Reproductive impacts of endocrine disrupting chemicals on wildlife species: implications for conservation of endangered species

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Abstract

Wildlife have proven valuable to our understanding of the potential effects of endocrine disrupting chemicals (EDCs) on human health by contributing considerably to our understanding of the mechanisms and consequences of EDC exposure. Yet the threats EDCs present to populations of wildlife species themselves are significant; particularly for endangered species whose existence is vulnerable to any reproductive perturbation. However, few studies address the threats EDCs pose to endangered species due to challenges associated with their study. Here, we highlight those barriers and review the available literature concerning EDC effects on endangered species. Drawing from other investigations in non-threatened wildlife species, we highlight opportunities for new approaches to advance our understanding and potentially mitigate the effects of EDCs on endangered species to enhance their fertility.

Introduction

The potential implications of living life in a contaminated world were largely made known with the publication of Rachel Carson's *Silent Spring* in 1962 (1). Her predictions about the effects of pesticides on wildlife, livestock, pets and humans captured the public's attention and played a central role in the rise of modern environmentalism. One example of *Silent Spring's* influence was the banning of the pesticide dichlorodiphenyltrichloroethane (DDT), which was a direct result of the public outcry galvanized by the book. Carson's writings also helped spur the development of the field of environmental toxicology as a whole (2). Today, this field is wide-ranging, complex and multi-faceted, reflecting the diverse biological consequences of contaminant exposure. In some cases, contaminant exposure leads to easily recognizable outcomes, such as mass mortality events, individuals exhibiting physical deformities, or

neurological impairment. On the other hand, many sub-lethal effects can be more difficult to identify, but nevertheless can have profound consequences for a species from the individual to population level.

Similar to how *Silent Spring* stimulated public awareness of the broad dangers associated with environmental chemicals, *Our Stolen Future* by Colburn et al. (1997) (3) emphasized the risks of exposure to chemicals in the environment known as endocrine disrupting chemicals (EDCs). In their book, the authors illustrate the capacity of these chemicals to interact with an organism's endocrine system and disrupt normal endocrine function, sexual development and ultimately reproduction. By weaving together examples of EDC-induced reproductive anomalies in humans, laboratory and livestock species, the book makes a compelling case for the global nature of the threats EDCs pose. However, a powerful theme of *Our Stolen Future's* narrative is how lessons learned from wildlife inform a basic understanding of EDC mechanisms of action, how EDCs affect humans and why levels of EDCs in the environment should be reduced.

Endocrine Disrupting Chemicals (EDCs) and Wildlife

In 1991, well before the publication of *Our Stolen Future*, scientists at the Wingspread work session in Racine, Wisconsin formally recognized the potential of environmental chemicals to disrupt "the endocrine systems of fish, wildlife, and humans" (4). Although Wingspread is often cited in the literature as the origin of research on EDCs, there were sufficient existing data for the attendees to conclude; 1) adequate numbers and quantities of potential EDCs were present in the environment; 2) wildlife populations were already being impacted by man-made (and natural) environmental chemicals and 3) increased and improved screening of potential EDCs was needed to mitigate future effects of EDCs on both wildlife and humans (4). Soon thereafter

in 1996, the Food Quality Protection Act was passed and the Safe Drinking Water Act was amended, mandating that the U.S. Environmental Protection Agency (USEPA) develop assays to evaluate the potential of suspected EDCs to interfere with human endocrine function. This led to the development of the USEPA Endocrine Disruptor Screening Program (EDSP). As one example of the USEPA-EDSP's efforts, data demonstrating the estrogenic and anti-estrogenic activity are currently available for more than 1,800 chemicals (5). In addition, 52 chemicals have been screened in the USEPA-EDSP's Tier 1 Screening Program, which assesses interactions with estrogen, androgen, thyroid and steroidogenic pathways using validated *in vivo* and *in vitro* assays (6). Today, due in large part to initial studies conducted in sentinel wildlife species and programs like the USEPA-EDSP, the potential role of EDCs in human disease is recognized globally (7-9).

