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Topic 4.7

Contaminant-induced endocrine and reproductive alterations in reptiles*

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Abstract: Many chemicals introduced into the environment by humans adversely affect embryonic development and the functioning of the vertebrate reproductive system. It has been hypothesized that many developmental alterations are due to the endocrine-disruptive effects of various environmental contaminants. The endocrine system exhibits an organizational effect on the developing embryo, altering gene expression and dosing. Thus, a disruption of the normal hormonal signals can permanently modify the organization and future functioning of the reproductive and endocrine system. We have worked extensively with contaminant-exposed and reference populations of the American alligator (*Alligator mississippiensis*) as well as performed a number of experimental studies exposing developing embryos to various persistent and nonpersistent pesticides. Using this species, we have described altered steroidogenesis, circulating hormone levels, and hepatic transformation of androgen and endocrine organ (gonad, thyroid) morphology in juvenile alligators living in polluted environments. Given the adverse observations reported to date, we recommend several important future needs:

1. Further development of "receptor zoos" and other molecular tools that include key reptiles from various major ecosystems, in addition to freshwater ecosystems.
2. Global studies extending the current knowledge base on crocodilians and freshwater turtles to comparable ecosystems on other continents, such as linked studies examining and extending current molecular to population-level studies in Florida (USA) to tropical and temperate regions of Africa, Australia, and South America.
3. Further studies of actual exposure, assimilation and excretion of contaminants by ectothermic vertebrates, especially reptiles that occupy high levels of the food chain.

INTRODUCTION

A central focus of comparative physiology and endocrinology has been the influence of environmental factors on the development and performance of various systems or whole organisms. Over the last century, it has been clearly established that such factors as temperature, pH, salinity, photoperiod, and gas tensions affect the endocrinology of vertebrates. As part of these studies, we have become aware of the influence of human activities on the biological performance of numerous species. The endocrine-dis-

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ruptive actions of various chemical contaminants have been a recent focus of much research [1–4]. This concern has become worldwide and goes beyond a focus on pesticides and industrial chemicals only. A number of pesticides, plasticizers or industrial chemicals and some naturally occurring plant or fungal compounds, have been shown to mimic naturally occurring steroids, act as hormone receptor antagonists or alter the enzymes responsible for hormone synthesis and degradation (Fig. 1). For example, hormones or chemicals with endocrine-like activity in food products and drinking water have become scientific and public concerns. Initial research from wildlife populations exposed to sewage or pesticides demonstrated endocrine disruptive activity from various classes of contaminants [2]. Recent studies have extended this concern to additional chemicals released from human activities such as antibiotics and a large number of pharmaceutical agents as well as chemicals listed as "inert" compounds in chemical formulations [5].

Contaminants can impact organisms in two fundamentally different ways, via organization or activation. First, embryonic exposure can cause "organizational" abnormalities by altering the chemical signals required for normal development and thus permanently changing the endocrine function and or response of an organ or organism [6]. Second, they can alter cellular signaling in mature systems, that is, disrupt "activational" signaling that would lead to altered organ system or organism performance. We will briefly overview our studies demonstrating both these effects in reptiles exposed chronically or acutely to various environmental contaminants or mixtures of contaminants.

Reptiles have received relatively little attention with regard to the effects of environmental contaminant exposure until recently [7], although they are valuable indicators of an ecosystem's condition and are exposed to environmental contaminants like other species [8]. Reptiles in general do not appear

