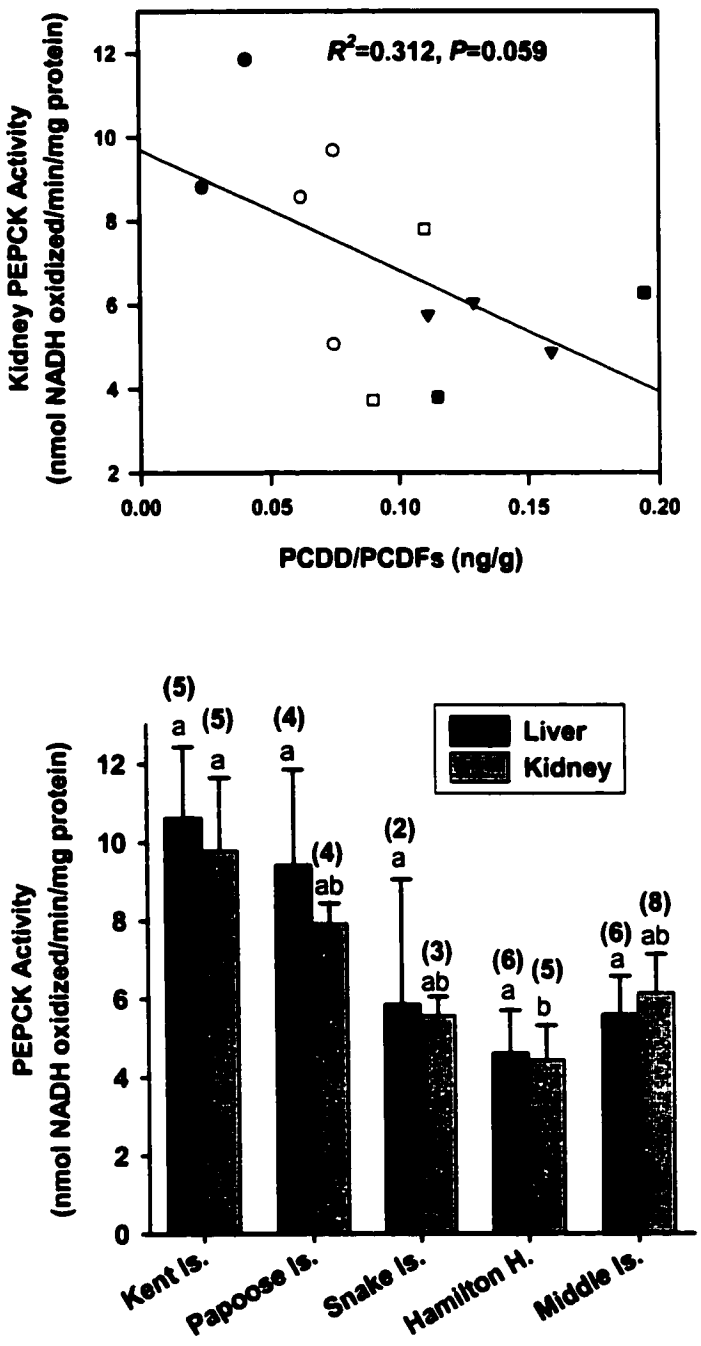


Figure 2.3 PEPCK activity of herring gull embryo liver and kidney cytosol. *Bar Graph:* Bars for each response identified with the same letter among sites are not significantly different from each other (ANOVA, Tukey's, P>0.05; (n); error bars = standard error of mean). *Scatter Graph:* Linear regression of individual kidney PEPCK activity data and individual yolk sac PCDD/PCDF concentrations is shown. Site symbols are described in Fig. 2.2, legend.

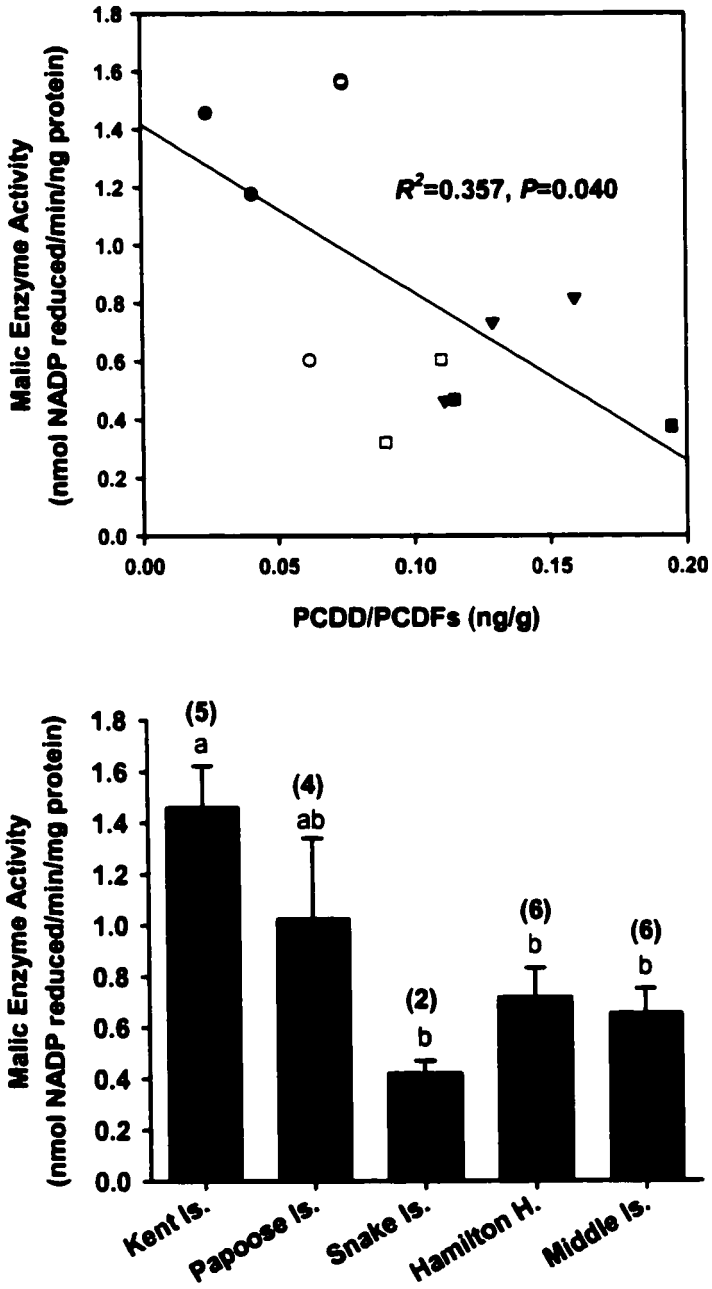


Figure 2.4 Malic enzyme activity of herring gull embryo liver cytosol. *Bar Graph:* Bars identified with the same letter among sites are not significantly different from each other (ANOVA, Tukey's P>0.05; (n); error bars = standard error of mean). *Scatter Graph:* Linear regression of individual ME activity data and individual yolk sac PCDD/PCDF concentrations is shown. Site symbols are as described in Fig. 2.2, legend.

concentrations with kidney cytosolic PEPCK also indicates a possible relationship that borders on statistical significance (P=0.059, Fig. 2.3, scatter graph; Table 2.1).

No statistically significant differences were observed among sites for either hepatic or renal ALAT or ASPAT activities (Fig. 2.5). Similarly, correlation coefficients obtained from regression data comparing individual organochlorine residues, or TEQs, against the activities of ALAT or ASPAT for individual embryos, did not reveal any significant relationships (P>0.05, Table 2.1).

2.6. Discussion

Studies were conducted to survey and detect differences in HPA axis-related endpoints in herring gull embryos environmentally exposed to various concentrations and mixtures of organochlorine contaminants *in ovo*. Although not apparent in site to site comparisons, regression analysis of individual yolk sac organochlorine residue concentrations against individual basal plasma corticosterone concentrations indicated statistically significant inverse relationships for PCDDs/PCDFs, total PCBs, and non-*ortho* PCBs. A similar trend was observed when basal plasma corticosterone concentrations were regressed against TEQs calculated for the individual yolk sac residue data using IEFs obtained for herring gull embryo hepatocyte cultures. The activity of malic enzyme was shown by regression analysis to be inversely related to yolk sac PCDD/PCDF concentrations. A similar, but weaker, relationship may also exist for renal PEPCK. Both of these intermediary metabolic enzymes are known to be regulated, at least in part, by glucocorticoids. In contrast, the activities of ALAT and ASPAT did not appear to be influenced by *in ovo* exposure of herring gull embryos to organochlorine

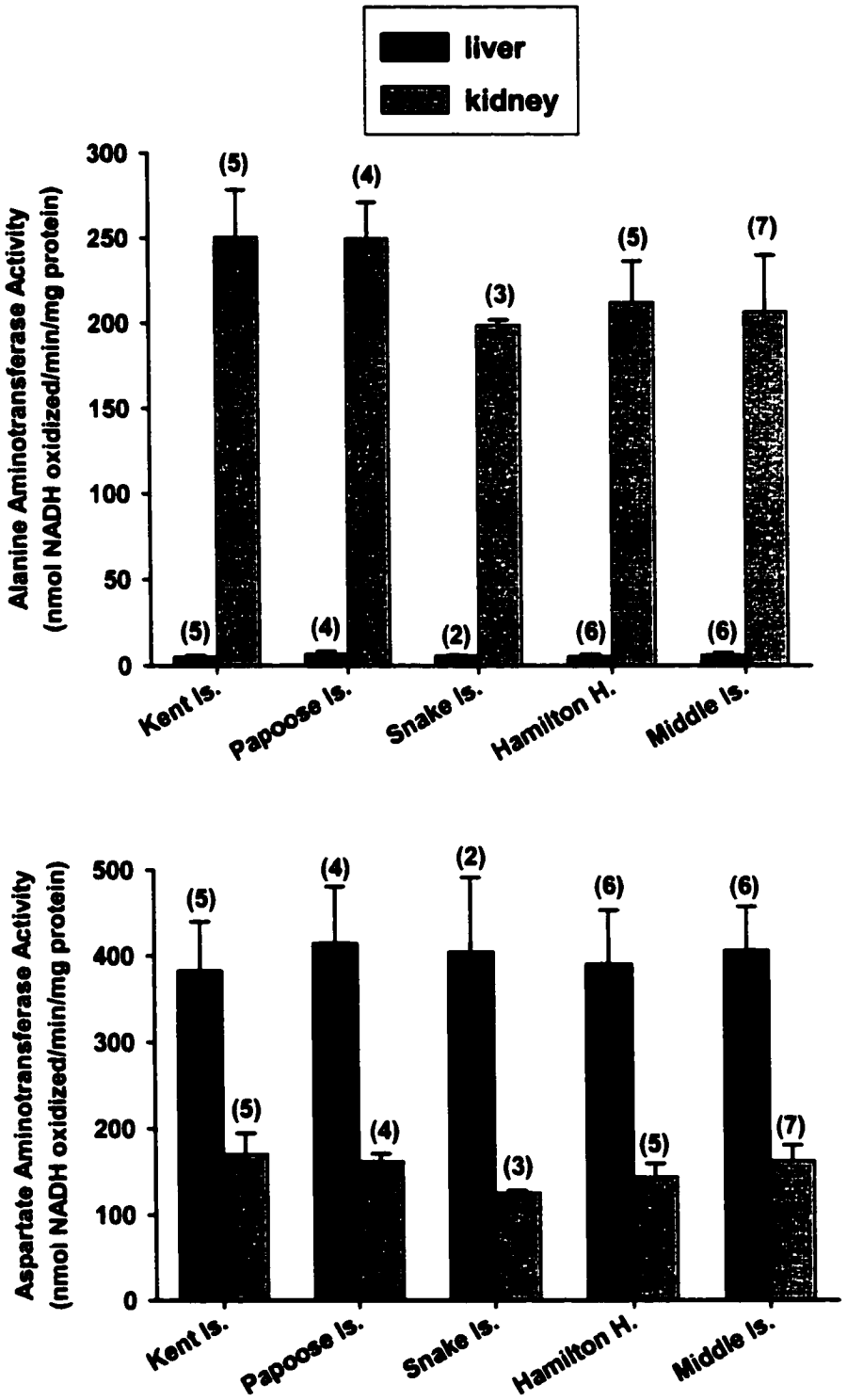


Figure 2.5 ALAT and ASPAT activity of herring gull embryo liver and kidney cytosol. There are no significant differences among sites (ANOVA, Tukey's, $P>0.05$; (n); error bars = standard error of mean).

contaminants. Overall, these data suggest that current organochlorine contamination may be affecting the HPA axis and associated intermediary metabolic pathways in environmentally-exposed herring gull embryos in the Great Lakes.

2.6.1. Plasma Corticosterone

Basal plasma corticosterone concentrations of individual herring gull embryos were found to negatively correlate with calculated TEQs and all major classes of organochlorine contaminants, except CHCs. This finding is interesting in light of previous studies in which metabolites of the predominant environmental CHC, DDT, were shown in laboratory studies to be directly toxic to the adrenal gland in birds (Jönsson *et al.*, 1994). Another study showed significant decreases in adrenal and plasma corticosterone concentrations in 5 week old chickens treated with as little as 5 ppm *p,p'*-DDT over the course of several weeks (Srebocan *et al.*, 1971). The primary DDT metabolites measured in brain, adrenal glands and liver of chickens during this feeding study were *p,p'*-DDE and *p,p'*-DDD (0.5 - 2.7 ppm depending on the tissue and metabolite). In comparison, the concentration of the predominant DDT metabolite, *p,p'*-DDE, measured in the herring gull embryo yolk sacs in the present study ranged from as low as 0.44 ppm (individual from Kent Island) to as high as 9.75 ppm (individual from Papoose Island). Thus it is conceivable that adrenotoxic effects due to DDT and its metabolites requires chronic (several weeks) exposure and would not become apparent until well after hatching.

The observation of reduced basal plasma corticosterone concentrations in herring gull embryos exposed to high environmental concentrations of

organochlorines is in contrast to recently published observations in amphibians. Mudpuppies environmentally exposed to a similar complex mixture of chemicals in the Ottawa River had *elevated* basal corticosterone levels compared to mudpuppies at a reference site on the same river (Gendron *et al.*, 1997). However, in this same study, it was also shown that exposure to organochlorine contaminants reduced the abilities of the mudpuppies to elevate blood corticosterone concentrations in response to an acute stress or ACTH challenge. Similarly, teleost fish environmentally exposed to complex mixtures of bleached kraft pulp mill effluent, PCBs, polycyclic aromatic hydrocarbons, mercury and other metals showed a reduced ability to elevate blood cortisol in response to an acute stress, and cellular atrophy was observed in tissues within the HPA axis (Hontela *et al.*, 1992, 1995, 1997).

Similar to fish and amphibians, measurement of plasma corticosterone is the most common endpoint used to assess the functional status of the HPA axis and/or adrenal gland in wild and domestic birds (Peakall *et al.*, 1979, 1981; Harvey *et al.*, 1980; Wentworth and Hussein, 1985; Fowler *et al.*, 1995). To assess adrenal function, ACTH is used to stimulate adrenal corticosterone production and secretion. This approach has been optimized and successfully used as a diagnostic test of adrenal insufficiency in racing pigeons (*Columbia livia domestica*) (Lumeij *et al.*, 1987) and black ducks (*Anas rubripes*) (Spelman *et al.*, 1995). Stressors, such as heat or cold exposure, effectively stimulate the entire HPA axis, also resulting in corticosterone synthesis and secretion. Several studies have shown that the HPA axis of the developing chicken embryo is sensitive to changes in environmental

temperatures (Avrutina *et al.*, 1985; Al-hassani, 1993; Jacobs, 1996). Furthermore, ACTH administration has been used successfully to induce corticosterone synthesis and secretion in avian embryos (Decuypere *et al.*, 1989; Rombauts *et al.*, 1994). Although experiments were conducted in 1997 to determine whether adrenal function (using ACTH stimulation) and HPA axis function (using heat exposure) were altered in environmentally-exposed herring gull embryos in the present study (data not shown), the sample sizes were insufficient to draw any conclusions.

2.6.2. *Intermediary Metabolic Enzymes*

This study also examined the impact of sampling site and contaminant levels on a number of intermediary metabolic enzymes thought to be regulated by HPA axis activation and subsequent adrenal steroid hormone release in mammals and, to some extent, in birds. Despite the lack of previous studies, corticosteroids are generally thought to be gluconeogenic in birds. Thus, the activity of PEPCK, a key regulatory enzyme in the gluconeogenic pathway, would be expected to increase in the presence of elevated corticosteroid levels. This prediction is supported by *in vitro* studies in which exposure of isolated chicken kidney tubules (Watford, 1989) or an avian hepatoma cell line (Savon *et al.*, 1993) resulted in induction of cytosolic PEPCK mRNA. However, in other studies, chickens and owls fed a high protein low glucose diet, showed only small increases in hepatic PEPCK activities (Myers and Klasing, 1995), while fed and 72 h-starved pigeons showed high, but unchanged activities of this enzyme (Söling and Kleineke, 1976). In the present study, site to site comparisons indicated a trend of reduced PEPCK activity for embryos collected

from the Great Lakes sites compared to the control site. However, in most cases the difference between sites was not statistically significant. On an individual basis, kidney cytosolic PEPCK activity appeared to be weakly inversely correlated with individual yolk sac PCDD/PCDF concentrations (P=0.059). Although the mechanism of action by which corticosteroids regulate PEPCK expression in different species is not fully understood, the results of the present study suggest that the reduced basal plasma corticosterone concentrations observed in the most highly contaminated embryos may be sufficient to impair regulation of PEPCK activity in these birds.

Gluconeogenesis produces glucose from non-carbohydrate precursors, especially amino acids. ALAT and ASPAT are necessary to provide carbon skeletons from alanine and aspartate to gluconeogenesis, such that under gluconeogenic conditions, and possibly corticosteroid regulation, activities of these two enzymes are increased. Activities of these enzymes may also be elevated due to their role in shuttling malate across mitochondrial membranes for utilization by cytosolic PEPCK, the form which has been shown to be corticosteroid-inducible in all animals studied (reviewed in Watford, 1989). However, based on previous studies, some caution must be taken when considering avian systems. Whereas avian embryo liver slices can incorporate ^{14}C amino acids into glucose (Nelson *et al.*, 1966; Yarnell *et al.*, 1966), it was also reported that fasted adult chickens cannot utilize aspartate to produce glucose (Sarkar, 1974). Furthermore, in another study, both chickens and owls fed a low protein low glucose diet showed a *reduced* level of ALAT (Myers and Klasing, 1995). These differences may relate to the species-specific cellular localization of PEPCK. For example, in pigeon liver, PEPCK is

essentially all mitochondrially localized, and thus amino acids are not a preferred gluconeogenic substrate (Söling and Kleineke, 1976). Whether this is the case with the herring gull is not known, but the relatively low ALAT activities in liver relative to kidney does support a limited hepatic alanine gluconeogenesis. The kidney PEPCK response to contaminants further supports an important role of amino acid gluconeogenesis in this tissue. In the present study, both ASPAT and ALAT activities were similar for all sites and individuals despite differences in organochlorine contaminant concentrations.

Malic enzyme provides NADPH for fatty acid synthesis. In mammals, corticosteroids generally stimulate lipolysis, whereas in birds, fatty acid synthesis and fat deposition appear to be favoured (Hazelwood, 1986). Thus, in the presence of elevated concentrations of corticosterone, one might predict up-regulation of the NADPH-generating enzymes in birds and down-regulation in mammals. Additionally, it has been reported that the pentose phosphate pathway may be an unimportant source of NAPDH in birds, further emphasizing the role of ME (Hazelwood, 1986). In the present study, the activity of ME was significantly reduced in all but one Great Lakes site (Papoose Island) compared to the control site. Regression analysis of individual yolk sac organochlorine residues and ME activity revealed a significant inverse relationship between PCDD/PCDF concentrations and ME activity.

2.6.3. CYP1A induction

Induction of CYP1A has commonly been used as a biomarker of exposure of birds to environmental contaminants, particularly PCDDs/PCDFs (Ellenton *et al.*,

1985; Boersma *et al.*, 1986; Sanderson *et al.*, 1994;; Elliott *et al.*, 1996). Since many organochlorine compounds are known to be ligands for the Ah receptor, this endpoint was included in the original study design. However, despite efforts to optimize the ethoxyresorufin-*O*-deethylase (EROD) assay for herring gull embryo liver microsomes, the activities were approximately 100-times lower than those previously reported for herring gull embryo liver fractions prepared by low-speed centrifugation (Boersma *et al.*, 1986). Since the formulation of the homogenization buffer used was optimized for measurement of cytosolic intermediary metabolic enzyme activities, and not for the measurement of microsomal EROD activities, it is possible that use of this buffer resulted in lower than expected absolute EROD activities. Thus, until further tissue is collected and prepared, it is unknown whether CYP1A induction is correlated with any of the endpoints measured in the present study.

2.7. Conclusions

The activities of two of four intermediary metabolic enzymes tested appear to be influenced by dioxins and furans deposited in the yolk sac. The observation of reduced kidney cytosolic PEPCK and hepatic ME activities in individuals exposed to the highest concentrations of yolk sac PCDDs and PCDFs is consistent with the finding that basal corticosterone concentrations were also reduced in similarly exposed individuals. Hence, reduced basal corticosterone production may result in impaired regulation of key intermediary metabolic enzymes. Although the long term consequences of down-regulation of intermediary metabolic enzymes were not

investigated in the present study, it is possible that deficits in gluconeogenic or lipogenic activity may play roles in the wasting observed in young chicks in highly contaminated areas within the Great Lakes basin (Harris *et al.*, 1993; Grasman *et al.*, 1996) Wasting, primarily characterized by loss of body weight and a voluntary decrease in food intake, is a well documented symptom of exposure to halogenated aromatic hydrocarbon, and in particular TCDD, in several species of laboratory animals (reviewed in Poland and Knustson, 1982). Furthermore, it has been shown in rats that TCDD inhibits several key gluconeogenic enzymes, including PEPCK, at doses that are in the same range as those causing TCDD-induced appetite suppression and weight loss (Gorski *et al.*, 1990; Weber *et al.*, 1991a, 1991b).

In conclusion, the data obtained in the present study provides the first evidence that current levels of organochlorine contamination in the Great Lakes basin may be affecting the HPA axis and associated intermediary metabolic pathways in environmentally-exposed herring gull embryos in the Great Lakes. Additional studies are required to determine whether the function of the adrenal gland or HPA axis are compromised in embryos exposed to organochlorine contaminants, and whether other species, or life stages, of birds show relationships between contaminant residues and HPA axis-related endpoints similar to those demonstrated here.

CHAPTER 3. COMPARISON OF THE ONSET OF HATCHING, PLASMA CORTICOSTERONE CONCENTRATIONS, INTERMEDIARY METABOLIC ENZYME ACTIVITIES, AND ORGANOCHLORINE CONTAMINANT RESIDUES IN HERRING GULL EMBRYOS FROM DIFFERENT GREAT LAKES SITES - 1999

3.1. Introduction

Results of studies conducted in 1997 to survey and detect differences in plasma corticosterone concentrations and intermediary metabolic enzyme activities in herring gull embryos exposed to organochlorine contaminants *in ovo* suggested that current levels of organochlorine contaminants in Great Lakes herring gull eggs may be affecting these HPA axis-related endpoints (Lorenzen *et al.*, 1999; see Chapter 2). Thus, it was of interest to confirm and extend these findings in a second study. To this end, experiments are described in which herring gull embryos collected from various Great Lakes sites were analyzed for basal plasma corticosterone concentrations, malic enzyme (ME) and phosphoenolpyruvate carboxykinase (PEPCK) activities. As well, the embryos were exposed to a heat stress to determine induced plasma corticosterone concentrations and the corticosterone stress response. To determine if there were any relationships between these biological endpoints and environmental contamination, yolk sacs from a subset of embryos were analyzed for total PCBs, non-*ortho* substituted PCBs, PCDDs/PCDFs and CHCs.

3.2. Hypothesis

Organochlorine contaminants deregulate the HPA axis in environmentally-exposed herring gull embryos resulting in altered function of the HPA axis and associated biochemical pathways.

3.3. Objectives

The objectives of this study were to compare basal and stressed plasma corticosterone concentrations and the activities of PEPCK and ME among herring gull embryos collected from six different Great Lakes sites. For a subset of embryos comparison of the biological responses to yolk sac organochlorine residues were made.

3.4. Materials and Methods

3.4.1. Egg Collection and Incubation

During April and May 1999, unincubated, fertile herring gull eggs were collected from Chantry Island (eastern side of Lake Huron), Fighting Island (Detroit River), Scotch Bonnet Island (eastern basin of Lake Ontario), Hamilton Harbour (western terminus of Lake Ontario), Middle Island (western basin of Lake Erie) and Mutton Island (near Thunder Bay, ON in Lake Superior). The eggs were brought to the National Wildlife Research Centre (Hull, QC, Canada) where they were artificially incubated until the initiation of hatching as indicated by shell starring, or for 26 days. Incubators and Incubation conditions were identical to those used in 1997 (see Chapter 2.4.1.).

3.4.2. Stress Test and Plasma Corticosterone Measurement

Blood was collected from individual herring gull embryos using the same technique as described for 1997 (Chapter 2.4.2), except that the eggs either had shells that were starred or had been incubated for 26 days. Windows were cut on day 24 of incubation, or earlier, if starring was present or anticipated for certain sites. For basal corticosterone analysis, a single blood sample (100 µl) was taken. This volume was replaced with an equivalent volume of saline (0.9% w/v NaCl). Each egg with the cannulated vessel was then placed in an incubator maintained at 41-41.5°C (approximately 4-5°C above the normal incubation temperature) for 3 h. After this stress period, 300-400 µl of blood was taken from each embryo. All embryos were sacrificed by decapitation and yolk sacs were frozen for organochlorine residue analysis. Plasma was prepared and analyzed for corticosterone as described previously (Chapter 2.4.2) except that the samples were analyzed using a Cobra II Auto-Gamma counter (Canberra Packard, Canada, Montreal, QC). Intra- and inter-assay coefficients of variation were 6.5% and 6.8%, respectively. Plasma dilutions of 1:20 were determined to be optimal for measurement of basal corticosterone concentrations and 1:50 for heat-stressed corticosterone concentrations in herring gull embryos. The corticosterone stress response refers to the difference between induced and basal plasma corticosterone concentrations for each embryo.

3.4.3. Tissue Preparation

Liver and kidneys were dissected from heat-stressed embryos after blood collection and decapitation, and frozen in liquid nitrogen. Homogenization of tissues and preparation of cytosol fractions were identical to the previous study (Chapter 2.4.3).

3.4.4. Intermediary Metabolic Enzymes

The activities of hepatic and renal PEPCK and hepatic ME were determined for each embryo using previous methods (Chapter 2.4.4), except a total cytosol protein concentration of 0.05 mg/well was used for hepatic PEPCK assays. Preliminary studies indicated that a 3 h heat stress did not affect the activities of ME or PEPCK (data not shown). Although the same instrumentation and reagent suppliers (where possible) were used, both hepatic and renal PEPCK activities were substantially higher for the 1999 than the 1997 samples. Furthermore, analysis of liver and kidney cytosol pools prepared and initially assayed in 1997 had, on average, 2.27 times greater PEPCK activity when re-analyzed in 1999. Thus to compare PEPCK data from 1999 to that obtained in 1997, a conversion factor of 2.27 is required. Data reported in both chapters represent raw data. The reason(s) for this difference is unknown.

3.4.5. Chemical Residue Analysis

Nineteen yolk sac samples from embryos that had not started to hatch (no starred shells) and were sampled on day 26 of incubation, were chosen at random

from three sites that had sufficient individuals for statistical analyses. The yolk sacs were analyzed for PCDDs, PCDFs, non-*ortho* substituted PCBs, organochlorine pesticides and other chlorinated hydrocarbons (CHCs), and total PCBs (sum of 59 congeners) as described previously (Chapter 2.4.5).

3.4.6. Statistical Analysis

Statistical analyses were conducted as previously described (see Chapter 2.4.6). A value of $P<0.05$ was considered statistically significant. For linear regressions, all data were tested for normality, and if necessary the independent and/or dependent variables were log transformed and normality tested again.

3.5. Results

3.5.1. Shell Starring

Many of the embryos collected in 1999 began hatching unexpectedly on or prior to day 26 of incubation as indicated by the presence of shell starring or pipping. Incidences of shell starring were particularly evident for fertilized eggs collected from Fighting Island and Scotch Bonnet Island with some embryos cracking the egg shell as early as day 23 of incubation (Fig. 3.1). Variation in incubation temperature probably contributed little to early starring since eggs from 2 to 3 sites were incubated within the same incubator. Furthermore, eggs collected from sites that were also studied in 1997 and for which no early starring had been observed in 1997 (Hamilton Harbour, Middle Island), did not show (Hamilton) or had low

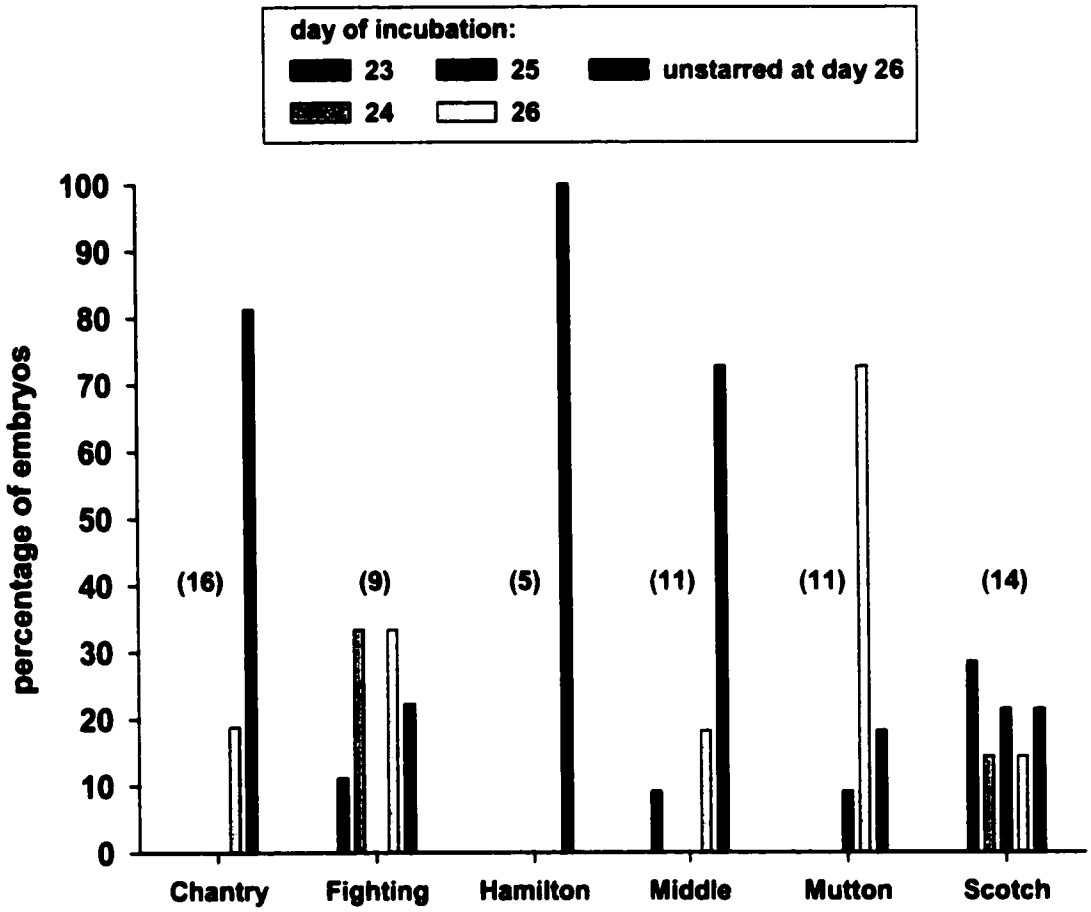


Figure 3.1 Incidences of shell starring. The percentage of embryos with starred egg shells at 23, 24, 25, 26 or unstarred at 26 days of incubation are shown for each site. Values enclosed in brackets indicate the number of embryos examined for each site.

incidences (Middle - 27%) of early starring in 1999 (Fig. 3.1). Thus the incidences of early starring may be related to the sampling site.

3.5.2. Biological Endpoints

Among the embryos sampled on day 26 of incubation, embryos with a starred shell had significantly lower induced corticosterone concentrations and corticosterone stress responses compared with those embryos that had not yet started the hatching process (Fig. 3.2; ANOVA, Tukey's $P<0.05$). The activities of ME, renal and hepatic PEPCK, or basal corticosterone concentrations were not significantly different between embryos from starred and unstarred eggs when sampled on day 26 of incubation. However, there was a minor trend towards higher ME, renal and hepatic PEPCK activities, or basal corticosterone concentrations in the embryos from the eggs with starred shells.