For wildlife, there is an extensive body of literature with examples of how EDCs disrupt nearly all hormone axes in both invertebrate and vertebrate species. A thorough synopsis of those studies is beyond the scope of this of this review. Here, we choose to concentrate on how EDCs interfere with reproduction in vertebrates, given the diversity of species sharing highly conserved mechanisms of endocrine function. A number of excellent reviews are already available on this topic (10-13). Therefore, this review will instead place particular emphasis on methods of assessing EDC effects on species that are threatened or endangered whose survival requires uncompromised reproductive function. Examining the effects of EDC exposure on species of conservation concern, with the goal of increasing fertility and population sustainability, is a nascent sub-discipline within the field of environmental toxicology. Consequently, we will discuss common mechanisms of EDC action in better-studied wildlife, as well as the few examples how reproductive effects of EDC exposure have been specifically evaluated in

threatened and endangered species. Finally, new technologies are providing fascinating insight into the mechanisms of EDC-mediated impairment of reproduction in humans, traditional laboratory species and wildlife. Thus, we will explore their potential applications to the study of EDC effects on the reproductive health threatened and endangered species.

Mechanisms of EDC action

The hypothalamic-pituitary-gonadal (HPG) axis is the primary signal producer and regulator of reproductive function in vertebrates (Fig. 1). Regulation is accomplished by the integration of environmental signals to synchronize the complex network of endocrine system processes, including hormone synthesis, activity (i.e., availability, signaling and transformation) and excretion. These processes rely on the coordination of enzymes, binding proteins and receptors, which represent the myriad of potential and known targets of EDCs. Although the evolutionary conservation of HPG control of reproduction in vertebrates suggests that EDCs will affect different species similarly, specific characteristics of endocrine function (e.g., the presence of multiple steroid receptor isoforms found in teleosts) dictate that EDCs can indeed elicit species-specific effects. Here, an overview of different mechanisms of endocrine disruption and how these physiological processes ultimately affect reproduction in wildlife species is provided.

There is growing evidence that neuroendocrine tissues are specific targets of EDCs (9, 14). Given the numerous processes that neuroendocrine tissues regulate, the mechanisms by which EDC-mediated effects on neuroendocrine function can impair reproduction are necessarily diverse. Within the hypothalamus, organochlorine pesticides (OCPs), phytochemicals and bisphenol A (BPA) have been shown to affect gonadotropin releasing hormone (GnRH) mRNA expression, GnRH responsiveness and morphology of GnRH expressing neurons in both rodents

and fish (15-17). These EDCs, in addition to polychlorinated biphenyls (PCBs), have also been shown to alter estrogen receptor expression within the hypothalamus or other regions of the brain (14, 18). Such alterations in expression of these hormones and receptors are likely to have adverse effects on crosstalk between hormone systems and feedback mechanisms regulating hormone secretion. However, a unique outcome of neuroendocrine EDC effects are behavioral changes that interfere with reproduction. For example, in two species of songbirds, the black capped chickadee (*Poecile atricapillus*) and the song sparrow (*Melospiza melodia*), non-lethal exposure to PCBs is associated with variations in song that appears to be a result of direct neuroendocrine modulation (19). It is reasonable to hypothesize that song variation could have reproductive consequences, but the study did not investigate any such relationship. Another example of how neuroendocrine disruption affects breeding behavior is that of EDC exposure in two species of freshwater fish from the southeastern United States; the native blacktail shiner (*Cyprinella venusta*) and the non-native, invasive red shiner (*Cyprinella lutrensis*). These species can hybridize, but males and females of each species display stronger breeding preference for conspecifics than heterospecifics, establishing an effective prezygotic barrier to hybridization (20). In a controlled laboratory experiment, Ward and Blum (2012) (21) examined the effects of BPA on mate preference in these two species and found that exposure to 1280 μg/L BPA for 14 days relaxed mate preference for both males and females of each shiner species. Although the exact mechanisms of changes in mate discrimination are unclear, this study suggests that EDC modulation of neuroendocrine function can lead to the erosion of prezygotic barriers that maintain species integrity. Usually it is assumed that species loss due to EDC exposure is the result of reproductive suppression or failure. However, this study highlights novel mechanisms by which these chemicals can eradicate individual species and reduce overall biodiversity.