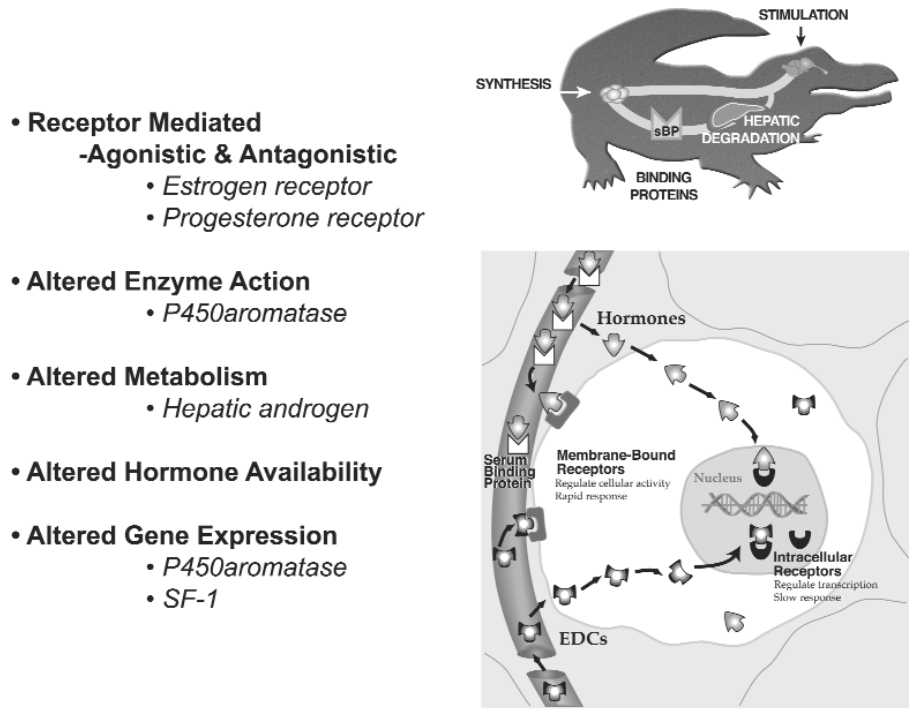

Fig. 1 Endocrine disruption in reptiles can occur at several levels of biological organization. To date, work has demonstrated that reptiles exposed to contaminants have the potential to alter the endocrine system by receptor-mediated agonistic or antagonistic actions. They can also alter enzyme action, such as alteration in P450aromatase activity or alterations in hepatic biotransformation of androgen. Recent studies have begun to demonstrate that alterations in gene expression also occur. See text for further explanations and references.

to be more or less affected by contaminants relative to other vertebrates, but given that the vast majority are predators or insectivores, they may be exposed to elevated levels of pesticides and other chemicals that bioaccumulate in food chains. Additionally, as with many other nonmammalian vertebrates, estrogens play an important and active role in the embryonic development of the ovary [9]; thus, reptiles could be susceptible to altered or abnormal gonadal development due to endocrine active contaminants that mimic or alter the estrogenic milieu of the developing embryo.

The American alligator is again common to many inland waters of the southeastern United States, following decades on the endangered species list. Recent studies from our laboratories have focused on this species because it is long-lived, a top-predator, easily obtained in large numbers (100s of neonates and juveniles can be obtained and sampled in a night), and individuals are large enough to provide adequate serum/urine samples for analysis of contaminants, hormones or other biomarkers. Further, an extensive history on population demography is available for this species in many lakes in Florida, USA. Further, in collaboration with the Florida Fish and Wildlife Conservation Commission, we have collected data on populations—recruitment and egg viability for the last 20 years—that continue to this day. Sex determination—temperature-induced sex—in alligators is different compared to that observed in mammals, but this mechanism of sex determination provides a very powerful tool for studying the role of contaminants capable of altering gender-specific steroid synthesis in wild animal populations. Additional studies have been performed on turtles, as they are an established model system for the study of environmental/temperature-induced sex determination [10].

STUDIES IN WILD POPULATIONS

We have examined seasonal and spatial (among lake) variation in plasma steroids, and have observed that sub-adult, juvenile animals (5–8 years of age) of both sexes exhibit seasonal (spring–fall) adult-like patterns of plasma sex steroids, even though they are not elevated to the same degree as in sexually mature individuals [11,12]. Sexual maturity occurs at approximately 12 years of age when they are almost 2 m in total length. Interestingly, this pattern suggests a multiyear period of puberty in alligators as is observed in humans. We have also studied pesticide interactions with the endocrine and reproductive systems in this species, allowing us to begin to understand the possible interactions between pesticide exposure and endocrine disruption.

