IDENTIFICATION OF CALIFORNIA CONDOR (*GYMNOGYPS CALIFORNIANUS*) ESTROGEN RECEPTORS 1 AND 2 AND THEIR ACTIVATION BY SUSPECTED ENDOCRINE DISRUPTING CONTAMINANTS

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

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a candidate for the degree of Master of Science

and hereby certify that, in their opinion, it is worthy of acceptance.

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Dr. Trista Strauch

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Dr. Lori Eggert

For my sister and best friend, Leslie Gerrard

Thank you for being a constant support

and inspiration

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ABSTRACT

The recovery of the critically endangered California condor (*Gymnogyps californianus*) has required significant human intervention and management. Inland condor populations primarily face challenges associated with lead poisoning, which contributed to this species nearly becoming extinct. Coastal condors scavenge marine megafauna that offer reduced lead exposure, but increased potential for exposure to endocrine disrupting contaminants (EDCs) such as DDTs, PCBs, and chlordanes. Specifically, EDCs found at high concentrations in southern California marine megafauna also appear elevated in condors, potentially leading to impaired reproductive function, such as the observed production of thin eggshells by coastal birds compared to inland birds. To investigate the effects of EDCs on condor reproduction we have cloned condor estrogen receptors 1 and 2 (ESR1 and ESR2). With regard to ESR2, this represents the first confirmed, full-length, coding sequence of this gene identified for any bird of prey. Condor ESR activation by suspected EDCs was characterized with the highest treatment of contaminant (10^{-4} M) resulting in the greatest response for both ESR1 and ESR2. In general, condor ESR2 was more sensitive to EDCs compared to ESR1; with PCB52, PCB138, PCB153, bisphenol A, dieldrin, *trans*-nonachlor, *p,p'* -DDD, and *p,p'* –DDE all stimulating significantly higher activation of ESR2 than of ESR1. Furthermore, phylogenetic analyses of condor ESRs revealed differences in rates of ESR1 and ESR2 evolution relative to other birds. While the concentrations EDCs are decreasing along the California coast the bioassay presented could be used as tool to screen food sources at release sites to determine if disruptive levels of EDCs are present.

NOMENCLATURE

AhR	Aryl hydrocarbon receptor
β-gal	β-galalactosidase
BPA	Bisphenol A
CA	Carbonic anhydrase
CYP	Cytochrome P450
DDE	Dichlorodiphenyldichloroethylene
DDD	Dichlorodiphenyldichloroethane
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DMSO	Dimethyl sulfoxide
E_1	Estrone
E_2	17β – Estradiol
E_3	Estriol
EC_{50}	Half maximal effective concentration
EE_2	Ethinyl estradiol
EDC	Endocrine disrupting contaminant

ERE	Estrogen response element
ESR	Estrogen receptor
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FBS	Fetal bovine serum
HEK293	Human embryonic kidney cells
MEM	Minimum essential media
ND	Non detectable
ORF	Open reading frame
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PPM	Parts-per-million
PP	Posterior probabilities
PPT	Propyl-pyrazole-triol
RACE	Rapid amplification of cDNA ends
RIN	RNA integrity number
SDZG	San Diego Zoo Global

SEM	Standard error of the mean
UTR	Untranslated region

LITERATURE REVIEW

California condor historical range and reintroduction

Before the mega fauna extinctions of the late Pleistocene, the California condor (*Gymnogyps californianus*) inhabited a large area of North America, feeding on carrion of the large mammals of the era (Chamberlain et al., 2005). After the mass extinctions, restricted food sources shifted the condor's population to North America's west coast as far south as Baja California and northward to British Columbia where marine mammal carcasses were plentiful (Koford, 1953; Wilbur 1978). By the start of the 20th century, hunting, collection of specimens, secondary poisoning from vermin control, and environmental contaminants had contributed to the condor's decline (Snyder and Snyder, 2005; D'Elia and Haig, 2013). During the early 1900s California condors persisted only in southern California and Baja California and by the 1950s the further shrinking condor population was confined to an area north of Los Angeles (Koford, 1953). As a result, the California condor received protection under the U.S. Endangered Species Act in 1972 and the California Condor Recovery Team formed in 1973. As their decline continued, conservation efforts shifted from *in situ* habitat preservation to *ex situ* captive breeding programs and in 1986 the decision was made to bring all individuals into captive breeding programs at the Los Angeles Zoo and the San Diego Wild Animal Park (now San Diego Zoo Safari Park; Walters et al., 2010).

Within less than a decade, the captive population steadily grew to over a hundred birds and in 1995 the first captive-reared condor was successfully

reintroduced to southern California (Walters et al., 2010). The California condor population now exceeds 400 individuals and continues to grow with approximately 200 birds living in captivity and the other half distributed among four release sites in North America. After release in Southern California (Bitter Creek National Wildlife Refuge) reintroduction continued at the Vermillion Cliffs National Monument (1996), Sierra de San Pedro Martir, National Park Baja California, Mexico (2002) and finally in 2003 to Pinnacles National Monument and Ventana Wilderness. The latter group, collectively known as the Big Sur population, was established so that the birds could comingle and forage along the California coast (Johnson et al., 2010), whereas the other populations inhabit primarily inland terrestrial environments.

Although the captive breeding program for the California condor made significant strides in the recovery of the species, the reintroduction of this species into its historical habitat continues to provide challenges for condor managers mainly due to anthropogenic impediments. The primary threat to the present wild population is lead poisoning from fragments of lead-based ammunition remaining in discarded carcasses. Although it likely played a role in the condor's original decline, the extent to which lead toxicosis threatens condor health and survival was not fully recognized until the 1980s (Snyder and Snyder 2005). Specifically, lead poisoning was recently reported responsible for the deaths of 26% of deceased juveniles and 67% of deceased adults from the beginning of the reintroduction program in 1992 through 2009 (Rideout et al., 2012). As a result, all reintroduction sites continue to provide supplemental feedings of lead-free carcass and routinely test all individuals

for accumulation of lead in the blood (Finkelstein et al., 2012). Due to the high mortality rate from lead poisoning, efforts are being made to release animals into areas of reduced lead exposure. Options include coastal habitat such as Big Sur where the diet is mainly composed of marine mammal (Burnett et al., 2013).

California condors are obligate scavengers, feeding strictly on carrion (Meretsky and Snyder, 1992). Inland populations feed primarily on mammalian carrion from mule deer (*Odocoileus hemionus*), tule elk (*Cervus canadensis nannodes)*, pronghorn antelope (*Antilocapra americana*), feral hogs, domestic ungulates, and smaller mammals (Koford, 1953). Along the coast, however, condors have the opportunity to feed on marine mammal carcasses, which reduces the incidence of lead exposure that is associated with inland scavenging (Johnson et al., 2010). The potential for less intensive management due to reduced lead levels in food sources is appealing; however, marine mammal carrion may present other health challenges to condors. For several decades, organochlorides such as dichloro-diphenyl- trichloroethane (DDT) and its metabolites, along with polychlorinated biphenyls (PCBs), have accumulated in the complex marine food web and persisted in the long-lived, apex predators such as the California sea lion (*Zalophus californianus*) as well as whales (order *Cetacea*) and marine fish that coastal condors scavenge (Koford, 1953). These marine species typically occupy higher trophic levels than the mostly herbivorous species that inland condors feed on and therefore put the coastal birds at greater risk for the bioaccumulation of environmental contaminants.

Endocrine disruption and estrogen receptor activation

Since World War II the widespread use of chemicals for industry, agriculture, and medicine has increased environmental pollution and vertebrate exposure to harmful endocrine-disrupting chemicals (EDCs; Colborn et al., 1993). EDCs such as pesticides, herbicides, and endocrine-active phytochemicals mimic endogenous hormones altering reproductive, thyroidal, metabolic, and immunological functions in vertebrate wildlife (Sonneschein and Soto, 1998). The focus of this work and most of the EDC research in wildlife is on organochlorides; mainly DDTs and PCBs, which persist in the environment for extended periods of time and become incorporated into the food chain (Colborn, 1993; Guillette, 2000). EDCs elicit their effects by binding and consequently activating or inhibiting hormone receptors. Receptors for the steroid hormone estrogen (i.e. estrogen steroid receptor: ESR) are particularity "promiscuous"; binding endogenous estrogens as well as structurally similarly EDCs (McLachlan et al., 2001; Iguchi and Katsu, 2008). The two nuclear membrane ESRs (1 and 2) are members of the nuclear receptor superfamily (Nelson and Habibi, 2013) and act as ligand activated transcription factors. Their differential expression in tissues suggests that ESR1 and ESR2 mediate different responses. Therefore, to fully understand the potential effects of EDCs on organisms, investigation of both ESRs is warranted (Hannafy, 2005; Zaho, 2008).

Given its importance in development and reproductive functions of vertebrates, EDCs that interfere with estrogen signaling can have significant negative affects on the reproductive capacity of exposed animals (Guillette, 2000). A prime example is exposure of juvenile alligators residing in Florida lakes to

pesticides that interact with ESRs resulting in decreased phallus size and altered sex hormone levels (Guillette, 1999; Vonier, 1996). During the 1970s, California gull (*Larus californicus*) populations on Santa Barbara Island had skewed sex ratios, later explained by exposure to the estrogen agonist DDT, which caused developmental feminization of male embryos (Fry and Toone, 1981).