The biological endpoints were further examined for embryos from Scotch Bonnet Island that had starred egg shells and that were sampled on day 23 to day 26 of incubation. For samples from this site, differences appeared to exist for each of the endpoints that may be related to the day of incubation (Fig. 3.3). Of particular note is the trend observed for induced corticosterone concentrations and the corticosterone stress response which appeared to peak on day 25 of incubation and decreased markedly by day 26 of incubation. However, due to low sample sizes a more rigorous interpretation of these data cannot be made.

Due to the evidence suggesting that the day of incubation on which the embryos were sampled may confound interpretation of the biological data, further

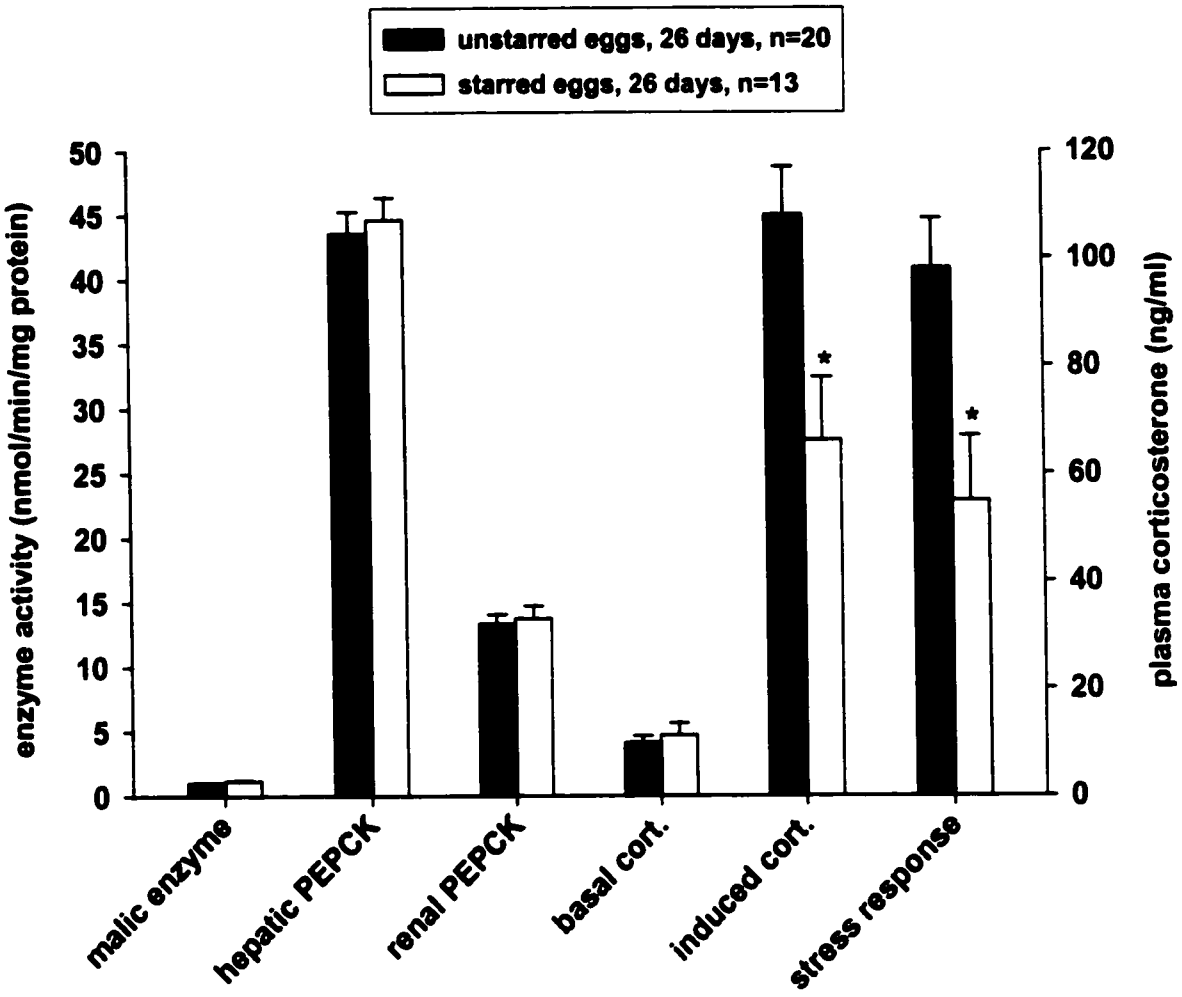


Figure 3.2 Effect of shell starring on biological endpoints. The mean activities of malic enzyme, hepatic PEPCK and renal PEPCK, or the mean concentrations of basal plasma corticosterone, induced plasma corticosterone and the corticosterone stress response are shown for eggs from all sites without starred shells sampled on day 26 of incubation. Error bars indicate standard error. Asterisks indicate statistically significant differences between starred and unstarred eggs for a given endpoint (ANOVA, Tukey's, $P<0.05$).

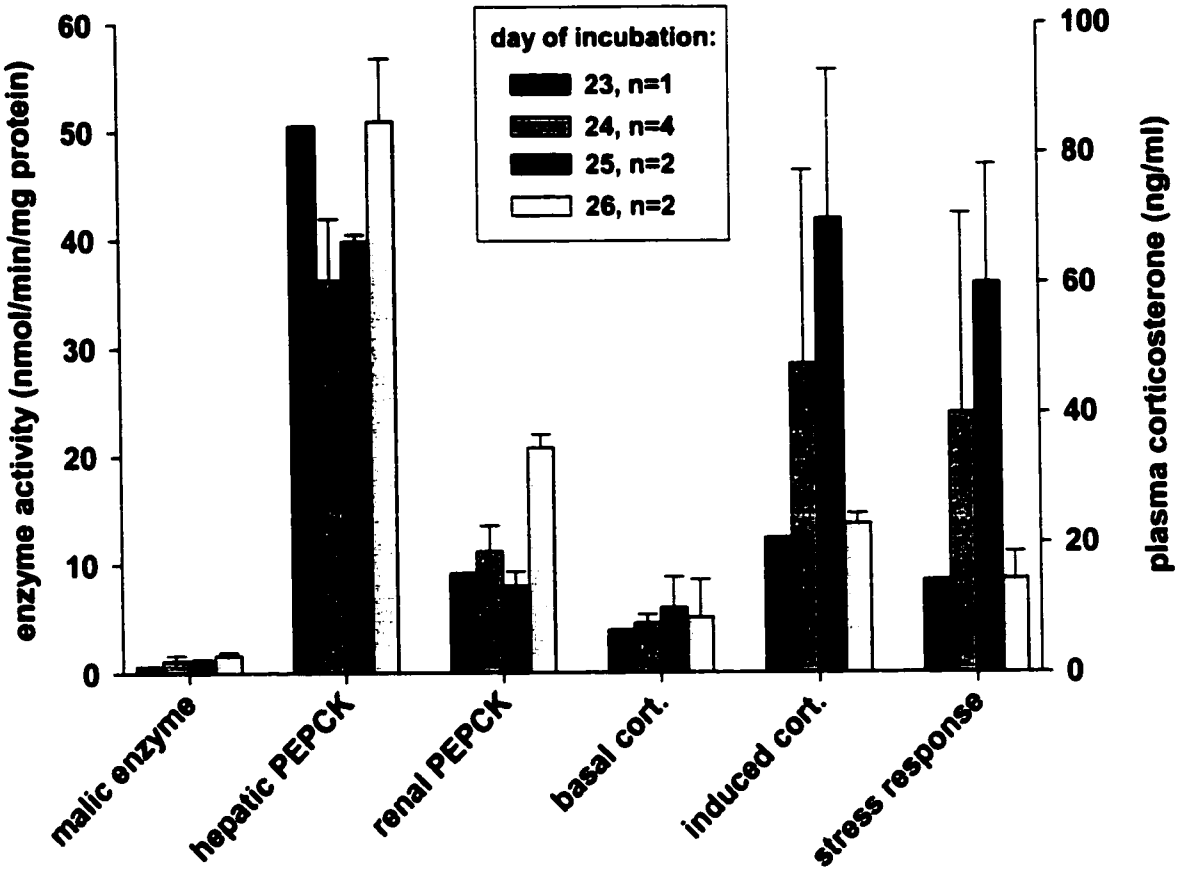


Figure 3.3 Effect of incubation time on biological endpoints. Bars show mean responses for embryos from Scotch Bonnet Island that had starred egg shells at the time of sampling. Legend indicates the day of incubation on which the embryos were sampled and the number of individuals sampled on each day. Error bars indicate standard deviation.

analyses of the data were confined only to embryos that had been sampled on day 26 of incubation. Furthermore, to account for variability due to the initiation of the hatching process, as indicated by the presence of a starred shell, these data were further subdivided into groups of embryos that had or had not starred the egg shell. However, no statistically significant differences (possibly due to the low sample sizes) were observed for any of the biological endpoints between unstarred and starred eggs (data not shown). Therefore embryos from both unstarred and starred eggs for each site were grouped for each endpoint.

Mean ME activities of embryos sampled on day 26 of incubation for each sampling site are shown in Fig. 3.4. Site to site comparisons revealed significant differences in mean ME activities between Mutton Island and Scotch Bonnet Island (ANOVA, Tukey's, $P<0.05$). No significant differences were observed for mean hepatic PEPCK activities among sites (Figure 3.5, top graph). Mean activities of renal PEPCK were significantly higher for Scotch Bonnet Island embryos compared to embryos from all other sites (Figure 3.5, bottom graph; ANOVA, Tukey's, $P<0.05$). Neither mean basal or induced plasma corticosterone concentrations, nor the mean corticosterone stress responses were statistically different among sites (Fig. 3.6). However, Scotch Bonnet Island embryos had the lowest mean induced plasma corticosterone concentrations and mean corticosterone stress responses compared with all other sites.

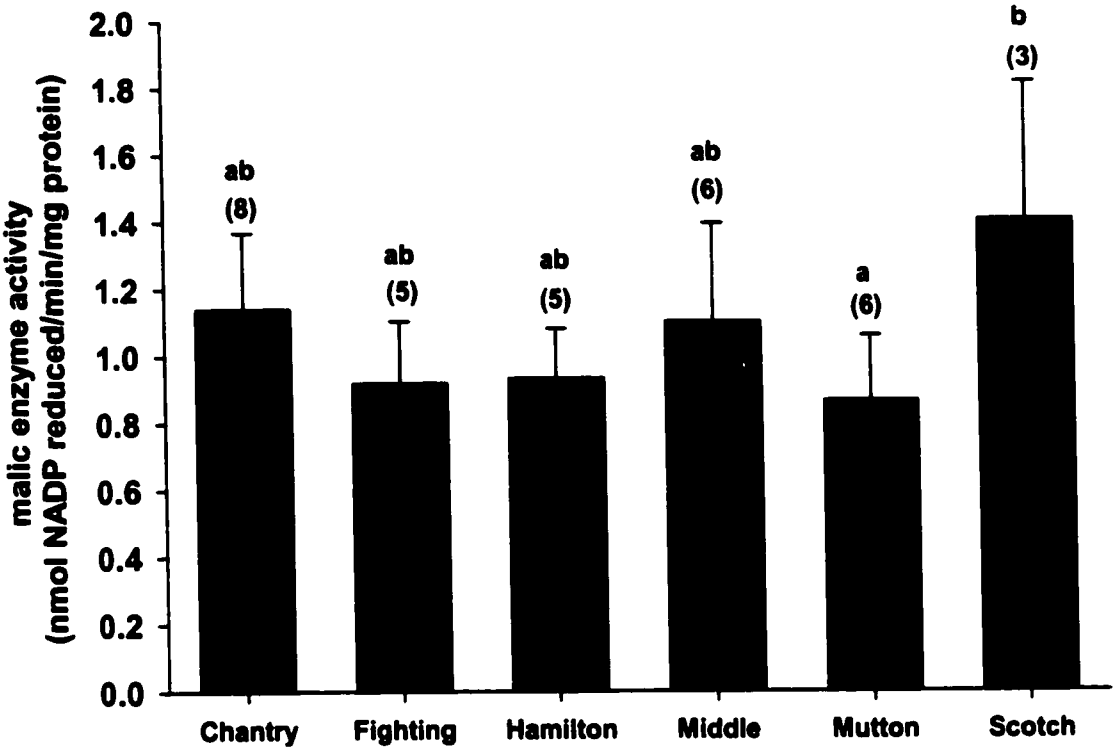


Figure 3.4 Site to site comparisons for malic enzyme activities. Mean malic enzyme activities for embryos sampled on day 26 of incubation are shown for each site. Values enclosed in brackets indicate the number of embryos examined for each site. Error bars indicate standard deviation. Sites identified with different letters were significantly different from one another (ANOVA, Tukey's, $P<0.05$).

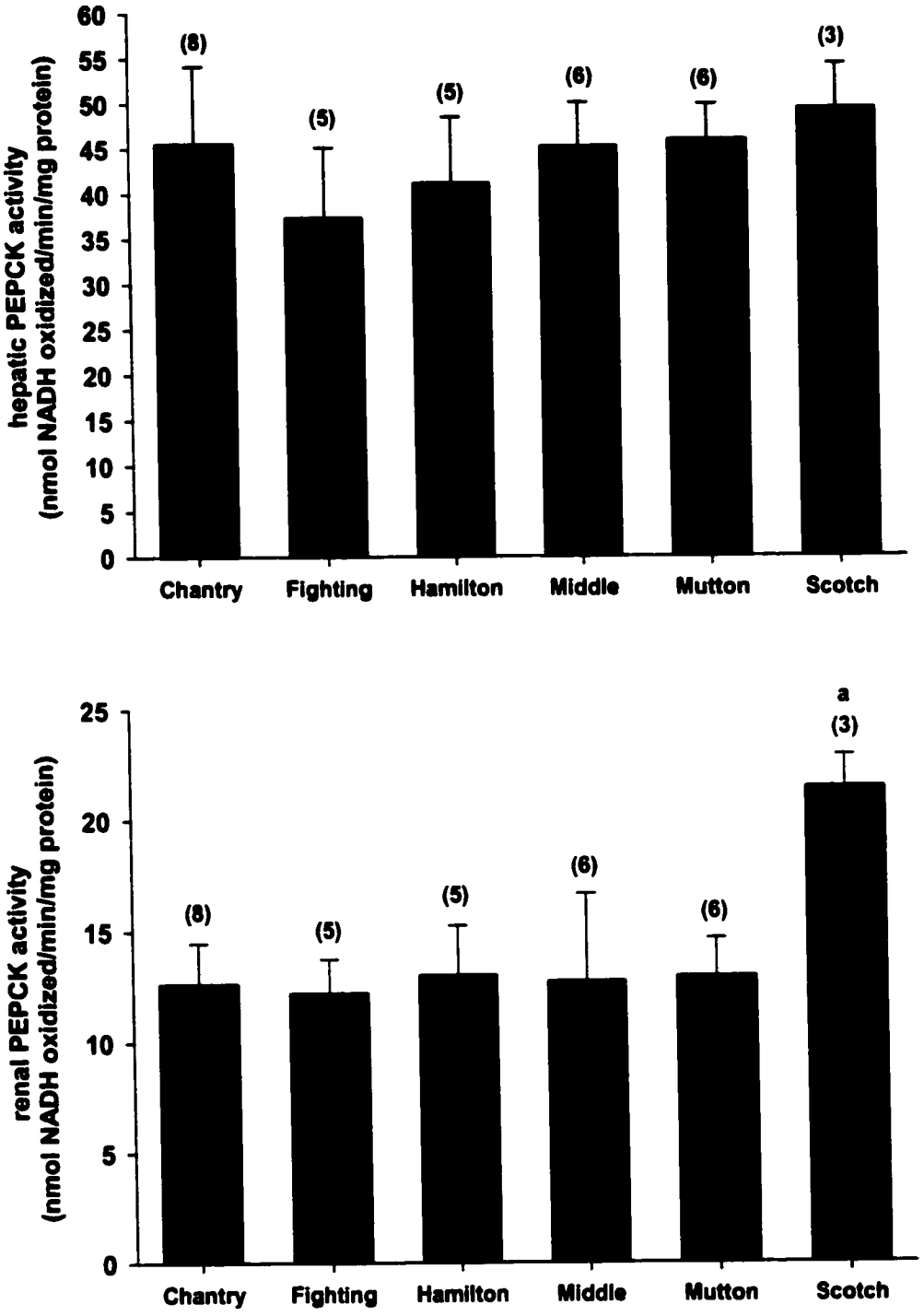


Figure 3.5 Site to site comparisons for PEPCK activities. Mean hepatic (top graph) and renal (bottom graph) PEPCK activities for embryos sampled on day 26 of incubation are shown for each site. Values enclosed in brackets indicate the number of embryos examined for each site. Error bars indicate standard deviation. Sites identified with different letters were significantly different from one another (ANOVA, Tukey's, $P<0.05$).

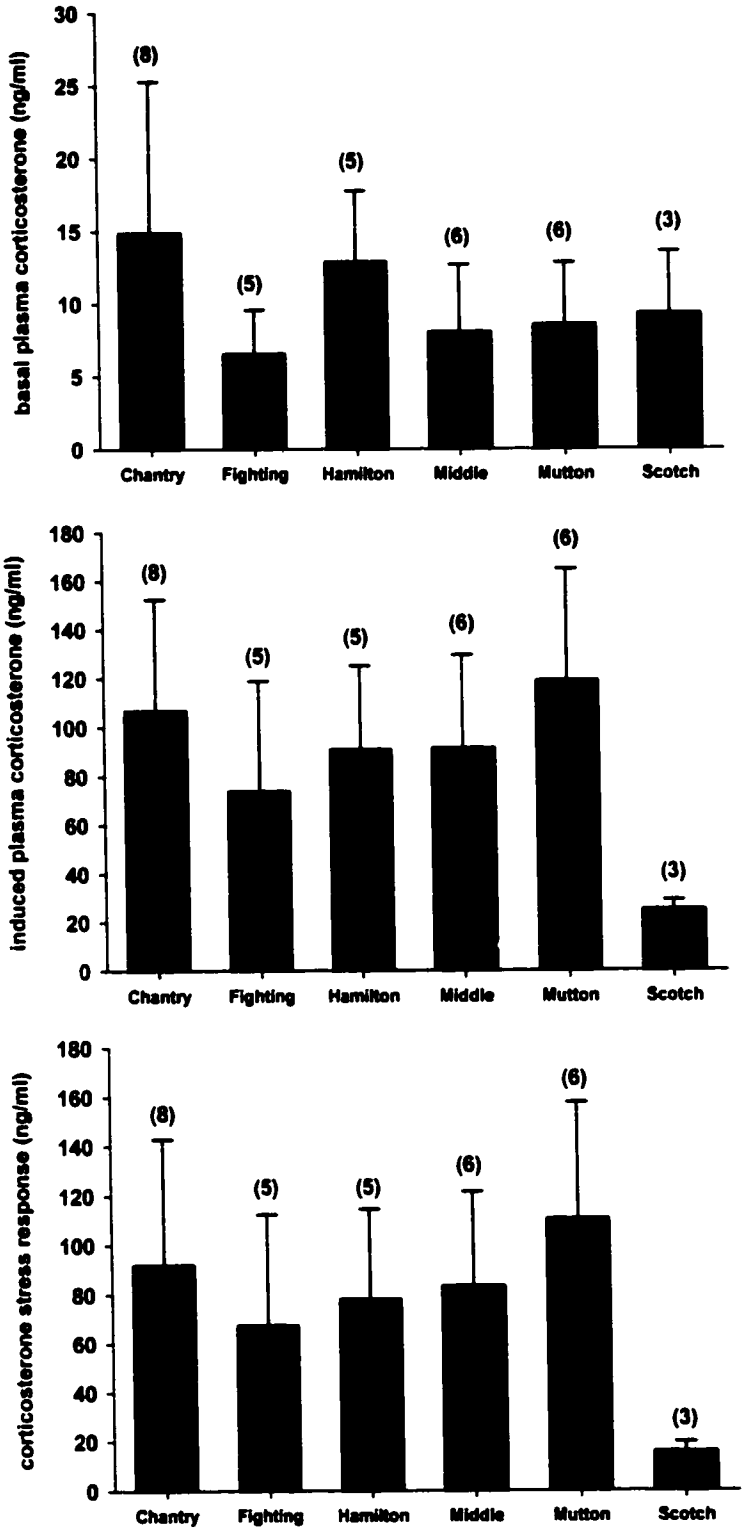


Figure 3.6 Site to site comparisons for basal and induced corticosterone and the corticosterone stress response. Mean basal plasma corticosterone concentrations (top graph), induced plasma corticosterone concentrations (middle graph) and the corticosterone stress response (bottom graph) for embryos sampled on day 26 of incubation are shown for each site. Values enclosed in brackets indicates number of embryos examined for each site. Error bars indicate standard deviation.

3.5.3 Correlation of Biological Endpoints

Linear regression analyses were conducted to determine if there were any significant relationships between ME or PEPCK activities and basal plasma corticosterone concentrations or the corticosterone stress response for embryos from starred or unstarred eggs sampled on day 26 of incubation. A significant inverse relationship (R^2=0.503, P=0.007) was observed for embryos from starred eggs for ME activities and the corticosterone stress response (Fig. 3.7, top graph). A significant positive relationship (R^2 = 0.289, P=0.014) was observed for embryos from unstarred eggs for log ME activities and basal corticosterone concentrations (Fig. 3.7, bottom graph). No correlations were found for PEPCK activities and either corticosterone endpoint.

3.5.4. Organochlorine Contaminant Analyses

Mean CHC, total PCB, PCDD/PCDF and non-*ortho* substituted PCB concentrations for a subset of herring gull embryo yolk sacs for Chantry Island, Hamilton Harbour and Middle Island are shown in Fig. 3.8. There were no statistically significant differences among the three sites for CHCs or PCDDs/PCDFs (ANOVA, Tukey's, P>0.05). Embryo yolk sacs from Middle Island had significantly higher concentrations of total PCBs and non-*ortho* PCBs than embryo yolk sacs from either Chantry Island or Hamilton Harbour (ANOVA, Tukey's, P<0.05). Regression analysis of individual embryo yolk sac CHC, total PCB, non-*ortho* PCB or PCDD/PCDF concentrations with each of the biological endpoints revealed a

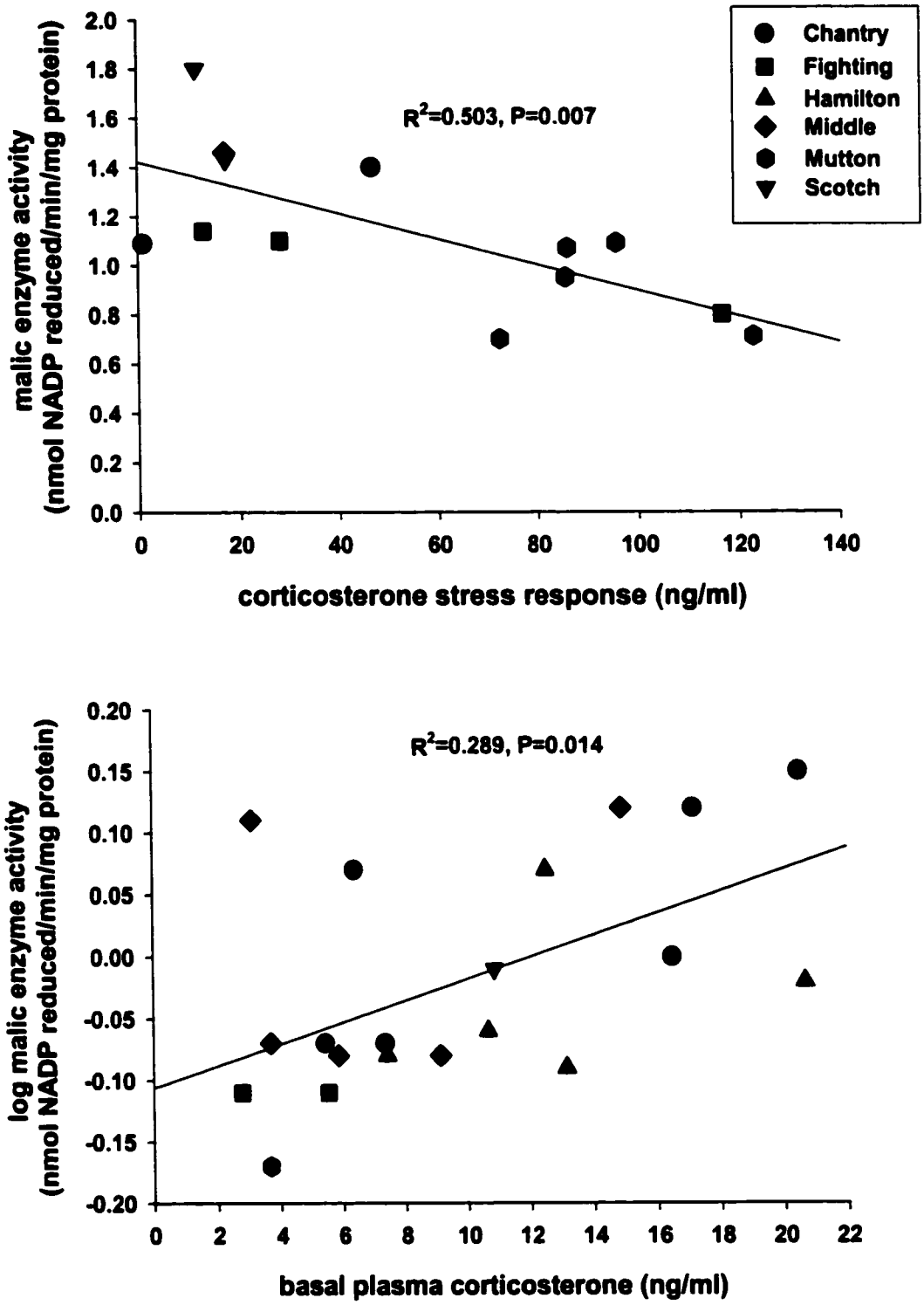


Figure 3.7. Correlation of biological endpoints. A significant inverse relationship between malic enzyme activities and corticosterone stress responses for embryos from starred eggs (top graph) and between log malic enzyme activities and basal plasma corticosterone concentrations for embryos from unstarred eggs (bottom graph) sampled on day 26 of incubation are shown. Legend indicates symbols used to represent each site. Lines indicate linear regression of the data for each graph; P and R^2 values are shown.

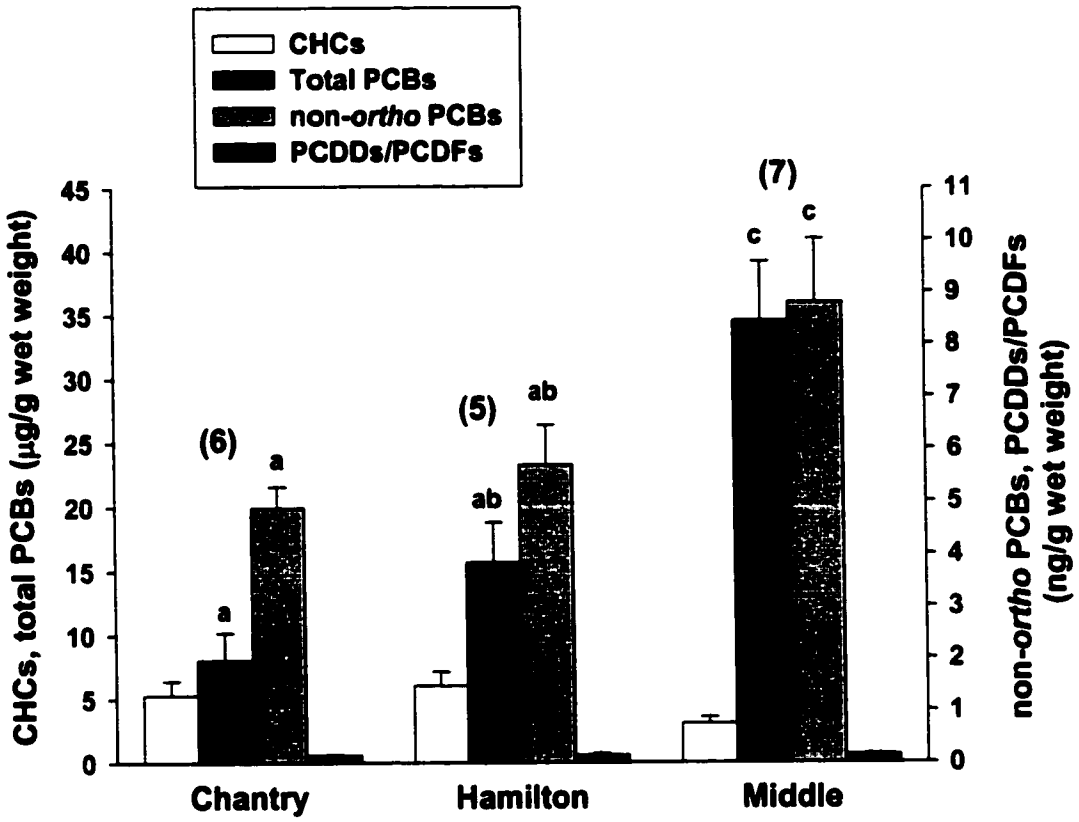


Figure 3.8 Mean organochlorine contaminant residues. Bars represent mean organochlorine concentrations determined for embryo yolk sacs from each of three sites as indicated in the legend. Bars identified with different letters were significantly different among sites (ANOVA, Tukey's $P<0.05$). The number of samples per site is indicated (n), and error bars represent standard error.

single statistically significant positive relationship between CHCs and basal plasma corticosterone concentrations ($P=0.001$, $R^2 = 0.479$; Fig. 3.9).

3.6. Discussion

3.6.1. Early Onset of Hatching

Initiation of the hatching process in birds first becomes evident by starring or fracture of the egg shell by the embryo. This is followed by complete puncture of the egg shell or "pipping". Both of these endpoints usually indicate that the embryo has broken through the shell membrane to access the air space of the egg and has begun to ventilate its lungs (Düttman *et al.*, 1998). Typically, starring and pipping precede the onset of active hatching by approximately 15 h for domestic chicken embryos (Freeman and Vince, 1974a). The results of the present study indicated that herring gull embryos from some of the Great Lakes sites examined had incidences of early shell starring. These observations were particularly notable for Scotch Bonnet Island (eastern basin of Lake Ontario) and Fighting Island (Detroit River) for which incidences of shell starring were observed as early as day 23 of incubation. The normal incubation period for the herring gull is 26-28 days.

Previous reports suggest that both the onset of breathing and starring of the egg shell result from a gaseous stimulus involving an increase in carbon dioxide concentrations (reviewed in Freeman and Vince, 1974a). In contrast, the stimulus which initiates active hatching is not thought to be gaseous, but may involve hormones (Freeman and Vince, 1974a). Both the onset of active hatching and pulmonary respiration in the avian embryo have been induced by exposure to

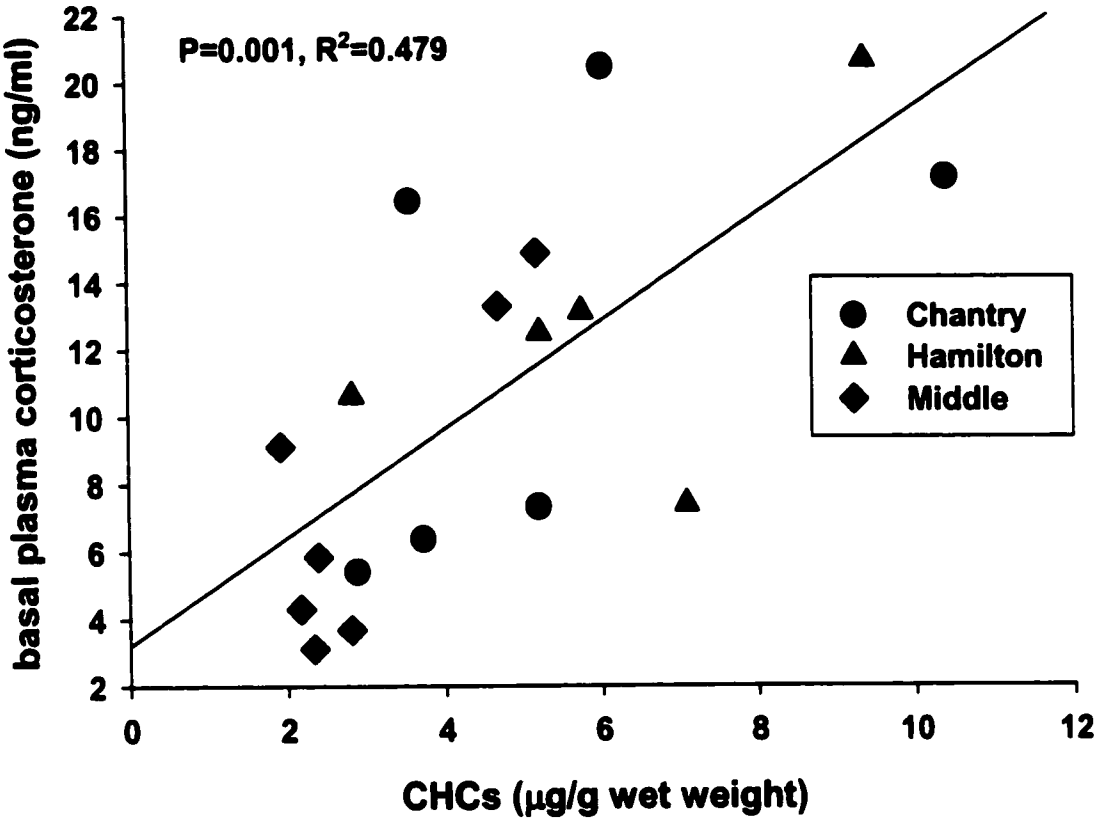


Figure 3.9 Relationship between basal plasma corticosterone concentrations and CHCs. A significant positive relationship between basal plasma corticosterone concentrations and CHCs is shown. Legend indicates symbol used to represent each site. Line indicates linear regression of the data; P and R^2 values are shown.

thyroid hormones (reviewed in Freeman and Vince, 1974a, 1974b). Furthermore, suppression of the natural secretion of thyroid hormones with substances such as thiouracil or thiourea has been shown to result in decreased growth rates, decreased metabolic rates, hypertrophy of the thyroid, and a delay in the time of hatching in avian embryos (reviewed in Freeman and Vince, 1974b).