The majority of EDC studies in wildlife species examine the consequences of exposure as it relates to alteration in gonadal structure, function (i.e., hormone production, gametogenesis) or the development of secondary sex characteristics. There are a myriad of examples of such effects across all vertebrate classes, of which a few classic examples are presented below. In fish, exposure to both estrogenic and androgenic EDCs in wastewater and pulp mill effluents can alter steroid production, cause the development of intersex gonads and lead to the development of sexually dimorphic phenotypes in the inappropriate sex (22-24). Exposure of *Xenopus* tadpoles to the herbicide atrazine results in the development of hermaphroditic adults and suppresses production of testosterone, though whether the concentrations of atrazine at which intersex occurs in laboratory studies are environmentally relevant has been the subject of much debate (25, 26). In reptiles, juvenile alligators from a lake contaminated with the pesticides difocol and DDT exhibit abnormal gonadal development, steroid production and reduced recruitment compared to juveniles from reference lakes (27). In freshwater turtles, *in ovo* exposure to environmentally relevant concentrations of certain OCPs and PCBs inhibits the process of temperature dependent sex determination (28). In birds, exposure to OCPs during embryonic development can lead to the development of ovarian tissue within the testis and likely decreased reproductive fitness in males (29). Adult females of some avian species exposed to the DDT metabolite *p,p'*-DDE (dichlorodiphenyldichloroethylene) produce eggs with shells thin enough to be crushed during incubation, which led to the near collapse of a number of wild bird populations in the mid-1900s (30, 31). Fewer examples exist for mammalian wildlife, especially with strongly supporting mechanistic data. However, elevated blubber levels of DDT and PCBs in ringed seals is associated uterine pathology, suppressed reproduction and population declines in heavily contaminated regions of the Baltic Sea (32). Taken together, these examples illustrate

that despite the varied nature of EDCs and reproductive endpoints affected, ultimately the reproductive consequences of EDC exposure across vertebrates are consistent and profound.

Most information regarding specific mechanisms of EDC action focuses on the interactions between environmental chemicals and estrogen, androgen and thyroid hormone systems and the receptors that regulate them (Fig. 1). Indeed, a chemical's ability to bind and either activate or antagonize a hormone receptor can result in significant physiological outcomes. Quantifying EDC-receptor interactions can therefore be a useful predictor of organismal responses (33). However, EDCs can disrupt numerous other processes that warrant further investigation (Fig. 1; 34). For example, differences in hormone levels resulting from exposure are commonly measured endpoints in EDC research. Differences in hormone concentrations can result from changes in rates of hormone synthesis or hormone degradation. Unfortunately, there are relatively few studies on the effects of EDCs on hormone synthesis and degradation compared to investigations on EDC interactions with hormone receptors. Mechanisms by which the herbicide atrazine functions as an EDC serves as an excellent example of how studying 'non-traditional' endpoints of EDC action can deepen our understanding of the mechanistic effects of these chemicals on wildlife. Early studies on atrazine's effects demonstrated its estrogenic potential (35). However, instead direct interaction with estrogen receptors (36), atrazine's estrogenic effects appear to be the result of increased endogenous estrogen concentrations the expression via increased expression of aromatase (CYP19; (35)); the enzyme responsible for conversion of androgens to estrogens. Atrazine-induced expression of aromatase might be explained through the herbicide's direct interaction with the orphan receptor, steroidogenic factor-1 (SF-1; (37)). Although the studies above elucidate a novel mechanism of EDC action, they nevertheless emphasize an estrogenic response to EDCs. Although estrogenic effects have

dominated the EDC literature since the early 1990s, many other pathways, systems and classes of EDCs are still waiting to be studied. Some potential open avenues for exploration in wildlife include the effects of EDCs on the hypothalamic-pituitary-adrenal axis (14), the study of emerging, potentially novel EDCs (38-40) and how EDCs and an organism's microbiome interact to influence EDC actions (41), among others (see (34) for review).