Therefore studies of endocrine disruption *in vitro* can be used as an accurate proxy for specific chemical sensitivity and subsequent reproductive harm in the species of interest (Kohno et al., 2008). ESR activation by EDCs has been studied *in vitro* in members of all vertebrate classes and produces species-specific physiological responses and consequences due to exposure events (Kuiper et al., 1998; Katsu et al., 2006; Milnes and Guillette, 2008; Iguchi and Katsu, 2008; Katsu et al., 2008; Katsu et al., 2010). *In vitro*-based approaches provide unique opportunities for molecular based research in threatened and endangered species for which biological samples are limited and *in vivo* EDC experiments are not possible (Kohno et al., 2008; Tubbs et al., 2014). For example, *in vitro* studies have been used to establish a relationship between dietary phytoestrogen levels, as measured by ESR activation, and low fertility in captive southern white rhinoceros (Tubbs et al., 2011). In avian species, ESR activation studies are surprisingly limited. Activation of South African White-backed vulture (*Gyps africanus*) ESR1 by *p,p'*-DDT, *o,p'*-DDT and *p,p'*-DDE, found circulating concentrations of EDCs were much lower than those needed to stimulate estrogenic responses *in vitro* and therefore could not be linked to the poor reproductive success of the species during the 20th century (Naidoo et al., 2008). Differences in fold activation of ESR1 by *p,p'*-DDE and

p,p' -DDT were demonstrated between a bird of prey, the vulture, and a domestic species, the chicken (Naidoo et al., 2008). Importantly in some species, ESR2 is known to show higher sensitivity to estrogenic ligands *in vitro* compared to ESR1, so a screening of only ESR1 would potentially result in falsely evaluating a chemical as not estrogenic (Hannafy 2005; Zaho et al., 2008; Katsu et al., 2010).

Endocrine disruption in avian species: eggshell thinning

Eggshell thinning in response to exposure to EDCs has been recorded in wild birds for almost a century now, though its exact mechanism is not clear. During the mid 20th century unregulated pesticide use in the United States and the reports of birds unable to fully incubate eggs due to thin eggshells encompassed North America from the Great Lakes to the coast of California (Cooke, 1973). Bald Eagles (*Haliaeetus leucocephalus*) present a classic example of EDC exposure and eggshell thinning in avian wildlife. Populations of bald eagles around the Great Lakes, feeding on anadromous fishes with high concentrations of *p,p'* -DDE, total PCBs, and 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD), had low rates of reproduction and increased incidence of eggshell thinning (Bowerman et al., 1995). On the west coast, bald eagles reintroduced to Santa Catalina Island averaged lethal levels of *p,p'* -DDE, two decades after the ban of DDT (Wiemeyer, 1993; Garcelon, 1997). A similar story occurs along California's northern Channel Islands. During the 1950s the Peregrine falcon disappeared and post reintroduction continued to have elevated levels of *p,p'* -DDE and eggshell thinning into the 1980s (Hunt, 1994).

Several experiments have been conducted on captive birds fed diets supplemented with organochlorides and have revealed eggshell thinning with the greatest response elicited by metabolites of DDT (Cooke, 1973). Experimental feeding trials using Japanese quail (*Coturnix japonica*) fed low calcium diets supplemented with *p,p'* -DDT and *o,p'* -DDT, layed eggs with thinner eggshells and a decreased percentage of shell calcium compared to controls (Bitman et al.,1969). The studies were repeated with quail fed adequate calcium diets supplemented with *p,p'* -DDT and *o,p'* -DDE. In the original study, quail fed low calcium diets experienced over 50% egg breakage, while quails fed an adequate calcium diet with added DDT experienced a lower 21% egg breakage (Cecil et al., 1971). American kestrels (*Falco sparverius*) suffering from *p,p'* -DDE exposure and eggshell thinning in the wild experienced similar eggshell thickness problems as did experimental birds fed a diet supplemented with *p,p'* -DDE (Lincer, 1975). Interestingly, domestic hens (*Gallus gallus*) fed *p,p'* -DDE experienced no eggshell thinning and seemed to be insensitive to the contaminant, supporting the hypothesis of interspecies specific responses (Cooke, 1973). Cooke placed avian species into three categories based on sensitivity to *p,p'* -DDE induced eggshell thinning: (1) birds insensitive to contaminants e.g., the domestic fowl and the Bengales finch (*Lonchura striata*); (2) birds experiencing intermediate thinning (5-15%) e.g., the American kestrel and the Japanese quail; and (3) birds highly sensitive to thinning (≥30%) e.g., the peregrine falcon (*Falco peregrinus*), the brown pelican (*Pelecanus occidentalis*), and some ducks (*Anas spp.*) (Cooke, 1973). Therefore birds of prey such as the California condor that not only feed on fish but on higher trophic levels including marine

mammals are likely more susceptible to *p,p'* -DDE induced eggshell thinning than other avian species.

Another critical factor to consider when evaluating the potential effects of EDCs on birds is timing of exposure. Activational responses to EDC exposure occur after puberty or sexual maturation, typically producing transient symptoms, while organizational responses are a result of exposure during critical developmental periods leading to permanent damage (Phoenix, 1959). Laboratory studies have therefore been divided: (1) EDC delivered by egg injection to explore embryonic effects vs. (2) EDC delivered in the diet to the laying female. In ducks, exposure to the primary metabolite of DDT, *p,p'* -DDE inhibits prostaglandin E_2 synthesis in the shell gland mucosa, thus decreasing calcium and bicarbonate transport across the mucosa (Lundholm, 1997). This mechanism is currently the strongest activational hypothesis for eggshell thinning particularly in light of the findings of identical studies in the domestic fowl that are insensitive to *p,p'* -DDE and showed none of the detrimental effects (Lundholm, 1997). Here the role of ESR is unclear, although there is a possibility that *p,p'* -DDE binds ESR producing an estrogenic response that inhibits prostaglandin synthesis. Regardless, a clear receptor mediated pathway has not been established.

Original studies of embryonic exposure of domestic hen to a synthetic estrogen, ethinyl estradiol (EE_2), produced a decrease in carbonic anhydrase (CA) activity in the shell gland capillaries. The subsequent response was thinner shells due to a lack of calcium and carbonate ions available for shell formation (Berg et al., 2004). CA is a key enzyme in eggshell formation and is directly linked to EDC

exposure and ESR regulation of eggshell thinning (Berg et al., 2004). Domestic chicken hens exposed to *o,p'* -DDT during development also experienced a decrease in CA activity in capillaries and thinning eggshells (Holm et al., 2006), suggesting that this functional and structural (organizational) change in the shell gland during embryonic exposure in the lab may be reflected in wild birds where one embryonic exposure could affect the eggshell production in a female for life (Berg et al., 2004).

History of eggshell thinning in California condors

Eggshell thinning in birds along the California coast has been linked to the discharge of chemicals into the Palos Verdes shelf from the 1940s-1980s (EPA, 1997; Glaser and Connolly, 2002). Most notably, effluent from the largest manufacturer of DDT combined with industrial PCB waste has been deposited directly into shelf sediments (EPA, 1997). Dumping of 1,700 tons of DDTs and hundreds of tons of PCBs has left sediments and the costal food web surrounding the Palos Verdes shelf contaminated decades later (EPA, 1997).

The effect of DDT on California condors during the 1950s-1960s is not well understood due to limited data available from such a small population of animals and only a few eggs produced every year. Nonetheless, condor eggshell fragments collected between 1964 and 1969 exhibited severe thinning, structural abnormalities, and significant residues of DDE similar to that of eggshells from the American kestrel experimentally fed DDE (Kiff et al., 1979). High levels of DDE were proposed to result in eggshell thinning, egg breakage, and ultimately egg failures in the population during the 1960s (Kiff et al., 1979). In 1986 the last California condor

female to breed in the wild laid 2 eggs that were 44% thinner than the historical mean thickness and contained high levels of DDE and DDT (Kiff, 1989).

Forty years after the ban of DDT, this persistent pesticide and its metabolites may continue to pose a threat to California condors. Recent studies found eggshells collected from coastal birds from 2006-2010 were 34% thinner than the inland bird eggs, leading to a 50% decrease in successful hatch among costal birds when compared to inland birds (Burnett et al., 2013). While DDE wasn't measured in this study, increased feeding on California sea lions, which the highest concentrations of DDTs compared to other marine mammals, was noted (Kannan et al., 2004; Le Boeuf, 2002; Finkelstein unpublished obs.). The degree to which eggshell thinning occurs is directly proportional to the concentrations to which the birds were exposed (Lundholm, 1997). Along with high concentrations in eggs there is also recent documentation of significantly higher levels of EDCs in the blood plasma of coastal birds of the Big Sur population than is found in inland birds of the Bitter Creek population (Finkelstein unpublished obs.).

Coastal reintroduction sites provide plentiful food sources for condors with low risk of lead exposure. In order to achieve a sustainable population, birds must be able to scavenge for food and reproduce without human intervention; and to ensure reproduction is not compromised, carrion must be sampled for EDCs and tested in receptor activation assays. Release sites will likely contain their own array of chemicals consisting of DDTs and PCBs, with some proving more detrimental to health than others. Laboratory results will be used to establish relationships

between environmental contaminants and maximize the reproductive success of released populations of the endangered California condor.

MATERIALS AND METHODS

Chemical reagents

17β-estradiol (E_2), 17α-ethynlestradiol (EE_2), diethylstilbesterol (DES), estrone (E_1), and estriol (E_3) were obtained from Steraloids (Newport, RI). Bisphenol A (BPA), dieldrin, *o,p'* -DDT, *p,p'* -DDT, *o,p'* -DDE, *p,p'* -DDE, *o,p'* -DDD, *p,p'* -DDD, PCB52, PCB138, PCB153, and *trans*-nonachlor were obtained from Sigma-Aldrich Corp. (St. Louis, MO). All chemicals were dissolved in dimethyl sulfoxide (DMSO) prior to use.