Interestingly, thyroid glands collected from adult herring gulls from various Great Lakes sites between 1974 and 1983 had greater masses, were microfollicular and frequently were hyperplastic compared with samples obtained from an Atlantic coast control site (Moccia *et al.*, 1986). It was concluded that herring gulls from the Great Lakes basin suffered from goiter that is typically associated with iodine deficiency or insufficient thyroid hormone concentrations. Comparison of spatial and temporal differences in the severity of the thyroid disfunction were consistent with the hypothesis that halogenated aromatic hydrocarbons (HAHs) were responsible for these effects (Moccia *et al.*, 1986). Further analyses of samples collected from Great Lakes herring gulls in the early 1990's, indicated only slight enlargement of the thyroid glands (Fox *et al.*, 1998). These findings were consistent with the decline in organochlorine residues observed in the Great Lakes by this time (Fox *et al.*, 1998). In other studies with common terns in the Netherlands, highest concentrations of HAHs were correlated with lower plasma thyroid hormone levels and *prolonged* incubation periods (Murk *et al.*, 1996). Thus, it is unlikely that the early onset of hatching observed for gull embryos collected at Scotch Bonnet Island or Fighting Island was related to *elevated* thyroid hormone levels. However, to confirm this, further experiments are required to determine thyroid hormone

concentrations at various stages of incubation in relation to the onset of hatching in herring gull embryos.

The onset of hatching, as indicated by the presence of a starred egg shell, was also shown to influence some of the biological endpoints measured in herring gull embryos in the present study. Those embryos that had starred the egg shell on day 26 of incubation had statistically significant lower induced corticosterone concentrations and a reduced stress response. Because hatching is an extremely complex process involving the onset of pulmonary respiration, muscle maturation, changes in circulation, withdrawal of the yolk sac, starring of the egg shell, and emergence of the chick (reviewed in Freeman and Vince, 1974a), it was not surprising to observe differences in the endpoints measured in embryos from starred and unstarred eggs in the present study.

3.6.2. Biological Endpoints

The technique used to take blood samples from the herring gull embryos is dependent on the association of the embryonic blood vessels with the shell membrane. This association only remains stable until shortly after the shell membrane is pierced by the embryo just prior to shell starring. Therefore, embryos were either sampled on day 26 of incubation as planned, or as soon as shell starring was noticed or anticipated for eggs from certain sites. Due to many incidences of shell starring, the herring gull embryos were sampled at several different incubation days. Therefore, it was important to determine what influence the day of sampling might have on the biological endpoints measured in this study. Comparison of the

biological endpoints for various days of incubation for Scotch Bonnet Island embryos showed what appeared to be time-dependent trends. In particular, induced corticosterone concentrations and the corticosterone stress response appeared to peak on day 25 of incubation.

Based on these observations, further comparisons of the biological data were only conducted for embryos that had been sampled on day 26 of incubation. Initially these embryos were further subdivided into groups of embryos for each site which had unstarred or starred egg shells. However, statistical analyses did not indicate any significant differences (possibly due to the low sample sizes) for any of the biological endpoints between unstarred and starred eggs for each site. Therefore embryos from both unstarred and starred eggs for each site were grouped for each endpoint. Despite the low sample sizes, and the possibility that starring may influence the biological responses that were measured, site to site comparisons indicated that herring gull embryos from Scotch Bonnet Island appeared to have different responses than the other sites. For example, renal PEPCK and ME activities were higher, and both the induced plasma corticosterone concentrations and stress responses were lower for Scotch Bonnet Island embryos compared with the other sites.

Interestingly, studies conducted in 1972 on herring gull embryos from Scotch Bonnet Island indicated high incidences (20%) of embryonic mortality (Gilbertson and Hale, 1974a). Further studies conducted in 1973 indicated that reproductive failure of herring gulls breeding on Scotch Bonnet Island was due to several factors including a high disappearance of nests and eggs, embryonic and chick mortality

and egg-shell breakage (Gilbertson and Hale, 1974b). Analyses of eggs collected from Scotch Bonnet Island and other nearby Lake Ontario colonies showed severe contamination with PCBs and the organochlorine insecticide metabolite, DDE (Gilbertson, 1974). Furthermore, eggshell thinning was correlated with the content of DDE in the eggs (Gilbertson, 1974). Thus, thirty years ago, the Scotch Bonnet Island colony of herring gulls was exposed to high concentrations of environmental contaminants that resulted in severe reproductive failure. Although concentrations of PCBs, DDE, Mirex and other contaminants have decreased dramatically in Lake Ontario herring gull eggs (reviewed in Ryckman *et al.*, 1997; Hebert *et al.*, 1999), it is possible that selective pressure due to environmental contaminant exposure may be related to the current observations of decreased renal PEPCK and ME activities, reduced corticosterone stress responses and early onset of hatching observed in herring gull embryos from this site in 1999.

3.6.3. Correlation of Biological Endpoints

In general, avian corticosteroids have the same major metabolic roles as in mammals. In particular, corticosterone plays an important role in carbohydrate and lipid metabolism (see Hodges, 1981). Results of the previous study (Chapter 2) indicated that both basal plasma corticosterone and metabolic enzymes (determined in different embryos) may be influenced by organochlorine contaminants. Therefore, it was of interest to determine whether there were any relationships between PEPCK or ME activities and those endpoints related to corticosterone determined for the same individual herring gull embryos in the present study. Two relationships

were observed: 1) a significant inverse relationship between ME activities and corticosterone stress responses for embryos from starred eggs, and 2) a significant positive relationship between log ME activities and basal plasma corticosterone concentrations for embryos from unstarred egg shells. These results suggest that corticosterone may modulate ME activity in herring gull embryos. Although little information is available on the hormonal, and more specifically, corticosteroid regulation of ME activity in birds, both nutritional and hormonal factors are generally regarded as the main controls of the amounts of fatty acids synthesized in birds (Stevens, 1996). The data obtained in this study for herring gull embryos suggest that under unstressed conditions, corticosterone may stimulate ME activity and hence promote lipogenesis. In contrast, ME activity may decrease under conditions of stress (onset of hatching plus heat) and lipogenesis would be limited. This would allow other physiological processes and metabolic pathways (e.g., gluconeogenesis) that are more critical for survival to dominate. Interestingly, results of the previous study (Lorenzen *et al.*, 1999, Chapter 2) indicated that high environmental concentrations of PCDDs/PCDFs were associated with low ME activities and basal plasma corticosterone concentrations in herring gull embryos. Based on the relationship observed between ME and basal plasma corticosterone within the same individuals in this study, it is possible that environmental contaminants, such as PCDD/PCDFs, may impair corticosterone regulation of ME and lipogenesis.

3.6.4. Organochlorine Contaminants

Although CHC and PCDD/PCDF concentrations in yolk sacs were not significantly different between the three sites examined (Chantry Island, Hamilton Harbour and Middle Island), concentrations of total PCBs and non-*ortho* PCBs in yolk sacs from gull embryos collected from Middle Isand were significantly higher than at Chantry Island or Hamilton Harbour. Despite the absence of site to site differences for CHCs, a significant positive relationship was observed between CHCs and basal plasma corticosterone concentrations determined for each embryo. These results suggest that exposure of herring gull embryos to high environmental concentrations of CHCs may result in elevated basal plasma corticosterone concentrations. These results are different from those obtained in the previous study (Lorenzen *et al.*, 1999; Chapter 2.5.2). In the 1997 study, no relationships were observed between any of the biological endpoints measured and CHC residues. However, *inverse* relationships were observed when total PCB, PCDD/PCDF, non-*ortho*-PCB residues or TEQs were regressed against herring gull embryo basal plasma corticosterone concentrations. Thus, CHCs may have different effects than the other classes of organochlorine contaminants .

The inverse relationships observed between PCDD/PCDF yolk sac residues and malic enzyme or renal PEPCK activities in the previous study (Lorenzen *et al.*, 1999; Chapter 2) were not apparent in the present study. Possible reasons for this include the smaller gradient of yolk sac PCDD/PCDF contamination detected in embryos from the three Great Lakes sites examined in this study compared to the four Great Lakes sites and east coast control site examined in the previous study. In

the present study, an east coast control site was not used to minimize genetic variation among study sites. As well, other Great Lakes sites which were included in the present study which historically have had high PCDD/PCDF (Scotch Bonnet Island) or low PCDD/PCDF contamination (Mutton Island) were not analyzed for organochlorine residues due to low sample sizes as a consequence of early onset of hatching.

3.7. Conclusions

Although incidences of early onset of hatching and limited organochlorine residue analyses compromised interpretation of the results obtained for the present study, several important observations were made. This is the first time early onset of hatching has been observed for herring gull embryos from some Great Lakes sites. To confirm these findings, further studies are planned to document the incubation periods of herring gull embryos in the field at various sites. As well, based on the high incidences of early shell starring, elevated renal PEPCK and ME activities, and decreased induced corticosterone concentrations and corticosterone stress responses, herring gull embryos from Scotch Bonnet Island will be more intensively studied to confirm and to fully characterize these observations. Results of these further studies should indicate whether current levels of organochlorine contaminants continue to adversely affect this herring gull colony.

CHAPTER 4. DEVELOPMENT OF AN RT-PCR BIOASSAY FOR AVIAN VITELLOGENIN mRNA[2]

4.1. Introduction

Since it is difficult to predict the hormonal activity of environmental contaminants based on their chemical structures, the estrogenic potency of a substance, or mixture of substances, must be determined and characterized by biological methods. Both *in vivo* and *in vitro* estrogenicity bioassays have been developed for fish and amphibians that utilize induction of the estrogen-inducible protein vitellogenin as an endpoint (see section 1.C.7). In contrast, attempts to produce a vitellogenic response in primary avian hepatocyte cultures have not been very successful. However, Strijker (1986) did detect induction of VTG mRNA by dot-blot hybridization in primary chicken embryo hepatocyte cultures after treatment with a semi-synthetic estradiol analogue, moxestrol. Thus, VTG induction may also be a useful endpoint for estrogenicity/anti-estrogenicity bioassays in avian primary hepatocyte cultures.

4.2. Hypothesis

In vitro VTG induction in primary avian hepatocyte cultures can be used as the basis of a bioassay for assessing species-specific estrogenic potencies of environmental contaminants.

---

[2] a manuscript based on this chapter has been accepted for publication in *Toxicol. Appl. Pharmacol.*

4.3. Objectives

The objectives of this study were:

1) to develop a semi-quantitative *in vitro* avian hepatocyte culture bioassay for the determination of the estrogenic effects of environmental contaminants; and,

2) to compare the sensitivities of herring gull and chicken embryo hepatocyte cultures to estrogen and the estrogenic effects of *o,p'*-DDT.

4.4. Materials and Methods

4.4.1. Liver Tissue and Hepatocyte Sources

Liver and/or hepatocyte culture samples used for PCR, cloning and sequencing were obtained from seven species of birds as follows: White Leghorn chicken (*Gallus domesticus*) - Agriculture Canada, Ottawa, ON - laying hen or embryo hepatocyte cultures treated with 1000 nM moxestrol; herring gull *(Larus argentatus)* - nesting female from Thunder Bay, ON, or embryo hepatocyte cultures treated with 1000 nM moxestrol; common tern *(Sterna hirundo)* - 1 mg/kg estradiol-injected adult from North Limestone Island, Lake Huron; Caspian tern *(Sterna caspia)* - juvenile from Saginaw Bay, Lake Huron; American kestrel *(Falco sparverius)* - 1 mg/kg estradiol-injected adult male from the Avian Science and Conservation Centre, MacDonald College, McGill University, Montreal QC; American robin (*Turdus migratorius*) - 1 mg/kg estradiol-injected adult male from the Okanagan Valley, BC; Zebra finch (*Taeniopygia guttata*) - breeding female from the University of Ottawa, Animal Care and Veterinary Services, in collaboration with Wildlife International (Easton, MD). Livers were either frozen immediately in liquid

nitrogen and stored at -80°C, or submerged in RNAlater (5 ml/g tissue; Ambion, Austin, TX) and stored at -20°C. Cell culture samples were lyzed in RLT buffer (Qiagen, Mississauga, ON) containing 10 μl/ml β-mercaptoethanol, frozen in liquid nitrogen and stored at -80°C. Sampling was approved and conducted in compliance with the "Guide to the Care and Use of Experimental Animals" and "Ethics of Animal Experimentation" of the Canadian Council on Animal Care.

4.4.2. Sample Homogenization and RNA Extraction

Liver samples (~30 mg) were homogenized in RLT buffer containing 10 μl/ml β-mercaptoethanol with a hand-held tissue grinder (Kontes). Hepatocyte lysates were homogenized with QIAshredder columns (Qiagen) as per the manufacturer's instructions. Total RNA was purified from lysates using a spin column method (RNeasy; Qiagen) according to the manufacturer's instructions. RNA samples were treated with DNAse I (Ambion) to remove contaminating genomic DNA and re-purified by spin column (RNeasy; Qiagen). RNA concentrations were determined spectrophotometrically (GBC, UV/VIS 918).

4.4.3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for Cloning and Sequencing

Tissue and hepatocyte culture total RNA samples (2 μg, except for Caspian tern which was 5 μg) were primed with oligo $(dT)_{12-18}$ and reverse transcribed with SuperSCRIPT™ II reverse transcriptase for first strand cDNA synthesis according to the suppliers instructions (GibcoBRL, Burlington, ON). Sequences for the chicken

VTG, β-actin and serum albumin precursor (pre-proalbumin, referred to in this thesis as albumin) coding sequences were obtained from the GenBank database (Benson *et al.*, 2000). PCR primers were designed using the software, Primer Designer 4 (Scientific and Education Software, Durham, NC) from the reported coding sequences of the chicken VTGII gene (Nardelli *et al.*, 1987; GenBank accession number M18060) and chicken β-actin gene (GenBank accession number L08165). Primers for chicken albumin were designed by R. Deeley (Queen's University, Kingston, ON) based on the reported mRNA sequence (GenBank accession number X60688). The primers used in this study are summarized in Table 4.1. The VTG primer pairs span at least one intron in the chicken (van het Schip *et al.*, 1987). PCR products for cloning were obtained using a high-fidelity enzyme mixture (Advantage®-HF, Clontech, Palo Alto, CA). Products were assessed based on predicted size by agarose gel electrophoresis (2%) and ethidium bromide (GibcoBRL) staining prior to ligation.

4.4.4. Cloning and Sequencing of PCR Products

All PCR products used for cloning were obtained from a single animal with the exceptions of chicken β-actin and albumin PCR products that were derived from chicken embryo hepatocyte cultures prepared from a pool of approximately 50 livers. Triplicate PCR products for each species were ligated directly into the TA cloning vector using the Original TA Cloning® Kit (Invitrogen, Carlsbad, CA). Positive colonies were screened to determine insert sizes by PCR. Briefly, bacterial colonies were marked and picked into 200 μl water, then frozen at -80°C to lyse the cells.

Table 4.1 PCR primers

gene	primer ID and sequence (5'-3')	position[1]	product size (bps)[2]	species cloned and sequenced; bioassay use
chicken VTGII	*CV4421F (forward, 21mer)* GCA CAA GTG AAG CTG GAG TGG *CV4748R (reverse, 21mer)* AAT TCT CGA GCA CGG CAG AGG	4421 4748	286	chicken, gull, terns, robin, kestrel
	CVF120 (forward, 21mer) GAA TCC TTC TCG ACA AGC CAG *CVR305 (reverse, 21mer)* GCT TCA GCA AAG ACG TTC CAG	4540 4705	144	chicken embryo, finch; used for bioassay
chicken β-actin	*BA666 (forward, 20mer)* TTC ACC ACC ACA GCC GAG AG *BA953 (reverse, 20mer)* CAC CAG AAG GCA CTG TGT TG	666 953	267	chicken, gull, finch
	BAF89 (forward, 18mer) CTG GAG AAG AGC TAT GAA *BAR194 (reverse, 18mer)* ACT CCA TAC CCA AGA AAG	774 862	70	used for bioassay
chicken albumin	*CALBF (forward, 20mer)* ACG CAT GGC TTG TTC TGA GG *CALBR (reverse, 22mer)* GGC TCT GCT TTG GAC TAT TAG G	1424 1814	370	chicken, gull, finch
	SAF116 (forward, 16mer) CTG AGC ATT GTG ATT C *SAR275 (reverse, 16mer)* TTC TTC TGT CAT CTG G	1449 1703	238	used for bioassay

[1]based on chicken sequences cited above; [2] does not include primers

Lysate (2 μl) was used in a PCR with primers spanning the insertion site on the vector. Plasmids were isolated using the QIAwell 8 ultra system (Qiagen). Inserts were sequenced in both directions using a fluorescent dye-terminator cycle sequencing chemistry (BigDye Terminator FS; Applied Biosystems, Streetsville, ON) and an automated sequencing system (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). At least three clones from different PCRs were sequenced for each species.

4.4.5. Design of Bioassay Primers

Sequences obtained for each species were aligned for each gene (VTG, β-actin, albumin) and primers were designed using Align Plus and Primer Designer 4 (Scientific and Educational Software), respectively. There were insufficient regions of homology across all species to design "universal" avian primers, so primers used in the bioassay were designed to hybridize to identical regions between at least the herring gull and chicken cDNAs. These homologous primers are summarized in Table 4.1 (VTG: CVF120, CVR305; β-actin: BAF89, BAR194; albumin: SAF116, SAR275).

4.4.6. Preparation of Test Substances

Moxestrol (11β-methoxy-19-nor-17α-pregn-1-3,5(10)-trien-20-yne-3,17-diol; NEN Life Science products, Boston, MA) was dissolved in absolute ethanol and DMSO was used to dissolve 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (*o,p'*-DDT; Accustandard Inc., New Haven, CT). Serial dilutions of moxestrol (0.1 -1000

nM) were stored at -20°C while serial dilutions of *o,p'*-DDT (1-10000 nM) were stored at room temperature. Test substances or solvent were added to cell culture media (1 µl/ml for moxestrol, 5 µl/ml for *o,p'*-DDT) and mixed thoroughly prior to addition to the cell culture dishes.

4.4.7. Hepatocyte Cultures

Hepatocyte cultures were prepared from white Leghorn chicken embryos (day 20 of incubation) and herring gull embryos (day 26 of incubation) using sterile conditions according to the original method of Fischer and Marks (1976) with several modifications. After all dissections (~25-50 embryos) and rinsing in cold Krebs-Ringer buffer (KRB), the pools of ~ 25 livers were transferred to a Petri dish containing 12.5 mg collagenase (Sigma, Oakville, ON) and 0.1 mg DNAse I (Roche, Laval, QC) dissolved in 25 ml KRB at 37°C. The livers were sliced with a sterile scalpel in this solution and transferred to a 250 ml capped erlenmeyer flask. The digestion volume was brought to ~75 ml with additional collagenase/DNase I/KRB solution (37°C). The flask was shaken (125 rpm) for 10 min in a 37°C water bath, then 25 ml of the supernatant was transferred to a sterile filtration apparatus (BioDesign Inc., Carmel, NY) containing 100 ml KRB containing 2% BSA (Sigma). The cells were passed through four layered filters of 200, 100, 50 and 25 µm pore sizes (BioDesign). Fresh collagenase/DNase solution (25 ml) was added to the digestion flask which was returned to the water bath to shake for another 10 min. This supernatant removal and collagenase/DNase I solution replacement procedure was repeated one additional time, except only 10 ml fresh collagenase/DNAse I

solution was added for the final digestion period. After the complete 30 min digestion period, all digestate was filtered through the filtration apparatus and light vacuum was applied as required. Small pieces of tissue remaining in the filtration apparatus were rinsed with ~10 ml of collagenase/DNAse I solution and filtered as above. The filtrate was transferred to four 50 ml centrifuge tubes and centrifuged at 300g for 5 min at room temperature. The cell pellets were pooled in 50 ml cell culture medium (Waymouth 752/1; GibcoBRL) supplemented with penicillin G (60 mg/l; Sigma), streptomycin sulfate (100 mg/l; Sigma), L-thyroxine (1 mg/l; Sigma) and insulin (1 mg/l; Sigma). The cells were thoroughly resuspended by gentle pipeting. The cell suspension was transferred to two 50 ml centrifuge tubes (25 ml each) containing 24 ml of a mixture of Percoll (90%; Amersham Pharmacia Biotech, Baie D'Urfé, QC) and 2.5 M sucrose (10%; Sigma). The tubes were gently inverted to mix then centrifuged at 50g for 10 min. The upper layer of cells from each tube was carefully transferred by glass pipet to pre-weighed 15 ml centrifuge tubes. DNase I (10 ml, 0.02mg/ml KRB, 37°C) was added to each tube. The tubes were shaken by hand or in a 37°C water bath until all clumps disappeared and then centrifuged at 50g for 5 min. The supernatant was removed and an additional 10 ml DNAse I solution was added to each tube and the tubes were shaken again to ensure complete dissociation of all aggregated cells. The tubes were centrifuged as before and the supernatant was removed. The cell pellets were rinsed two times with the supplemented culture medium. The resulting pellet was weighed and a volume of supplemented culture medium (ml) equivalent to 32 times the mass of the pellet (g) was used to resuspend the cells in a sterile beaker equipped with a

magnet and stirred slowly using a magnetic stirrer. The cell suspension was added at 1.3 ml/plate to 100 mm treated tissue culture dishes (Falcon, 353003) containing 10 ml medium pre-treated with solvent or test substances at appropriate concentrations. The cultures were maintained at 37°C and 5% CO_2 in a humidified cell culture incubator (Forma Scientific, Marieta, OH). After 24 h, the medium from each plate was removed by aspiration and replaced with medium pre-treated with solvent or test substances. This procedure was repeated at 48 h. At 72 h, the medium was removed by aspiration and the cells were lysed and stored as described above.

4.4.8. Bioassay Development

Template cDNA for the bioassay was prepared by reverse transcription of RNA extracted from hepatocyte cultures as described above. When the reverse transcription reaction was run without reverse transcriptase, followed by PCR, no products were formed indicating absence of genomic DNA (data not shown). The homologous primers designed to specifically hybridize to identical regions of chicken and herring gull VTG, β-actin and albumin cDNAs were thoroughly tested to determine optimal annealing temperatures using a gradient thermocycler (MJ Research PTC-200, Waltham, MA). Optimal concentrations of $MgCl_2$ and Taq DNA polymerase were determined empirically. For each primer pair, experiments were conducted to determine the linear phase of amplification and the dynamic range for various amounts of cDNA template. Due to different optimal annealing temperatures or primer-dimer interferences, each primer pair was run in a separate tube. The final

PCR reaction mixture used for each primer pair was the same: 1XPCR Gold® Buffer, 2 mM $MgCl_2$, 200 µM each dNTP, 2U AmpliTaq Gold®, 200 nM each forward and reverse primer, 0.75 µl cDNA template, brought to a final volume of 50 µl with RNase-free water. PCR Gold® buffer, $MgCl_2$ and AmpliTaq Gold® DNA polymerase were supplied by Applied Biosystems, dNTPs were supplied by Roche and primers were supplied by GibcoBRL. All reactions were hotstart (94°C for 10 min prior to cycling) and two thermocyler programs run in calculated mode were used. For the VTG primers, melting temperature was 94°C for 10 s, and annealing and extension steps were combined at a temperature of 68°C for 2 min for a total of 33 cycles. For β-actin and albumin primers, melting temperature was 94°C for 10 s, annealing temperature was 51°C for 45 s, and extension temperature was 72°C for 45 s for a total of 26 cycles. All reactions for a complete concentration-response curve for each of the primer pairs were prepared using a common master mix (without primers) and run at the same time using dual blocks (MJ Research, 48/48 Dual Alpha). PCR products were electrophoresed on 11X14 cm 3% agarose gels (Horizon® 11•14 Gel Electrophoresis Apparatus, Agarose 1000, GibcoBRL) for 1 hour at 150V. Products were visualized by ethidium bromide staining (GibcoBRL) and quantitated using a gel documentation system (UVP GDS 7500 or BioChemi System, San Gabriel, CA).

4.4.9. Data Analysis

The optical density data obtained using the gel documentation system were analyzed to determine both absolute and relative PCR product abundance. To

standardize the assay for RNA extraction and reverse transcription, VTG induction was expressed relative to β-actin and as a percentage of maximal induction. Analysis of variance was used to determine responses that differed significantly from solvent controls for each test substance and species. Both β-actin and albumin, that are constitutively expressed "house-keeping" genes, were used as gene expression standards to assess the possible effects of moxestrol or *o,p'*-DDT in interfering with overall gene expression. The errors reported indicate standard error of the bioassay replicates.

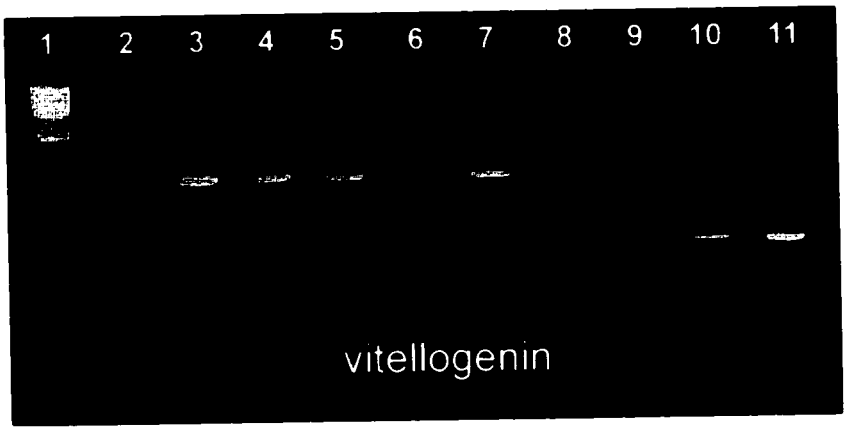
4.5. Results

4.5.1. Species-Specific PCR Products

PCR products obtained for each species using primers designed from the published coding sequences for the chicken vitellogenin II, β-actin, or albumin genes are shown in Fig. 4.1. All species gave a single major PCR product for each primer pair that was cloned and sequenced as described in section 4.4.4.

4.5.2. Sequences of PCR Products

a) VTG primers. Using the primers CV4421F and CV4748R, 286 bases of the VTG gene were cloned and sequenced for five species of birds (Fig. 4.2). A 144 base sequence was also determined for the zebra finch using primers CVF120 and CVR 305 (Table 4.1, Fig. 4.2). Compared with the published coding sequence (Nardelli *et al.*, 1987), the sequence obtained for the domestic chicken in this study differed by two bases at positions 180 (G/A) and 256 (T/C). These differences were



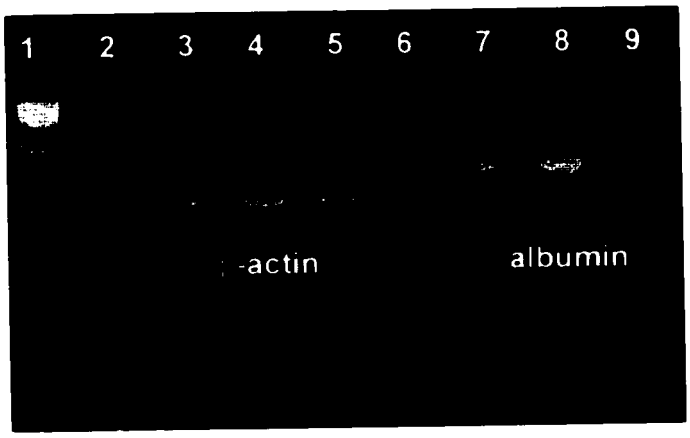


Figure 4.1 PCR products obtained for each species using chicken-based primers. *Top panel* shows PCR products obtained using VTG primers CV4421F/CV4748R (lanes 3-7) or CVF120/CVR305 (lanes 10,11). Lane 1 was loaded with 100 bp DNA ladder and lanes 2 and 9 represent control reactions in which the cDNA template was replaced with water. Lanes 3-7 and 10,11 show PCR products obtained using cDNA templates from the following species: 3 chicken (embryo hepatocytes); 4 herring gull; 5 common tern; 6 Caspian tern; 7 American kestrel; 8 American robin; 10 chicken (embryo hepatocytes); 11 zebra finch. *Bottom panel* shows PCR products obtained using β-actin primers BA666/BA953 (lanes 3-5) or albumin primers CALBF/CALBR (lanes 7-9). Lane 1 was loaded with 100 bp DNA ladder and lanes 2 and 6 represent control reactions in which the cDNA template was replaced with water. Lanes 3-5 and 7-9 show PCR products obtained using cDNA templates from the following species: 3,7 chicken (embryo hepatocytes); 4,8 herring gull; 5,9 zebra finch. Liver tissues and hepatocyte sources were as described in *Material and Methods*.