Over the last decade, a common observation in laboratory and wildlife studies is an alteration in androgen synthesis and function in males [13,14]. For example, fish downstream from sewage treatment plants display elevated yolk protein—an estrogenic response—as well as exhibiting reduced androgen plasma concentrations, and increased estrogen concentrations [15–17]. Alligators living in several Florida lakes exhibit alterations of the reproductive and endocrine systems [18]. Many of these modifications are detectable at hatching, and persist throughout juvenile and sub-adult life. Examinations of the reproductive and endocrine systems of juvenile male alligators from Lake Apopka, have demonstrated elevations in plasma estradiol, reductions in androgen (T and DHT) and altered thyroxin concentrations (Fig. 2) as well as morphological abnormalities of the testis [11,19]. The alterations in plasma hormone concentrations persist to date, and also occur in females [18]. Altered endocrine parameters in juvenile alligators also occur in other Florida wetlands not associated with significant pesticide spills but that have a history of non-point source contamination, such as Lake Okeechobee and Lake Griffin, FL, USA [11,20,21].

Various factors have been shown to alter plasma androgen concentrations in alligators, the most common being seasonal variations due to temperature and photoperiod. However, depression in plasma androgen concentration has also been reported due to stress [22], and exposure to contaminated lakes could induce a stress response. We have examined basal and stress-induced (2 h capture stress) corticosterone levels in alligators from contaminated and reference lakes and found no differences among exposure groups in plasma corticosterone but significant differences in plasma testosterone concentrations [23]. This study, as well as a 20 h capture-stress study [12], suggests that the depressed testos-

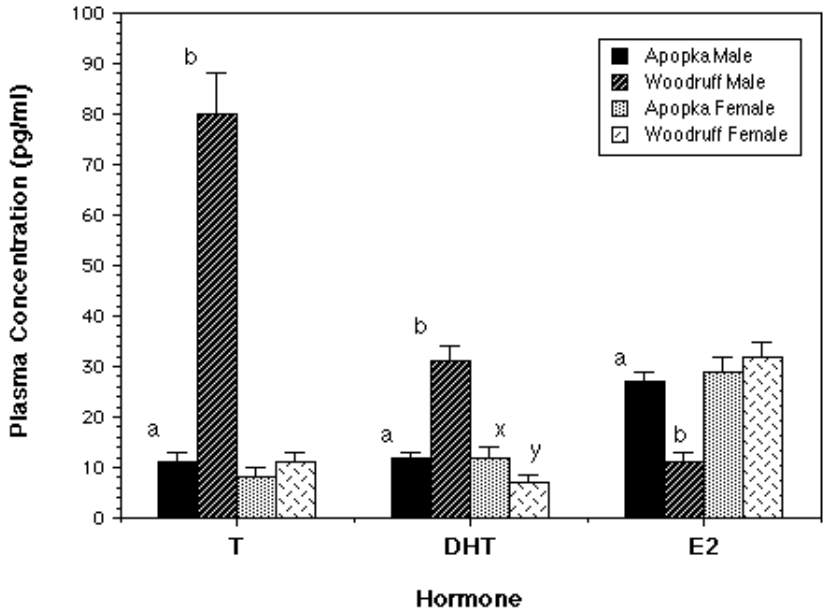

Fig. 2 Plasma concentration of three hormones, testosterone (T), dehydrotestosterone (DHT) and estradiol-17β (E2) in juvenile alligators from two Florida lakes, Lake Apopka and Lake Woodruff. Within a sex, differing superscripts indicate a significant difference. If no superscript is present, no significant difference was observed. [Data from refs. 11,19.]

terone concentrations reported in alligators from contaminated lakes are not due directly to stress, but does not rule it out as one of many factors influencing plasma sex steroid concentrations. Additional studies are required to examine ACTH as well as other parameters.