Tissue collection

All procedures in this study were approved by San Diego Zoo Global's (SDZG's) Institutional Animal Care and Use Committee. California condor tissues (ovary, n=1 and testis, n=1) were collected at necropsy by SDZG's pathology staff and placed immediately in RNAlater (Ambion, Austin, TX). Total RNA was extracted by tissue homogenization (PowerGen125, Fisher Scientific, Pittsburgh, PA), followed by a phenol:choloroform precipitation. All samples were stored at -80°C until assessed for RNA quality using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA). Samples yielding the highest RNA

Integrity Number (RIN; ovary 5.4, testis 6.3) were used for receptor cloning procedures described below.

	Forward primer	Reverse primer
condor ESR1	5'-TACCGAGCTC**GGATCC**ACCATGACCCTTCACACCAAAAC-3'	5'-ATATCTGCA**GAATTC**GGTAGTACACTGCTGGGTTTC-3'
	BAMHI	ECORI
condor ESR2	5'-CTT**GGTACC**AAAATGTCTCTGTGTGCATCTTC-3'	5'-ATATCTGCA**GAATTC**TTACTGGGCTGTCCCGTTTCG-3'
	KPNI	ECORI

Table 1. Plasmid specific primers for generation of full length condor ESR genes. Plasmid and vector specific restriction sites were used for subcloning condor ESR1 and ESR2 into expression vector pcDNA 3.1(+). Restriction site indicated in bold and condor specific start and stop sites are underlined.

Cloning of estrogen receptors

Approximately one microgram of total RNA was reverse transcribed using the SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA). Nested PCR was performed using primers designed against highly conserved regions of avian estrogen receptors and SMART RACE adaptor oligo specific primers to identify 5' UTRs and start sites. Partial condor ESR1 3' UTRs and the stop site was identified using primers directed against a region of condor ESR1 3' UTR available on the UCSC Genome Browser (Romanov 2008). Full-length PCR using Platinum PCR Supermix Hi Fidelity (Life Technologies, Carlsbad, CA) was performed with primers designed from nested PCR identified start and stop sites to capture entire open reading frame. Next, condor ESRs were subcloned into a pCR2.1 TA cloning vector (Life Technologies), transformed into TOP10 competent *Escherichia coli* (Life Technologies), positive colonies were selected, and purified using a Rneasy QIAquick Miniprep (Qiagen, Valencia, CA). Five plasmids, per gene, were selected to identify a consensus sequence from two individual condors (Studbook; 298, 238). Plasmids were sequenced by a commercial vendor (Eton Bioscience, San Diego, California) and edited using 4Peaks software (http://nucleobytes.com/index.php/4peaks).

Full-length ESR coding sequences, identified above, were then subcloned into a pcDNA3.1 (+) expression vector (Life Technologies) using a ligase dependent method. To ensure cleavage of insert, PCR was performed on consensus sequence plasmids using primers designed with appropriate restriction enzymes: ESR1, BAMHI and ECORI, and ESR2, KPNI and ECORI (See Table 2). Plasmid (pcDNA 3.1

(+)) and insert containing ESR entire open reading frame were digested with designated restriction enzymes in 10X Buffer K (Life Technologies) and ligated into expression vector using T4 DNA Ligase (Life Technologies).

Phylogenetic analyses

All vertebrate ESR nucleotide and amino acid sequences, except by California condor were obtained from GenBank (Benson, 2013) and Ensembl (Flicek et al. 2014). Nucleotide sequences were aligned using Clustal W2 (Labarga, 2007) and adjusted by eye following the codon reading frame. Amino acid sequences were aligned using Muscle (Edgar, 2004; (See Table 2 for GenBank accession numbers). All sequences were imported into BioEdit (http://www.mbio.ncsu.edu/ BioEdit/bioedit.html) and edited by eye.

Aligned nucleotide sequences were analyzed using ModelTest 3.7 (Posada & Crandall, 1998) to determine the model of molecular evolution of ESRs according to the Akaike Information Criterion. The model identified for phylogenetic analyses was the general time reversible (GTR) + gamma distribution (G) + proportion invariant sites (I). Probabilistic analyses were performed using PhyML 3.0 (Guindon et al., 2010) with 1000 bootstrap replicates, and MrBayes v. 3.1.1. The Bayesian inference consisted of two concurrent runs with four Markov chains (one cold and seven heated chains with a temperature of 0.2), ten million generations (sampled every 1000 generations), and a 50% burn-in. Posterior probabilities were calculated for each node.

Species name	GenBank accession number ESR1	GenBank accession number ESR2
Alligator sinensis	XM_006024833*	XM_006018673*
Alligator mississippiensis	AB115909	AB548298
Anas platyrhynchos	XM_005024788*	XM_005022664*
Anolis carolinensis	XM_003224679*	XM_003214282*
Chrysemys picta	FJ195592.1	XM_005285890
Columba livia	NM_001282825*	NM_001282841*
Coturnix japonica	AF442965	AF045149
Falco cherrug	XM_005446565*	XM_005437288*
Falco peregrinus	XM_005230194*	XM_005241812*
Ficedula albicollis	XM_005043647*	XM_005047707*
Gallus gallus	NM_205183	NM_204794
Geospiza fortis	XM_005420121*	XM_005417937*
Gymnogyps californianus	**KJ651952**	**KJ651953**
Homo sapiens	BC128573	NG_011535
Hynobius tokyoensis	AB524910	AB524911
Meleagris gallopavo	XM_003204124*	XM_003206778*
Melopsittacus undulatus	XM_005149610*	XM_005150080
Pelodiscus sinensis	XM_006114975*	XM_006134078*
Protobothrops flavoviridis	AB548294	XM_006134078
Pseudemys nelsoni	AB301060	AB548299
Pseudopodoces humilis	XM_005518407*	XM_005520059*
Rattus norvegicus	AB477039	NM_012754
Taeniopygia guttata	NM_001076701	XM_002200595*
Protopterus dolloi	AB435629	AB435630
Xenopus (silurana) tropicalis	NM_203535	NM_001040012

Table 2. GenBank accession numbers for nucleotide sequences of estrogen receptor 1 and 2 (ESR 1 and 2) used for phylogenetic analysis. (* Indicates predicted sequence).

Aligned protein sequences were analyzed using ProtTest 2.4 (Abascal et al., 2005) to determine the best-fit model of protein evolution of ESRs. The model identified for phylogenetic analyses was the Jones-Thornton-Taylor (JTT) matrix (Jones et al., 1992) + gamma distribution (G) + observed amino acid frequencies (F). Phylogenetic analyses were performed using PhyML 3.0 (Guindon et al., 2010) and MEGA 4.0 (Tamura, 2007), both with 1,000 bootstrap replicates. All trees were visualized and rooted in Tree view v1.6.6 (Page, 1996) using slender lungfish (*Protopterus*) as outgroup.

Positive selection analyses

Positive selection analyses were performed with the codeml program from PAML (Yang, 1997), using codons aligned by ClustalW2 and phylogenetic trees obtained from the Bayesian inference. The site model approach among codons was used to identify significant differences in likelihood values between nearly neutral (model 7) and positive selection models with unconstrained omega (model 8) and omega constrained to 1.0 (model 8a). The branch-site models were used to detect positive selection affecting a few sites along a particular lineage, among codons and branches on the tree, by comparing differences in likelihood values between nearly neutral (model 1) and positive selection models with unconstrained omega (model 2) and omega constrained to 1.0 (model 2a). The total number of nucleotide substitutions and species pairwise comparisons of synonymous and non-synonymous substitutions were estimated using MEGA 4.0 (Tamura, 2007).

Cell culture and receptor activation assays

Receptor activation assays were performed as previously described with minor modifications (Tubbs et al., 2011). Briefly, human embryonic kidney (HEK293) cells were maintained in minimum essential medium (MEM; Corning™cellgro™) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Cells (10 x10^4 cells) were added to each well of a 96-well plate. After 24 h each plate of cells was co-transfected using TransIT 2020 transfection reagent (Mirus Bio LLC, Madison, WI) with 3 plasmids: 5 µg pCMX-β-galactosidase (β-gal), 5 µg pGL2-3xERE luciferase reporter plasmid (Addgene plasmid 11354 (Hall J.M., 1999)), and condor 0.5 µg ESR-pcDNA3.1 (+). After 1 h, transfection media was removed and replaced with MEM supplemented with 10% charcoal-resin stripped FBS containing 10^{-12}-10^{-7} M of endogenous hormone or 10^{-9}-10^{-4} M of test compound or appropriate vehicle control (0.01% EtOH or 0.1% DMSO), as done previously (Naidoo et al., 2012; Katsu et al., 2006; Chang et al., 1999). After 48 h cells were lysed and each well assayed for luciferase and β-gal activity as described previously (Grün et al., 2002; Tubbs et al., 2011). Luciferase activity per well was standardized to β-gal activity and fold activation was determined from the normalized luciferase values relative to the vehicle control. The test compounds fold-activation was normalized to 1 nM 17β-estradiol, which was run in triplicate on every plate. Finally, sigmoidal dose-response curves for relative activation by each compound were determined using GraphPad Prism Software (Version 6, San Diego, CA) and used to calculate each compound's EC_{50} and maximal activation relative to 1nM E_2. All experiments were run independently at least three times.