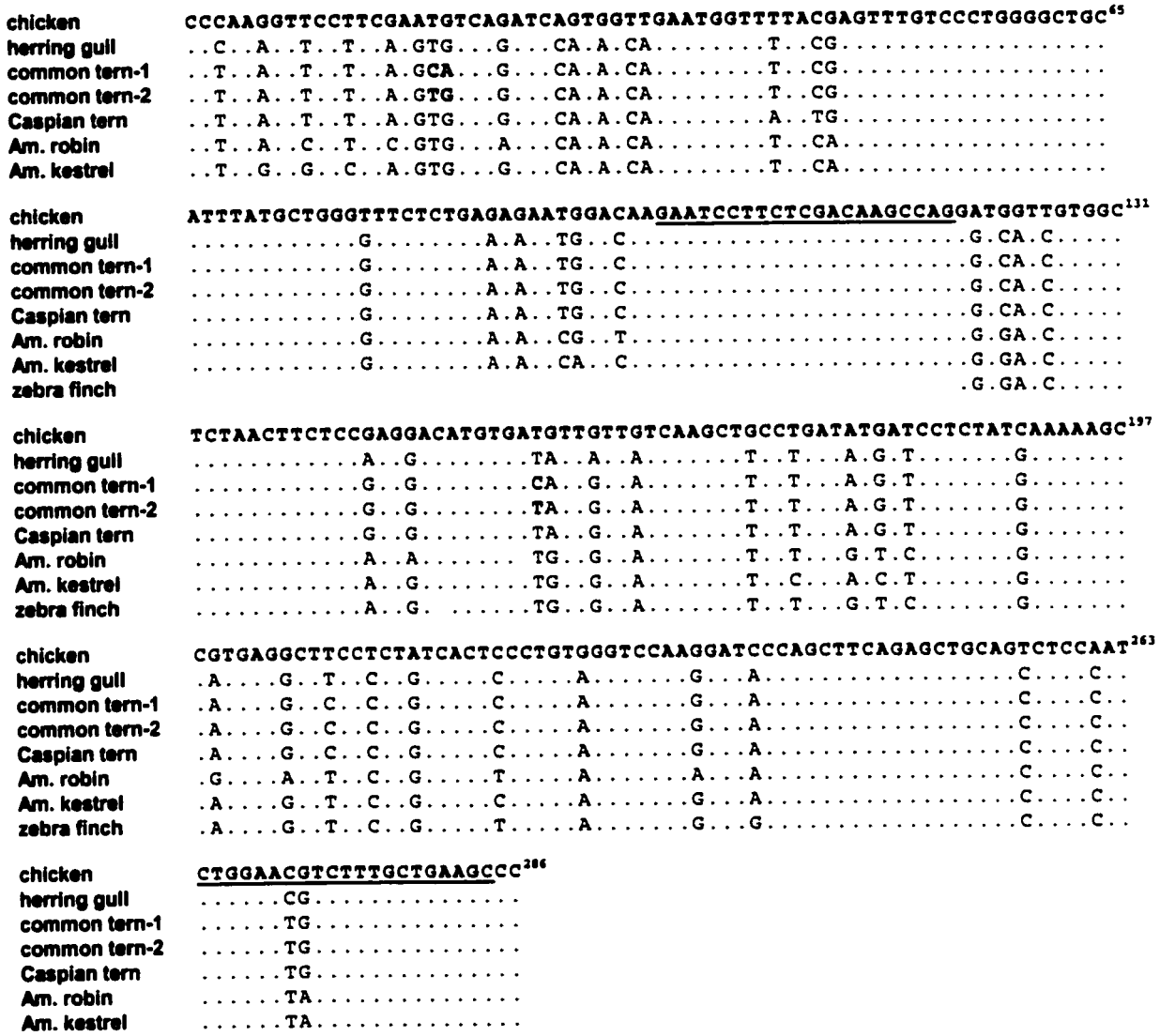


Figure 4.2 DNA sequences of PCR products obtained for VTG. Primers CV4421F/CV4748R were used for all species except the zebra finch for which the sequence was obtained using primers CVF120/CVR305. Bases identified with dots were conserved for all species tested. Underlined bases indicate hybridization positions of the forward and reverse primers (CVF120/CVR305) used for the bioassay and shaded bases indicate polymorphisms in the common tern. GenBank accession numbers for these sequences are: herring gull - AY045719; common tern - AY045728; Caspian tern - AY045720; American robin - AY045721; American kestrel - AY045722; zebra finch - AY045723.

also present in sequences obtained from moxestrol-treated chicken embryo hepatocyte cultures and may be indicative of different strains of chickens used in the two studies (data not shown). Among the different species of birds examined, the chicken showed the least homology with all other species for the VTG sequence that was obtained (86.4% to 90.2%; Table 4.2). Other species showed 92.3% or greater similarity among their sequences. Translation of the DNA sequences to amino acid sequences using the reading frame established for the chicken vitellogenin II gene indicates slightly poorer homology in most cases (Fig. 4.3, Table 4.3). Two sequences are reported for the common tern that showed polymorphisms at bases 18-19 (CA/TG) and base 156 (C/T) (compare common tern-1 with common tern-2 in Fig. 4.2). The presence of these polymorphisms was confirmed by the detection of heterozygosity at these positions in directly sequenced PCR products. The CA_{18-19} genotype was shown to be in coupling with the C_{156} genotype, based on sequencing of individual clones. New primers were designed that would hybridize to areas of the chicken and herring gull VTG cDNAs that were identical for these two species (Fig. 4.2). These primers (CVF120 and CVR305) were used in further studies for the development of the VTG mRNA bioassay for both chicken and herring gull embryo hepatocyte culture samples.

b) β-actin primers. Using the primers BA666 and BA953, 267 bases of the β-actin gene were cloned and sequenced for 3 species of birds (Fig. 4.4). Chicken β-actin PCR products for cloning were prepared from total hepatocyte RNA from a pool of ~50 embryos. Sequencing of these clones identified two sequences, one of which was identical to the reported sequence (GenBank accession number

Table 4.2 DNA sequence homology. Base identity for clones of VTG, β-actin and albumin PCR products for each species of bird analyzed. Separate entries are shown for each species where polymophisms were identified. For β-actin, chicken-1 is identical to the sequence reported in GenBank (accession number L08165). Values enclosed in brackets indicate percentage homology between species.

VITELLOGENIN	chicken	herring gull	common tern-1	common tern-2	Caspian tern	Am. robin	Am. kestrel	zebra finch
chicken	**286/286 (100)**							
herring gull	258/286 (90.2)	**286/286 (100)**						
common tern-1	254/286 (88.8)	278/286 (97.2)	**286/286 (100)**					
common tern-2	257/286 (89.9)	281/286 (98.3)	283/286 (99.0)	**286/286 (100)**				
Caspian tern	255/286 (89.2)	279/286 (97.6)	281/286 (98.3)	284/286 (99.3)	**286/286 (100)**			
Am. robin	252/286 (88.1)	272/286 (95.1)	270/286 (94.4)	273/286 (95.5)	271/286 (94.8)	**286/286 (100)**		
Am. kestrel	247/286 (86.4)	266/286 (93.0)	264/286 (92.3)	267/286 (93.4)	265/286 (92.7)	270/286 (94.4)	**286/286 (100)**	
zebra finch	126/144 (87.5)	136/144 (94.4)	134/144 (93.1)	135/144 (93.8)	135/144 (93.8)	138/144 (95.8)	139/144 (96.5)	**144/144 (100)**

β-ACTIN	chicken-1	chicken-2	herring gull	zebra finch-1	zebra finch-2
chicken-1	**267/267 (100)**				
chicken-2	265/267 (99.3)	**267/267 (100)**			
herring gull	256/267 (95.9)	256/267 (95.9)	**267/267 (100)**		
zebra finch-1	255/267 (95.5)	257/267 (96.3)	256/267 (95.9)	**267/267 (100)**	
zebra finch-2	256/267 (95.9)	256/267 (95.9)	255/267 (95.5)	266/267 (99.6)	**267/267 (100)**

ALBUMIN	chicken	herring gull	zebra finch-1	zebra finch-2
chicken	**370/370 (100)**			
herring gull	331/370 (89.5)	**370/370 (100)**		
zebra finch-1	279/370 (75.4)	294/370 (79.5)	**370/370 (100)**	
zebra finch-2	281/370 (75.9)	294/370 (79.5)	368/370 (99.5)	**370/370 (100)**

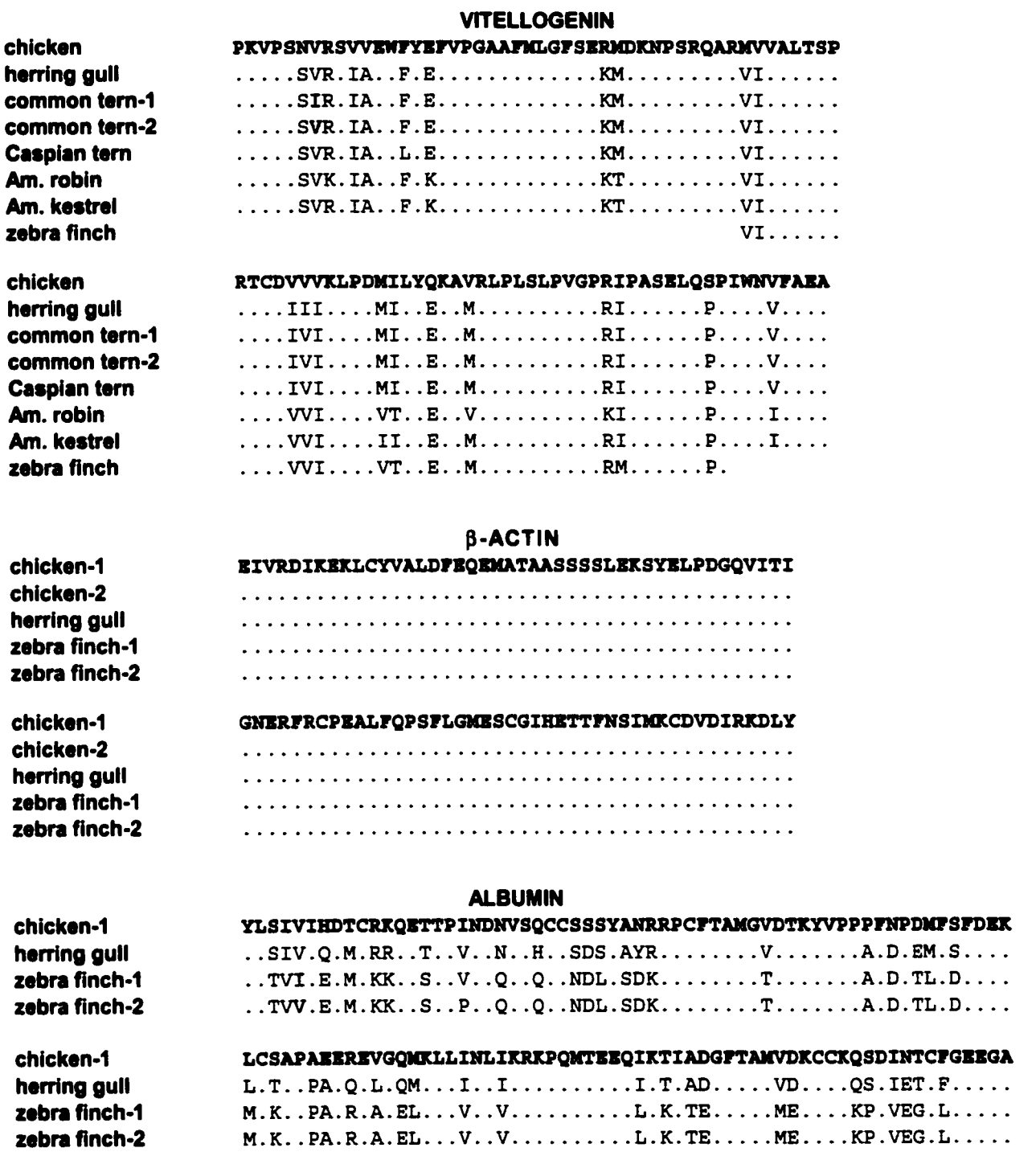


Figure 4.3 Amino acid sequences of PCR products. *Top panel* shows sequences obtained using the VTG primers CV4421F/CV4748R for all species indicated except the zebra finch for which the sequence was obtained using primers CVF120/CVR305. *Middle panel* shows sequences obtained using the β-actin primers BA666/BA953. *Bottom panel* shows sequences obtained using the albumin primers CALBF/CALBR. Amino acids identified with dots were conserved for all species examined and shaded bases indicate polymorphisms.

Table 4.3 Amino acid sequence homology. Amino acid identity for sequences obtained for clones of VTG, β-actin and albumin PCR products for each species of bird analyzed. Separate entries are shown for each species where polymorphisms were identified. For β-actin, chicken-1 is identical to the sequence reported in GenBank (accession number L08165). Values enclosed in brackets indicate percentage homology between species.

VITELLOGENIN	chicken	herring gull	common tern-1	common tern-2	Caspian tern	Am. robin	Am. kestrel	zebra finch
chicken	**95/95 (100)**							
herring gull	83/95 (87.4)	**95/95 (100)**						
common tern-1	83/95 (87.4)	93/95 (97.9)	**95/95 (100)**					
common tern-2	84/95 (88.4)	94/95 (98.9)	94/95 (98.9)	**95/95 (100)**				
Caspian tern	83/95 (87.4)	93/95 (97.9)	93/95 (97.9)	94/95 (98.9)	**95/95 (100)**			
Am. robin	79/95 (83.2)	85/95 (89.5)	85/95 (89.5)	86/95 (90.5)	85/95 (89.5)	**95/95 (100)**		
Am. kestrel	81/95 (85.3)	89/95 (93.7)	89/95 (93.7)	90/95 (94.7)	89/95 (93.7)	90/95 (94.7)	**95/95 (100)**	
zebra finch	38/47 (80.9)	42/47 (89.4)	43/47 (91.5)	43/47 (91.5)	43/47 (91.5)	44/47 (93.6)	44/47 (93.6)	**47/47 (100)**

β-ACTIN	chicken-1	chicken-2	herring gull	zebra finch-1	zebra finch-2
chicken-1	**88/88 (100)**				
chicken-2	88/88 (100)	**88/88 (100)**			
herring gull	88/88 (100)	88/88 (100)	**88/88 (100)**		
zebra finch-1	88/88 (100)	88/88 (100)	88/88 (100)	**88/88 (100)**	
zebra finch-2	88/88 (100)	88/88 (100)	88/88 (100)	88/88 (100)	**88/88 (100)**

ALBUMIN	chicken	herring gull	zebra finch-1	zebra finch-2
chicken	**122/122 (100)**			
herring gull	106/122 (86.9)	**122/122 (100)**		
zebra finch-1	80/122 (65.6)	85/122 (69.7)	**122/122 (100)**	
zebra finch-2	81/122 (66.4)	86/122 (70.5)	121/122 (99.2)	**122/122 (100)**

Figure 4.4 DNA sequences of PCR products obtained for β-actin. Primers BA666 and BA953 were used for all species. Bases identified with dots were conserved for all species tested. Underlined bases indicate hybridization positions of the forward and reverse primers (BAF89/BAR194) used for the bioassay and shaded bases indicate polymorphisms. Chicken-1 is identical to the sequence reported in GenBank (accession number L08165). New GenBank accession numbers are: herring gull - AY045724; zebra finch - AY045726.

L08165), and a second that contained two single nucleotide polymorphisms (C/T at base 157 and T/C at base 182; compare chicken-1 with chicken-2 in Fig. 4.4). The sequence obtained for the zebra finch showed a single nucleotide polymorphism (T/C at base 157; Fig. 4.4). DNA sequence homology among all three species for this region of the β-actin gene exceeded 95% (Table 4.2). Translation of the DNA sequences to amino acid sequences using the reading frame established for the chicken β-actin gene indicates 100% homology across all species (Fig. 4.3, Table 4.3). New primers were designed that would hybridize to areas of the chicken and herring gull β-actin cDNAs that were identical for the gull and one form of the chicken (Fig. 4.4). These primers (BAF89 and BAR194) were used in further studies for the development of the VTG mRNA bioassay for both chicken and herring gull embryo hepatocyte culture samples.

c) albumin primers. Using the primers CALBF and CALBR, 370 bases of the albumin gene were cloned and sequenced for three species of birds (Fig. 4.5). Compared to the coding sequence reported previously (GenBank accession number X60688), the sequence determined experimentally for this region of the albumin gene for the domestic chicken showed 100% homology. Two sequences are reported for the zebra finch that showed two single nucleotide polymorphisms (A/G at base 15 in coupling with C/T at base 140; compare zebra finch-1 with zebra finch-2 in Fig. 4.5). Homology among all three species for this region of the albumin gene was higher for the chicken and gull (89.5%; Table 4.2, bottom panel) than for either of these species and the zebra finch (75.4 - 79.5%; Table 4.2). Translation of the DNA sequences to amino acid sequences using the reading frame established for

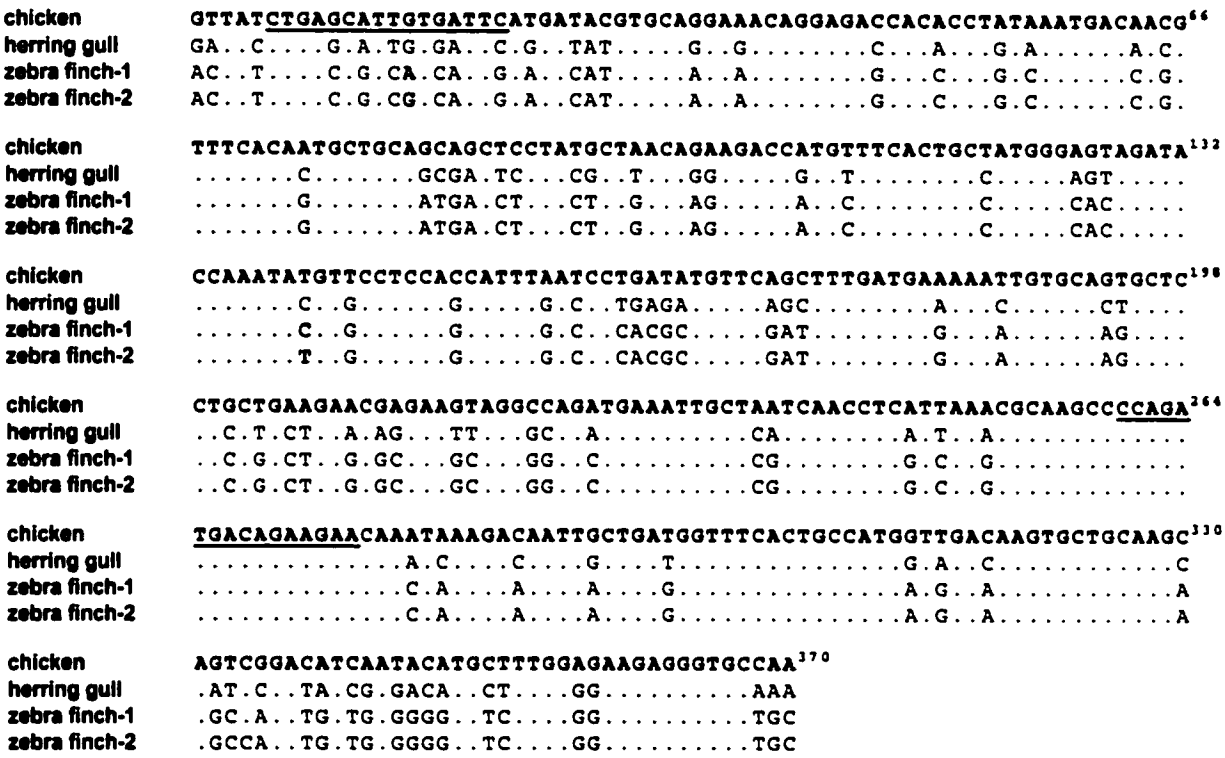


Figure 4.5 DNA sequences of PCR products obtained for albumin. Primers CALBF and CALBR were used for all species. Bases identified with dots were conserved for all species tested. Underlined bases indicate hybridization positions of the forward and reverse primers (SAF116, SAR275) used for the bioassay and shaded bases indicate polymorphisms. GenBank accession numbers are: herring gull - AY045725; zebra finch - AY045727.

the chicken albumin gene indicates a pattern of homology similar to that observed for the DNA sequences (Fig. 4.3, Table 4.3). New primers were designed that would hybridize to areas of the chicken and herring gull albumin cDNAs that were identical for these two species (see Fig. 4.5). These primers (SAF116 and SAR275) were used in further studies for the development of the VTG mRNA bioassay for both chicken and herring gull embryo hepatocyte culture samples.

4.5.3. Development of Bioassay

As described in section 4.4.8, the bioassay primers for VTG, β-actin and albumin were first thoroughly tested to determine optimal annealing temperatures, $MgCl_2$ and Taq DNA polymerase concentrations in both gull and chicken embryo hepatocyte cultures treated with 1000 nM moxestrol. Results of dynamic range and cycle number experiments for each pair of primers are shown in Fig. 4.6. Based on these data, 0.75 μl input cDNA, corresponding to 0.75 ng total RNA input, gave a signal for all primer pairs that was within the linear range of detection afforded by UV detection of PCR products electrophoresed in ethidium bromide stained gels. For the VTG primers at an optimal annealing temperature of 68°C, 33 cycles resulted in the production of a PCR product within the linear phase of amplification. For β-actin and albumin primer pairs at an optimal annealing temperature of 51°C, 26 cycles resulted in the production of PCR products within the linear phase of amplification. Attempts to duplex the β-actin and albumin reactions were not successful due to primer dimerization. Final bioassay reaction conditions are reported in section 4.4.8.

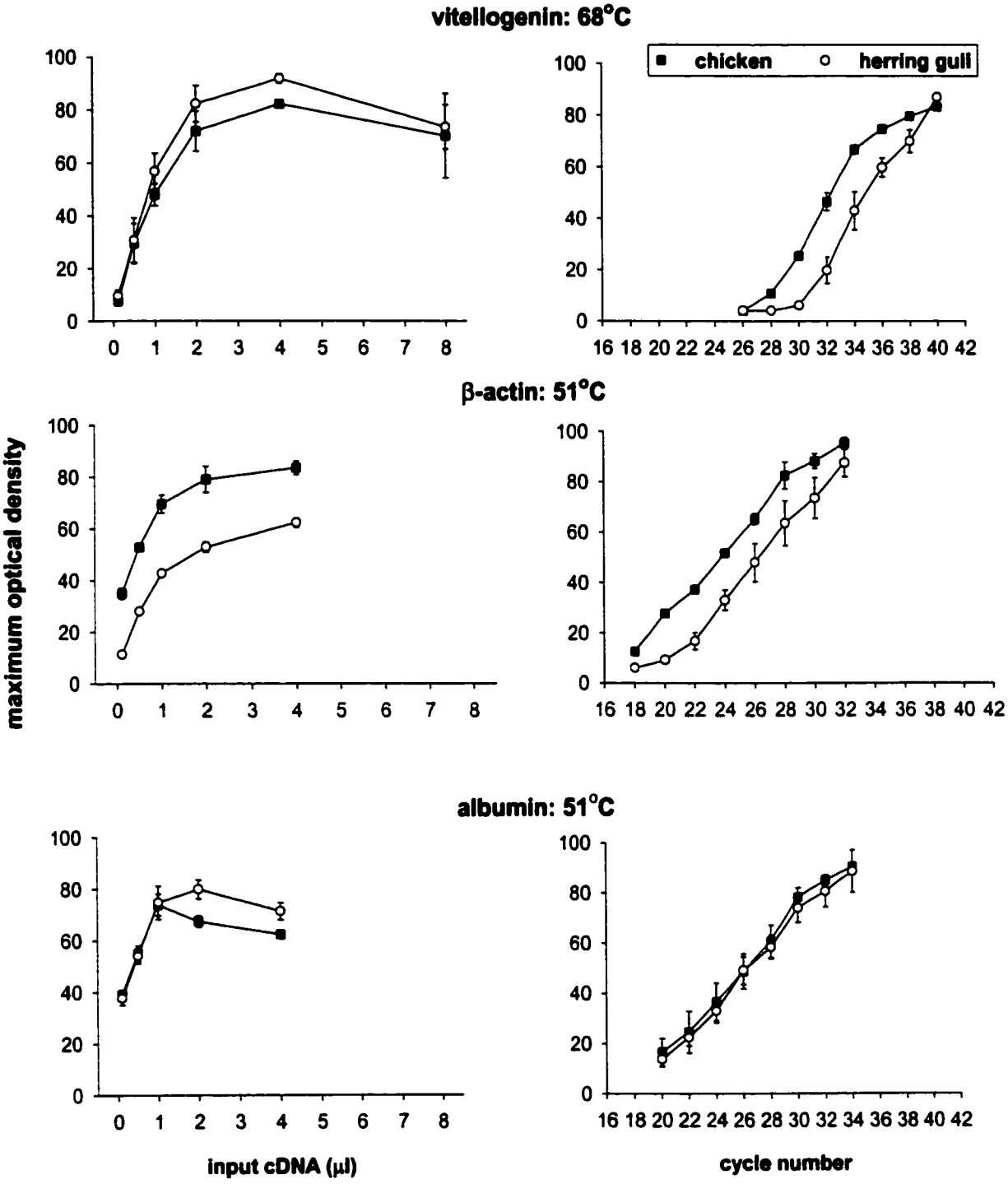


Figure 4.6 Input cDNA dynamic range and cycle experiments. PCR conditions for chicken and gull embryo hepatocyte culture samples treated with 1000 nM moxestrol were optimized for each primer pair used in the bioassay. Each primer pair was run in separate PCR reactions at the annealing temperatures indicated. For the dynamic range experiments, VTG reactions were amplified for 33 cycles and β-actin and albumin reactions were amplified for 26 cycles. For the cycle experiments, 2 µl input cDNA was used. PCR products were electrophoresed and quantified as described in Materials and Methods. Each point represents triplicate determinations and error bars indicate standard error of the mean.

4.5.4. Bioassay Reproducibility

Experiments were conducted to determine the reproducibility of the VTG mRNA bioassay in both chicken and herring gull embryo hepatocyte cultures treated with solvent (control) or various concentrations of moxestrol. Results of these experiments are shown in Figs. 4.7 and 4.8. For the chicken embryo hepatocyte cultures, statistically significant differences ($P<0.05$) in vitellogenin mRNA induction among three culture experiments within a single treatment group were only observed for 100 nM moxestrol (Fig. 4.8, top graph). Hepatocyte cultures prepared from herring gull embryos showed more variability than the chicken cultures in terms of VTG mRNA induction. In this case, statistically significant differences in induction were observed within four treatment groups: solvent control, 0.1, 1, and 1000 nM moxestrol (Fig. 4.8, bottom graph).

Table 4.4 summarizes the VTG mRNA induction responses observed in moxestrol-treated chicken or herring gull embryo hepatocyte cultures. Overall, the gull embryo hepatocyte cultures appear to be ~10 times more sensitive than chicken embryo hepatocyte cultures to moxestrol. This is reflected in the lowest dose at which VTG mRNA induction became significantly greater than that observed for the solvent control, the dose of moxestrol at which VTG mRNA induction was greater than 50% of the maximum response, and also the dose at which maximum induction was observed.

Data shown in Fig. 4.9 indicate that treatment of embryo hepatocyte cultures with moxestrol had no apparent detrimental influence on β-actin or albumin gene expression. Both β-actin and albumin are considered to be "housekeeping"

Figure 4.7 Agarose gel electrophoresis of bioassay PCR products. Bioassay PCR products for each primer pair (as indicated) were electrophoresed and quantitated as described in *Materials and Methods*. Top two panels show PCR products obtained for chicken (*upper*) and herring gull (*lower*) embryo hepatocyte cultures at each moxestrol concentration as indicated. Bottom two panels show PCR products obtained for chicken (*upper*) and herring gull (*lower*) embryo hepatocyte cultures at each *o,p'*-DDT concentration as indicated.

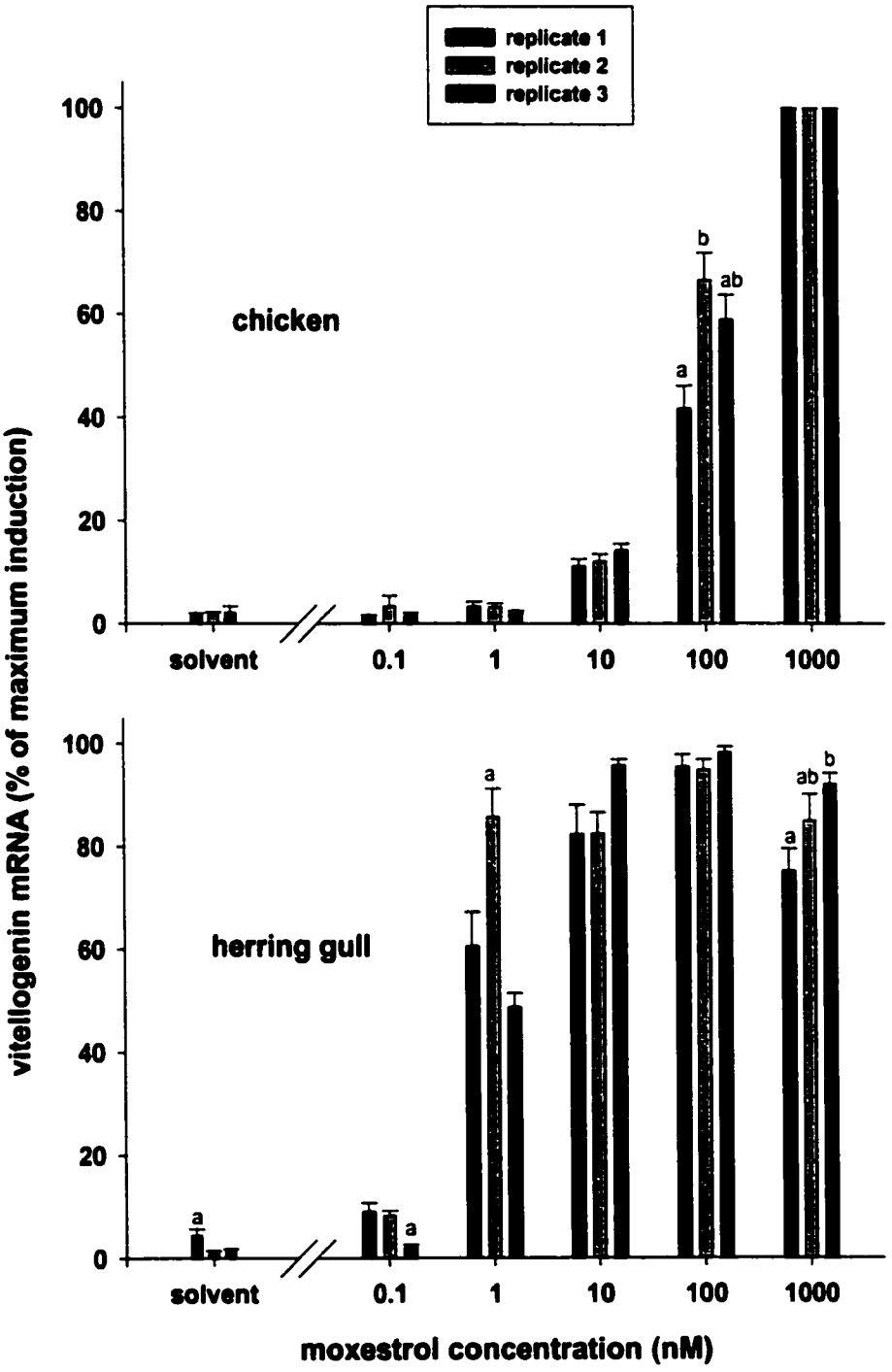


Figure 4.8 Reproducibility of VTG mRNA bioassay. Triplicate preparations of chicken or herring gull embryo hepatocyte cultures were treated with solvent (ethanol) or various concentrations of moxestrol and VTG mRNA induction was determined using the bioassay. Significant differences among replicate cell culture preparations for the solvent control or moxestrol are indicated (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of triplicate samples from one of three cell culture experiments for each species. Error bars indicate standard error of the bioassay.

Table 4.4 VTG mRNA induction by moxestrol

embryo hepatocyte culture	VTG mRNA induction significantly > control (P<0.05, nM)	VTG mRNA induction > 50% maximum (nM)	concentration at maximum induction (nM)
chicken			
replicate 1	10-1000	1000	1000
replicate 2	10-1000	100	1000
replicate 3	10-1000	100	1000
herring gull			
replicate 1 (L. Ontario)	1-1000	1	100
replicate 2 (Atlantic)	1-1000	1	100
replicate 3 (L. Huron)	1-1000	10	100

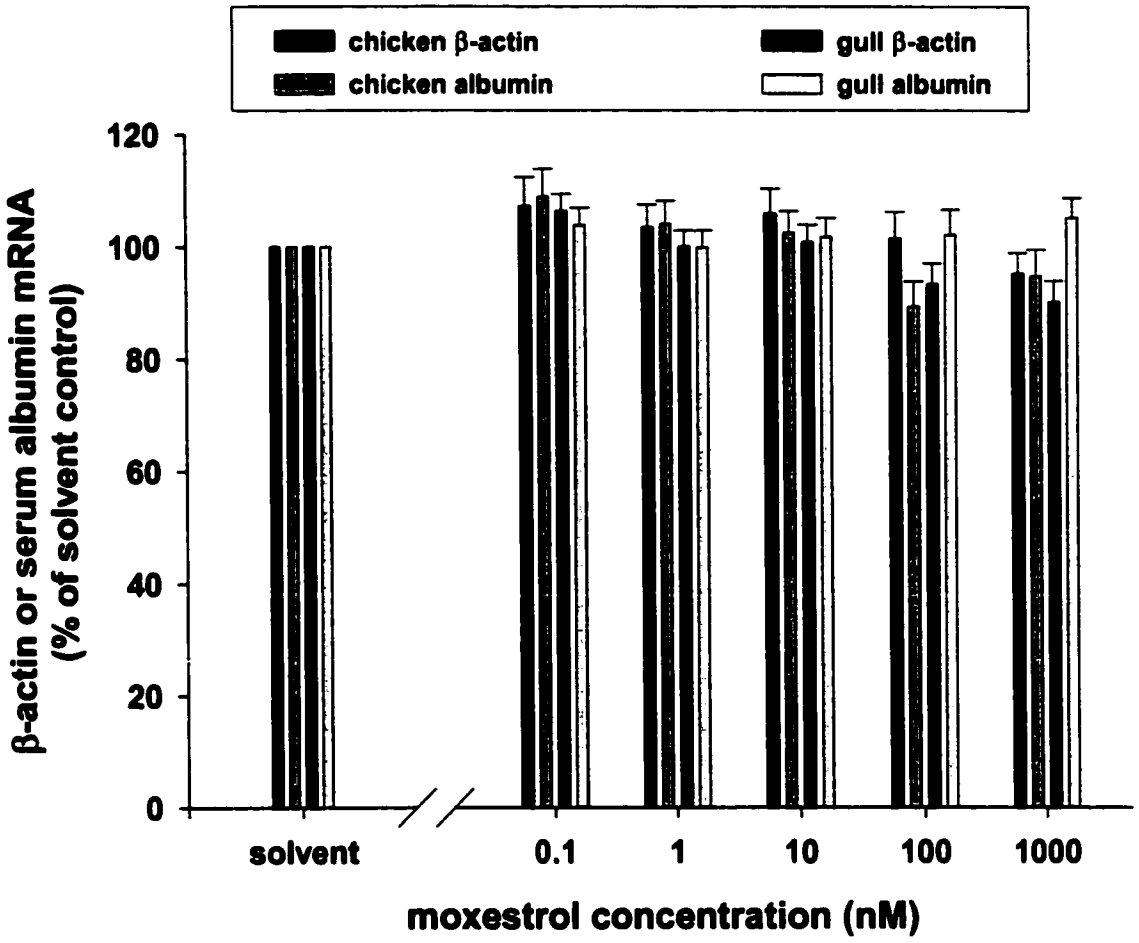


Figure 4.9 Gene expression standards for moxestrol. Triplicate preparations of chicken or herring gull embryo hepatocyte cultures were treated with solvent (ethanol) or various concentrations of moxestrol and β-actin and albumin mRNA levels were determined using the bioassay. There were no significant differences in β-actin or albumin mRNA levels between moxestrol-treated samples and the solvent controls (ANOVA, Tukey's, $P>0.05$). Each bar represents the mean of triplicate bioassays of triplicate plates for 3 cell culture experiments. Error bars indicate standard error of the bioassay.

genes which are constitutively expressed at relatively constant levels.