The study of EDC effects on endangered species

The most compelling research on EDC effects on wildlife species results from the ability to conduct controlled laboratory studies to generate strong causal evidence in support of observations from wild populations. An excellent example of this is two studies of fathead minnows (FHM; *Pimephales promelas*) living near feedlots where cattle are given androgenic supplements to increase beef production. In populations of FHMs living downstream from feedlots, females exhibit morphological evidence of masculinization and altered gonadal steroid production compared to females living upstream (42, 43). In one study, the presence of suspected androgenic EDCs was confirmed in an *in vitro* laboratory experiment by treating CV-1 cells expressing human androgen receptor with water collected downstream from cattle feedlots (43). In another study, Ankley and colleagues (42) focused specifically on the anabolic steroid metabolite, 17-β-trenbelone. By treating laboratory-reared FHMs with environmentally relevant concentrations of 17-β-trenbelone they observed evidence of masculinization of females similar to observations in wild populations living downstream of feedlots (42, 43). In addition, they demonstrated that 17-β-trenbelone binds the FHM androgen receptor and does so with higher affinity that its endogenous ligand, testosterone. Taken together, these studies illustrate how field

and laboratory studies, employing sub-cellular and organismal approaches, can give clear mechanistic insight to the effects of EDC exposure in wildlife species.

In some ways, scientists studying the effects of EDCs on endangered species face challenges similar to those imposed upon researchers interested in studying the effects of EDCs on humans. When working with endangered species, ethical and legal constraints result in restricted access to biological samples and the types of experimental approaches that can be used. In addition, where researchers focused on human health issues can rely heavily upon epidemiological data, the comparatively small population sizes of endangered species and restricted access to individuals in those populations limit the application of epidemiological approaches. This problem is further confounded when working with a species for which little is known about their basic endocrine function or reproductive physiology. As a result of the challenges of working with endangered species, alternative techniques and experimental approaches must be considered to gain an understanding of the potential for EDCs to compromise successful reproduction.

The development of techniques to measure hormones in samples collected in a non-invasive, or minimally invasive manner, has given rise to the field of conservation endocrinology and increased our understanding of species which would otherwise remain unstudied (see (44) for review; (45, 46)). One of the first examples of the application of this approach for conservation purposes was the use of fecal hormone assays to determine the sex of sexually monomorphic birds in an effort to enhance captive breeding efforts (47). Today, nearly 40 years later, techniques that measure hormones and their metabolites have been developed and validated for a variety of biological sample types (e.g., urine, feces, saliva, hair and feathers). These minimally invasive approaches are the centerpiece of most endocrine studies in threatened and

endangered species (44, 48). Although examples are limited, applying these techniques in paired field and laboratory studies to specifically evaluate changes in hormone production associated with EDC exposure in wildlife appears promising (49, 50).

While non- and minimally-invasive methods certainly have value in broadening our understanding of endocrine function in species of conservation concern, the mechanistic insight they can provide into biological phenomena, such as the effects of EDC exposure, are limited. Hence, there is a recognized opportunity to utilize novel approaches based on cellular and molecular techniques to further our understanding of the endocrine systems in endangered species (45, 46). Many of these approaches have the additional benefit of shedding light on the possible effects of EDCs on endangered species without exposing organisms to potentially harmful chemicals. For example, in our lab we examine interactions between suspected EDCs and steroid receptors cloned from endangered species to predict mechanistic responses and species sensitivity (46, 51, 52). Prior to the advent of recombinant DNA technology, such approaches in an endangered species would be impossible due to the large quantities of tissue and numbers of individuals needed to perform receptor binding or gene transcription studies. However, now only small pieces of tissue, which can be collected opportunistically during necropsies, veterinary examinations or remotely via biopsy darts, are needed to isolate sufficient genetic material for receptor cloning. Moreover, as more species' genomes are sequenced (53), the highly conserved nature of steroid receptors will permit identification of coding sequences *in silico* and synthesis of recombinant receptors, thereby eliminating the need to collect tissue altogether. Similar approaches could be used to isolate and/or generate sequence for potentially any receptor, enzyme, or other protein suspected of being an EDC target.