PCB inhibition assays

Receptor activation assays were performed as previously described with modifications to treatment protocol to detect inhibition of E_2 activation of ESRs in the presence of PCB congeners 138 and 153. ESRs were treated with a range, 10^{-12}M to 10^{-4}M, of PCBs immediately followed by E_2 (10^{-10}M) for all cotreatment wells. E_2 treatment concentration was determined from dose response curves of E_2 in previous experiments where 10^{-10}M produced a response below that of maximum activation of receptors. Log transformed transactivation of ESRs, normalized to 10^{-10}M E_2, were fit to a one site competition, non-linear curve using Graph Pad Prism Software. All experiments were run independently three times.

RESULTS

Identification of California condor ESR

Full-length sequences of ESR1 and ESR2 were amplified from *Gymnogyps californianus* ovary and testis. Condor ESR open reading frames are represented by 1761- and 1655-bp for ESR1 and ESR2 respectively, which show high homology to other avian and vertebrate ESRs (*Gymnogyps californianus* ESR1 GenBank accession no. KJ651952, ESR2 GenBank accession no. KJ651953).

Condor ESR2 is the first confirmed full-length coding sequence cloned from a bird of prey. Using the nomenclature by Mangelsdorf (1995) the five functional domains for California condor ESRs are illustrated (Fig. 1 A/B, - F), and compared to other available avian and reptilian ESR amino acid sequences (Fig. 1). The DNA-

binding domain for ESR1 (Fig. 1C) and ESR2 (Fig. 2C) shared the highest homology with 100% identity between birds and reptiles. Ligand binding domain identity is 99-96% for ESR1 (Fig. 1E) and for ESR2 (Fig. 2E) across birds and reptiles. Between avian species the F domain presented the most divergence, 100-92% for ESR1 (Fig. 1F) and 100-62% for ESR2 (Fig. 2F), while avian transactivation (Fig. 1, 2A/B) and hinge (Fig. 1, 2D) domains also exhibit divergence; >97% for ESR1 and >90% for ESR2. -Overall percent identity for condor ESR1 and ESR2 is 46.6% with variability in the A/B (22%), D (42%), E (61%), and F (23%) domains (Fig. 3).

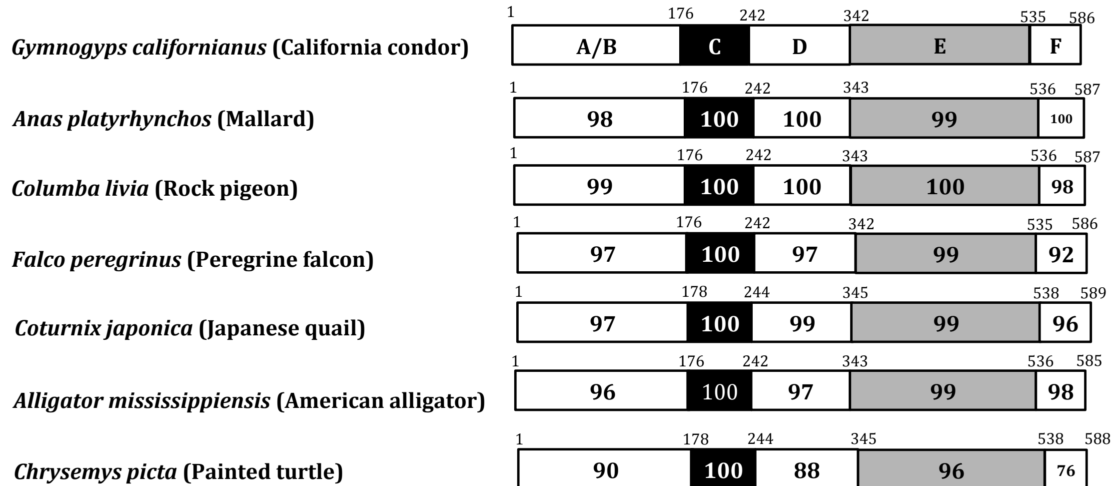

FIG. 1. Comparison of functional A/B - F domains of bird and reptile ESR1 protein sequence to California condor functional domains. Values above ESR1 diagrams represent number of amino acid per domain, and values within ESR1 diagrams represent percent similarity to condor ESR1 of the individual domain.

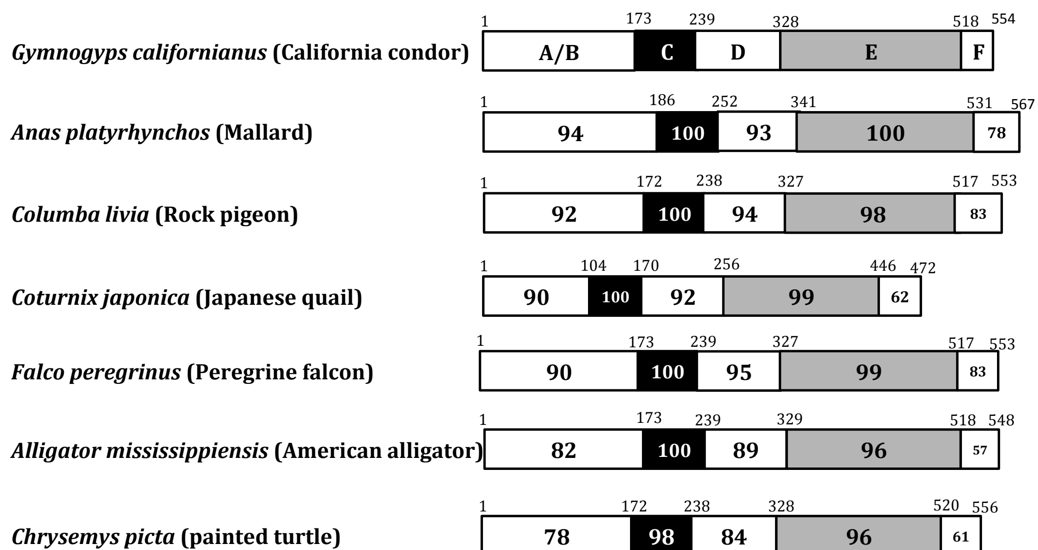

FIG. 2. Comparison of functional A/B - F domains of bird and reptile ESR2 protein sequence to California condor functional domains. Values above ESR2 diagrams represent number of amino acid per domain, and values within ESR2 diagrams represent percent similarity to condor ESR2 of the individual domain.

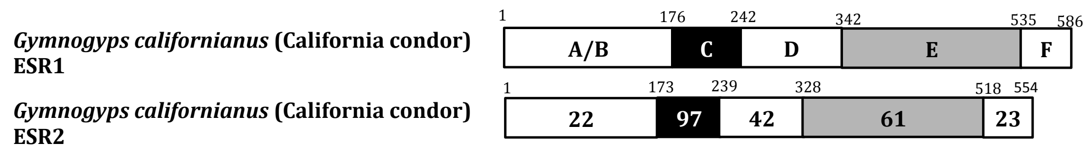

Fig. 3. Comparison of functional A/B - F domains of California condor ESR1 and ESR2. Values above ESR2 diagrams represent number of amino acid per domain, and values within ESR2 diagrams represent percent similarity to condor ESR2 of the individual domain.

Phylogenetic analyses

Phylogenetic analyses of 25 tetrapod ESRs including representatives from five vertebrate classes, shared similar numbers of synonymous and non-synonymous nucleotide substitutions in ESR1 and ESR2 genes across all included species (Fig. 4, Fig. 5). However, amongst avian ESRs, for which full length coding sequences are available, there were twice as many non-synonymous mutations (mutations producing amino acids changes) in ESR2 than in ESR1 (Fig. 4, Fig. 5). Further analysis did not detect any significant differences ($p < 0.05$) in codon or nucleotide usage to determine a positive selection site for these amino acid changes. Condor ESR1 placement on the tetrapod ESR1 nucleotide tree clusters with other birds of prey supported by 0.74 posterior probabilities and 59% bootstraps (Fig. 4). Of particular interest, placement of condor ESR2 on the same clade as the pigeon revealed a higher posterior probability, 0.93, but similar bootstrap support 58% to that of condor ESR1. Phylogenetic analyses of 25 taxa, 13 birds, predict avian ESR1 genes are more closely related to mammals and squamates than other reptiles and amphibians, while avian ESR2 genes are more similar to reptiles and amphibians genes and less closely related to mammalian genes (Fig. 4, Fig. 5).