4.5.5. o,p'-DDT

Hepatocyte cultures prepared from chicken and herring gull embryos were treated with the chlorinated insecticide by-product, *o,p'*-DDT, which is known to be an estrogenic environmental contaminant. Data shown in Fig. 4.10 (top graph) indicate that no significant differences in vitellogenin mRNA induction within a single treatment group were observed among duplicate culture experiments for chicken embryo hepatocyte cultures. Statistically significant differences in VTG mRNA induction within treatment groups were observed among duplicate herring gull embryo culture experiments for the solvent control, 1, 10, and 100 nM *o,p'*-DDT ($P<0.05$; Fig. 4.10, bottom graph).

Table 4.5 summarizes the VTG mRNA induction responses observed in *o,p'*-DDT-treated chicken and herring gull embryo hepatocyte cultures. Only one replicate of the gull embryo hepatocyte cultures showed small, but statistically significant induction at a dose of 1000 nM *o,p'*-DDT. The other gull replicate and both chicken embryo hepatocyte culture replicates showed significant induction only at 10000 nM *o,p'*-DDT. Data shown in Fig. 4.11 indicate that the expression of albumin mRNA was significantly decreased in chicken embryo hepatocyte cultures treated with 10000 nM *o,p'*-DDT ($P<0.05$). These data suggest that high concentrations of *o,p'*-DDT may adversely affect overall gene expression in chicken embryo hepatocyte cultures. No other effects on β-actin or albumin gene expression were observed for *o,p'*-DDT-treated chicken or gull embryo hepatocyte cultures.

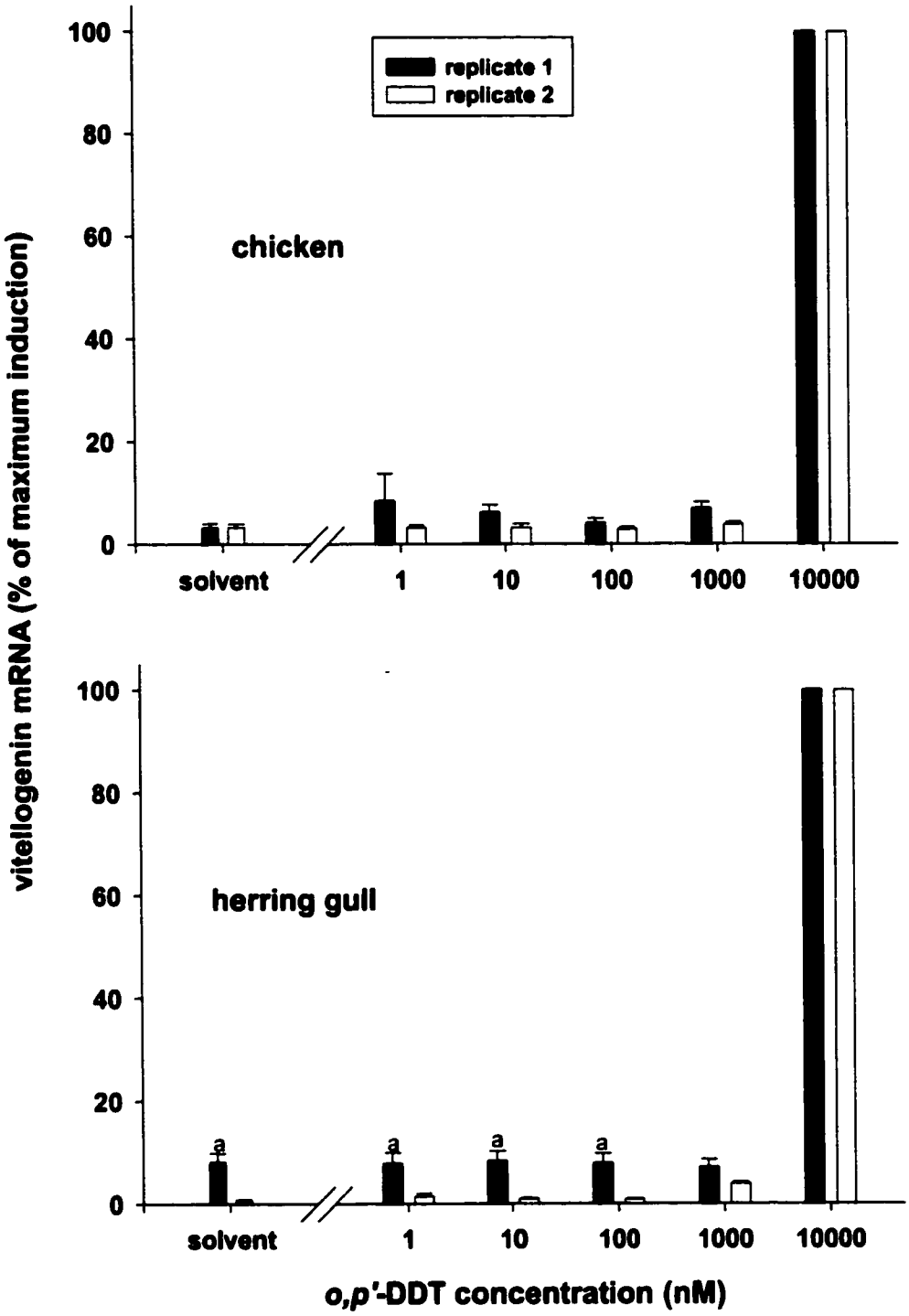


Figure 4.10 Estrogenicity of *o,p'*-DDT. Duplicate preparations of chicken or herring gull embryo hepatocyte cultures were treated with solvent (DMSO) or various concentrations of o,p'-DDT and VTG mRNA induction was determined using the bioassay. Significant differences among replicate cell culture preparations for the solvent control or *o,p'*-DDT are indicated (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of triplicate (replicate 1) or duplicate (replicate 2) samples from one of two cell culture experiments for each species. Error bars indicate standard error of the bioassay.

Table 4.5 VTG mRNA induction by *o,p'*-DDT

embryo hepatocyte culture	VTG mRNA induction significantly > control (P<0.05, nM)	VTG mRNA induction > 50% maximum (nM)	concentration at maximum induction (nM)
chicken			
replicate 1	10000	10000	10000
replicate 2	10000	10000	10000
herring gull			
replicate 1 (L. Ontario)	10000	10000	10000
replicate 2 (Atlantic)	1000,10000	10000	10000

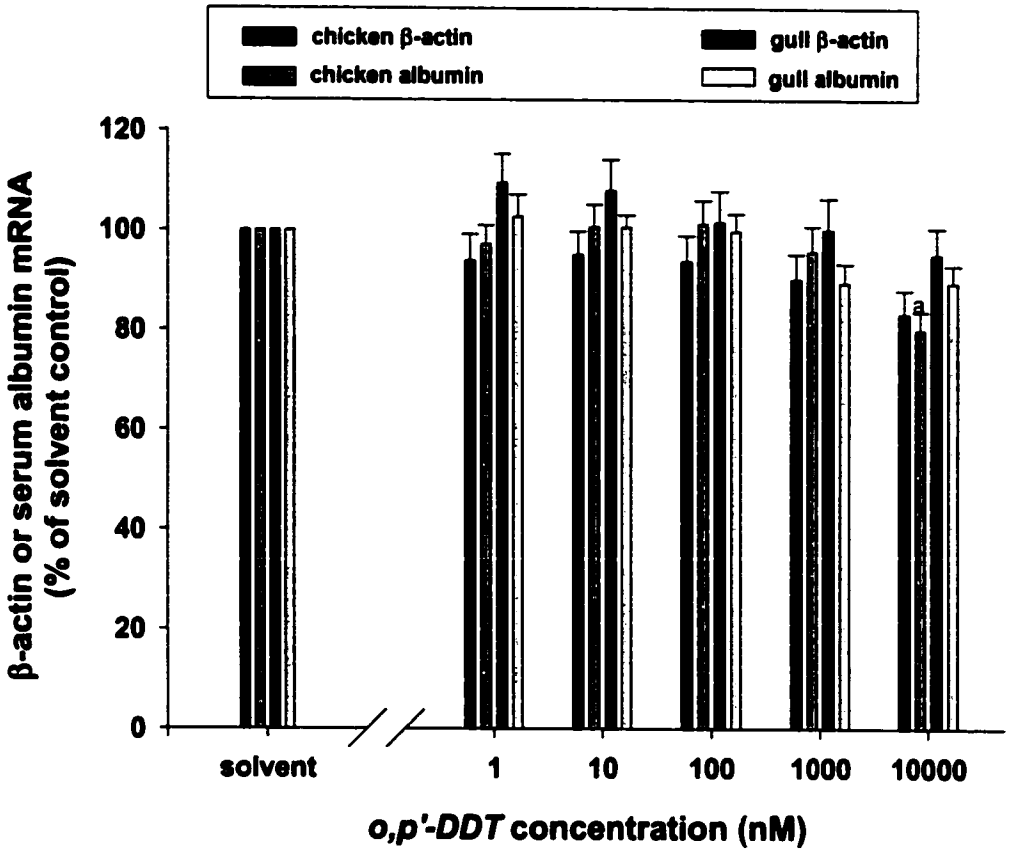


Figure 4.11 Gene expression standards for *o,p'*-DDT. Duplicate preparations of chicken or herring gull embryo hepatocyte cultures were treated with solvent (DMSO) or various concentrations of *o,p'*-DDT and β-actin and albumin mRNA levels were determined using the bioassay. No significant differences for β-actin mRNA levels between *o,p'*-DDT-treated samples and the solvent controls were observed for either species (ANOVA, Tukey's $P>0.05$). A significant reduction in albumin mRNA levels between 10000 nM *o,p'*-DDT-treated samples and the solvent control was observed only for chicken embryo hepatocyte cultures (ANOVA, Tukey's, $P<0.05$). Each bar represents the mean of triplicate bioassays of triplicate or duplicate plates for 2 cell culture experiments. Error bars indicate standard error of the bioassay.

4.6. Discussion

4.6.1. Sequences of PCR Products

To develop PCR primers for a semi-quantitative avian VTG mRNA bioassay, species-specific DNA sequences were required. To date, the domestic chicken is the only avian species for which sequences of VTG, β-actin and albumin genes have been published (Nardelli *et al.*, 1987; van het Schip *et al.*, 1987; and GenBank submissions). Under the assumption that these genes may be fairly well conserved among all avian species, primers were designed to specifically hybridize to the chicken sequences and applied to other species using RT-PCR. This strategy was very effective, such that single DNA sequences (except for incidences of polymorphisms within a species) were obtained for each species.

The short nucleotide sequences obtained from each cDNA and species allowed the design of the bioassay primers. Due to limited regions of homology of the VTG sequences among the species of birds examined and constraints on primer design, development of "universal" avian VTG primers was not possible. Therefore, bioassay primers were designed that would hybridize to areas of the chicken and herring gull cDNAs that were identical for at least these two species. This choice of species was based on preliminary experiments where methods to prepare chicken and herring gull embryo hepatocyte cultures were developed and optimized. Furthermore, there is some evidence to indicate that herring gulls may be susceptible to the feminizing influence of environmental contaminants (Boss, 1943; Boss and Witschi 1947; Fry and Toone 1981; Fry *et al.*, 1987). Bioassay primers for β-actin and albumin were designed in a similar manner.

4.6.2. Development of Bioassay

In a previous study, induction of VTG mRNA was detected by dot-blot hybridization in hepatocyte cultures prepared from 14.5 day old chicken embryos after exposure for 72 h to estradiol or moxestrol, a metabolically stable semi-synthetic estradiol analogue (Strijker, 1986). These results were reproduced in preliminary experiments for the present study using primary hepatocyte cultures prepared from chicken embryos that were incubated for 20 days (data not shown). To choose the most appropriate standard to compare VTG mRNA induction responses to, preliminary experiments were also conducted to compare induction responses in chicken embryo hepatocyte cultures treated with either 17β-estradiol or moxestrol. Similar to results obtained by Strijker (1986), moxestrol produced a much stronger vitellogenic response than 17β-estradiol (data not shown). Previous studies have shown that estradiol is rapidly metabolized to water soluble conjugates in the cell culture medium of both fish and chicken embryo hepatocyte cultures (Schneider *et al.*, 1983; Strijker, 1986; Anderson *et al.*, 1996). Furthermore, examination of the receptor binding kinetics for moxestrol, estradiol and ethynylestradiol in chicken embryo and adult liver cytosol preparations has shown that although the binding affinities of all three of these compounds were similar, moxestrol took longer to saturate the estrogen receptor, the receptor-moxestrol complex had a much longer half life, and there was less non-specific binding of moxestrol (De Boer *et al.*, 1982). Based on these findings, moxestrol was used as a standard for all cell culture experiments conducted for this study.

4.6.3. Bioassay Reproducibility

All VTG mRNA induction responses were confirmed by triplicate bioassays of each sample. Although the pattern of VTG mRNA induction relative to β-actin mRNA levels determined for the same cDNA sample was very consistent among the triplicate assays, the maximal level of induction often differed widely. As common reagent master mixes including cDNA, but not the gene-specific primers were used, this phenomenon can most likely be attributed to differences in amplification efficiencies for the VTG and β-actin PCRs that were run in separate tubes. To overcome this problem, final VTG induction was expressed as a percentage of maximal relative induction. Future studies using real-time PCR methods or development of primers that can be multi-plexed are planned.

In culture to culture comparisons, moxestrol-treated chicken embryo hepatocytes showed highly reproducible patterns of VTG mRNA induction. This confirms earlier findings reported by Strijker (1986). The pattern of moxestrol-mediated VTG mRNA induction was less consistent for the gull embryo hepatocyte cultures. However, because the gull hepatocyte cultures were prepared from fertile eggs collected from three different free-living gull colonies (replicate 1: Lake Ontario, replicate 2: Atlantic coast, replicate 3: Lake Huron), it is possible that genetic variation and/or environmental or dietary contamination of the eggs with estrogenic or anti-estrogenic substances may have contributed to the different patterns of response.

4.6.4. Species Comparisons

Herring gull embryo hepatocytes were ~10 times more sensitive than chicken embryo hepatocyte cultures to moxestrol-mediated VTG mRNA induction. Concentrations of moxestrol required to significantly induce VTG mRNA were 10 nM for the chicken and 1 nM for the gull. In comparison, 1 - 2 nM estradiol was required to induce VTG protein synthesis in carp and channel catfish primary hepatocyte cultures (Monteverdi and Di Giulio 1999; Smeets *et al.*, 1999b). On the other hand, aggregate cultures of rainbow trout hepatocytes responded with VTG protein production after treatment with ~10 nM 17β-estradiol for 6 days (Pelissero *et al.*, 1993). Based on these limited data, it is possible that there are differences in the sensitivities of various species of both birds and fish to pharmacological estrogens. Although further studies are required to determine if these differences observed *in vitro* are predictive of the relative sensitivities of species to estrogenic chemicals *in vivo*, results of earlier studies conducted with California and western gulls suggested that feminization of male embryos and abnormal development of the right oviduct in females occurred at 10-50 fold lower concentrations of estradiol than that required for similar effects in chicken and duck embryos (Romanoff, 1960; Fry *et al.*, 1987). This difference in species sensitivity between chickens and gulls is supported by the *in vitro* findings in the present study. Interestingly, chicken embryo hepatocyte cultures are *more* sensitive than all other species of birds tested to the CYP1A-inducing effects of HAHs (Kennedy *et al.*, 1996a). Thus caution must be taken when using the chicken as a model for predicting the biological effects of environmental contaminants in wild birds.

4.6.5. o,p'-DDT

Experiments were conducted to determine whether the avian VTG mRNA bioassay could be used to assess the estrogenic potency of the environmental contaminant *o,p'*-DDT. As per moxestrol, culture to culture variation was greater for the gull than the chicken. Although minor, but statistically significant, VTG mRNA induction was observed for one replicate of gull embryo hepatocyte cultures treated with 1000 nM *o,p'*-DDT, substantial induction for both species was only observed at a concentration of 10000 nM *o,p'*-DDT. In comparison, concentrations of 1000, 1000 - 10000, or 25000 nM *o,p'*-DDT were required to induce VTG protein synthesis in rainbow trout, catfish and carp hepatocyte cultures, respectively (Sumpter and Jobling 1995; Monteverdi and Di Giulio 1999; Smeets *et al.*, 1999b).

Unlike the results obtained for moxestrol, the sensitivities of the gull and chicken to *o,p'*-DDT-mediated VTG induction appeared to be very similar. However, it is possible that the logarithmic phase of the dose-response curve is much steeper for *o,p'*-DDT. In this case, the concentrations of *o,p'*-DDT tested may not have been adequate for the separation of species differences in sensitivity to this compound. Further experiments incorporating additional test concentrations between 1000 and 10000 nM *o,p'*-DDT are required to resolve this issue.

4.7. Conclusions

An *in vitro* semi-quantitative RT-PCR bioassay based on the induction of VTG mRNA in chicken and herring gull embryo hepatocyte cultures has been described.

To my knowledge this is the first *in vitro* VTG bioassay to be developed for birds and the first RT-PCR based hepatocyte culture VTG bioassay for any species.

Initial application of this bioassay to assess the potencies of the pharmacological estrogen, moxestrol and, to a lesser extent, the chlorinated insecticide, *o,p'*-DDT, suggest that there may be substantial differences regarding the sensitivities of various species of birds to these and other estrogenic compounds. This is of particular importance when considering the effects of exposure of wild species of birds to environmental contaminants that are known, or suspected, to have estrogenic properties. It is possible that sensitivity to estrogenic contaminants may contribute to the reproductive impairment and population declines observed for some species of highly exposed fish-eating birds.

Development of the avian estrogenicity bioassay entailed the determination of the sequences of short regions of the vitellogenin, β-actin and albumin coding sequences from a number of different species of birds. This novel sequence information is valuable in itself in that it can be used to develop species-specific PCR primers for biomarker applications. For example, liver samples collected from male, juvenile or non-breeding female birds suspected of being exposed to estrogenic contaminants could be analyzed for the presence of VTG mRNA. The abundance of VTG mRNA detected could then be quantitatively compared to matched control birds to assess whether exposure to estrogenic contaminants has occurred.

Further experiments are currently underway to improve the avian estrogenicity bioassay and to expand its use to include the determination of the anti-

estrogenic potencies of environmental contaminants. Proposed improvements include reduction of the cell culture plate size to a 12- or 24-well format and the inclusion of more concentrations of test substance to allow accurate determination of EC50 values. As well, real-time PCR techniques will be investigated to improve assay to assay variation in amplification efficiencies and to increase sample throughput. Finally, sequencing of longer regions of the VTG, β-actin and albumin genes in the herring gull and other wild species of birds should allow the development of primers that can be multiplexed or run under the same cycling conditions.

CHAPTER 5. ESTROGENIC AND ANTI-ESTROGENIC EFFECTS OF ENVIRONMENTAL CONTAMINANTS AND AN EGG EXTRACT IN CHICKEN EMBRYO HEPATOCYTE CULTURES

5.1. Introduction

The most commonly reported endocrine-related effects of environmental contaminant exposure in wildlife involve altered reproductive systems and decreased reproductive success. Both "feminizing" and "masculinizing" effects due to environmental contaminant exposure have been documented (see section 1.A.3). An estrogenic chemical is a chemical that can elicit primary effects normally caused by estrogens, such as cell proliferation in the cells and tissues of the genital tract and induction of mating behaviour and reproduction in females (Janssen *et al.*, 1998). Anti-estrogenic chemicals inhibit or reduce the normal response to estrogen. Many chlorinated and non-chlorinated compounds manufactured intentionally or as industrial by-products are suspected, or known, to have estrogenic or anti-estrogenic properties (see section 1.A.4). Although less intensively studied than fish, birds are also subject to the estrogenic or anti-estrogenic effects of environmental contaminants (see section 1.A.4) However, only recently has a bioassay been developed to determine the estrogenic or anti-estrogenic potencies of environmental contaminants in avian hepatocyte cultures (Chapter 4; Lorenzen *et al., in press*). Thus, determination of the estrogenic or anti-estrogenic activities of environmental contaminants in chicken embryo hepatocyte cultures using the avian VTG mRNA RT-PCR bioassay should indicate whether this bioassay provides results that are

predictive of estrogenic or anti-estrogenic responses previously observed *in vivo* or *in ovo* in birds.

5.2. Hypothesis

In vitro vitellogenin mRNA induction in primary chicken embryo hepatocyte cultures can be used to predict the estrogenic or anti-estrogenic effects of environmental contaminants in birds.

5.3. Objective

The objective of this study was to determine the estrogenic or anti-estrogenic effects of various environmental contaminants and an egg extract in chicken embryo hepatocyte cultures using the avian VTG mRNA RT-PCR bioassay.

5.4. Materials and Methods

5.4.1. Hepatocyte Culture

Hepatocyte cultures were prepared from white leghorn chicken embryos (day 20 of incubation) according to the original method of Fischer and Marks (1976) with several modifications as described in Chapter 4.4.7. Culture treatment and medium changes were identical to those described in Chapter 4.4.7.

5.4.2. Preparation of Test Substances and Culture Treatment

Moxestrol (11β-methoxy-19-nor-17α-pregn-1-3,5(10)-trien-20-yne-3,17-diol; NEN Life Science products, Boston, MA), 4-*tert*-octylphenol (OP; Sigma-Aldrich,

Oakville, ON), bisphenol A (BPA; Sigma-Aldrich) methoxychlor (MXCL; 1,1'-(2,2,2-trichloroethylidene)-bis[4-methoxybenzene]; Accustandard, New Haven, CT) and tamoxifen ([Z]-2-[4-(1,2-diphenyl-1-butenyl)-phenoxyl-N,N-dimethylethanamine; Sigma-Aldrich) were each dissolved in absolute ethanol. DMSO was used to dissolve 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Dr. J. J. Ryan, Health Canada, Ottawa, ON), benzo[k]fluoranthene (B[k]F; Sigma-Aldrich), 3,3',4,4',5-pentachlorobiphenyl (PCB 126; Ultra Scientific, Kingston, RI), halogenated dimethyl bipyrroles (HDBPs: Br4 - 1,1'-dimethyl-5,5'-dichloro-3,3',4,4'-tetrabromo-2,2'-bipyrrole; Br5 - 1,1'-dimethyl-5-chloro-3,3',4,4',5-pentabromo-2,2'-bipyrrole; Br6 - 1,1'-dimethyl-3,3',4,4',5,5'-hexabromo-2,2'-bipyrrole; Dr. G. Gribble, Dartmouth College, Hanover, NH), and an organochlorine extract from a common tern (*Sterna hirundo*) egg collected from Bird Island, MA. The egg extract was prepared as described previously (Hart, 2000) and would contain all organochlorine contaminants including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenozofurans (PCDFs), PCBs, structurally related halogenated aromatic hydrocarbons (HAHs) and chlorinated hydrocarbon pesticides that were present in the egg. Serial dilutions of moxestrol and tamoxifen were stored at -20°C. Serial dilutions of all other compounds were stored at room temperature. Cell culture medium was treated with 1 µl/ml solvent or each concentration of test substance, with the exception of the anti-estrogenicity experiments for TCDD in which the medium was treated with 5 µl/ml solvent or each concentration of TCDD. All treated medium was mixed thoroughly prior to addition to the cell culture dishes. For estrogenicity bioassays, one culture plate (100 mm diameter) per concentration was

prepared for TCDD, B[k]F, Br4, Br5, Br6 and the tern egg extract, and two culture plates per concentration were prepared for OP, BPA and MXCL. For anti-estrogenicity bioassays all medium was pre-treated with moxestrol (0.1 µl/ml 1000 nM stock, final concentration 100 nM) prior to addition of test substances. For determination of the EC50 of moxestrol, two culture plates were prepared and analyzed for each of 9 concentrations or solvent control. The number of replicates for each concentration of the other test substance is indicated in Table 5.1.

5.4.3. RNA Extraction and Vitellogenin mRNA RT-PCR Bioassay

Hepatocyte culture samples were homogenized and total RNA was extracted as described in Chapter 4.4.2. The RT-PCR VTG mRNA bioassay was conducted as described in Chapter 4.4.8.

5.4.4. Data Analysis

Bioassay data were analyzed as described in Chapter 4.4.9. A logistic curve was fitted to the concentration-response curve data obtained for moxestrol and the concentration at which the response was half maximal (EC50) was determined as previously described by Kennedy *et al.*, (1993).

5.5 Results

5.5.1. Estrogenicity Bioassays

Substances tested for estrogenicity in chicken embryo hepatocyte cultures using the avian VTG mRNA RT-PCR bioassay were TCDD (0.0002 - 2 nM), B[k]F

(0.2 - 2000 nM), OP (1 nM - 200 μM), BPA (1 -10000 nM), Br4 (1 -10000 nM), Br5 (1 - 10000 nM), Br6 (1-10000 nM), MXCL (1 - 50 μM), and an egg extract (0.025 - 250 mg egg homogenate equivalents). Of these 9 test substances, only 100-200 μM concentrations of OP exhibited any estrogenic activity (Fig. 5.1). However the levels of VTG mRNA induction for these concentrations of OP were only ~9% of the level of induction typically observed with 1 μM moxestrol and were not significantly different from the solvent control (ANOVA, Tukey's, $P>0.05$; Fig. 5.1). Furthermore, examination of the culture samples treated with 100 or 200 μM OP showed substantial cell detachment and RNA yields were lower than for the solvent control. However, for equivalent RNA concentrations, expression of neither β-actin nor albumin mRNA appeared to be impaired compared with the solvent control (ANOVA, Tukey's, $P>0.05$).

Although no VTG mRNA induction was observed for any concentrations of the other substances tested, β-actin gene expression was determined to be significantly reduced in cultures treated with 10000 nM BPA or 0.0002 and 0.002 nM TCDD compared with the solvent controls (ANOVA, Tukey's, $P<0.05$). The expression of albumin was significantly reduced in cultures treated with 1000 nM BPA compared with the solvent control (ANOVA, Tukey's, $P<0.05$). However, because some of these decreases in β-actin or albumin gene expression were not observed within a concentration-response context, it is difficult to assess whether these test substances actually did affect overall gene expression. Further experiments are required to confirm these observations. Additional experiments conducted with 100 and 200 μM BPA resulted in significant cell detachment,

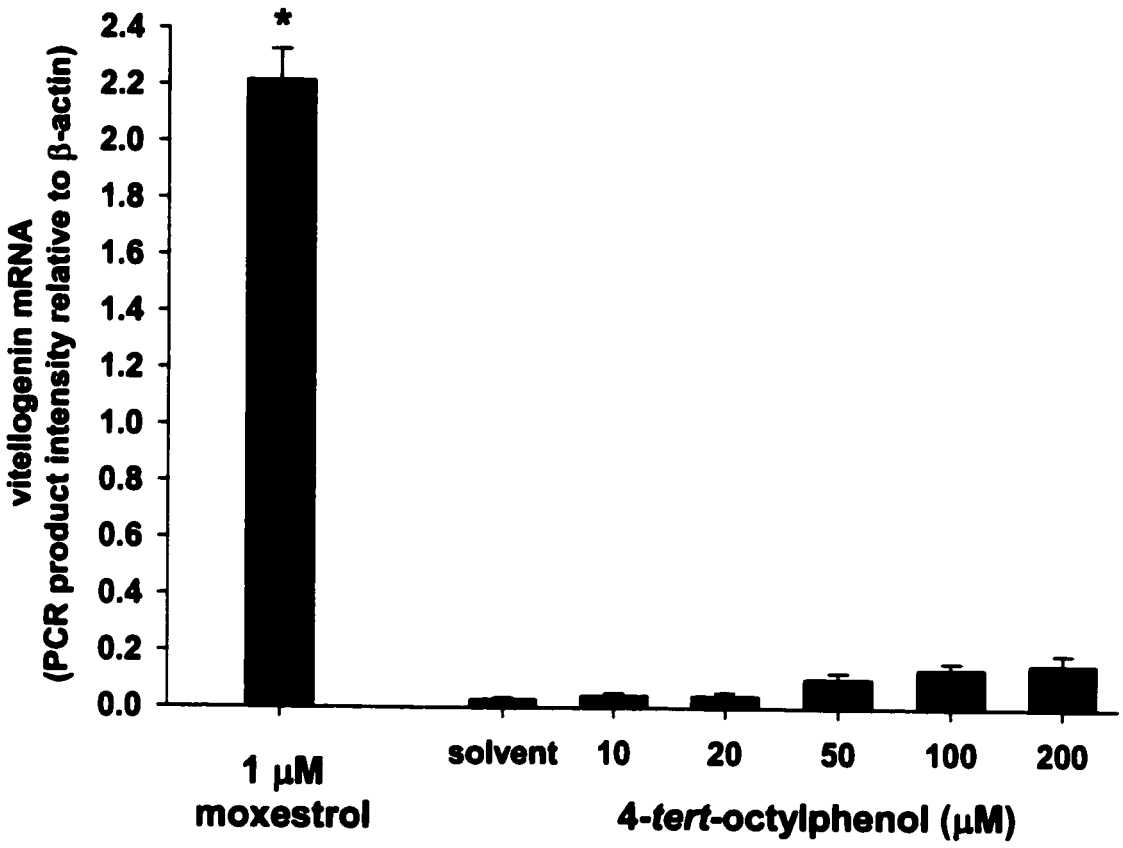


Figure 5.1 Comparison of induction responses observed for moxestrol and OP. VTG mRNA induction, relative to β-actin expression, is shown for chicken embryo hepatocyte cultures treated with either 1 μM moxestrol, solvent, or various concentrations of OP. Asterisk indicates a response significantly greater than the solvent control (ANOVA, Tukey's, $P<0.05$). Bars indicate standard error of the bioassay (n=6 for moxestrol, n=4 for OP and solvent control).

suggesting that these concentrations are overtly toxic to the chicken embryo hepatocyte cultures.

5.5.2. Anti-estrogenicity Bioassays

To accurately determine the concentration of moxestrol required to sub-maximally induce VTG mRNA for the anti-estrogenicity bioassay, chicken embryo hepatocyte cultures were exposed to varying concentrations of moxestrol and the cell culture samples were analyzed for VTG mRNA induction using the avian VTG mRNA RT-PCR bioassay. As shown in Fig. 5.2, a sigmoidal curve was fitted to the concentration-response data and the concentration of moxestrol that caused half-maximal induction (EC50) was estimated to be 67 nM. Based on these results, the serial dilution closest to the EC50 value (100 nM) was chosen for pre-treatment of cell culture medium to be used for the anti-estrogenicity bioassay.