Altered plasma hormone concentrations can also result from alterations in hepatic biotransformation and metabolism of hormones [24]. We have recently documented alterations in hepatic testosterone biotransformation in juvenile alligators exposed to elevated organochlorines relative to lesser-exposed, reference individuals [25]. For example, hepatic oxido-reduction of testosterone displays a sexually dimorphic pattern—females higher than males—in alligators from Lake Woodruff, FL, a population with low organochlorine exposure, whereas populations with greater exposure, such as Lakes Apopka and Okeechobee, FL, show a lack of dimorphism (Fig. 3). Interestingly, the lack of dimorphism is due to females from the contaminated populations exhibiting a male-like pattern of androgen metabolism. Patterns in other enzyme activities are also altered. For example, sexual dimorphism is acquired in the activity of UDP-glucuronosyltransferase enzyme in one exposed population that is not observed in the reference population [25]. These data demonstrate that wildlife exposed to contaminants can display altered hepatic metabolism of hormones that appears to be a specific rather than a general response; that is, the animals exhibiting altered hepatic metabolism of testosterone do not have elevated EROD activities, indicative of a generalized heightened hepatic response to toxins.

Recent collaborative studies from our laboratories have begun to focus on alterations in the expression of genes essential for normal spermatogenesis or endocrine action, such as P450aromatase ($P450_{arom}$), steroidogenic factor-1 (SF-1) and the androgen and estrogen receptors. The genes for $P450_{arom}$ and SF-1 were previously cloned from alligator [26,27] and we have recently developed and validated a quantitative RT-PCR technique for assessing transcript number of the mRNA generated from these genes in alligators from Lakes Apopka, Woodruff, and Orange, FL (Bermudez, Katsu, Iguchi, and Guillette, unpublished data). Additionally, we have clones of alligator estrogen receptor alpha (aERα), aERβ and an androgen receptor (aAR), and recently validated quantitative RT-PCR tech-