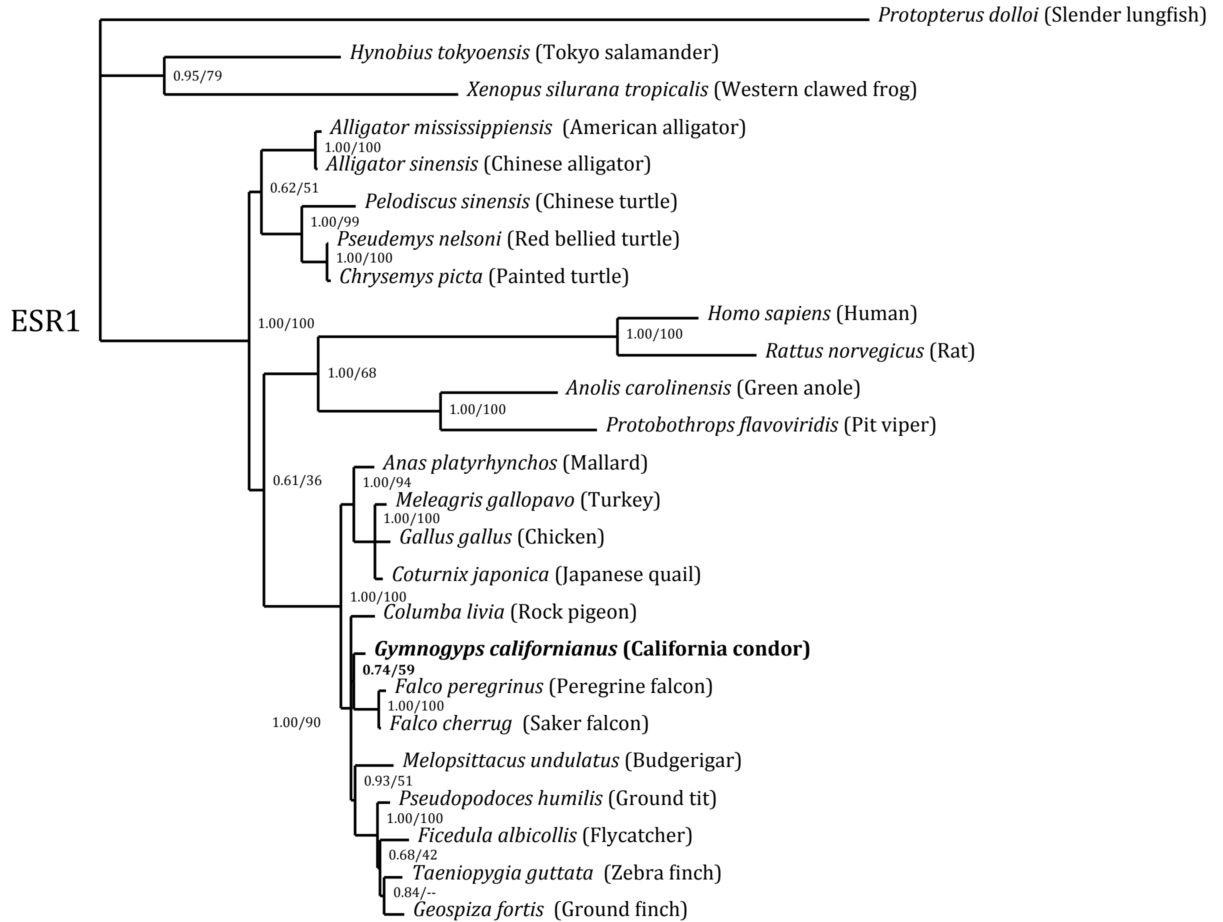

FIG.4. Bayesian tree of tetrapod ESR1phylogenetic relationships. Maximum likelihood (ML) posterior probabilities (PP) and bootstraps and are indicated for all nodes. Highest support for each approach PP = 1.00, BP_{ML} = 100%, . The scale bar represents 0.1 expected amino acid substitutions per site.

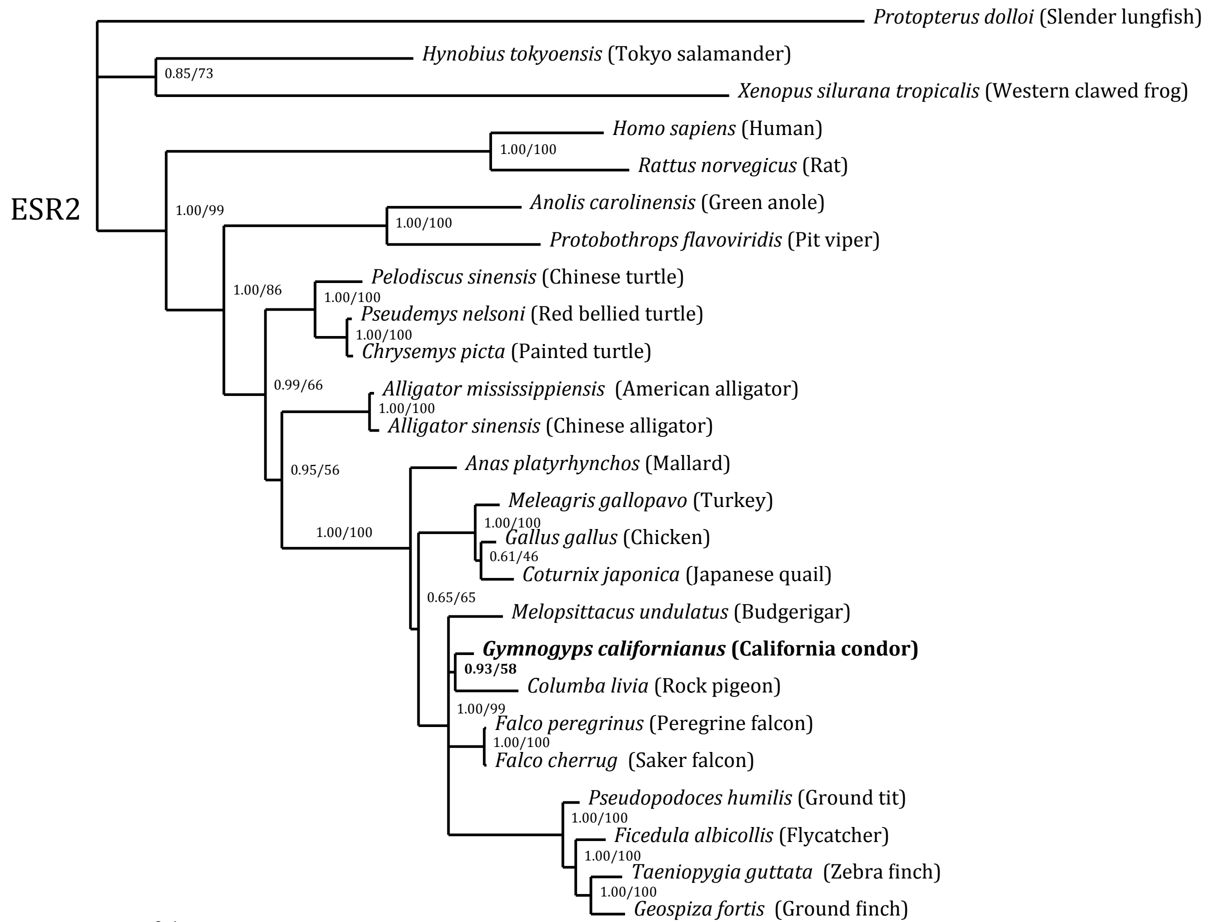

FIG. 5

FIG.5. Bayesian tree of tetrapod ESR2 phylogenetic relationships. Maximum likelihood (ML) posterior probabilities (PP) and bootstraps and are indicated for all nodes. Highest support for each approach PP = 1.00, BP_{ML} = 100%, . The scale bar represents 0.1 expected amino acid substitutions per site.

Transcriptional activity of California condor estrogen receptor

Examination of California condor ESR1 and ESR2 transcriptional activity was determined using a luciferase reporter plasmid, pGL2-3xERE, containing three estrogen response elements in tandem. For the three endogenous estrogens the concentration to reach EC_{50} were: E_2=3.45 x 10^{-11}M > E_1=8.04 x 10^{-10}M > E_3=1.6 x10^{-11}M (Fig. 6 A, B, Table 3). Condor ESR1 activity was slightly more sensitive to DES (EC_{50}=6.2x10^{-11}M) than to EE_2 (2.6x10^{-11}M), and both synthetic estrogens maximally activate ESR1 above 1nm E_2 (Table 3). While condor ESR2 was less sensitive to DES (EC_{50}=1.13 x 10^{-10}M), and EE_2 (3.16 x 10^{-10}M) than to E_2 (Table 3). The endogenous hormones, corticosterone, progesterone, and testosterone, failed to activate condor estrogen receptors (Table 3).

DDT and its metabolites, known to persist in the California coastal food web, were evaluated in relation to their effect on ESR1 and ESR2 transcriptional activity. All DDTs and metabolites evaluated in this experiment activated condor ESR1 and ESR2 at 10^{-6} M concentrations (Fig. 7 A, B). Overall, the highest treatment of EDC, 10^{-4} M, resulted in the largest ESR response (Fig. 7). The most potent DDT agonist of ESR1, determined by percent maximum activation relative to 1nM E_2 (± SEM), was *o,p'* –DDT (85.45 ± 2.7) , while, *p,p'* -DDT maximally activated ESR2 at 117.6 ± 7.9, and was the only chemical to surpass 1nM E_2 activation of ESR2. ESR2 maximum activation (% 1nM E_2 ± SEM) by *p,p'* –DDE was 80% higher and activation by *p,p'*-DDD was 40% higher than determined with ESR1 (Fig. 9). Environmental chemicals bisphenol A (BPA), dieldrin, and *trans*-nonachlor induced a significantly ($p<0.05$) higher activation at 10^{-4}M of ESR2 (BPA = 91.67 ± 2.4, dieldrin = 35.25 ± 1.5, *trans*-

nonacholor = 84.92 ± 3.3) than ESR1 (BPA = 28.09 ± 1.6, dieldrin = 19.56 ±0.9, *trans*-nonacholor = 66.89 ± 9.0) (Fig.8, 9). The activation of condor ESR2 was significantly ($p<0.05$) higher, than the activation of condor ESR1 at 10^{-4}M for PCB52, 138, and 153 (Fig. 8, 9). The activation of condor ESR2 was significantly ($p<0.05$) higher, than the activation of condor ESR1 at 10^{-4}M for PCB52, 138, and 153 (Fig. 7,8). Cotreatment of ESRs with PCB138 and PCB153 resulted in decreased E_2 induced transcriptional activity of ESRs at the highest concentration (10^{-4}M) tested (Fig. 10). ESR2 also experienced greater inhibition of E_2 activity at 10^{-5}M by both PCB congeners when compared to ESR1.