Substances tested for anti-estrogenicity in chicken embryo hepatocyte cultures using the avian VTG mRNA RT-PCR bioassay were tamoxifen (0.1 - 1000 nM), TCDD (0.001 - 10 nM), PCB 126 (0.02 - 200 nM), B[k]F (0.2 - 2000 nM), Br4 (1 - 10000 nM), Br5 (1 - 10000 nM), Br6 (1 - 10000 nM), and an egg extract (0.025 - 250 mg egg homogenate equivalents). Chicken embryo hepatocyte cultures were treated with 100 nM moxestrol plus either solvent (control) or serial dilutions of each test substance. As shown in Fig. 5.3, tamoxifen at a concentration of 1000 nM significantly decreased moxestrol-mediated VTG mRNA induction (ANOVA, Tukey's, $P<0.05$). TCDD was observed to be a potent anti-estrogen, significantly inhibiting moxestrol-mediated VTG mRNA induction at concentrations as low as

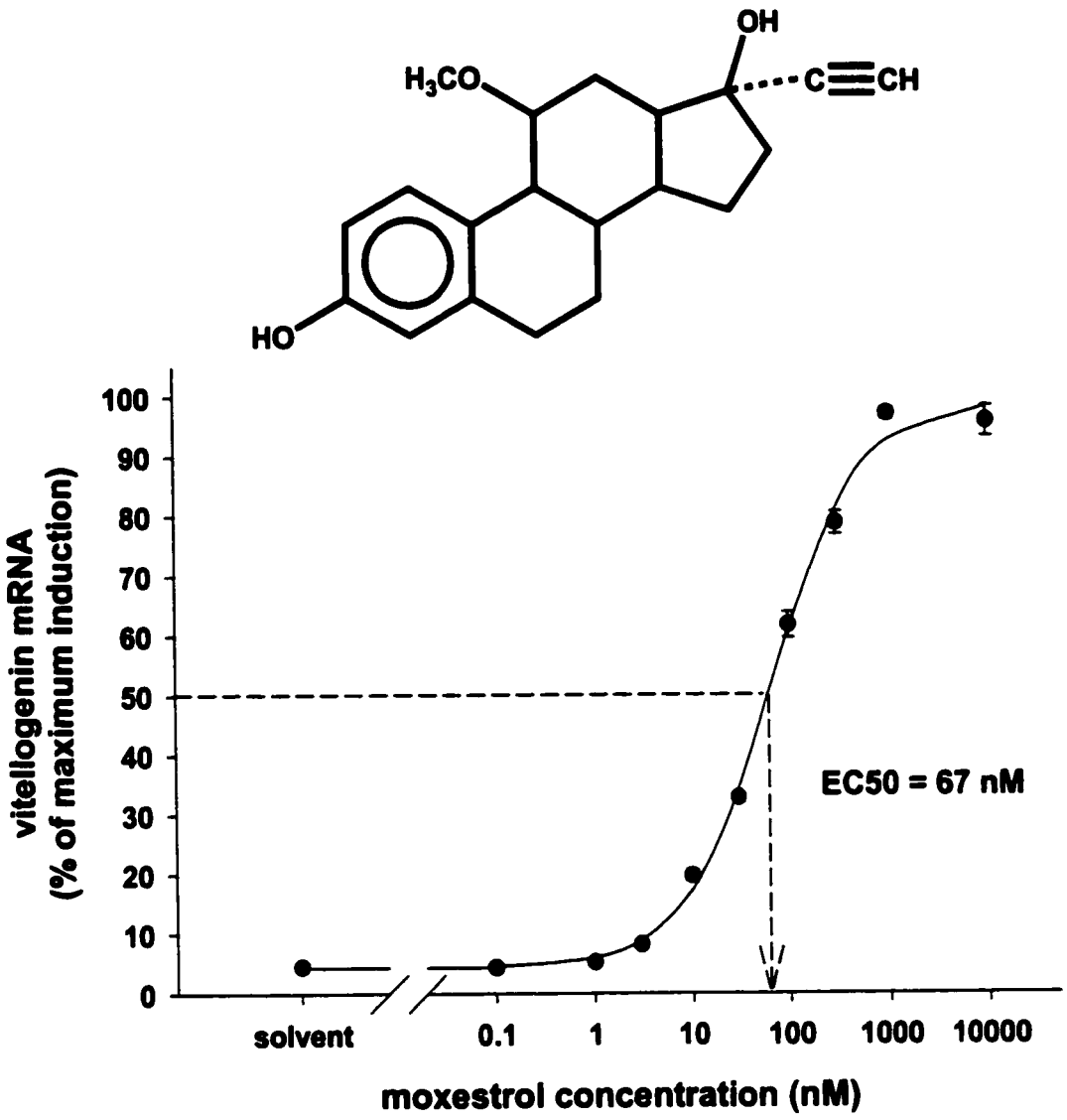


Figure 5.2 Moxestrol concentration-response curve. Chicken embryo hepatocyte cultures were treated with various concentrations of moxestrol or solvent. Each point represents triplicate bioassays of duplicate plates from one cell culture experiment. EC50 was determined by fitting a logistic curve to the data. Error bars indicate standard error of the bioassay.

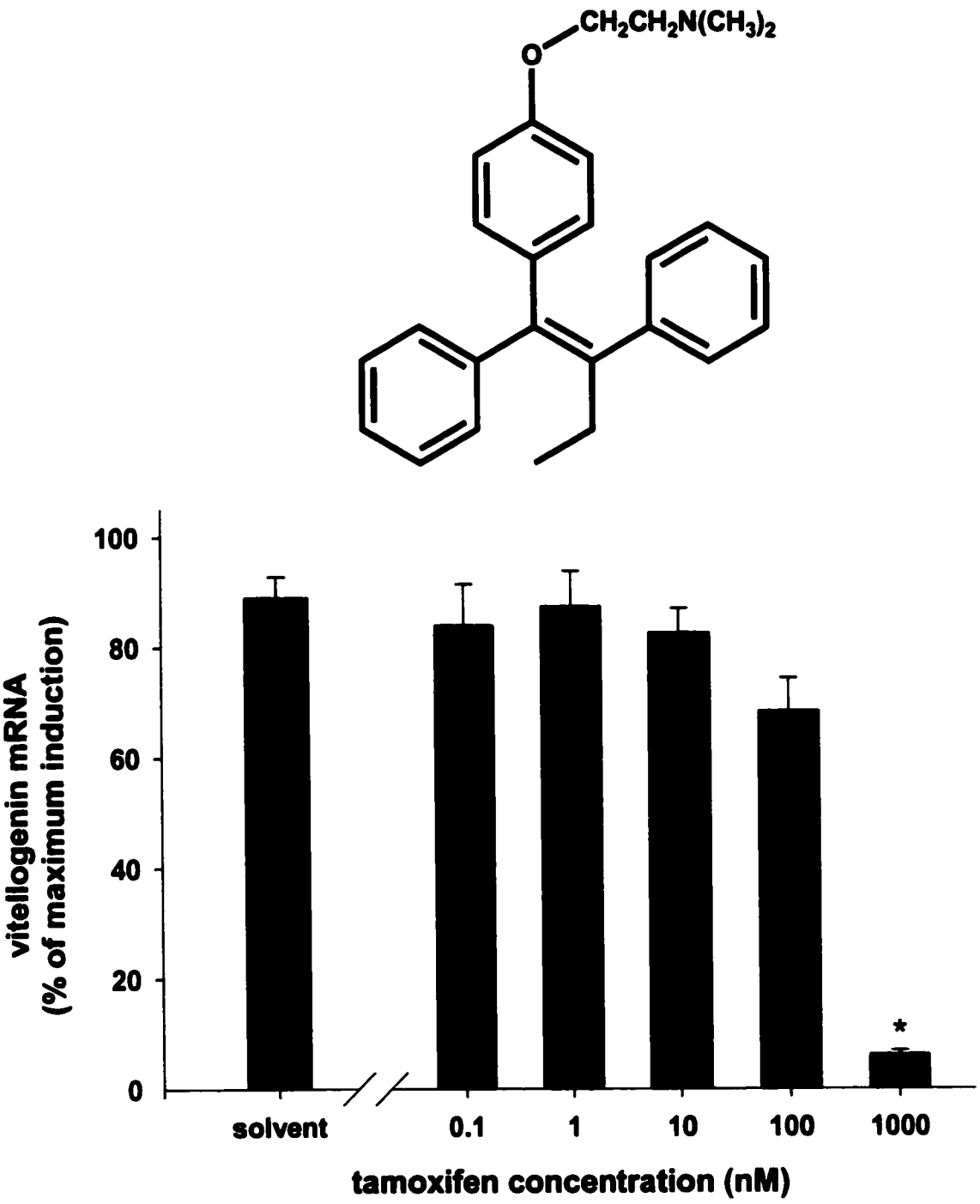


Figure 5.3 Anti-estrogenicity bioassay of tamoxifen. Chicken embryo hepatocyte cultures were treated with 100 nM moxestrol plus various concentrations of tamoxifen or solvent. Significant inhibition of moxestrol-mediated vitellogenin mRNA induction was observed for cells treated with 1000 nM tamoxifen (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of duplicate plates from one cell culture experiment. Error bars indicate standard error of the bioassay.

0.01 nM (Fig. 5.4, top graph; ANOVA, Tukey's, $P<0.05$). PCB 126 also caused significant inhibition of moxestrol-mediated VTG mRNA induction at a concentration of 0.2 nM, but higher concentrations of this compound did not cause significant anti-estrogenic effects (Fig. 5.4, bottom graph). B[k]F was weakly anti-estrogenic as indicated by significant inhibition of moxestrol-mediated VTG mRNA induction at 2000 nM (Fig. 5.5, ANOVA, Tukey's $P<0.05$). All three HDBPs also caused significant inhibition of moxestrol-mediated VTG mRNA induction at the highest concentration tested, 10000 nM (Fig. 5.6, ANOVA, Tukey's, $P<0.05$). Finally, the extract prepared from the common tern egg showed concentration-dependent anti-estrogenicity in the chicken embryo hepatocyte cultures (Fig. 5.7). Significant inhibition of moxestrol-mediated VTG mRNA induction was observed at 250 mg egg homogenate equivalents (ANOVA, Tukey's, $P<0.05$).

β-actin and albumin gene expression were also determined for each concentration of test substance and compared with the expression determined for the solvent controls. No significant decrease in albumin mRNA concentrations were observed for any of the substances tested. For tamoxifen, B[k]F, Br4 and the tern egg extract, no significant decreases in β-actin mRNA concentrations were observed (data not shown, ANOVA, Tukey's, $P>0.05$). Significant inhibition of β-actin gene expression, however, was observed for TCDD (10 nM), PCB 126 (200 nM), Br5 (10000 nM), and Br6 (1000 and 10000 nM) (see Figs. 5.4 and 5.6, ANOVA, Tukey's, $P<0.05$). Because these declines in β-actin gene expression were observed at the highest concentrations of the compounds tested, it is possible that high concentrations of these compounds are overtly toxic to the chicken embryo

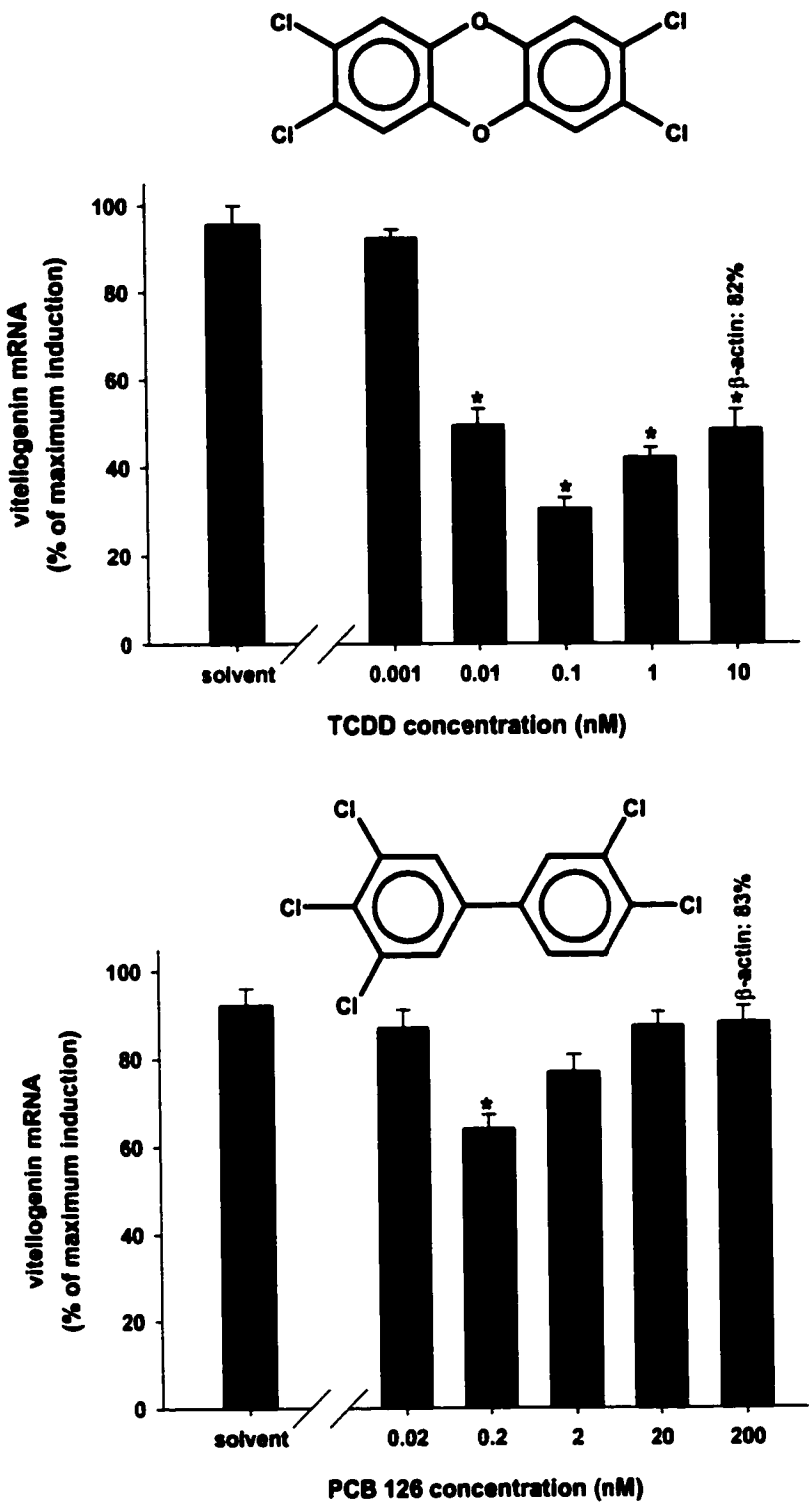


Figure 5.4 Anti-estrogenicity bioassay of TCDD and PCB 126. Chicken embryo hepatocyte cultures were treated with 100 nM moxestrol plus various concentrations of TCDD or solvent (top graph), or PCB 126 or solvent (bottom graph). Significant inhibition of moxestrol-mediated vitellogenin mRNA induction was observed for cells treated with 0.01 - 10 nM TCDD, or 0.2 nM PCB 126 (ANOVA, Tukey's, $P<0.05$). Significant decreases in β-actin gene expression were observed for 10 nM TCDD (expressed at 82% of solvent control) and 200 nM PCB 126 (expressed at 83% of control) (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of duplicate plates from one cell culture experiment. Error bars indicate standard error of the bioassay.

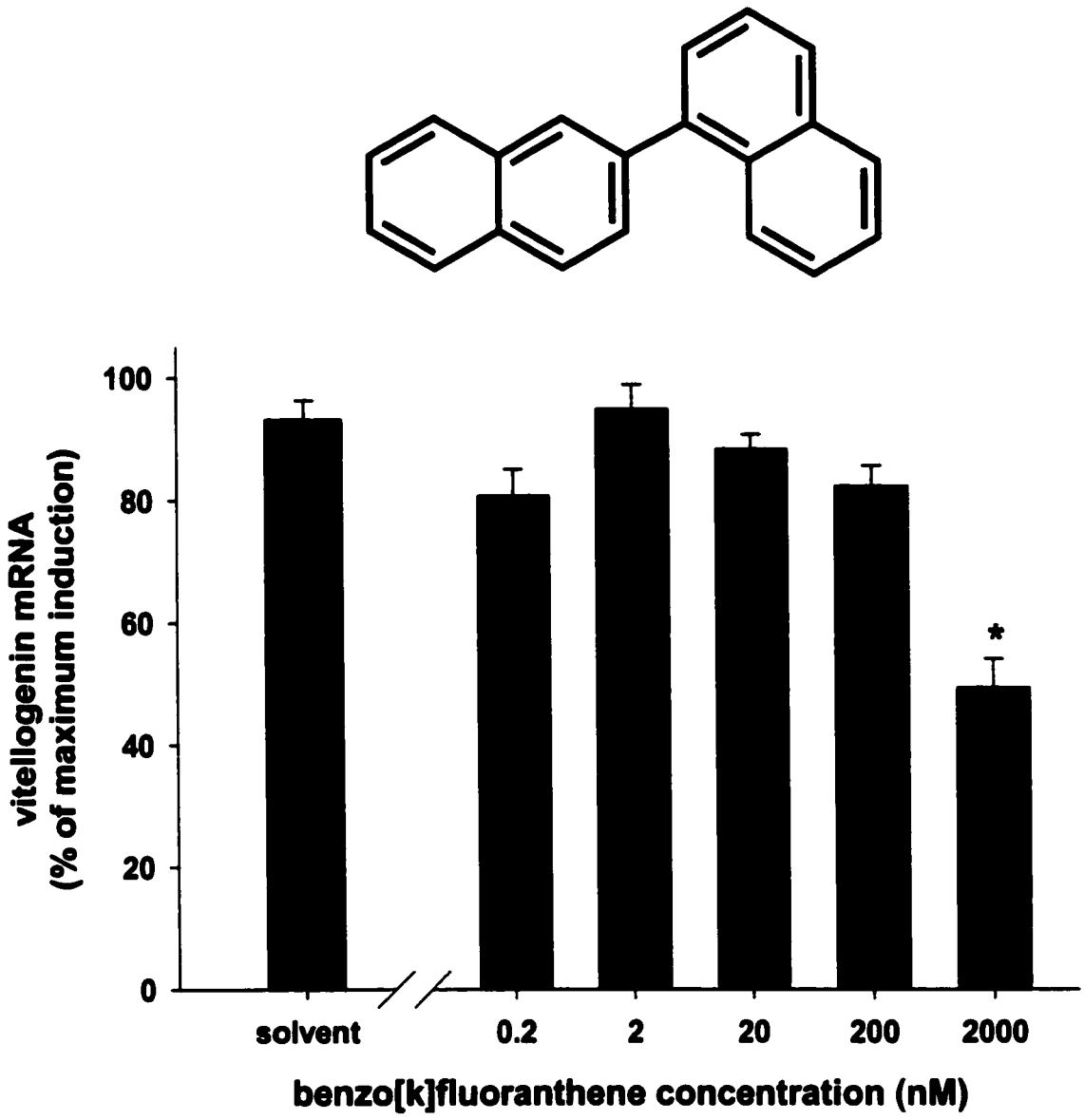


Figure 5.5 Anti-estrogenicity bioassay of benzo[k[fluoranthene. Chicken embryo hepatocyte cultures were treated with 100 nM moxestrol plus various concentrations of B[k]F or solvent. Significant inhibition of moxestrol-mediated vitellogenin mRNA induction was observed for cells treated with 2000 nM B[k]F (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of duplicate plates from one cell culture experiment. Error bars indicate standard error of the bioassay.

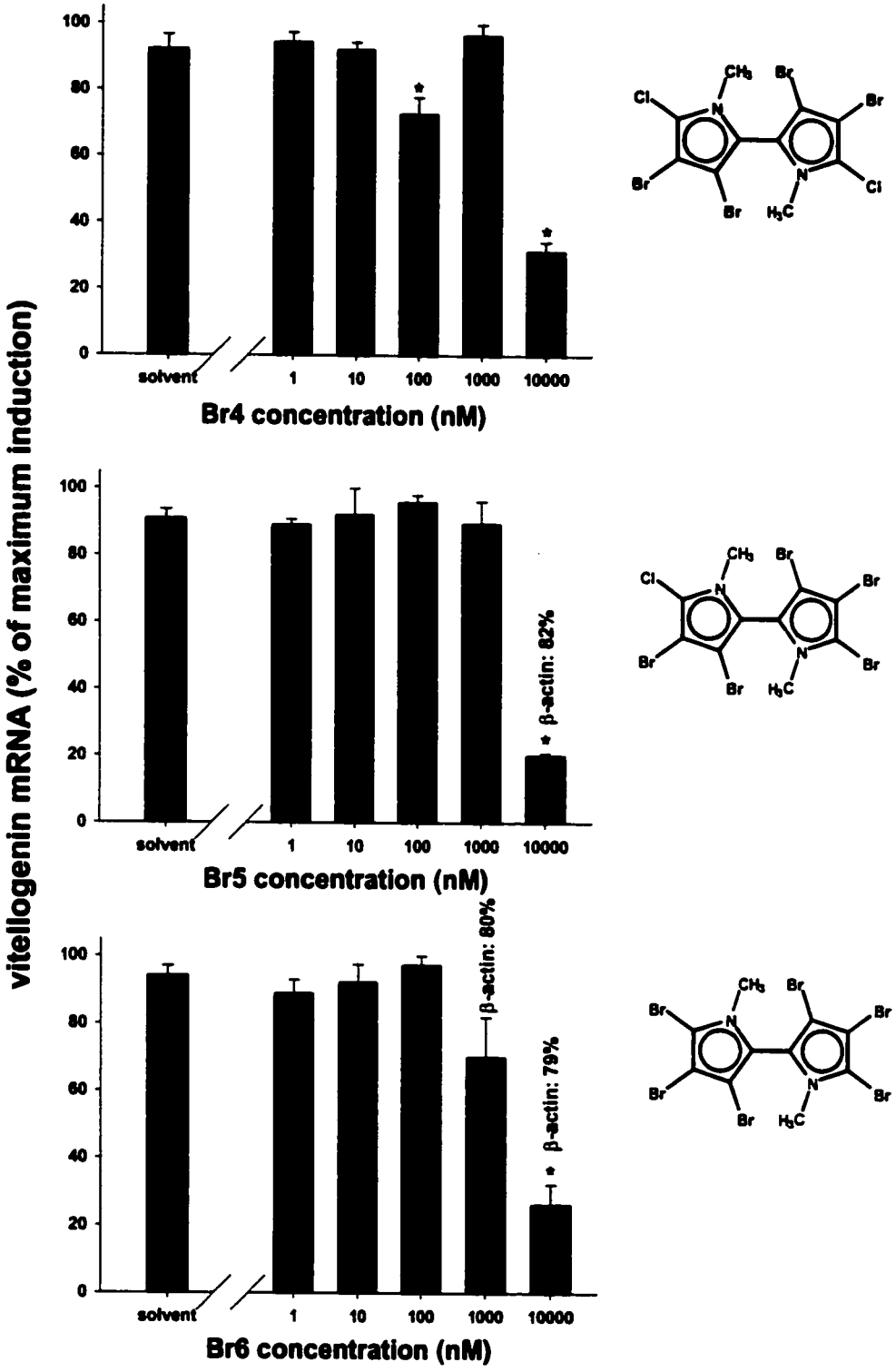


Figure 5.6 Anti-estrogenicity bioassay of halogenated dimethyl bipyrroles. Chicken embryo hepatocyte cultures were treated with 100 nM moxestrol plus various concentrations of Br4 or solvent (top graph), Br5 or solvent (middle graph), or Br6 or solvent (bottom graph). Significant inhibition of moxestrol-mediated vitellogenin mRNA induction was observed for cells treated with 10000 nM Br4, Br5, or Br6 (ANOVA, Tukey's, $P<0.05$). Significant decreases in β-actin gene expression were observed for 10000 nM Br5 (expressed at 82% of control), 1000 nM and 10000 nM Br6 (expressed at 80% and 79% of control, respectively) (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of a single plate from one cell culture experiment. Error bars indicate standard error of the bioassay.

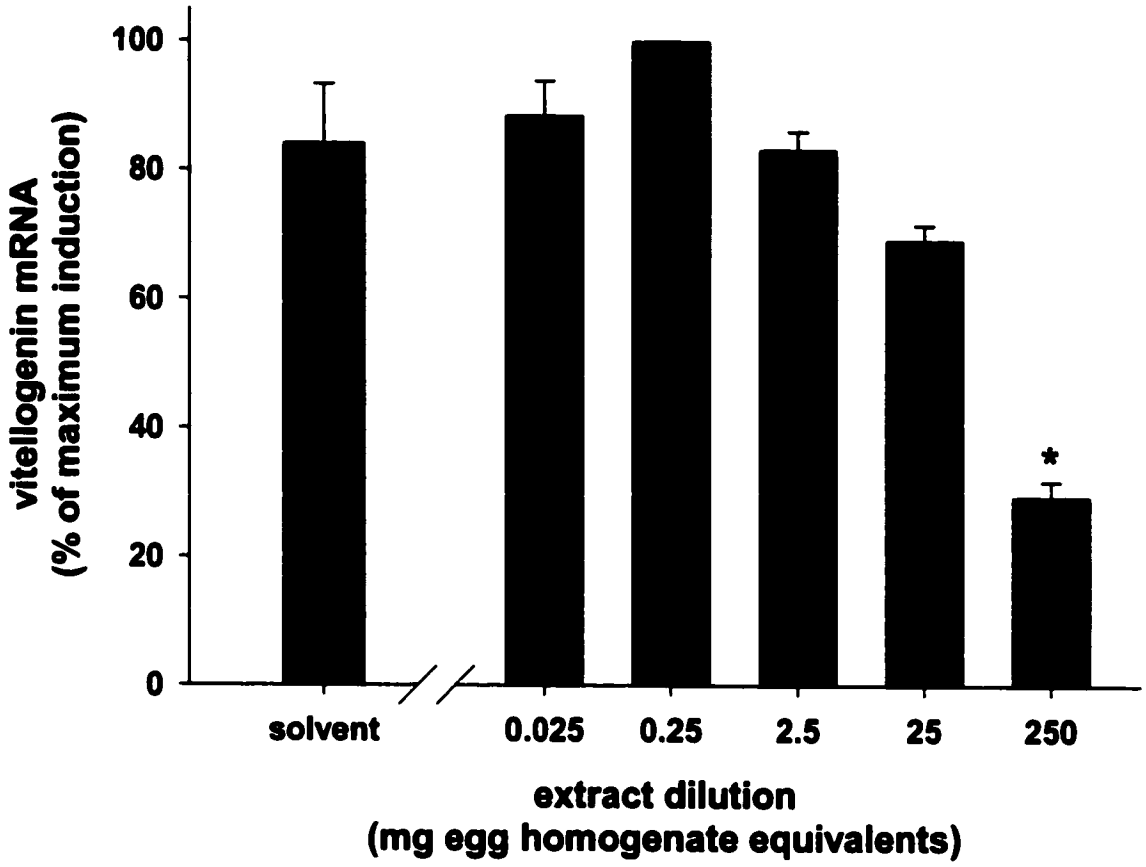


Figure 5.7 Anti-estrogenicity bioassay of a common tern egg extract. Chicken embryo hepatocyte cultures were treated with 100 nM moxestrol plus various dilutions of the egg extract or solvent. Significant inhibition of moxestrol-mediated vitellogenin mRNA induction was observed for cells treated with 250 mg egg homogenate equivalents (ANOVA, Tukey's, $P<0.05$). Each bar represents triplicate bioassays of a single plate from one cell culture experiment. Error bars indicate standard error of the bioassay.

hepatocyte cultures. Table 5.1 summarizes the results obtained for the anti-estrogenicity application of the avian VTG mRNA RT-PCR bioassay.

5.6 Discussion

5.6.1. Estrogenicity Bioassays

Of the compounds tested for estrogenicity in the chicken embryo hepatocyte cultures using the avian VTG mRNA RT-PCR bioassay, only OP gave a weak response at high concentrations (100-200 μM). Maximal VTG mRNA induction observed for OP corresponded to approximately 9% of that typically observed in chicken embryo hepatocyte cultures treated with 1000 nM moxestrol. Although no studies to date have indicated that OP or other alkylphenols have estrogenic effects in birds, estrogenic effects have been demonstrated in mammals and fish for these compounds. In rats, several studies have shown that alkyl phenol exposure can result in stimulation of estrogen-dependent uterine growth (reviewed in Janssen *et al.*, 1998). Similarly, *in vitro* measurement of the proliferative effect of estrogenic compounds in the MCF-7 human mammary tumor cell line indicated that OP and other alkyl phenols are estrogenic (White *et al.*, 1994; Soto *et al.*, 1995). In fish caged downstream from sewage treatment plants, elevated levels of VTG were correlated with effluent containing high levels of alkyphenolic chemicals (Harries *et al.*, 1997). Further *in vitro* assays with rainbow trout hepatocytes confirmed that several alkylphenol compounds, including OP, were weakly estrogenic (Jobling and Sumpter, 1993; White *et al.*, 1994).

Table 5.1 Anti-estrogenicity bioassay results

test substance + 100 nM moxestrol	embryo hepatocyte culture (plates/concentration[1])	VTG mRNA induction significantly < control (P<0.05, nM)	β-actin or albumin mRNA significantly < control (P<0.05, nM)
tamoxifen (0.1-1000 nM)	***chicken*** (2)	1000	-
TCDD (0.001-10 nM)	***chicken*** (2)	0.01, 0.1, 1, 10	β-actin: 10
PCB 126 (0.02-200 nM)	***chicken*** (2)	0.2	β-actin: 200
benzo[*k*] fluoranthene (0.2-2000 nM)	***chicken*** (2)	2000	-
Br4 (1-10000 nM)	***chicken*** (1)	100, 10000	-
Br5 (1-10000 nM)	***chicken*** (1)	10000	β-actin: 10000
Br6 (1-10000 nM)	***chicken*** (1)	10000	β-actin: 1000, 10000
tern egg extract (0.025-250 mg homogenate)	***chicken*** (1)	250	-

[1] plates/concentration refers to the number of cell culture plates (100 mm diameter) used to test each concentration of the test substance

Although no VTG mRNA induction was observed in chicken embryo hepatocyte cultures, MXCL has been shown to be weakly estrogenic in bird embryos after *in ovo* exposure. In these studies MXCL treatment of gull eggs resulted in feminization of male embryos and abnormal development of the right oviduct in female embryos (Fry and Toone, 1981; Fry *et al.*, 1987). These effects were similar to those observed after estradiol treatment of gull eggs, but estradiol was found to be at least 100-fold more potent than MXCL (Fry and Toone, 1981; Fry *et al.*, 1987). Thus results of these experiments indicate that MXCL is weakly estrogenic in gull embryos.

MXCL has also been shown to be weakly estrogenic in mammals and fish. The minimum effective dose required to produce an estrogenic response in the rat uterus was determined to be 4-times higher for pure MXCL (*p,p'*-substituted) than for technical MXCL which also contains other contaminants (Bitman and Cecil, 1970). For either preparation, MXCL was at least 1000-times less estrogenic than natural estrogens (Bitman and Cecil, 1970). Studies with carp hepatocyte cultures indicated that MXCL (reported as 98% pure) caused significant VTG induction at concentrations as low as 5 μM (Smeets *et al.*, 1999b).

A possible reason why no estrogenic activity was observed in chicken embryo hepatocyte cultures treated with concentrations of MXCL as high as 50 μM may be due to the "pro-estrogenic" nature of this compound. Several studies have shown that pure (*p,p'*-substituted) MXCL does not bind to rat uterine estrogen receptor, nor cause estrogenic effects, but technical grade formulations of MXCL do (reviewed in Kupfer and Bulger, 1987). However, pure preparations of MXCL do exhibit

estrogenic activity when tested *in vivo* (reviewed in Kupfer and Bulger, 1987). Further studies indicated that, although MXCL itself was not estrogenic, metabolites of this compound were (Kupfer and Bulger, 1987)). Therefore, it is possible that the chicken embryo hepatocyte cultures used in the avian estrogenicity bioassay do not effectively metabolize MXCL to estrogenic metabolites and hence no estrogenic response was observed. Alternatively, an impure grade of MXCL may have been used in the gull experiments described above.

Of the other compounds tested in the avian estrogenicity bioassay, BPA (an industrial plasticizer) has consistently been shown to be weakly estrogenic *in vitro* in mammalian and fish systems. In MCF-7 cells, 10-25 nM BPA induced progesterone receptors, and 25 nM BPA increased the rate of cell proliferation (Krishnan *et al.*, 1993). In carp and rainbow trout hepatocyte cultures, BPA was weakly estrogenic, inducing VTG at concentrations of 50 μM or greater, or 5 μM or greater, respectively (Sumpter and Jobling, 1995; Smeets *et al.*, 1999a). In the present study, no estrogenic response was observed in chicken embryo hepatocyte cultures treated with 1-10 μM BPA. VTG mRNA induction was also not observed in additional experiments with chicken embryo hepatocyte cultures treated with higher concentrations of BPA (10-200 μM). In these latter experiments significant cell detachment was observed for cultures treated with 100 and 200 μM BPA indicating that these concentrations were overtly toxic to the cells. Interestingly, BPA was shown to have "anti-estrogenic" effects in birds as indicated by degradation of apolipoprotein II mRNA in liver extracts obtained from roosters treated with a combination of estrogen and BPA (Ratnasabapathy *et al.*, 1997). Based on these

results, it was concluded that BPA prevented expression of an estrogen-regulated mRNA stabilizing factor that is normally expressed in response to estrogen in avian liver. Clearly, further studies are required to determine if BPA is anti-estrogenic in birds.