The application of *in vitro* molecular techniques provides novel insight into how EDCs may affect endangered species, but like non-invasive hormone monitoring, they are not without limitations and caveats. Although they have proven useful in our efforts to study and address the low fertility of female southern white rhinoceros (SWR; *Ceratotherium simum simum*) in captivity (54), *in vitro*-derived results are not entirely predictive of *in vivo* responses. For example, in a study investigating interactions between estrogen receptors cloned from the critically endangered California condor (*Gymnogyps californianus*) and persistent organochlorine contaminants, we showed that both the DDT isomer *p,p'*-DDT and *p,p'*-DDE activated both estrogen receptors α and β (51). This suggests that in condors these two EDCs are estrogenic and, in the case of *p,p'*-DDE which activated receptors at physiologically relevant concentrations, may be disrupting endocrine function in wild birds (51, 55). However, there is little evidence from *in vivo* studies in other avian species that either of these two chemicals is strongly estrogenic. Conducting *in vivo* EDC-treatment studies in this species, of which there are fewer than 450 living individuals, is not possible. Thus, determining if these data represent a unique mechanism of EDC action, or an *in vitro* artifact, may only become clear with long term population monitoring (55). Despite their shortcomings, the use of cellular and molecular approaches in the field of conservation endocrinology and EDC research should serve to further our understanding of endocrine function and assist efforts to conserve threatened and endangered species.

Organizational and Activational Effects of EDCs

Perhaps the best-known example of the deleterious effects of EDC exposure is that of diethylstilbestrol (DES). Given to women in the mid-20th century to prevent premature abortion

and miscarriage, this potent synthetic estrogen was later realized to have devastating effects on the developing fetuses of mothers who took the drug. Although evidence suggests that DES can disrupt development of fetuses of both sexes, the consequences of DES exposure are best studied in females. Women whose mothers took DES (DES daughters) during their pregnancy suffer from a number of pathologies including altered hormone cycles, malformations of the reproductive tract and in many cases, the post-pubertal early onset of vaginal clear cell adenocarcinoma (see (11, 56) for review). DES sons show an increased incidence in the development of genital abnormalities, such as hypospadias (56). A similar, albeit slight, increase in hypospadias has also been documented in DES grandsons, suggesting the effects of DES exposure may be transgenerational (56). Similar outcomes have been proposed for DES granddaughters, though more investigation is needed to confirm this hypothesis. Interestingly, there is some evidence suggesting women who themselves took DES have a modest increased risk of developing breast cancer, but they do not suffer from the extreme reproductive impairments afflicting daughters and possibly granddaughters (56).

The summarized version of Paracelsus' central maxim of toxicology-*sola dosis facit venenum*-states that *the dose makes the poison*. While this is certainly true in many regards, the story of DES highlights the importance of the timing in determining the consequences of exposure to EDCs. The effects of EDC exposure are typically categorized as either organizational or activational, which differ from one another in terms of when exposure occurs, the severity and duration of the effects, and the implications for the exposed individuals. Organizational effects, like those observed in DES daughters and sons, are the result of exposure to EDCs during times of critical sensitivity, such as embryogenesis or fetal development, and are typically permanent (Fig. 2; 57, 58). On the other hand, activational effects are those brought on

by EDC exposure outside of these critical periods, resulting in effects that are typically temporary. Thus, eliminating the source of exposure can be effective in mediating activational outcomes of EDCs, but may not be helpful in alleviating organizational effects.