	EC_{50}(M)	Maximum (% 1nM E_2)	EC_{50}(M)	Maximum (% 1nM E_2)
Hormone				
E_1	8.04×10^{-10}	89.22 ± 2.9	1.18×10^{-9}	102.4 ± 2.2
E_2	3.45×10^{-11}	102.2 ± 1.6	7.78×10^{-11}	106.1 ± 1.7
E_3	1.60×10^{-9}	110.2 ± 4.4	2.52×10^{-10}	104.7 ± 5.2
Corticosterone	ND	ND	3.13×10^{-5}	ND
Progesterone	ND	ND	3.69×10^{-3}	ND
Testosterone	ND	ND	ND	ND
DES	6.2×10^{-11}	136.8 ± 3.6	1.18×10^{-10}	108.5 ± 2.8
EE_2	2.60×10^{-11}	122.6 ± 2.6	3.16×10^{-10}	97.3 ± 3.1

Table 3. EC_{50} (M) concentration, and maximal activation of endogenous hormones and synthetic estrogens relative to 1nM E_2 treatment (% +/- SEM) for California condor ESR1 and ESR2.

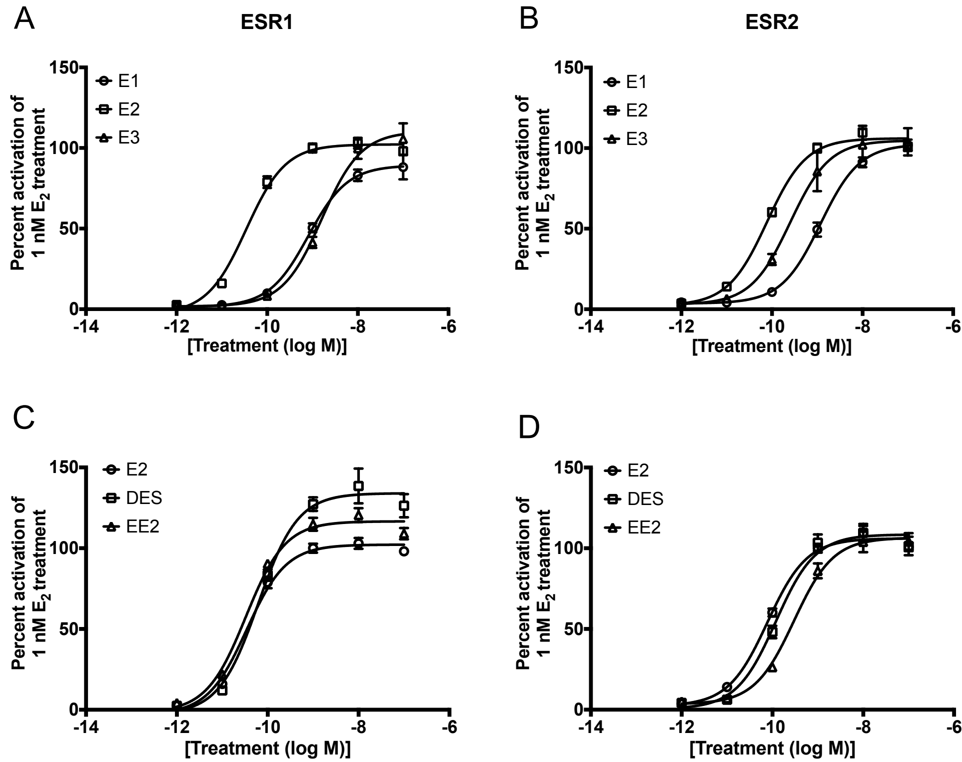

FIG. 6. Transcriptional activity of recombinant California condor ESRs in response to endogenous and synthetic estrogens. HEK293 cells were transiently cotransfected with condor ESR, luciferase reporter plasmid (pGL2-3XERE), and pCMX-β-galactosidase (β-gal) reporter plasmid. Cells were then treated in triplicate with increasing concentrations (10^{-12} to 10^{-6}M) of E_1, E_2, E_3, DES, EE_2 or vehicle (0.01% DMSO or EtOH). Fold receptor activation was calculated from luciferase activity of treatments relative to vehicle only treatment and normalized to β-gal activity. Results are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of 1nM E_2 treatment; n = 3.

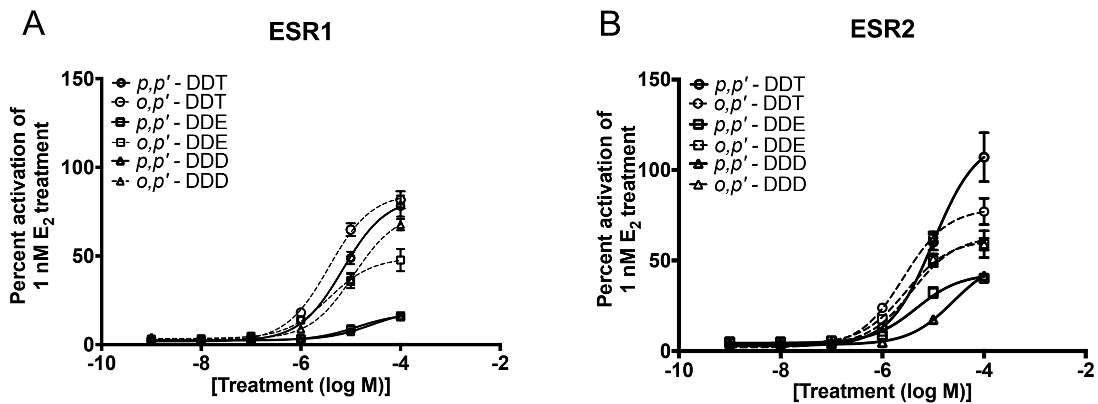

FIG. 7. Transcriptional activity of recombinant California condor ESR1 (A) or ESR2 (B) in response to DDT and DDE isoforms. HEK293 cells were transiently cotransfected with condor ESR, luciferase reporter plasmid (pGL2-3XERE), and pCMX-β-galactosidase (β-gal) reporter plasmid. Cells were then treated in triplicate with increasing concentrations (10^{-9} to 10^{-4}M) of, *p,p'* – DDT, *o,p'* – DDT, *p,p'* – DDE, *o,p'* – DDE, *p,p'* – DDD, *o,p'* - DDD, or vehicle (0.1% DMSO). Fold receptor activation was calculated from luciferase activity of treatments relative to vehicle only treatment and normalized to β-gal activity. Results are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of 1nM E_2 treatment; n = 3.

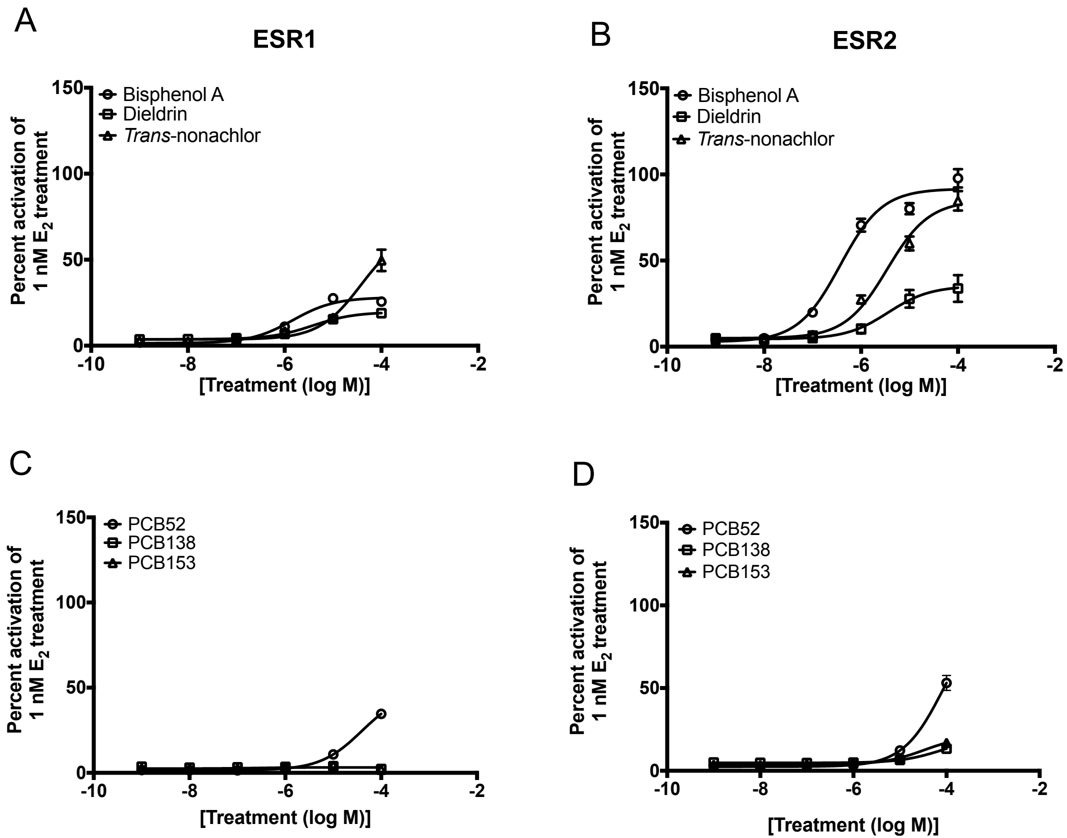

FIG. 8. Transcriptional activity of recombinant California condor ESRs in response to environmental contaminants. HEK293 cells were transiently cotransfected with condor ESR, luciferase reporter plasmid (pGL2-3XERE), and pCMX-β-galactosidase (β-gal) reporter plasmid. Cells were then treated in triplicate with increasing concentrations (10^{-9} to 10^{-4}M) of bisphenol A (BPA), dieldrin, PCB52, PCB138, PCB153, *trans*-nonachlor, or vehicle (0.1% DMSO). Fold receptor activation was calculated from luciferase activity of treatments relative to vehicle only treatment and normalized to β-gal activity. Results are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of 1nM E_2 treatment; n = 3.