The VTG mRNA induction responses observed in this study and the results described in Chapter 4.5.5 for *o,p'*-DDT for chicken embryo hepatocyte cultures are compared with the estrogenic responses observed in previously developed fish hepatocyte VTG bioassays in Table 5.2. Although the data are variable and incomplete, with the exception of *o,p'*-DDT, chicken embryo hepatocyte cultures appeared to be less sensitive to VTG mRNA induction after treatment with either OP or BPA than the fish hepatocyte cultures. Furthermore, among the fish hepatocyte cultures, the rainbow trout appeared to be the most sensitive to both estradiol and the environmental contaminants shown.

A possible reason for the differences observed between the results of the avian and fish VTG bioassays may involve differences in estrogen receptor binding affinities. Results of a recent study indicated that bacterially expressed glutathione-*S*-transferase estrogen receptor fusion proteins for the chicken exhibited approximately 5-fold lower binding affinities for a number of environmental contaminants (including OP, BPA, MXCL and *o,p'*-DDT) than observed for rainbow trout estrogen receptor fusion proteins (Matthews *et al.*, 2000). In contrast, both chicken and rainbow trout estrogen receptor fusion proteins bound estradiol with a similar affinity (Matthews *et al.*, 2000). As well, MXCL was considered to be an extremely weak ligand for estrogen receptor fusion proteins from all species tested

Table 5.2 Comparison of results from avian and fish hepatocyte VTG bioassays

test substance	chicken embryo		carp[3]		channel catfish[4]		rainbow trout[5]	
	LOEC[1] (nM)	relative potency[2]	LOEC (nM)	relative potency	LOEC (nM)	relative potency	LOEC (nM)	relative potency
moxestrol or estradiol	10	100	2	100	1	100	0.1	100
o,p'-DDT	10000	0.1	25000	0.008	1000	0.1	1000	0.01
4-*tert*-octylphenol	>100000	<0.01	nd	-	10	10	10	1
bisphenol A	nr	-	50000	0.004	nd	-	1000	0.01

[1]LOEC = lowest observed effect concentration
[2]relative potency = (LOEC moxestrol or estradiol)/(LOEC test substance)X100
nr - no response; nd = not determined

[3]Smeets *et al.*, 1999a,1999b,1999c
[4]Monteverdi and Di Giulio, 1999
[5]Jobling and Sumpter, 1993; Sumpter and Jobling, 1995

except the rainbow trout (Matthews *et al.*, 2000). Based on these observations, the authors of this study concluded that estrogen receptor fusion proteins from the rainbow trout bind environmental contaminants with high affinity, and suggested that the trout may be particularly sensitive to the estrogenic effects of these contaminants.

5.6.2. Anti-estrogenicity Bioassays

Experiments were conducted to determine whether the avian VTG mRNA RT-PCR bioassay could also be used to assess the effects of anti-estrogenic compounds. The pharmacological anti-estrogen tamoxifen effectively inhibited moxestrol-mediated VTG mRNA induction in chicken embryo hepatocyte cultures without altering β-actin or albumin gene expression at a concentration of 1000 nM. Tamoxifen has been shown to compete for estrogen-receptor nuclear binding sites in mammals and has been used to treat some forms of breast cancer in humans (Erlichman and Kerr, 1989). In carp hepatocyte cultures, concentrations of tamoxifen in excess of 600 nM also caused concentration-dependent reduction of VTG secretion (Smeets *et al.*, 1999a).

The dioxin, TCDD, was observed to be a potent anti-estrogen in the chicken embryo hepatocyte cultures, causing a significant decrease in moxestrol-mediated VTG mRNA induction at concentrations as low as 0.01 nM. In comparison, the results obtained for the "dioxin-like" PCB 126 were not as clear. For PCB 126 a significant reduction of moxestrol-mediated VTG mRNA induction was observed at a concentration of 0.2 nM, but higher concentrations were not effective. TCDD has

repeatedly been shown to be a potent anti-estrogen in both mammals and fish, and in a limited number of studies in birds (reviewed in Janssen *et al.*, 1998). For example, exposure of adult chickens to TCDD resulted in significant delays in egg production in female offspring and reduced sperm numbers and volume in male offspring (Alonso *et al.*, 1996). In other studies, treatment of pigeon embryos *in ovo* with TCDD during late incubation resulted in a significant decrease in 17β-estradiol concentrations and elevated hepatic estrogen receptor concentrations at hatch (Janz and Bellward, 1996).

In vitro studies conducted with carp hepatocyte cultures showed that TCDD inhibited estradiol-mediated VTG secretion at concentrations as low as 0.02-0.06 nM (Smeets *et al.*, 1999a). These results are in good agreement with the results obtained using the avian bioassay. In both cases, TCDD was observed to be about 10000 times more potent than tamoxifen. In contrast to the avian bioassay results, the same investigators also found that PCB 126 was a relatively potent anti-estrogen in carp hepatocyte cultures (Smeets *et al.*, 1999c). In this case PCB 126 caused a concentration-dependent decrease in VTG secretion at concentrations as low as 0.6 nM (EC50 = 1 nM). However, other studies with rainbow trout hepatocyte cultures indicated that 10 nM PCB 126 was not anti-estrogenic as indicated by lack of inhibition of estradiol-mediated VTG secretion (Anderson *et al.*, 1996). For *in vitro* mammalian-based systems, both TCDD (EC50 = 8.6 nM) and PCB 126 (EC50 = 180 nM) were shown to be anti-estrogenic in MCF-7 cells as indicated by inhibition of estradiol-induced secretion of procathepsin D (Krishnan and Safe, 1993). Because PCB 126 did not cause a concentration-dependent decrease in moxestrol-

mediated VTG mRNA induction in chicken embryo hepatocyte cultures in the present study, but was possibly anti-estrogenic at 0.2 nM, further studies are required to clarify whether PCB 126 is anti-estrogenic (or estrogenic) *in vitro* in avian hepatocyte cultures, and *in vivo/in ovo* in birds.

The PAH, B[k]F was shown to be anti-estrogenic in avian embryo hepatocyte cultures at the highest concentration tested, 2000 nM. Although previous studies have shown embryotoxic effects of PAHs in bird embryos (Brunstrom *et al.*, 1990; Brunstrom, 1991), to my knowledge this is the first evidence of an anti-estrogenic effect of a PAH in an avian system. In comparison, PAHs have been shown to cause decreases in estrogen receptor concentrations and interfered with the binding of estradiol to estrogen receptors in rat uteri (reviewed in Janssen *et al.*, 1998). The anti-estrogenic effects of PAHs have also been documented in fish. Exposure of female Atlantic croaker to benzo[a]pyrene resulted in a reduction in circulating hormone levels and decreased ovarian growth (Thomas, 1990). Exposure to PAHs was also shown to result in decreased estradiol secretion by salmon ovarian follicles (Afonso *et al.*, 1997). Furthermore, both benzo[a]pyrene and benzo[a]anthracene produced small, but concentration-dependent suppression of VTG secretion in carp hepatocyte cultures at concentrations exceeding 6 μM (Smeets *et al.*, 1999c). Thus, it is likely that PAHs, such as B[k]F are weakly anti-estrogenic in birds.

The HDBPs, Br4, Br5 and Br6 also showed anti-estrogenic activities in chicken embryo hepatocyte cultures at 10000 nM. However, significant inhibition (~20%) of β-actin gene expression was also observed for two of the three HDBPs tested at 1000 and/or 10000 nM. Therefore, these data should be interpreted

cautiously as these compounds may impair overall gene expression at these concentrations. As the HDBPs are a recently discovered class of halogenated environmental contaminants (Tittlemier *et al.*, 1999), their toxicology has not previously been investigated in any species. HDBPs are structurally similar to other organohalogen compounds such as dioxins and PCBs, but it is thought that HDBPs are naturally produced (Tittlemier *et al.*, 1999). Furthermore, it has been shown that HDBPs can bioaccumulate in seabird eggs. Further testing of these compounds should provide additional insight into their possible anti-estrogenic effects in birds and other animals.

An organochlorine extract prepared from a common tern egg collected from a site highly contaminated with PCBs and heavy metals (Bird Island, MA) also showed concentration-dependent inhibition of moxestrol-mediated VTG mRNA induction in chicken embryo hepatocyte cultures. Although chemical residues in the extract were not determined, based on the method of preparation the extract should contain dioxins, furans, PCBs, other halogenated aromatic hydrocarbons, and organochlorine insecticides. Interestingly roseate terns (*Sterna dougallii*) residing at Bird Island have shown abnormal reproductive behaviour as indicated by a sex ratio skewed towards females, female-female pairing and supernormal clutches of eggs (reviewed in Hart, 2000). However, studies to date have not shown a convincing relationship between these reproductive effects and contaminant exposure (Nisbet *et al.*, 1996; Hart, 2000). Although it is not clear whether the reproductive effects observed would be considered "feminizing" or "masculinizing", results from the avian bioassay suggest that the terns may be exposed to anti-estrogenic substances.

Further studies of egg extracts obtained from this and other sites should provide interesting information regarding exposure of wild populations of birds to potentially estrogenic or anti-estrogenic environmental contaminants.

5.6.3. Mechanism of Anti-estrogenicity

The mechanism of anti-estrogenicity of TCDD and structurally related environmental contaminants is not thought to involve binding of these contaminants to the estrogen receptor. Rather these compounds are thought to elicit anti-estrogenic effects by binding to another protein, the aryl hydrocarbon (Ah) receptor (reviewed in Safe *et al.*, 1991; Chapter 1.A.4). Reasonable correlations have been observed between the order of potency of agonists binding to the Ah receptor, induction of CYP1A1 and anti-estrogenic effects (Anderson *et al.*, 1996; Smeets *et al.*, 1999c). Although the precise mechanism(s) by which Ah receptor ligands elicit their anti-estrogenic effects is not known, possible pathways have been proposed. These include induction of CYP1A1 resulting in increased metabolism of 17β-estradiol, binding of the activated Ah receptor to repressor sites on promoter regions of estrogen-inducible genes, synthesis of modulatory proteins that can bind to repressor sites on promoter regions of estrogen-inducible genes, down-regulation of estrogen receptor levels, inhibition of binding of estradiol to the estrogen receptor, and/or mutual inhibition of activated estrogen or Ah receptors for binding to DNA (reviewed in Smeets *et al.*, 1999c). Of these mechanisms, increased metabolism of estradiol by CYP1A1 is thought to be least likely since studies in fish hepatocyte cultures demonstrated a poor relationship between the actual concentrations of Ah

receptor ligands required to cause anti-estrogenic effects and CYP1A1 induction (Smeets *et al.*, 1999c). Furthermore, other studies show that CYP1A1 does not catalyze oxidative metabolism of 17β-estradiol (Snowberger and Stegeman, 1987).

All of the substances tested for anti-estrogenicity in the present study (tamoxifen, TCDD, PCB126, B[k]F, HDBPs, egg extract), except tamoxifen, induce CYP1A in chicken embryo hepatocyte cultures (Kennedy *et al.*, 1996a; Hart, 2000; Jeffery, 2001; Tittlemier *et al.*, unpublished data). As discussed previously, tamoxifen is a classic anti-estrogen that elicits its effects by binding to estrogen receptors antagonistically or with partial agonism (Erlichman and Kerr, 1989). All of the other compounds, with the possible exception of PCB 126, were determined to be anti-estrogenic as indicated by suppression of moxestrol-mediated VTG mRNA induction in chicken embryo hepatocyte cultures. Thus, it is possible that the environmental contaminants tested in the avian bioassay are exerting anti-estrogenic effects through a pathway(s) that involves Ah receptor binding.

5.7 Conclusions

Overall, the results of this study show that the avian VTG mRNA bioassay can be used to determine both the estrogenic and anti-estrogenic effects of a variety of pharmacological agents and environmental contaminants. The literature was reviewed in an attempt to assess the utility of the bioassay for predicting the estrogenic or anti-estrogenic activities of environmental contaminants in birds *in ovo* or *in vivo*. Although experimental data are extremely limited for birds, it appears that the avian VTG mRNA bioassay can predict the *direct* estrogenic or anti-estrogenic

effects of environmental contaminants in birds. However, it is more difficult to interpret these data in terms of the actual concentration of test substance required to produce an effect. *In vitro* bioassays are designed for rapid throughput and screening and do not take into account effects of chronic exposure and, in some systems, potential metabolism of the parent compound. Therefore, the actual concentrations of test substances determined to produce an estrogenic or anti-estrogenic effect *in vitro* should not be extrapolated or rigorously compared with similar data obtained from *in vivo* systems.

Further studies are required to characterize the metabolic capacity of the chicken embryo hepatocyte cultures to understand why the pro-estrogen, MXCL, produced no estrogenic response in the bioassay. Finally, results obtained with the egg extract indicate that the avian VTG mRNA bioassay may be extremely useful for predicting whether wild populations of birds are exposed to estrogenic or anti-estrogenic environmental contaminants.

CHAPTER 6. GENERAL CONCLUSIONS

The effects of environmental contaminants on components of two endocrine pathways - the HPA axis and the HPO axis - were examined in herring gull embryos and herring gull and chicken embryo hepatocyte cultures. Studies on the HPA axis provided the first evidence that current levels of organochlorine contaminants in the Great Lakes may be affecting this endocrine axis and associated intermediary metabolic pathways in environmentally-exposed herring gull embryos. More specifically, inverse relationships were observed between PCDDs/PCDFs, total PCBs, non-*ortho* PCBs, or TEQs and gull embryo basal plasma corticosterone concentrations. Similarly, the activities of two intermediary metabolic enzymes, PEPCK and ME, were inversely correlated with PCDDs/PCDFs. Further studies provided previously undocumented observations that embryos from some of the Great Lakes sites may have shorter than average incubation periods. In particular, early onset of hatching, elevated renal PEPCK activities, and lower than average corticosterone stress responses were observed for embryos from Scotch Bonnet Island (Lake Ontario). This site was seriously contaminated with PCBs, DDE, Mirex and other contaminants in the 1970's. Thus it is possible that this historical exposure to contaminants is continuing to influence herring gull embryos from this site due to genetic selection, or other mechanisms. Studies are planned to document the incubation periods of herring gull embryos in the field at Scotch Bonnet Island and other Great Lakes sites. Furthermore, Scotch Bonnet Island embryos will be more

intensively studied to determine if contaminant exposure is continuing to affect the health of the herring gulls in this colony.

Studies related to the HPO axis resulted in the development of a novel semi-quantitative RT-PCR bioassay for avian vitellogenin mRNA. This is the first hepatocyte culture vitellogenin bioassay to be developed for birds and is the first use of RT-PCR technology for hepatocyte culture VTG mRNA detection for any species. Development of the RT-PCR VTG mRNA bioassay involved cloning and sequencing regions of the VTG, β-actin and albumin genes for several species of birds for which no sequence data had previously been published. This novel sequence information is valuable in itself in that it can be used to develop species-specific PCR primers for biomarker or bioassay applications.

Application of the RT-PCR bioassay allowed detection and quantitation of VTG mRNA from herring gull embryo hepatocyte cultures for the first time. Comparison of the VTG mRNA induction responses between chicken and herring gull embryo hepatocyte cultures treated with moxestrol indicated that herring gull embryo hepatocyte cultures were significantly more sensitive to this pharmacological estrogen. These results suggest that species differences in sensitivity to estrogenic substances can exist for birds. Data obtained from chicken embryo hepatocyte cultures indicate that the bioassay can be used to determine both the estrogenic and anti-estrogenic potencies of pharmacological agents, specific environmental contaminants, and biological extracts containing complex mixtures of environmental contaminants. Furthermore, it appears that the bioassay can predict the direct estrogenic or anti-estrogenic effects of environmental contaminants previously

documented in *in ovo* or *in vivo* studies of birds. Overall, the avian VTG mRNA bioassay may be extremely useful for predicting whether wild populations of birds are exposed, or may be sensitive to, estrogenic or anti-estrogenic environmental contaminants.

REFERENCES

Afonso, L. O. B., Campbell, P. M., Iwama, G. K., Devlin, R. H., and Donaldson, E. M. (1997). The effect of the aromatase inhibitor fadrozole and two polynuclear aromatic hydrocarbons on sex steroid secretion by ovarian follicles of Coho salmon. *Gen. Comp. Endocrinol.* **106**, 169-174.

Al-hassani, D. (1993). Production and biochemical studies on chicken embryos under cold stress. *Ind. J. Animal. Sci* **63**, 46-49.

Alonso, K. R., Peden, M. M., Henshel, D., and Dickerson, R. L. (1996). *In ovo* exposure to 2,3,7,8-TCDD results in delayed reproductive maturation in the domestic chicken. *Organohalogen Compounds* **29**, 195-199.

Anderson, M. J., Miller, M. J., and Hinton, D. E. (1996). *In vitro* modulation of 17-B-estradiol-induced vitellogenin synthesis: Effects of cytochrome P4501A1 inducing compounds on rainbow trout (*Oncorhyncus mykiss*) liver cells. *Aquat. Toxicol.* **34**, 327-350.

Avrutina, A. J., Kislyuk, S. M., Soltitskaya, L. P., and Tatarchuk, V. V. (1984). Influence of the genotype on the reactivity of the hypothalamo-pitutitary-adrenal system of chick embryos. *J. Evol. Biochem. Physiol.* **20**, 391-394.

Avrutina, A. J., Galpern, I. L., and Kisljuk, S. M. (1985). Stimulation of adrenals during the critical periods of development and production in fowls. *World's Poultry Sci. J.* **41**, 108-114.

Beaudry, C., Bellabarba, D., and Lehoux, J.-G. (1983). Corticosteroid receptors in the kidney of chick embryo. *Gen. Comp. Endocrinol.* **50**, 292-304.

Bellabarba, D., Beaudry, C., and Lehoux, J.-G. (1983). Corticosteroid receptors in the kidney of chick embryo II. Ontogeny of corticosterone receptor and cellular development. *Gen. Comp. Endocrinol.* **50**, 305-312.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., Rapp, B. A., Wheeler, D. L. (2000). GenBank. Nucl. Acids Res. **28**, 15-18.

Bergink, E. W., Kloosterboer, H. J., Gruber, M., and Ab, G. (1973). Estrogen-induced phosphoprotein synthesis in roosters. Kinetics of induction. *Biochim. Biophys. Acta* **294**, 497-506.

Bergink, E. W., and Wallace, R. A. (1974). Estrogen-induced synthesis of yolk proteins in roosters. *Amer. Zool.* **14**, 1177-1193.

Bergman, A., and Olsson, M. (1985). Pathology of Baltic grey seal and ring seal females with special reference to adrenocortical hyperplasia: is environmental pollution the cause of a widely distributed disease syndrome? *Finn. Game Res.* **44**, 47-62.

Binder, R., MacDonald, C. C., Burch, J. B. E., Lazier, C. B., and Williams, D. L. (1990). Expression of endogenous and transfected apolipoprotein II and vitellogenin II genes in an estrogen responsive chicken liver cell line. *Mol. Endocrinol.* **4**, 201-208.

Birnbaum, R. S., and Goodman, H. M. (1977). Studies on the mechanisms of epinephrine stimulation of lypolysis. *Biochim. Biophys. Acta* **496**, 292-301.

Bitman, J., and Cecil, H. C. (1970). Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agr. Food Chem.* **18**, 1108-1112.

Boehm, K. D., Hood, R. L., and Ilan, J. (1988). Induction of vitellogenin in primary monolayer cultures of cockerel hepatocytes. *Proc. Natl. Acad. Sci. USA* **85**, 3450-3454.

Boersma, D. C., Ellenton, J. A., and Yagminas, A. (1986). Investigation of the hepatic mixed-function oxidase system in herring gull embryos in relation to environmental contaminants. *Environ. Toxicol. Chem.* **5**, 309-318.

Boss, W. R. (1943). Hormonal determination of adult characters and sex behavior in herring gulls (*Larus argentatus*). *J. Exp. Zool.* **94**, 181-209.

Boss, W. R., and Witschi, E. (1947). The permanent effects of early stilbestrol injections on the sex organs of the herring gull (*Larus argentatus*). *J. Exp. Zool.* **105**, 61-77.

Brooks, W. S., and Ungar, F. (1967). Effect of C_{21}-methyl steroids on the musculus complexus and hatching of the chick. *Proc. Soc. Exp. Biol. Med.* **125**, 488-492.

Brunstrom, B., and Reutergardh, L. (1986). Differences in sensitivity of some avian species to the embryotoxicity of a PCB, 3,3',4,4'-tetrachlorobiphenyl, injected into the eggs. *Environ. Pollut.* **42**, 37-45.

Brunstrom, B. (1988). Sensitivity of embryos from duck, goose, herring gull and various chicken breeds to 3,3',4,4'-tetrachlorobiphenyl. *Poultry Sci.* **67**, 52-57.

Brunstrom, B., Broman, D., and Naf, C. (1990). Embryotoxicity of polycyclic aromatic hydrocarbons (PAHs) in three domestic avian species, and of PAHs and coplanar polychlorinated biphenyls (PCBs) in the common eider. *Environ. Pollut.* **67**, 133-143.

Brunstrom, B. (1991). Embryolethality and induction of 7-ethoxyresorufin O-deethylase in chick embryos by polychlorinated biphenyls and polycyclic aromatic hydrocarbons having Ah receptor affinity. *Chem. Biol. Interact.* **81**, 69-77.

Bryan, G. W., Gibbs, P. E., Hummerstone, L. G., and Burt, G. R. (1986). The decline of the gastropod *Nucella lapillus* around south-west England: Evidence for the effect of tributyltin from antifouling plants. *J. Mar. Biol. Assoc. U. K.* **67**, 524-544.

Burch, J. B. E., Evans, M. I., Friedman, T. M., and O'Malley, P. J. (1988). Two functional estrogen response elements are located upstream of the major chicken vitellogenin gene. *Mol. Cell. Biol.* **8**, 1123-1131.

Burley, R. W., and Vadehra, D. V. (1989). *The Avian Egg. Chemistry and Biology.* John Wiley & Sons, New York.

Carsia, R. V., Morin, M. E., Rosen, H. D., and Weber, H. (1987). Ontogenic corticosteroidogenesis of the domestic fowl: Response of isolated adrenocortical cells. *Proc. Soc. Exp. Biol. Med.* **184**, 436-445.

Carsia, R.V. and Harvey, S. (2000). Adrenals. In Sturkie's Avian Physiology (G.C. Whittow, Ed.), pp. 489-537. Academic Press, San Diego.

Cato, A. C. B., Heitlinger, E., Ponta, H., Keline-Hitpass, L., Ryffel, G. U., Bailly, A., Rauch, C., and Milgrom, E. (1988). Estrogen and progesterone receptor-binding sites on the chicken vitellogenin II gene: Synergism of steroid hormone action. *Mol. Cell. Biol.* **8**, 5323-5330.

Chou, W.-Y., Huang, S.-M., Liu, Y. H., and Chang, G.-G. (1994). Cloning and expression of pigeon liver cytosolic $NADP^+$-dependent malic enzyme cDNA and some of its abortive mutants. *Arch. Biochem. Biophys.* **310**, 158-166.

Colby, H. D., and Longhurst, P. A. (1996). Toxicology of the Adrenal Gland. In Endocrine Toxicology (C. K. Atterwill, and J. D. Flack, Eds.), pp. 243-281. Cambridge University Press, Cambridge.

De Boer, W., Snippe, L., Ab, G., and Gruber, M. (1982). Estrogen receptor in avian embryo and adult liver: Estrogen receptor activation and dissociation kinetics of estradiol, ethynylestradiol and moxestrol. *Endocrinology* **110**, 1217-1224.

Decuypere, E., Darras, V. M., Vermiljen, K., and Kühn, E. R. (1989). Developmental changes in the corticosterone response to corticotrophin and in the adrenal corticosterone content of rapid and slow growing strains of chickens (*Gallus domesticus*). *Br. Poultry Sci.* **30**, 699-709.

Deeley, R. G., Burtch-Wright, R. A., Grant, C. E., Hoodless, P. A., Ryan, A. K., and Schrader, T. J. (1993). Synthesis and deposition of egg proteins. In Manipulation of

the Avian Genome (R. J. Etches, and A. M. Verrinder Gibbins, Eds.), pp. 205-222. Boca Raton.

Deeley, R. G., Udell, D. S., Burns, A. T. H., Gordon, J. I., and Goldberger, R. F. (1977). Kinetics of avian vitellogenin messenger RNA induction. *J. Biol. Chem.* **252**, 7913-7915.

DeRosa, G., and Swick, R. W. (1975). Metabolic implications of the distribution of the alanine aminotransferase isoenzymes. *J. Biol. Chem.* **250**, 7961-7967.

Düttman, H., Bergmann, H.-H., and Engländer, W. (1998). Development of Behaviour. In Avian Growth and Development (J. M. Starck, and R. E. Ricklefs, Eds.), pp. 223-246. Oxford University Press, New York.

Edinger, R. S., Mambo, E., and Evans, M. I. (1997). Estrogen-dependent transcriptional activation and vitellogenin gene memory. *Mol. Endocrinol.* **11**, 1985-1993.

Ellenton, J. A., Brownlee, L. J., and Hollebone, B. R. (1985). Aryl hydrocarbon hydroxylase levels in herring gull embryos from different locations on the Great Lakes. *Environ. Toxicol. Chem.* **4**, 615-622.

Elliott, J. E., Norstrom, R. J., Lorenzen, A., Hart, L. E., Philibert, H., Kennedy, S. W., Stegeman, J. J., Bellward, G. D., and Cheng, K. M. (1996). Biological effects of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls in bald eagle (*Haliaeetus leucocephalus*) chicks. *Environ. Toxicol. Chem.* **15**, 782-793.

Erlichman, C., and Kerr, I. G. (1989). Antineoplastic Drugs. In Principles of Medical Pharmacology (H. Kalant, and W. H. E. Roschlau, Eds.), pp. 604-614. B.C. Decker Inc, Toronto.

Etches, R. J. (1996). *Reproduction in Poultry*. CAB International, Wallingford.

Evans, M. I., O'Malley, P. J., Krust, A., and Burch, J. B. E. (1987). Developmental regulation of the estrogen receptor and the estrogen responsiveness of five yolk protein genes in the avian liver. *Proc. Natl. Acad. Sci. USA* **84**, 8493-8497.

Evans, M. I., Silva, R., and Burch, J. B. E. (1988). Isolation of chicken vitellogenin I and III cDNAs and the developmental regulation of give estrogen-responsive genes in the embryonic liver. *Genes Develop.* **2**, 116-124.

Exton, J. H. (1979). Regulation of gluconeogenesis by glucocorticoids. In Glucocorticoid Hormone Action (J. D. Baxter, and G. G. Rousseau, Eds.), pp. 535-546. Springer, New York.

Fain, J. N. (1979). Inhibition of glucose transport in fat cells and activation of lipolysis by glucocorticoids. In Glucocorticoid Hormone Action (J. D. Baxter, and G. G. Rousseau, Eds.), pp. 547-560. Springer, New York.

Felicioli, R. A., Gabrielli, F., and Rossi, C. A. (1967). The synthesis of phosphoenolpyruvate in the gluconeogenesis. Enzyme levels of chick embryo livers. *Eur. J. Biochem.* **3**, 19-24.

Fischer, P. W. F., and Marks, G. S. (1976). Chick embryo liver cells maintained in serum-free Waymouth MD 705/1 medium. *Tissue Culture Association Manual* **2**, 449-452.

Fowler, G. S., Wingfield, J. C., and Boersma, P. D. (1995). Hormonal and reproductive effects of low levels of petroleum fouling in Magellanic penguins (*Spheniscus magellanicus*). *Auk* **112**, 382-389.

Fox, G. A., Kennedy, S. W., Norstrom, R. J., and Wigfield, D. C. (1988). Porphyria in herring gulls: A biochemical response to chemical contamination of Great Lakes food chains. *Environ. Toxicol. Chem.* **7**, 831-839.

Fox, G. A., Trudeau, S., Won, H., and Grasman, K. A. (1998). Monitoring the elimination of persistent toxic substances from the Great Lakes: Chemical and physiological evidence from adult herring gulls. *Environ. Monitor. Assess.* **53**, 147-168.

Freeman, B. M., and Vince, M. A. (1974a). Hormones in Development. In Development of the Avian Embryo (B. M. Freeman, and M. A. Vince, Eds.), pp. 208-236. Chapman and Hall, London.

Freeman, B. M., and Vince, M. A. (1974b). Hormones in Development. In Development of the Avian Embryo (B. M. Freeman, and M. A. Vince, Eds.), pp. 208-236. Chapman and Hall, London.

Fry, D. M., and Toone, C. K. (1981). DDT-induced feminization of gull embryos. *Science* **213**, 922-924.

Fry, D. M., Toone, C. K., Speich, S. M., and Peard, R. J. (1987). Sex ratio skew and breeding patterns of gulls: demographic and toxicological considerations. *Stud. Avian Biol.* **10**, 26-43.

Gendron, A. D., Bishop, C. A., Fortrin, R., and Hontela, A. (1997). In vivo testing of the functional integrity of the corticosterone-producing axis in mudpuppy (amphibia) exposed to chlorinated hydrocarbons in the wild. *Environ. Toxicol. Chem.* **16**, 1694-1706.

Gilbertson, M. (1974). Pollutants in breeding herring gulls in the lower Great Lakes. *Can. Field-Natur.* **88**, 273-280.

Gilbertson, M., and Hale, R. (1974a). Early embryonic mortality in a herring gull colony in Lake Ontario. *Can. Field-Natur.* **88**, 354-356.

Gilbertson, M., and Hale, R. (1974b). Characteristics of the breeding failure of a colony of herring gulls on Lake Ontario. *Can. Field-Natur.* **88**, 356-358.

Gilbertson, M., Kubiak, T., Ludwig, J., and Fox, G. (1991). Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDS) in colonial fish-eating birds: Similarity to chick-edema disease. *J. Toxicol. Environ. Health* **33**, 455-520.

Gilman, A. P., Fox, G. A., Peakall, D. B., Teeple, S. M., Carroll, T. R., and Haynes, G. T. (1977). Reproductive parameters and egg contaminant levels of Great Lakes herring gulls. *J. Wildlife Manage.* **41**, 458-468.

Girouard, R. J., and Hall, B. K. (1973). Pituitary-adrenal interaction and growth of the embryonic avian adrenal gland. *J. Exp. Zool.* **183**, 323-332.

Goldberg, A. L., Tischler, M., DeMartino, G., and Griffin, G. (1980). Hormonal regulation of protein degradation and synthesis in skeletal muscle. *Federation Proc.* **39**, 31-36.

Goodridge, A. G. (1968). Citrate-cleavage enzyme, 'malic' enzyme and certain dehydrogenases in embryonic and growing chicks. *Biochem. J.* **108**, 663-666.

Goodridge, A. G., Jenik, R. A., McDevitt, M. A., Morris, S. M., and Winberry, L. K. (1984). Malic enzyme and fatty acid synthase in the uropygial gland and liver of embryonic and neonatal ducklings. Tissue-specific regulation of gene expression. *Arch. Biochem. Biophys.* **230**, 82-92.

Gorski, J. R., Weber, L. W. D., and Rozman, K. (1990). Reduced gluconeogenesis in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Arch. Toxicol.* **64**, 66-71.

Grasman, K. A., Fox, G. A., Scanlon, P. F., and Ludwig, J. P. (1996). Organochlorine-associated immunosuppression in prefledgling Caspian terns and herring gulls from the Great Lakes: An ecoepidemiolgical study. *Environ. Health Perspect.* **104**, 829-842.

Greenman, D. L., and Zarrow, M. X. (1961). Steroids and carbohydrate metabolism in the domestic bird. *Proc. Soc. Exp. Biol. Med.* **106**, 459-462.

Griminger, P., and Scanes, C. G. (1986). Protein Metabolism. In Avian Physiology (P. D. Sturkie, Ed.), pp. 326-344. Springer-Verlag, New York.

Gruber, M., Bos, E. S., and Ab, G. (1976). Hormonal control of vitellogenin synthesis in avian liver. *Mol. Cell. Endocrinol.* **5**, 41-50.

Guillette, L. J., Jr., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F., and Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **102**, 680-688.

Guillette, L. J., Jr., Pickford, D. B., Crain, D. A., Rooney, A. A., and Percival, H. F. (1996). Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. *Gen. Comp. Endocrinol.* **101**, 32-42.

Gumbleton, M., and Nicholls, P. J. (1996). Molecular and systems pharmacology of glucocorticosteroids. In The Adrenal in Toxicology (P. W. Harvey, Ed.), pp. 81-128. Taylor & Francis, London.

Haché, R. J. G., Tam, S.-P., Cochrane, A., Nesheim, M., and Deeley, R. G. (1987). Long-term effects of estrogen on avian liver: Estrogen-inducible switch in expression of nuclear, hormone-binding proteins. *Mol. Cell. Biol.* **7**, 3538-3547.