There are numerous examples strongly suggestive of organizational effects resulting from EDC exposure in wildlife (see (58) for review). However, one of the most striking examples is that of the western gull (*Larus occidentalis*) and the resulting abnormalities that were observed following *in ovo* exposure to DDT (29). In the early 1970's the western gull population residing in proximity to a DDT hotspot near the Channel Islands off the coast of California exhibited reproductive failure. In contrast to other bird species in the same area that experienced population declines due to *p,p'*-DDE associated eggshell thinning, the western gull breeding failure was the result of a highly skewed sex ratio due to the absence of adult males within the population. Fry and Toone (29) provide compelling evidence that this population level effect was the result of feminization of male embryos by the estrogenic isomer of DDT, *o,p'*-DDT. Following treatment of western gull eggs collected from non-DDT contaminated sites with environmentally relevant concentrations of *o,p'*-DDT, they observed the presence of ovarian tissue and oviducts in male embryos. Based on studies in other avian species, where *in ovo* treatment with estrogen resulted in feminization of male behaviors, the authors suggest that altered migratory behavior of male western gulls exposed to DDT could partially explain the low numbers of males in the Channel Island population (29).

Organizational effects of EDC exposure in wildlife pose a number of challenges for the species they affect and researchers attempting to study them. Most notably, the developmental alterations that result from EDC exposure are often not readily observable and can therefore go unnoticed for some time. In addition, because they interfere with developmental processes,

organizational effects are often permanent and can persist even if EDC exposure is mitigated. Moreover, recent studies in laboratory species have demonstrated that organizational effects resulting from developmental EDC exposure can persist for multiple generations ((59); *Epigenetic effects of EDC exposure* section below). The possible long-term, irreversible implications of organizational effects of EDC exposure are particularly problematic for threatened and endangered species, especially if they are part of a managed breeding program where reproductive success is a primary goal. Since it is difficult to know precisely the timing, the extent and the type of EDC exposure in wildlife species, discrimination of observed effects as organizational, activational or both can be difficult.

Our recent studies have addressed the effects of developmental exposure to phytoestrogens in the southern white rhinoceros (SWR; *Ceratotherium simum simum*). In the mid-20th century, *ex situ* captive SWR breeding programs were established in an effort to conserve the species. Initial breeding efforts were largely successful, however, it soon became clear that female SWR born in captivity exhibited a marked decrease in fertility compared to their wild-born female counterparts (60). For decades, the specific cause of this problem remained elusive, but was attributed to some unknown effect resulting from developing in captivity (61). In managed settings, SWR are typically fed diets containing soy and alfalfa; plants that produce high levels of phytoestrogens. Many captive SWR exhibit reproductive pathologies similar to those reported in DES daughters and laboratory and livestock species consuming foods rich in phytoestrogens so we focused on phytoestrogens as a likely cause of SWR fertility issues (52, 62). Our hypothesis was that gestational exposure to phytoestrogens consumed through the maternal diet resulted in the reproductive failure of captive-born female SWR (52, 62). The fact that the dramatic decrease in fertility in captive-born females compared to wild-born females

persists even when captive-born females are moved to institutions feeding low phytoestrogen diets points strongly to an organizational effect of phytoestrogen exposure. We therefore questioned the benefit of transferring affected animals to a low phytoestrogen regimen. Nevertheless, at our institution we began feeding SWR a low phytoestrogen diet and soon thereafter observed three pregnancies in two females that had never reproduced before (54). This finding was encouraging and suggests that the reduced fertility resulting from developmental exposure to phytoestrogens may not be permanent. However, it should be noted that four female SWR within our herd have failed to reproduce following the diet change, suggesting both organizational and activational effects are associated with phytoestrogen exposure in SWR. Thus, the relationship between organizational and activational effects of EDC exposure appears to not be straightforward, and that the severity and specific outcomes within a particular species are likely to vary individually.

Recent advances in EDC research and their applicability to the study of endangered species

The development of new technologies is providing novel insight into the mechanisms of EDC action. Although initially applied to common laboratory species in the context of addressing human health concerns, these advances are generally applicable to the study of EDCs and their effects on reproduction in most vertebrates. Although there are few instances of their application explicitly to endangered species, they show a great deal of potential as a tool for conservation endocrinologists and some examples are highlighted below.