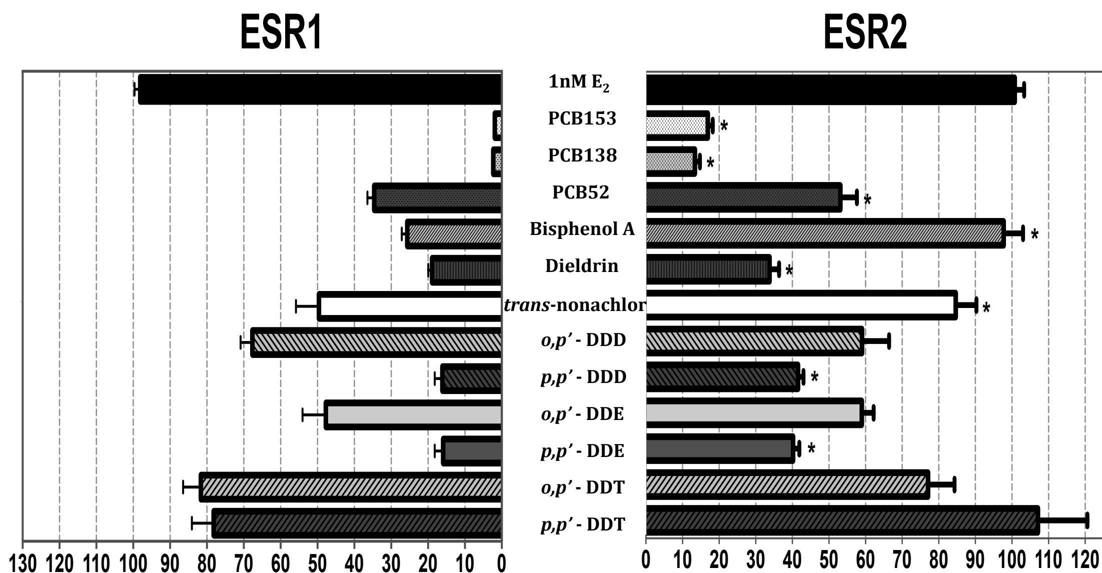

FIG. 9. Maximal activation of California condor ESR1 compared to ESR2 in response to environmental chemicals (10^{-4}M) relative to 1nM E_2 treatment (% +/- SEM). Significant differences between mean ESR activation was determined using a student's t-test ($p < 0.05$). * indicates ESR2 activation significantly greater than ESR1 activation by environmental chemical.

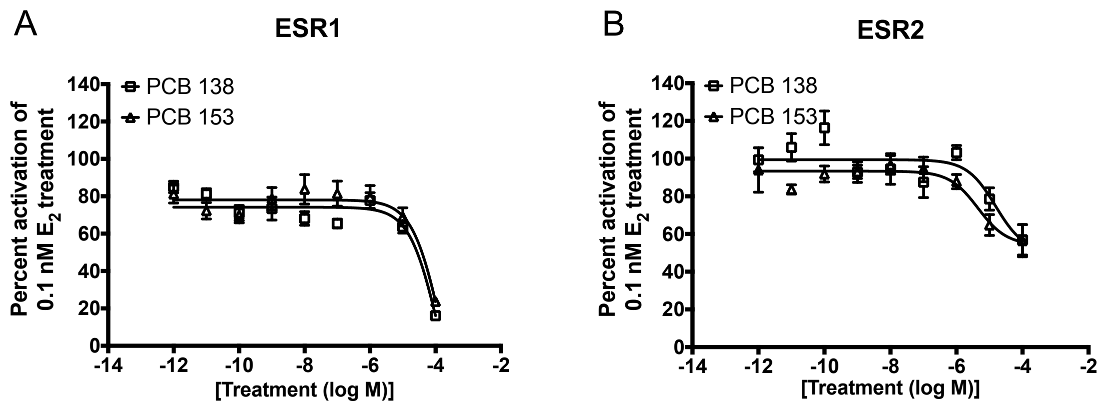

FIG. 10. Transcriptional activity of recombinant California condor ESR1 (A) ESR2 (B) in response to cotreatment with PCBs and E_2. HEK293 cells were transiently cotransfected with condor ESR, luciferase reporter plasmid (pGL2-3XERE), and pCMX-β-galactosidase (β-gal) reporter plasmid. Cells were then treated in triplicate with increasing concentrations (10^{-12} to 10^{-4}M) of PCB138 and PCB153 followed by E_2 (10^{-10}M) to determine inhibition. Fold receptor activation was calculated from luciferase activity of treatments relative to vehicle only treatment and normalized to β-gal activity. Results are presented as mean ± SEM of the fold activation of each treatment divided by the fold activation of 0.1nM E_2 treatment; n = 3.

DISUCUSSION

Currently the success of reintroduced condors weighs heavily on reducing exposure of birds to lethal levels of lead present in the food they scavenge. In addition to tracking of released birds at presently established reintroduction sites managers also provide supplemental feedings of lead-free carcass and routinely test all individuals for lead toxicosis. In 1997 two breeding pairs of condors were released in Big Sur, California to establish a coastal population with access to adequate food in the form of marine mammals carcasses that presumably contain less lead than terrestrial food sources (Johnson et al., 2010). The discovery of increased eggshell thinning of the coastal condor population (Burnett et al., 2013) and high concentrations of a mixture of contaminants in their plasma (Finkelstein, unpublished obs.) is evidence for endocrine disruption in this population. The present study investigated activation of condor ESR1 and ESR2 by endocrine disrupting contaminants (EDCs) known to persist in marine mammals along the California coast (Kannan et al., 2004) and found at elevated levels in circulation in coastal condors. Estrogenic EDCs present in coastal condors could act as agonists to condor ESRs and produce adverse effects such as the severe eggshell thinning discovered by Burnett et al. (2013) in the Big Sur population, the only reintroduced population feeding on marine mammals.

In general, activation by EDCs was significantly greater for condor ESR2 than ESR1 (see Figure 8). This pattern differs from that observed in humans (Kuiper et al., 1998) and some reptile species(Katsu in preparation), in which ESR1 activation by EDCs is greater than activation of ESR2 by the same contaminants. The pattern

presented in this manuscript is similar to fish (Katsu et al., 2008) and amphibian (Katsu et al., 2011) ESRs activated by EDCs. Katsu et al. (2011) suggested that non-mammalian vertebrate species have ESR2s that are more sensitive to estrogens than ESR1, which corroborates findings in condors. Whether this pattern is consistent among all avian species would be purely speculative since no previous reports of avian ESR2 activation by EDCs exists.

In vitro avian ESR comparisons are limited, despite the historical susceptibility of birds of prey to reproductive impairment following exposure to EDCs. This report constitutes the first cloning and characterization of ESR2 from a member of the group: birds of prey. Condor ESR1 sensitivity to DDT and its metabolites at high concentrations (10^{-5}-10^{-4}M) responds with the same pattern of activation as the African white-backed vulture (WB-vulture) and chicken ESR1s in the only other avian ESR activation study, where *o,p'* –DDT > *p,p'* -DDT > *p,p'* –DDE (Naidoo et al., 2008). In WB-vulture, circulating levels of DDEs (7.67-20.12 μg/l; Wyk et al., 2001), were lower than the concentrations needed to produce a response from vulture ESR1, and in the vulture study ESR2 was not tested (Naidoo et al., 2008). Condor ESR2 shows a different response than ESR1 with the highest activation by *p,p'* –DDT followed by, *o,p'* –DDT, and lastly *p,p'* –DDE activation. On average *p,p'* -DDE is present in circulation at levels (Finkelstein unpublished obs.) that coincide with those that result in in coastal condors 20% and 40% maximum activation by E_2 *in vitro*. Levels of DDEs found in WB-vulture during the 20th century (Wyk et al., 2001) are lower than levels found in coastal condor plasma in the 21st century (Finkelstein unpublished obs). Unlike vulture ESRs, the condor ESRs were

significantly activated by levels present *in vivo*. Whether the circulating concentration in the plasma of costal California condors is enough to produce an adverse reproductive response is not clear, however other species along the coast allow for speculation. The persistence of DDT and its metabolites in the food web of the Southern California Bight is well documented (Glaser and Connolly 2002). Thirty years after the ban of DDT, concentrations of *p,p'* –DDE were found to be the dominant metabolite in the surface sediments of the Southern California Bight (Connolly and Glaser, 2002). Sediment persistence resonated in high concentrations of *p,p'* –DDE documented in thin eggs of peregrine falcon (20μg/g; Hunt, 1994), bald eagle (36μg/g; Wiemeyer et al., 1993), and double crested cormorants (8μg/g; Gress, 1994), all feeding in the same coastal food web as the California condor. Contaminant levels present in coastal condor plasma 40 years after the ban of DDT are higher than those found in eggs of birds of prey mentioned above (Finkelstein unpublished obs.). Levels of DDE measured in 5 failed eggs of the coastal condor population ranged from 170 to 500 parts per million-lipid weight similar to levels associated with reproductive decline in bald eagle (Wiemeyer et al 1984, 1993). Measurements of contaminant levels in condor eggshells are in preparation (D. Crane, K. Regalado, L. Burnett, and R. Risebrough, unpublished data) and will provide a link between EDC concentrations in the eggshell, in the plasma of laying females, and subsequent eggshell thinning. Studies investigating maternal deposition of EDCs are limited, leaving potential for species differences in maternal transfer that are not yet described. Tissue concentrations of EDCs in herring gulls, peregrine falcons, and Adelie penguins (*Pygoscelis adeliae*) were found to be directly

proportional to the normalized egg-to-whole body chemical ratios for EDCs (Russell et al., 1999). Circulating concentrations in the blood are representative of those in the tissue (Norstrom et al., 2007) so it is likely that EDCs present in laying female condors are directly proportional to those present in eggs.