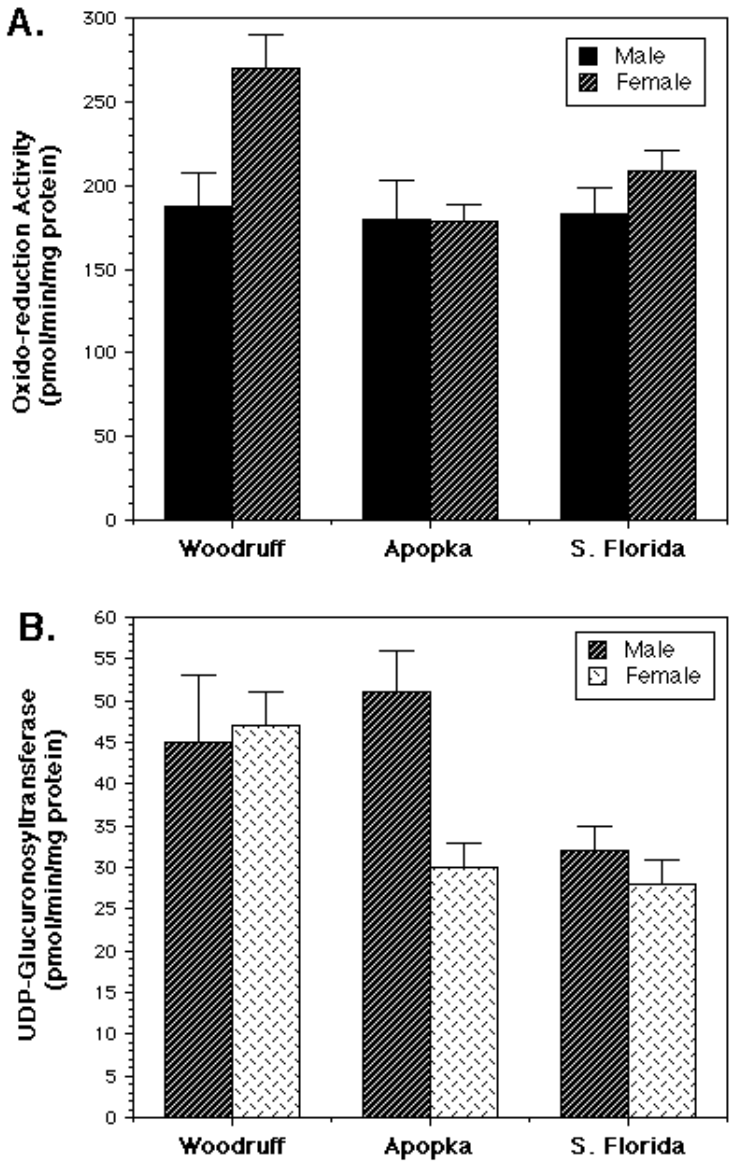

Fig. 3 Activity of hepatic enzymes on testosterone biotransformation in juvenile alligators from three regions in Florida, Lake Apopka, Lake Woodruff, and South Florida (the Lake Okeechobee watershed). Within a sex, differing superscripts indicate a significant difference. A star within a lake system indicates sexual dimorphism in testosterone biotransformation. [Data from ref. 25.]

niques to begin studies of gene expression in exposed and reference populations (Katsu, Guillette, Miyagawa, and Iguchi, unpublished data). Initial studies using quantitative RT-PCR suggest alterations in hepatic $P450_{arom}$ and SF-1 induction in animals from Lake Apopka (Bermudez and Katsu, unpublished data). Future studies will examine the relationship between gene induction/transcript number, cellular enzyme activity and plasma hormone concentrations in animals obtained from lakes having different pollution sources and histories.

LABORATORY-BASED STUDIES

Contaminants can impact organisms during embryonic development. Embryonic exposure can cause organizational abnormalities by altering the chemical signals associated with normal development [4,6]. Many of the abnormalities reported in the contaminant-exposed alligator populations appear to be organizational, in part, as they are present at birth and apparently continue throughout life. It has been proposed that many, but not all, of the actions of contaminants on embryos are a response to xenobiotic compounds interacting with hormone receptors. In order to support this hypothesis, chemicals that bind the receptor(s) must do so and must be present in concentrations high enough to induce effects.

A number of contaminants have been identified in alligator eggs [28], serum [29], and body tissues [30,31]. Concentrations of these compounds range from ppm concentrations of various organochlorine compounds in the eggs and or tissue of alligators to ppb concentrations of like compounds in the serum. Many of the compounds identified in the body exhibit an affinity for alligator estrogen (ER) and/or progesterone (PR) receptor proteins purified from the uterus [32,33]. Although the affinity of only a few of these compounds is relatively high (e.g., *o,p′*-DDT, DDOH, *trans*-nonachlor) for the aER, many compounds show an ability to displace estradiol from the aER, if present in high enough concentrations [32,33]. A recent study has also cloned an ERα from a crocodilian, *Caimen crocodylus* [34]. Studies using this cloned receptor transfected into mammalian HeLa cells demonstrated that at least one environmental estrogen, bisphenol A, was capable of binding to the receptor and inducing gene expression. We have recently cloned two estrogen receptors (ERα, ERβ), an androgen receptor and progesterone receptor from the American alligator (Katsu, Guillette, Miyagawa, and Iguchi, unpublished data). Using these recently cloned alligator receptors, we plan to transfect cells and test the ability of various chemicals, known to contaminate wild populations, to induce estrogenic, androgenic or progestogenic actions at the gene level.

In vitro system are useful as screens, but ultimately we need to address whether contaminants at known exposure levels in embryos alter development and the potential health of the individuals exposed. We have performed such studies on alligator embryos. Alligators have environmental sex determination in that temperature influences whether the gonad develops as an ovary or testis (Fig. 4). Recent studies examining alligator sex determination have begun to identify a number of genes associated with this phenomenon. Many of the genes commonly associated with sex determination in mammals and birds are also active during gonadal differentiation in alligators [35]. In other words, although differentiation is driven, in part, by temperature, sex determination in reptiles is not so unique as to be uninformative for