Epigenetic effects of EDC exposure

Defined as mitotically and meiotically heritable changes to DNA that do not result in alterations in DNA sequence, epigenetic modifications can have significant effects on gene expression and phenotype. Epigenetic modifications are influenced by a number of environmental factors including diet, social interactions and both physical and chemical environmental stressors (63). The ability of EDCs to modify the epigenome is a growing area of interest within the field of endocrine disruption research. One especially interesting aspect of EDC-mediated effects on the epigenome is the evidence that the effects can be transgenerational, suggesting that EDC exposure can affect populations long after the actual exposure occurs (59). There are a number of different types of epigenetic modifications, including histone modification, chromatin remodeling and non-coding RNA and DNA methylation. Of these, DNA methylation is the best understood global patterns and levels of DNA methylation are generally conserved across vertebrates (64). DNA methylation regulates a number of critical, wide-ranging biological processes such as temperature-dependent sex determination (65, 66), reproductive behaviors (67) and gamete function (68) and thus any disruption of methylation could have diverse implications within and across species. However, the majority of investigations into the epigenetic consequences of EDC exposure have been performed in laboratory species and few studies in wildlife species are available.

Perhaps the best known example how EDC exposure can modify the epigenome to negatively affect endocrine and reproductive function comes from laboratory studies in rodents (59, 68). In male rats, exposure to the anti-androgenic fungicide vinclozolin or the estrogenic pesticide methoxychlor, during the testicular development stage of fetal development results in reduced sperm production and viability as adults (68). This is associated with altered DNA

methylation patterns within the male germ line (68), which have been shown to affect the expression of genes associated with sperm function like olfactory transduction and calcium signaling (69). What is most compelling, however, is that reduced testicular function is maintained in the embryonically exposed male's progeny for at least three subsequent generations (68). In these particular studies similar epigenetic transgenerational effects were absent in female mice exposed to vinclozolin or methoxychlor *in utero* (68). However, others have shown epigenetic transgenerational effects on gene expression and behavior in both sexes of mice following embryonic exposure to BPA (70). Taken together, these studies and others clearly demonstrate the sex specificity of epigenetic transgenerational effects resulting from EDC exposure (see (59) for review).

EDC-mediated epigenetic effects that transcend generations could have potentially profound and persistent effects on wildlife species and thus are an emerging topic of interest in the field of ecotoxicology (64, 71, 72). However, examining this phenomenon is challenging in wildlife species. One confounding factor is that, in contrast to laboratory studies where the timing of exposure is controlled and subsequent generations can be outcrossed to non-exposed individuals, it is unlikely for a single, well-defined exposure to occur in the wild. Thus, the criteria required for an observed effect to be considered transgenerational are not met in many cases (59, 73). In addition, unlike controlled laboratory experiments, wildlife is more likely exposed to mixtures of EDCs, which are known to have additive, subtractive or synergistic effects on other endpoints of EDC action (74, 75). Nonetheless, a number of studies clearly demonstrate epigenetic responses in wildlife species to EDC exposure, primarily in the form of alteration in patterns of methylation. For example, in alligator erythrocytes, global methylation is inversely correlated with the degree of environmental mercury contamination (76). In another

study, alligators from lakes with varying levels and types of EDC contamination exhibited alterations in the patterns of differentially methylated regions of erythrocyte DNA (77). Analysis of the gene networks associated with these differentially methylated regions of DNA reveal a collection of genes that regulate both endocrine and non-endocrine processes, suggesting the physiological outcomes of these epigenetic response are likely diverse (77).