Although DDTs are the leading contaminants present in circulation (Finkelstein unpublished obs.) there are several other estrogenic contaminants present in the diets of coastal condors. From 1999-2010 the coastal condors marine mammal carcass intake consisted of 84% California sea lions (Burnett et al., 2012), contaminated with a wide variety of EDCs including dieldrin and *trans*-nonachlor (Kannan et al., 2004). Both compounds activate ESRs, inducing greater activation in ESR2 at high concentrations in the present study, which is the first to examine transcriptional activation in avian species. Importantly, *trans*-nonachlor not only represents the highest percentage of chlordane forms in California sea lions (Kannan et al., 2003) it was also found at the highest concentration of chlordanes in coastal condor plasma (Myra Finkelstein unpublished obs). Captive-reared released condors often mistake microtrash for shells or bone fragments, and offer them as sources of calcium for juveniles (Mee et al., 2007) providing another avenue for endocrine disruption. From 2001 to 2005 plastic microtrash constituted 34.8% of the "junk" found in 18 southern California nests (Mee et al., 2007). This aberrant behavior of ingesting microtrash, often including plastics, and subsequently bisphenol A (BPA), which was found to be the most potent agonist of condor ESR2 at 10^{-6}M, of all suspect EDCs tested. Other EDCs such as dieldrin, *trans*-nonachlor, and

BPA, are often overshadowed by DDTs, but they are present at levels in the coastal environment that could also lead to adverse reproductive outcomes in the condor.

Understanding the effects of EDCs in avian species is particularly difficult due to several variables: opportunity for multiple exposures along migration routes, diverse life strategies, and a range of life spans as well as estrogen dependent sexual differentiation and an array of potential mechanisms of eggshell thinning. It is also challenging to extrapolate from experimental outcomes in lab species like the quail and chicken (precocial species), to the lifetime EDC exposure of a wild, bird of prey, like the condor (altricial species). Phylogenetic evaluation of avian estrogen receptors may assist in clarifying species-specific differences in ER mediated effects of EDCs. Previous phylogenetic studies place condor with other scavengers in the Family *Cathartidae*, or New World vultures, and the order *Accipitriformes*, which includes most diurnal birds of prey (Genome,K. Community of Scientists, 2009). Recently genetic studies suggest convergent evolution of the two orders encompassing the birds of prey: *Accipitriformes* and *Falconiformes.* Clustering of condor ESRs with other birds of prey would be expected as represented in the ESR1 tree, however, analyses suggest condor ESR2, evolutionarily, is more similar to pigeon (*Columba livia*) than other birds of prey. Nevertheless a robust analysis including additional avian ESRs, specifically from the orders *Accipitriformes* and *Falconiformes,* is needed to further elucidate the phylogenetic and functional similarities of these two genes. ESRs are highly conserved across vertebrates so any significant changes to structure of this tightly regulated system would be important to the species involved, and their response to EDCs (Thornton, 2001). Moreover, all

of the approximately 400 California condors were founded from 16 individuals from three genetically distinct populations (Romanov et al., 2009). California condors' low genetic diversity could increase their susceptibility to the adverse effects of EDCs (Brown et al., 2009) and enforces the need for more conservation genomics studies, to molecularly define the relationships between species difference and physiological function.

In the wild, condors are not exposed to single chemicals (EDCs) as reported in this manuscript, but instead are exposed to multiple chemicals in addition to endogenous hormones. This paradigm often leads to responses that differ from treatment with a single chemical alone. For example, interactions of multiple chemicals on alligator (Vonier et al., 1996) and human ER (Arnold et al., 1996) have produced synergistic effects much greater than that of single compound treatment. As a result, in vitro approaches employing mixtures of multiple chemicals combined with endogenous hormones would best replicate physiological conditions of wild animals (Crews and Willingham, 2000). Future studies should focus direct examination of ESR activation by serum or plasma from costal vs. inland birds thus isolating physiologically relevant mixtures of EDCs. The intensive monitoring of California condors will allow for blood sampling before and after reintroduction, and more controlled studies of EDC accumulation in the blood and potential estrogenic effects.

There is evidence that birds have a reduced EDC metabolism leading to the higher bioaccumulation and added sensitivity to EDCs. Deficiency in expression of the family of detoxifying enzymes, the cytochrome P450s (CYP), are suspected to

play a role in decreased metabolism of EDCs of avian species (Jönsson et al., 2011). Avian species lack sex hormone-binding proteins in the blood (Wingfield et al., 1983), which in other species bind EDCs and sequester their effects leaving higher concentrations of unbound endogenous hormone (Emeville et al., 2013; Ikonomopoulou et al., 2009). Increased sensitivity of birds to EDCs could be due to the lack of sex hormone binding proteins and higher concentrations of estrogenic chemicals in circulation. In contrast to exposure during mammalian development, in avian embryos environmental contaminants are maternally transferred to the yolk and then stay in circulation throughout development, with no mode of excretion (Carere and Balthazart, 2007). Birds of prey, especially those feeding at high trophic levels, such as coastal condors scavenging carcasses of predatory marine mammal are more susceptible to bioaccumulation of persistent environmental chemicals (Naso et al., 2003). CYP genes are biomarkers for the cytoplasmic transcription factor, aryl hydrocarbon receptor (AhR), which mediates toxic effects of EDCs (Yasui et al., 2007). Therefore, future studies of the condor AhR mediated pathway to investigate which EDCs induce toxicity in coastal condors could be accomplished by cloning and characterizing condor AhR. Reduced metabolism of EDCs may explain why avian species have greater sensitivity to EDC exposure as well as provide another mode for species differences in contaminant effects.

The timing of EDC exposure can be divided into two types of responses: transient symptoms in response to exposure after puberty or sexual maturation (activational effects) and developmental changes that are permanent in response to embryonic exposure (organizational effects; Phoenix et al., 1959). The

current coastal population consists of fifty-eight free-flying, captive reared condors and their wild born offspring. The current accounts of eggshell thinning therefore (Burnett et al., 2013), likely occur through incidences of activational endocrine disruption since during development in the captive environment chances of exposure to EDCs was low. The role of ESR in acute eggshell thinning is yet to be resolved. The current theory is that *p,*p'-DDE specifically inhibits prostaglandin synthesis, thus disrupting formation of the eggshell gland (Lundholm, 1997) . Organizational effects of estrogenic exposure have been documented across several avian species, including wild (bald eagle, gull) and captive birds (quail and chicken) (Bowerman et al., 1991; Fry et al., 1981; Berg et al., 2004; Holm et al., 2006). Addressing the potential for organizational disruption in coastal condor chicks is critical before the condor population is faced with life long reproductive alterations whether or not eggshell thinning is an outcome. In the gull, estrogen exposure of male embryos in ovo causes feminization of the embryo and the formation of ovotestes, while exposure to female embryos results in malformed right oviduct (Fry 1981). ESR1 stimulation by ESR specific agonist propyl-pyrazole-triol (PPT) in the gonads of quails and chickens impaired sexual differentiation, while functions of ESR2 have been linked to sexual behavior (Mattsson et al., 2008, 2011). In ovo exposure to 37 and 75 μg of *o,p'* -DDE resulted in significantly thinner eggshells, 5 and 10% respectively, in the domestic hen, therefore establishing the association between developmental exposure, eggshell gland abnormalities, and adult production of thin eggshells (Berg et al., 2006). Even though environmental levels of EDCs are decreasing, the potential exists for adverse effects of these chemicals to

persist in the population of these long-lived birds due to developmental exposure when environmental levels remain elevated.

CONCLUSION

When planning the reintroduction of a threatened/endangered species the main objective is to ensure the original threat to the population is removed, thus providing a sustainable habitat for recovery. In the case of the California condor, an original cause of extinction in the wild was not identified; instead there were several threats anthropogenic in nature. The goal is to move condors away from lead and intensive management practices, but not into another health compromising situation as presented by coastal scavenging. The bioassay presented in this work could be used to screen EDCs existing in food sources at potential release sites and test their ability to activate/inhibit condor ESRs and possibly reproduction. Equipping managers with strong tools to make confident management decisions without testing a critically endangered mammal as the levels of chemicals continue to decrease along the California coast.

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VITA

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