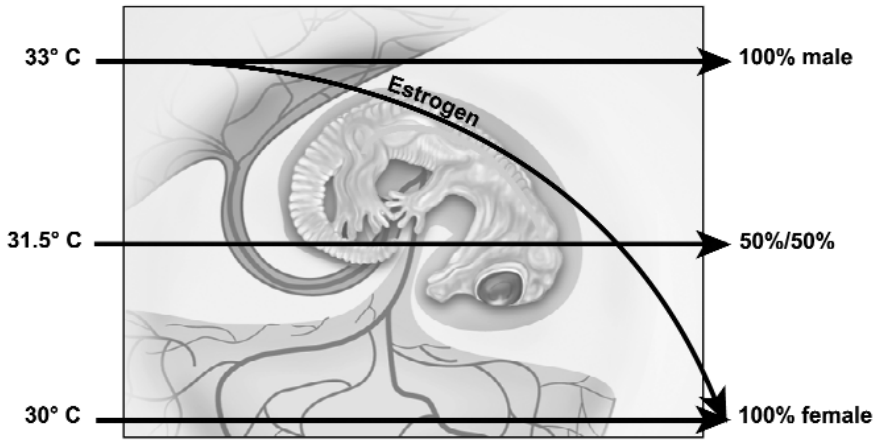

Fig. 4 Sex determination in alligators is temperature sensitive but estrogens can overcome male-producing temperatures and generate females. Several recent studies have demonstrated that environmental estrogens, such as *trans*-nonachlor and *p,p′*-DDD can act as estrogens in this system.

other vertebrates. In fact, many of the gene patterns seem to be identical to those observed during sex determination in birds, even though birds do not exhibit temperature-induced sex determination.

A number of studies using alligators and turtles have shown that various environmental contaminants are capable of altering (male to female) sex determination in reptilian embryos at concentrations as low as 100 ppt [12,36–38]. Using the average concentration of organochlorine pesticides or their metabolities (OCs) found in alligator eggs from Lake Apopka, FL, Willingham and Crews (1999) showed that sex reversal (male to female) could be induced in turtle embryos. They have also shown that steroidogenesis could be influenced by embryonic exposure to OCs. The concentrations used in these studies are clearly ecologically relevant as many studies examining contaminants in reptile eggs report ppb to ppm concentrations. The observation of altered steroidogenesis in OC-exposed embryos is important given recent data from our laboratories showing that alligators that were sex reversed by exogenous estrogens, in this case the naturally occurring estrogen estradiol-17β (E2), exhibited altered $P450_{arom}$ enzyme activity in the gonad and brain relative to reference animals developing under the influence of temperature alone [39]. That is, reference female embryos had elevated gonadal $P450_{arom}$ enzyme activity relative to females induced by E2 whereas E2-treated females had brain $P450_{arom}$ enzyme activity similar to males (Fig. 5). Previous studies with alligators have demonstrated that neonatal and