Hamada, T., and Matsumoto, M. (1984). Effects of nutrition and ontogeny on liver cytosolic and mitochondrial phosphoenolpyruvate carboxykinase activity of the rat, hamster, guinea-pig, pig, kid, calf and chick. *Comp. Biochem. Physiol.* **77B**, 547-550.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Tylor, T., and Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ. Toxicol. Chem.* **16**, 534-541.

Harris, H. J., Erdman, T. C., Ankley, G. T., and Lodge, K. B. (1993). Measures of reproductive success and polychlorinated biphenyl residues in eggs and chicks of Forster's terns on Green Bay, Lake Michigan, Wisconsin - 1988. *Arch. Environ. Contam. Toxicol.* **25**, 304-314.

Hart, C. A. (2000) Feminization in common terns (*Sterna hirundo*): Relationship fo persistent organic contaminants. Ph.D. thesis. Massachusetts Institute of Technology, Woods Hole Oceanographic Institution.

Harvey, S., Merry, B. J., and Phillips, J. G. (1980). Influence of stress on the secretion of corticosterone in the duck (*Anas platyrhyncos*). *J. Endocrinol.* **87**, 161-171.

Hazelwood, R. L. (1986). Carbohydrate Metabolism. In Avian Physiology (P. D. Sturkie, Ed.), pp. 303-325. Springer-Verlag, New York.

Hebert, C. E., Norstrom, R. J., and Weseloh, D. V. C. (1999). A quarter century of environmental surveillance: The Canadian Wildlife Service's Great Lakes herring gull monitoring program. *Environ. Rev.* **7**, 147-166.

Ho, S.-M. (1991). Vitellogenesis. In Vertebrate Endocrinology. Fundamentals and Biomedical Implications pp. 91-126. Academic Press, New York.

Hodges, R. D. (1981). Endocrine glands. In Form and Function in Birds (A. S. King, and J. McLelland, Eds.), Academic Press, London.

Holmes, W. N., Cronshaw, J., and Gorsline, J. (1978). Some effects of ingested petroleum on seawater-adapted ducks (*Anas platyrhyncos*). *Environ. Res.* **17**, 177-190.

Holmes, W. N., Gorsline, J., and Cronshaw, J. (1979). Effects of mild cold stress on the survival of seawater-adapted mallard ducks (*Anas platyrhyncos*) maintained on food contaminated with petroleum. *Environ. Res.* **20**, 425-444.

Hontela, A., Rasmussen, J. B., Audet, C., and Chevalier, G. (1992). Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs and mercury. *Environ. Contam. Toxicol.* **22**, 278-283.

Hontela, A., Dumont, P., Duclos, D., and Fortrin, R. (1995). Endocrine and metabolic dysfunction in yellow perch, *Perca flavescens*, exposed to organic contaminants and heavy metals in the St. Lawrence river. *Environ. Toxicol. Chem.* **14**, 725-731.

Hontela, A., Daniel, C., and Rasmussen, J. B. (1997). Structural and functional impairment of the hypothalamo-pituitary-interrenal axis in fish exposed to bleached kraft mill effluent in the St. Maurice River, Quebec. *Ecotoxicol.* **6**, 1-12.

Hontela, A. (1997). Endocrine and physiological responses of fish to xenobiotics: Role of glucocorticosteroid hormones. *Rev. Toxicol.* **1**, 1-46.

Hylka, V. W., and Doneen, B. A. (1983). Ontogeny of embryonic chicken lung: Effects of pituitary gland, corticosterone and other hormones upon pulmonary growth and synthesis of surfactant phospholipids. *Gen. Comp. Endocrinol.* **52**, 108-120.

Jacobs, J. (1996). Regulation of Life History Strategies Within Individuals in Predictable and Unpredictable Environments. Ph.D. Thesis. University of Washington.

Jansen, H. T., Cooke, P. S., Porcelli, J., Liu, T.-C., and Hansen, L. G. (1993). Estrogenic and antiestrogenic actions of PCBs in the female rat: *In vitro* and *in vivo* studies. *Reprod. Toxicol.* **7**, 237-248.

Janssen, P. A. H., Faber, J. H., and Bosveld, A. T. C. (1998). (Fe)male? How contaminants can disrupt the balance of sex hormones and affect reproduction in terrestrial wildlife species. 13, 1-150. 1998. Wageningen, DLO Institute for Forestry and Nature Research (IBN-DLO). IBN Scientific Contributions.

Janz, D. M., and Bellward, G. D. (1996). *In ovo* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure in three avian species. 2. Effects on estrogen receptor and plasma sex steroid hormones during the perinatal period. *Toxicol. Appl. Pharmacol.* **139**, 292-300.

Jeffery, R. W. (2001). Ethoxyresorufin-O-deethylase (EROD) inducing potency of polycyclic aromatic hydrocarbons (PAHs) in avian hepatocyte cultures: Investigations of the ability of an *in vitro* bioassay to predict the toxic potency of PAHs. M.Sc. thesis, University of Ottawa.

Jobling, S., and Sumpter, J. P. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat. Toxicol.* **27**, 361-372.

Jönsson, C.-J., Lund, B.-O., Brunstrom, B., and Brandt, I. (1994). Toxicity and irreversible binding of two DDT metabolites - 3-methylsulfone-DDE and *o,p'*-DDD - in adrenal interrenal cells in birds. *Environ. Toxicol. Chem.* **13**, 1303-1310.

Jost, J.-P., Ohno, T., Panyim, S., and Schuerch, A. R. (1978). Appearance of vitellogenin mRNA sequences and rate of vitellogenin synthesis in chicken liver following primary and secondary stimulation of 17β -estradiol. *Eur. J. Biochem.* **84**, 355-361.

Jost, J.-P., Saluz, H.-P., McEwan, I., Feavers, I. M., Hughes, M., Reiber, S., Liang, H. M., and Vaccaro, M. (1990). Tissue specific expression of avian vitellogenin gene is correlated with DNA hypomethylation and *in vivo* specific protein-DNA interaction. *Phil. Trans. Roy. Soc. London* **326**, 231-240.

Jost, J.-P., Saluz, H.-P., and Pawlak, A. (1991). Estradiol down regulates the binding activity of an avian vitellogenin gene repressor (MDBP-2) and triggers a gradual demethylation of the mCpG pair of its DNA binding site. *Nucleic Acids Res.* **19**, 5771-5775.

Juurlink, B. H., and Gibson, M. A. (2000). Histogenesis of the yolk sac in the chick. *Can. J. Zool.* **51**, 509-519.

Kalliecharan, R., and Hall, B. K. (1974). A developmental study of the levels of progesterone, corticosterone, cortisol and cortisone circulating in plasma of chick embryos. *Gen. Comp. Endocrinol.* **24**, 364-372.

Kalliecharan, R., and Hall, B. K. (1976). A developmental study of progesterone, corticosterone, cortisol, and cortisone in the adrenal glands of the embryonic chick. *Gen. Comp. Endocrinol.* **30**, 404-409.

Karnofsky, D. A., Ridgway, L. P., and Patterson, P. A. (1951). Growth-inhibiting effect of cortisone acetate on the chick embryo. *Endocrinology* **48**, 596-616.

Kennedy, S.W., Lorenzen, A., James, C.A. and Collins, B.T. (1993). Ethoxyresorufin-O-deethylase and porphyrin accumulation in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. *Anal. Biochem.* **211**, 102-112.

Kennedy, S. W., Lorenzen, A., Jones, S. P., Hahn, M. E., and Stegeman, J. J. (1996a). Cytochrome P4501A induction in avian hepatocyte cultures: A promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **141**, 214-230.

Kennedy, S. W., Lorenzen, A., and Norstrom, R. J. (1996b). Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. *Environ. Sci. Technol.* **30**, 706-715.

Kennedy, S. W., Fox, G. A., Trudeau, S., Bastien, L. J., and Jones, S. P. (1998). Highly carboxylated porphyrin concentration: a biochemical marker of PCB exposure in herring gulls. *Mar. Environ. Res.* **46**, 65-69.

Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993). Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279-2286.

Krishnan, V., and Safe, S. (1993). Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: quantitative structure-activity relationships. *Toxicol. Appl. Pharmacol.* **120**, 55-61.

Kupfer, D., and Bulger, W. H. (1987). Metabolic activation of pesticides with proestrogenic activity. *Fed. Proc.* **46**, 1869.

Langslow, D. R. (1978). Gluconeogenesis in birds. *Biochem. Soc. Trans.* **6**, 1148-1152.

Larsson, D. G. J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P.-E., and Forlin, L. (1999). Ethinyloestradiol - an undesired fish contraceptive? *Aquat. Toxicol.* **45**, 91-97.

Lazier, C. B. (1978). Ontogeny of the vitellogenic response to oestradiol and of the soluble nuclear oestrogen receptor in embryonic-chick liver. *Biochem. J.* **174**, 143-152.

Lorenzen, A., Moon, T. W., Kennedy, S. W., and Fox, G. A. (1999). Relationships between environmental organochlorine contaminant residues, plasma corticosterone concentrations, and intermediary metabolic enzyme activities in Great Lakes herring gull embryos. *Environ. Health Perspect.* **107**, 179-186.

Ludwig, J. P., Auman, H. J., Kurita, H., Ludwig, M. E., Campbell, L. M., Giesy, J. P., Tillitt, D. E., Jones, P., Yamashita, N., Tanabe, S., and Tatsukawa, R. (1993). Caspian tern reproduction in the Saginaw Bay ecosystem following a 100-year flood event. *J. Great Lakes Res.* **19**, 96-108.

Lumeij, J. T., Boschma, Y., Mol, J., de Kloet, E. R., and Van den Brom, W. E. (1987). Action of $ACTH^{1-24}$ upon plasma corticosterone concentrations in racing pigeons (*Columbia livia domestica*). *Avian Pathol.* **16**, 199-204.

Lutz-Ostertag, Y., and David, D. (1973). Embryologie Expérimentale. Action du DDT sur le tractus urogénital de l'embryon de poulet et de caille. *C. R. Acad. Sc. Paris* **276**, 1213-1216.

Mac, M. J., and Edsall, C. C. (1991). Environmental contaminants and the reproductive success of lake trout in the Great Lakes: an epidemiological approach. *J. Toxicol. Environ. Health* **33**, 375-394.

Malbon, C. C., Rapiejko, P. J., and Watkins, D. C. (1988). Permissive hormone regulation of hormone-sensitive effector systems. *Trends Pharmacol. Sci.* **9**, 33-36.

Martin, B., Gasc, J. M., and Thibier, M. (1977). C_{21}-steroid binding proteins and progesterone levels in chicken plasma during ontogenesis. *J. Steroid Biochem.* **8**, 161-166.

Matthews, J., Celius, T., Halgren, R., and Zacharewski, T. (2000). Differential estrogen receptor binding of estrogenic substances: a species comparison. *J. Steroid Biochem.* **74**, 223-234.

McCaffrey, J., and Hamilton, J. W. (1994). Developmental regulation of basal and hormone-inducible phosphoenolpyruvate carboxykinase gene expression in chick embryo liver *in vivo*. *Arch. Biochem. Biophys.* **309**, 10-17.

McEwan, I. J., Saluz, H.-P., and Jost, J.-P. (1991). *In vivo* and *in vitro* protein-DNA interactions at the distal oestrogen response element of the chicken vitellogenin gene: Evidence for the same protein binding to this sequence in hen and rooster liver. *J. Steroid Biochem. Molec. Biol.* **38**, 275-283.

McMahon, M., Gerich, J., and Rizza, R. (1988). Effects of glucocorticoids on carbohydrate metabolism. *Diabetes Metab. Rev.* **4**, 17-30.

McMaster, M. E., Port, C. B., Munkittrick, K. R., and Dixon, D. G. (1992). Milt characteristics, reproductive performance, and larval survival and development of white sucker exposed to bleached kraft effluent. *Ecotoxicol. Environ. Safety* **23**, 103-117.

McMaster, M. E., Van Der Kraak, G. J., and Munkittrick, K. R. (1996). An epidemiological evaluation of the biochemical basis for steroid hormonal depressions in fish exposed to industrial wastes. *J. Great Lakes Res.* **22**, 153-171.

McWhinnie, D. J., and Thommes, R. C. (1973). The influence of hypophysectomy on bone growth and alkaline phosphatase activity in the chick embryo. *J. Embryo. Exp. Morphol.* **29**, 515-527.

Mineau, P., Fox, G. A., Norstrom, R. J., Weseloh, D. V., Hallett, D. J., and Ellenton, J. A. (1984). Using the herring gull to monitor levels and effects of organochlorine contamination in the Canadian Great Lakes. In Toxic Contaminants in the Great Lakes (J. O. Nriagu, and M. S. Simmons, Eds.), pp. 426-452. John Wiley and Sons., Inc., Toronto.

Moccia, R. D., Fox, G. A., and Britton, A. (1986). A quantitative assessment of thyroid histopathology of herring gulls (*Larus argentatus*) from the Great Lakes and a hypothesis on the causal role of environmental contaminants. *J. Wildlife Dis.* **22**, 60-70.

Monteverdi, G. H., and Di Giulio, R. T. (1999). An enzyme-linked immunosorbent assay for estrogenicity using primary hepatocyte cultures from the channel catfish (*Ictalurus punctatus*). *Arch. Environ. Contam. Toxicol.* **37**, 62-69.

Moog, F., and Richardson, D. (1955). The functional differentiation of the small intestine. *J. Exp. Zool.* **130**, 29-55.

Moog, F. (1961). The functional differentiation of the small intestine IX. The influence of thyroid function on cellular differentiation and accumulation of alkaline phosphatase in the duodenum of the chick embryo. *Gen. Comp. Endocrinol.* **1**, 416-432.

Morris, S. M., Winberry, L. K., Fisch, J. E., Back, D. W., and Goodridge, A. G. (1984). Developmental and nutritional regulation of the messenger RNAs for fatty acid synthase, malic enzyme and albumin in the livers of embryonic and newly hatched chicks. *Mol. Cell Biochem.* **64**, 63-68.

Murk, A. J., Boudewijn, T. J., Meininger, P. L., Bosveld, A. T. C., Rossaert, G., Ysebaert, T., Meire, P., and Dirksen, S. (1996). Effects of polyhalogenated aromatic hydrocarbons and related contaminants on contaminants on common tern reproduction: Integration of biological, biochemical, and chemical data. *Arch. Environ. Contam. Toxicol.* **31**, 128-140.

Myers, M. R. and Klasing, K. C. (1995). Metabolic basis for glucose intolerance in raptors (abstract). *Journal of Raptor Research* **29**, 63-63.

Nagra, C. L., and Meyer, R. K. (1963). Influence of corticosterone on the metabolism of palmitate and glucose in cockerels. *Gen. Comp. Endocrinol.* **3**, 131-138.

Nardelli, D., van het Schip, F. D., Gerber-Huber, S., Haefliger, J.-A., Gruber, M., Ab, G., and Wahli, W. (1987). Comparison of the organization and fine structure of a chicken and a *Xenopus laevis* vitellogenin gene. *J. Biol. Chem.* **262**, 15377-15385.

Nelson, P., Yarnell, G., and Wagle, S. R. (1966). Biochemical studies of the developing embryo. II. Studies on CO_2 fixation enzymes. *Arch. Biochem. Biophys.* **114**, 543-546.

Nicolas, J.-M. (1999). Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. *Aquat. Toxicol.* **45**, 77-90.

Nisbet, I. C. T., Fry, D. M., Hatch, J. J., and Lynn, B. (1996). Feminization of male common tern embryos is not correlated with exposure to specific PCB congeners. *Bull. Environ. Contam. Toxicol.* **57**, 895-901.

Norstrom, R. J., Hallett, D. J., and Sonstegard, R. A. (1978). Coho Salmon (*Oncorhynchus kisutch*) and herring gulls (*Larus argentatus*) as indicators of organochlorine contamination in Lake Ontario. *J. Fish. Res. Board Can.* **35**, 1401-1409.

Norstrom, R. J., and Won, H. T. (1985). Long-term preservation of egg and tissue homogenates for determination of organochlorine compounds: Freezing versus freeze-drying. *J. Assoc. Off. Anal. Chem.* **68**, 129-135.

Norstrom, R. J., and Simon, M. (1991). Determination of specific polychlorinated dibenzo-*p*-dioxins and dibenzofurans in biological matrices by gel-permeation-carbon chromatography and gas chromatography-mass spectrometry. In Environmental Carcinogens Methods of Analysis and Exposure Measurement (C. Rappe, H. R. Buser, B. Dodet, and I. K. O'Neill, Eds.), pp. 281-297. IARC, Lyon.

Ochs, R. S., and Harris, R. A. (1980). Aminooxyacetate inhibits gluconeogenesis by isolated chicken hepatocytes. *Biochim. Biophys. Acta* **632**, 260-269

Palmer, B. D., and Palmer, S. K. (1995). Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. *Environ. Health Perspect.* **103**, 19-25.

Peakall, D. B., Miller, D. S., and Kinter, W. B. (1979). Physiological techniques for assessing the impact of oil on seabirds. *Am. Soc. Testing Materials* **693**, 52-60.

Peakall, D. B., Tremblay, J., Kinter, W. B., and Miller, D. S. (1981). Endocrine dysfunction in seabirds caused by ingested oil. *Environ. Res.* **24**, 6-14.

Peakall, D. B., Norstrom, R. J., Rahimtula, A. D., and Butler, R. D. (1986). Characterization of mixed-function oxidase systems of the nestling herring gull and its implications for bioeffects monitoring. *Environ. Toxicol. Chem.* **5**, 379-385.

Pedernera, E. A. (1971). Development of the secretory capacity of the chick embryo adrenal glands. *J. Embryo. Exp. Morphol.* **25**, 213-222.

Pelissero, C., Flouriot, G., Foucher, J. L., Bennetau, B., Dunogues, J., Le Gac, F., and Sumpter, J. P. (1993). Vitellogenin synthesis in cultured hepatocytes; an *in vitro* test for the estrogenic potency of chemicals. *J. Steroid Biochem.* **44**, 263-272.

Petrescu, I., Bojan, O., Saied, M., Barzu, O., Schmidt, F., and Kuhnle, H. F. (1979). Determination of phosphoenolpyruvate carboxykinase activity with deoxyguanosine 5'-diphosphate as nucleotide substrate. *Anal. Biochem.* **96**, 279-281.

Pilkis, S. J., and El-Maghrabi, M. R. (1988). Hormonal regulation of hepatic gluconeogenesis and glycolysis. *Annu. Rev. Biochem.* **57**, 755-783.

Poland, A., and Knutson, J. C. (1982). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**, 517-554.

Ratcliffe, D. A. (1967). Decrease in eggshell weight in certain birds of prey. *Science* **215**, 208-210.

Ratnasabapathy, R., Tom, M., and Post, C. (1997). Modulation of the hepatic expression of the estrogen-regulated mRNA stabilizing factor by estrogenic and antiestrogenic xenobiotics. *Biochem. Pharmacol.* **53**, 1424-1434.

Rattner, B. A., Eroschenko, V. P., Fox, G. A., Fry, D. M., and Gorsline, J. (1984). Avian endocrine responses to environmental pollutants. *J. Exp. Zool.* **232**, 683-689.

Redshaw, M. R., and Follett, B. K. (1976). Physiology of egg yolk production by the fowl: The measurement of circulating levels of vitellogenin employing a specific radioimmunoassay. *Comp. Biochem. Physiol.* **55A**, 399-405.

Ribelin, W. E. (1984). The effects of drugs and chemicals upon the structure of the adrenal gland. *Fund. Appl. Toxicol.* **4**, 105-119.

Romanoff, A. L. (1960). *The Avian Embryo*. The Macmillan Company, New York.

Rombauts, L., Vanmontfort, D., Berghman, L. R., Decuypere, E., and Verhoeven, G. (1994). Contribution of the fetal adrenal to circulating immunoactive inhibin in the chicken embryo. *Biol. Reprod.* **51**, 926-933.

Ryckman, D. P., Weseloh, D. V., and Bishop, C. A. Contaminants in herring gull eggs from the Great Lakes: 25 years of monitoring levels and effects. 1-12. 1997. Environment Canada Great Lakes Fact Sheet.

Saadoun, A., and LeClercq, B. (1983). Comparison of the *in vivo* fatty acid synthesis of the genetically lean and fat chickens. *Comp. Biochem. Physiol.* **75B**, 641-644.

Safe, S., Astroff, B., Harris, M., Zacharewski, T., Dickerson, R., Romkes, M., and Biegel, L. (1991). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related

compounds as antioestrogens: characterization and mechanism of action. *Pharmacol. Toxicol.* **69**, 400-409.

Sanderson, J. T., Norstrom, R. J., Elliott, J. E., Hart, L. E., Cheng, K. M., and Bellward, G. D. (1994). Biological effects of polychlorinated dibenzo-p-dioxins,dibenzofurans, and biphenyls in double-crested cormorant chicks (*Phalacrocorax auritus*). *J. Toxicol. Environ. Health* **41**, 247-265.

Sarkar, N. K. (1974). Alanine and aspartate aminotransferase activities in serum and various subcellular fractions from the livers of different species. *Int. J. Biochem.* **5**, 375-381.

Savon, S., Hakimi, P., and Hanson, R. W. (1993). Expression of the genes for the mitochondrial and cytosolic forms of phosphoenolpyruvate carboxykinase in avian liver during development. *Biol. Neonate* **64**, 62-68.

Schlenk, D., Stresser, D. M., McCants, J. C., Nimrod, A. C., and Benson, W. H. (1997). Influences of β-napthoflavone and methoxychlor pretreatment on the biotransformation and estrogenic activity of methoxychlor in channel catfish (*Ictalurus punctatus*). *Toxicol. Appl. Pharmacol.* **145**, 349-356.

Schneider, J., Sassa, S., and Kappas, A. (1983). Metabolism of estradiol in liver cell culture. *J. Clin. Invest.* **72**, 1420-1426.

Scott, T. R., Johnson, W. A., Satterlee, D. G., and Gildersleeve, R. P. (1981). Circulating levels of corticosterone in the serum of developing chick embryos and newly hatched chicks. *Poultry Sci.* **60**, 1314-1320.

Segal, H. L., and Matsuzawa, T. (1970). L-Alanine aminotransferase (rat liver). *Meth. Enzymol.* **17A**, 153-159.

Shapiro, D. (1982). Steroid hormone regulation of vitellogenin gene expression. *Crit. Rev. Biochem.* **12**, 187-203.

Shen, C. S., and Mistry, S. P. (1979). Development of gluconeogenic, glycolytic, and pentose-shunt enzymes in the chicken kidney. *Poultry Sci.* **58**, 663-667.

Sherry, J., Gamble, A., Fielden, M., Hodson, P., Burnison, B., and Solomon, K. (1999). An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. *Sci. Total Environ.* **225**, 13-31.

Siegel, H. S., and Gould, N. R. (1976). Chick embryonic plasma proteins and binding capacity for corticosterone. *Dev. Biol.* **50**, 510-516.

Simon, M. and Wakeford, B. J. (2000). Multiresidue method for the determination of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and non-*ortho* substituted polychlorinated biphenyls in wildlife tissue by HRGC/HRMS. 336, 1-41. Hull, Quebec, Canadian Wildlife Service. Technical Report Series.

Slater, E. P., Redeuilh, G., and Beato, M. (1991). Hormonal regulation of vitellogenin genes: An estrogen-responsive element in the *Xenopus* A2 gene and a multihormonal regulatory regions in the chicken II gene. *Mol. Endocrinol.* **5**, 386-396.

Smeets, J. M. W., Rouhani Rankouhi, T., Nichols, K. M., Komen, H., Kaminski, N. E., Giesy, J. P., and van den Berg, M. (1999a). *In vitro* vitellogenin production by carp (*Cyprinus carpio*) hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. *Toxicol. Appl. Pharmacol.* **157**, 68-76.

Smeets, J. M. W., van Holsteijn, I., Giesy, J. P., Seinem, W., and van den Berg, M. (1999b). Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. *Toxicol. Sci.* **50**, 206-213.

Smeets, J. M. W., van Holsteijn, I., Giesy, J. P., and van den Berg, M. (1999c). The anti-estrogenicity of Ah receptor agonists in carp (*Cyprinus carpio*) hepatocytes. *Toxicol. Sci.* **52**, 178-188.

Snowberger, E. A., and Stegeman, J. J. (1987). Patterns and regulation of estradiol metabolism by hepatic microsomes from two species of marine teleosts. *Gen. Comp. Endocrinol.* **66**, 256-265.

Söling, H.-D., Kleineke, J., Willms, B., Janson, G., and Kuhn, A. (1973). Relationship between intracellular distribution of phosphoenolpyruvate carboxykinase, regulation of gluconeogenesis, and energy cost of glucose formation. *Eur. J. Biochem.* **37**, 233-243.

Söling, H.-D., and Kleineke, J. (1976). Species-dependent regulation of hepatic gluconeogenesis in higher animals. In Gluconeogenesis: Its Regulation in Mammalian Species (R. W. Hanson, and M. A. Mehlman, Eds.), pp. 369-462. J. Wiley and Sons, New York.

Sonstegard, R. A., and Leatherland, J. F. (1976). The epizootiology and pathogenesis of thyroid hyperplasia in coho salmon (*Onchorhyncus kisutch*) in Lake Ontario. *Cancer Res.* **36**, 4467-4475.

Soto, A. M., Lin, T.-M., Justicia, H., Silvia, R. M., and Sonnenschein, C. (1992). An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-screen). In Chemically Induced Alterations in Sexual and Functional Development (T. Colborn, and C. Clement, Eds.), pp. 295-309. Princeton Scientific Publishing Co. Inc., Princeton.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**, 113-122.

Speake, B. K., Murray, A. M. B., and Noble, R. C. (1998). Transport and transformations of yolk lipids during development of the avian embryo. *Prog. Lipid Res.* **37**, 1-32.

Spelman, L. H., Fleming, W. J., Davis, G. S., and Stoskopf, M. K. (1995). Effect of exogenous adrenocorticotropic hormone administration on plasma corticosterone concentrations in American black ducks (*Anas rubripes*). *J. Wildlife Dis.* **31**, 136-141.

Srebocan, V., Pompe-Gotal, J., Adamovic, V., Sokic, B., and Delak, M. (1971). Effect of technical grade DDT and p,p'-DDT on adrenocortical function in chicks. *Poultry Sci.* **50**, 1271-1278.

Stamler, J., Pick, R., and Katz, L. N. (1954). Effects of cortisone, hydrocortisone and corticoptrophin on lipemia, glycemia and atherogenesis in cholesterol-fed chicks. *Circulation* **10**, 237-246.

Stanchfield, J. E., and Yager, J. D. (1980). Primary induction of vitellogenin synthesis in monolayer cultures of amphibian hepatocytes. *J. Cell Biol.* **84**, 468-475.

Stevens, L. (1996). *Avian Biochemistry and Molecular Biology*. Cambridge University Press, Cambridge.

Straznicky, K., Hajós, F., and Bohus, B. (1966). Relationship between the ultrastructure and cortical activity of the embryonic adrenal gland in the chicken. *Acta Biol. Hung.* **16**, 261-274.

Strijker, R. (1986). Transcription of genes coding for yolk proteins. 1-105. Ph.D. thesis, University of Gröningen.

Sumpter, J. P. (1985). The purification, radioimmunoassay and plasma levels of vitellogenin from the rainbow trout, *Salmo gairdneri*. In Current Trends in Comparative Endocrinology (B. Lofts, and W. N. Holmes, Eds.), pp. 355-357. Hong Kong University Press, Hong Kong.

Sumpter, J. P., and Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect. Suppl.* **103**, 173-178.

Thomas, P. (1990). Teleost model for studying the effects of chemicals on female reproductive endocrine function. *J. Exp. Zool. Suppl.* **4**, 126-128.

Tinker, D. A., Brosnan, J. T., and Herzberg, G. R. (1986). Interorgan metabolism of amino acids, glucose, lactate, glycerol and uric acid in the domestic fowl (*Gallus domesticus*). *Biochem. J.* **240**, 829-836.

Tittlemier, S. A., Simon, M., Jarman, W. M., Elliott, J. E., and Norstrom, R. J. (1999). Identification of a novel $C_{10}H_6N_2Br_4Cl_2$ heterocyclic compound in seabird eggs. A bioaccumulating marine natural product? *Environ. Sci. Technol* **33**, 26-33.

Tyler, C. R., van Aerle, R., Hutchinson, T. H., Maddix, S., and Trip, H. (1999). An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ. Toxicol. Chem.* **18**, 337-347.

van den Boogaart, P., Mulder, J., Halsema, I., Gruber, M., and Ab, G. (1981). Estradiol-induced vitellogenin synthesis in duck liver. *Biochim. Biophys. Acta* **654**, 1-10.

van het Schip, F. D., Samallo, J., Broos, J., Ophuis, J., Mojet, M., Gruber, M., and Ab, G. (1987). Nucleotide sequence of a chicken vitellogenin gene and derived amino acid sequence of the encoded yolk precursor protein. *J. Mol. Biol.* **196**, 245-260.

Watford, M., Hod, Y., Chiao, Y.-B., Utter, M. F., and Hanson, R. W. (1981). The unique role of the kidney in gluconeogenesis in the chicken. *J. Biol. Chem.* **256**, 10023-10027.

Watford, M. (1985). Gluconeogenesis in the chicken: Regulation of phosphoenolpyruvate carboxykinase gene expression. *Fed. Proc.* **44**, 2469-2474.

Watford, M. (1989). Hormonal and nutritional regulation of phosphoenolpyruvate carboxykinase mRNA levels in chicken kidney. *J. Nutr.* **119**, 319-322.

Weber, L. W. D., Lebofsky, M., Greim, H., and Rozman, K. (1991a). Key enzymes of gluconeogenesis are dose-dependently reduced in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Arch. Toxicol.* **65**, 119-123.

Weber, L. W. D., Stahl, B. U., Lebofsky, M., Alper, R. H., Kerecsen, L., and Rozman, K. (1991b). Inhibition of phosphoenolpyruvate carboxykinase activity appears to be the key biochemical lesion in the acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Chemosphere* **23**, 1957-1962.

Weldon, S. L., Rando, A., Matathias, A. S., Hod, Y., Kalonick, P. A., Savon, S., Cook, J. S., and Hanson, R. W. (1990). Mitochondrial phosphoenolpyruvate carboxykinase from the chicken. *J. Biol. Chem.* **265**, 7308-7317.

Wentworth, B. C., and Hussein, M. O. (1985). Serum corticosterone levels in embryos, newly hatched, and young turkey poults. *Poultry Sci.* **64**, 2195-2201.

White, R., Jobling, S., Hoare, S. A., Sumpter, J. P., and Parker, M. G. (1994). Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135**, 175-182.

Wingfield, J. C. (1994). Modulation of the adrenocortical response to stress in birds. In Perspectivies in Comparative Endocrinology (National Research Council of Canada, Ed.), pp. 520-528. National Research Council of Canada, Ottawa.

Wise, E. M., Jr., and Ball, E. G. (1964). Malic enzyme and lipogenesis. *Proc. Natl. Acad. Sci. USA* **52**, 1255-1263.

Wise, P. M., and Frye, B. E. (1973). Functional development of the hypothalamo-hypophyseal-adrenal cortex axis in the chick embryo, *Gallus domesticus*. *J. Exp. Zool.* **185**, 277-292.

Wishart, G. J., Leakey, J. E. A., and Dutton, G. J. (1977). Differential effects of hormones on precocious yolk sac retraction in chick embryos following administration by a new technique. *Gen. Comp. Endocrinol.* **31**, 373-380.

Won, H. T., Mulvihill, M. J., and Wakeford, B. J. (2001). Multiresidue methods for the determination of chlorinated pesticides and polychlorinated biphenyls (PCBs) in wildlife tissues by gas chromatography/mass spectrometry. 335, 1-1-2-18. Hull, Quebec, Canadian Wildlife Service. Technical Report Series.

Woods, J. E., De Vries, G. W., and Thommes, R. C. (1971). Ontogenesis of the pituitary-adrenal axis in the chick embryo. *Gen. Comp. Endocrinol.* **17**, 407-415.

Yamashita, N., Tanabe, S., Ludwig, J. P., Kurita, H., Ludwig, M. E., and Tatsukawa, R. (1993). Embryonic abnormalities and organochlorine contamination in double-crested cormorants (*Phalacrocorax auritus*) and Caspian terns (*Hydroprogne caspia*) from the upper Great Lakes in 1988. *Environ. Pollut.* **79**, 163-173.

Yarnell, G. R., Nelson, P. A., and Wagle, S. R. (1966). Biochemical studies of the developing embryo. 1. Metabolism of gluoconeogenic precursors. *Arch. Biochem. Biophys.* **114**, 539-542.

Zacharewski, T. (1997). *In vitro* bioassays for assessing estrogenic substances. *Environ. Sci. Technol.* **3**, 613-623.