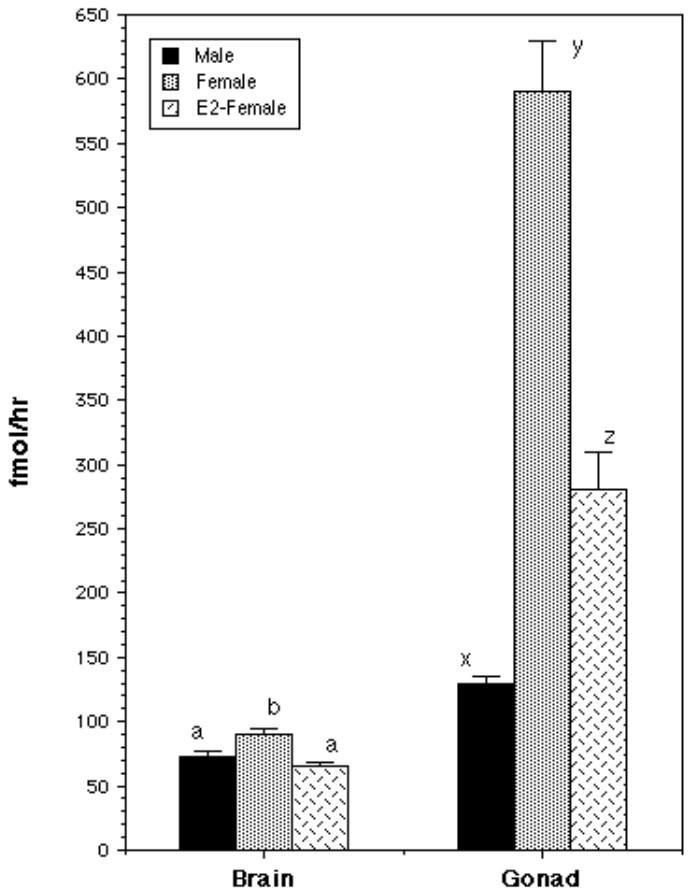

Fig. 5 Aromatase activity in the brain and gonad of stage 24 embryonic alligators. Stage 24 represents the stage immediately after the thermal sensitive period of sex determination. Differing superscript indicate a significant difference. E2 females where produced by exposing embryos to estradiol but incubating embryos at a male-producing temperature. Reference males and females were produced by incubating eggs and temperatures that produce 100 % males or females. [Data from ref. 39.]

juvenile alligators obtained from Lake Apopka had altered plasma sex steroid concentrations (reviewed in [18]) and that exposure to xenobiotics either experimentally or naturally during embryonic development can alter $P450_{arom}$ enzyme activity [40]. Thus, differentiation of a morphologically normal gonad can occur following exogenous hormone or xenobiotic exposure, but development of a normal endocrinology in that gonad may not occur.

Recent studies have begun to document the many mechanisms by which steroidogenesis can be altered in the gonad [41–43]. Any of these sites are potential targets for disruption. These can include alterations in the stimulus and inhibitory pathways involving gonadotropins, activins, and inhibins and as well as alterations in the molecular level activities involving receptor-specific actions, StAR protein, and the enzymes $P450_{scc}$ and 3β-HSD. Several recent papers clearly demonstrate that contaminants can alter the StAR-$P450_{scc}$-3β-HSD pathway [44,45]. Whether exposure of the embryo to endocrine active contaminants induces similar alterations in the StAR-$P450_{scc}$-3β-HSD pathway, causing permanent modifications in gonadal steroidogenesis, is unknown.

FUTURE NEEDS AND RECOMMENDATIONS

The current data on reptiles indicates that exposed populations, either due to polluted environments or in laboratory-controlled experiments, display altered reproductive, endocrine and immune systems (see [18,46]. Further, contamination need not be excessive, as low ppm or ppb concentrations are associated with alterations in the endocrine and reproductive systems. Further, even lower concentrations (parts per trillion) of various pesticides are capable of altering sex determination in reptiles, such as freshwater turtles and alligators. Much of the work to date has been performed on alligators from Florida, USA, but studies from the Lauretian Great Lakes of the United States and Canada, Australia, Africa, and Central America confirm that reptiles are contaminated with pollutants such as heavy metals and OC pesticides or their metabolites at levels seen in other vertebrates [8]. Their position in the food chain will influence bioaccumulation and biomagnification as it does with other vertebrates. Thus, reptiles are not unique in exposure or response. However, crocodilians may be very important ecological sentinels given their position at the top of most tropical and subtropical wetland ecosystems. Further, major advances in the endocrinological, reproductive and developmental biology of these animals at the molecular, organismal and population levels are providing important tools to further our understanding of the impacts of pollution on these important keystone species.

Given the above, we recommend several important future needs:

1. Further development of "receptor zoos" and molecular tools that include key reptiles from various major ecosystems, in addition to freshwater ecosystems.
2. Global studies extending the current knowledge base on crocodilians and freshwater turtles to comparable ecosystems on other continents, such as linked studies examining and extending current molecular to population level studies in Florida (USA) to tropical and temperate regions of Africa, Australia, and South America.
3. Further studies of actual exposure, assimilation and excretion of contaminants by ectothermic vertebrates, especially reptiles that occupy high levels of the food chain.

Receptor zoos

Although only one of several possible mechanisms by which endocrine disruption can occur, receptor-contaminant interactions are important as they can help identify chemicals that may be of major concern to a specific species of concern. Receptors from various species exhibit similar affinities for native hormones, such as that found between E2 and ERα from various species (see [47]). However, binding between the human ERα (hERα) or ERα from other species with environmental "estrogenic" chemicals can vary greatly—over several orders of magnitude. For example, a recent study examining the

affinities between hERα and an ERα from a teleost fish, the medaka, indicates that affinities can be 100X or more different [48] suggesting that use of the receptor from one species, or even a few species, could greatly misrepresent the risk in other species. Several studies in reptiles have provided conflicting evidence of the estrogenicity of *p*,*p*′-DDE. For example, *p*,*p*′-DDE causes sex reversal in embryonic freshwater turtles and alligators in some studies [36,37], but has no effect at similar dose ranges in snapping turtles or green sea turtles [49,50]. Further, we have shown that *p*,*p*′-DDE shows some affinity for aER [32], but other studies have shown this chemical has no affinity for the hERα [51]. Given the relative power of modern molecular biology, it is relatively straightforward to develop transfected cell lines with receptors from a variety of species. We propose that reptiles be included in this approach because of their important positions as keystone species in many ecosystems and their position in the evolutionary biology of vertebrates. Several studies have begun this approach and these studies should be continued.

Further, it is important to note that this could also be a powerful approach in determining if endangered species, whether reptiles or other vertebrates, are at risk to endocrine disruption via receptor-based mechanisms. The difficulty, if not impossibility, of studying toxicology in whole organisms or even their tissues when that animal is in danger of extinction, suggests an approach of harvesting tissues from one individual and developing a receptor "zoo". Given that pollution is global and no ecosystem is pristine, this approach should be considered for all major endangered species, not just reptiles.

Global studies

One of the major weaknesses of current ecotoxicology is that many major studies are limited to specific geographical areas. For example, extensive field and laboratory-based studies have been performed on fish below sewage treatment works in the United Kingdom [17], on fish exposed to pulp mill effluents in Canadian waters [52,53], and on alligators living in Florida's freshwater lakes [18]. Other studies have been done on fish below sewage works or exposed to pulp mill effluent and other reptile studies are available. But linked studies, using similar approaches and interdisciplinary teams as well as international teams, are exceedingly rare. Many of the initial studies in endocrine disruption involved studies of wildlife at specific geographical locations. This generated a great deal of criticism suggesting these were isolated studies, based on isolated conditions and not representative of other populations. Today, available wildlife studies from diverse areas indicate that the original studies were not unique and do represent global problems. However, the same criticisms put forth against early wildlife studies of endocrine disruption are now leveled on studies examining human health effects. For example, our studies of alligators in central Florida were suggested to be unique and isolated to one lake. Studies have shown that criticism to be unwarranted as similar problems are seen on other Florida lakes [11,20]. Although a growing literature has documented endocrine disruption in wildlife from Europe, North America, and Japan, only a few studies have reported studies on wildlife from other areas. Given the large use of "older" chemicals, especially organochlorine pesticides in many tropical countries, their use throughout the year as well as the growing use of chemicals in every day activities in those nations, a greatly expanded effort to understand the extent of endocrine disruption in those countries is needed. Of particular concern, is the lack of a resource database on the fate, distribution and exposure of animals, including humans, in tropical areas. Current ecological risk and health policy is based largely on studies performed in northern temperate climates. Expanded studies in the tropics would add greatly to our understanding of ecotoxicology and endocrine disruption. Reptiles are important models given their abundance at all levels of the food chain. Further, reptiles are a protein source for many human populations in the tropics and thus serve as a source of exposure to contaminants.

Exposure, assimilation, and excretion

There are very few studies from even fewer ecosystems that document contaminant exposure in reptiles. Although the studies that do exist indicate that reptiles are not obviously different when compared to other vertebrates, there is little exposure data. Further, studies of hepatic degradation are relatively rare, although some important studies have been done (for examples, see [54]). In addition to basic studies of contaminant metabolism, work examining how contaminants alter hepatic biotransformation of hormones is essential. Work on a variety of species indicates that contaminant exposure can alter the endocrine system by modifying hepatic transformation of hormones [55,56]. We have recently reported that hepatic androgen metabolism was altered in contaminant-exposed alligators whereas enzymes responsible for generalized hepatic metabolism of toxins was not altered [25]. A real need is further studies, in reptiles and other vertebrates, on the role of hepatic biotransformation on hormone concentrations and the role of contaminants in altering these activities.

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