To our knowledge, there are no studies to date that explicitly address epigenetic responses to EDCs in endangered species. Similar to other approaches described, conducting such studies with limited access to potentially meaningful biological samples will be challenging. However, methylation studies utilizing alligator erythrocytes demonstrate that epigenetic signatures of EDC exposure can be identified in samples collected in a minimally invasive manner. While such samples may not provide a complete understanding of the reproductive consequences of exposure, they can begin to further our understanding about whether particular EDC exposures act at the level of the epigenome, and may even serve as useful biomarkers of exposure (75, 77). Other possible means of studying epigenetic responses to EDC exposure is to make use of cells and tissues preserved from endangered species in the growing number of cryobanks established in an effort to preserve biodiversity. In our lab, we have utilized fibroblasts from two rhinoceros species to examine the mechanisms by which phytoestrogens may affect reproduction (46). We observed that expression levels of genes associated with endocrine function varied between cell lines. Although not yet assessed, it is possible that the variations in gene expression are the result of differential epigenetic signatures across cell lines. If so, cryobanks represent an accessible source of biological material that could be used gain a better understanding of basic epigenetic information for endangered species.

Applications of '*-omics*'

The number of studies applying transcriptomic, proteomic and/or metabolomics approaches (collectively referred to as '*-omics*') to investigate the effects of EDCs on wildlife species research is rapidly growing (78, 79). This is due to a number of factors that make these approaches more accessible to researchers studying non-traditional species. For example, recent initiatives to sequence reference genomes of thousands of species is proving largely successful (53). In addition, costs associated with different '*-omics*' approaches (e.g., high throughput sequencing, non-targeted chemical analyses) are decreasing while the number of computing platforms, and bioinformatics researchers, able to process large amounts of data generated are growing. As a result, '*-omics*' technologies are providing valuable and intriguing insight into the effects of EDCs on wildlife and their use in the field in certain to become more prevalent.

Numerous studies have quantified changes in transcriptomes, using microarray or RNA-seq analyses, to investigate changes in global gene expression in response to exposure to EDCs and other contaminants (78-80). The use of RNA-seq, and similar non-targeted technologies, can be particularly advantageous in that they eliminate potential biases inherent in the *a priori* assumptions made with targeted approaches (78). This is demonstrated nicely by Martyniuk et al (2016) (81), in which largemouth bass were stocked into natural ponds containing high levels of organochlorine pesticides near Lake Apopka, FL. Compared to control fish, those inhabiting the Lake Apopka site showed elevated levels of organochlorine pesticides in their tissues and lower circulating levels of 17β-E_2 (81). In contrast, the authors observed no differences between control and exposed fish in two other endpoints traditionally used to identify EDC exposure; gonadosomatic index and vitellogenin production. When characterizing differences in ovarian gene expression using RNA-seq, however, the authors discovered deregulation of gene networks

associated with reproductive and immune function, which paralleled findings from more controlled laboratory studies (81). Thus, when assessment of traditional endpoints fail to detect an effect of exposure to EDCs, non-targeted approaches like transcriptome profiling may demonstrate otherwise, and highlight other endpoints for future study.

Transcriptomic approaches are certainly informative and becoming more feasible to conduct, but they are not without limitations. Namely, levels of gene expression may not necessarily correlate well with expression levels of the proteins they encode (82). Consequently, a putative physiological response to EDC exposure may not actually occur. Proteomic approaches, therefore, may provide a clearer understanding of the physiological pathways and processes altered by EDC exposure. Similar to transcriptomic approaches, proteomic studies can be non-targeted, though a lack of well-annotated genomes for non-traditional species can make protein identification challenging (78). Furthermore, like genomes and transcriptomes, the proteins comprising the proteome vary in sequence across species, potentially limiting the use of commercially available tools (e.g., antibodies) in non-traditional species.

Studying the metabolome potentially alleviates the species-specific limitations of transcriptomic and proteomic approaches. The metabolome is the collection of biomolecules (e.g., amino acids, lipids, carbohydrates, etc.) within a biological sample associated with various metabolic processes, which in many cases are identical across diverse species. These approaches couple chromatography and mass spectrometry to quantify the biochemical milieu that ultimately results from changes in transcriptomes, proteomes and other biological responses. This coupled with their ability to directly measure altered physiological pathways and processes, makes metabolic approaches attractive techniques for the study of EDCs effects on wildlife species. However, as is becoming clear, data generated from *'-omics'* studies possesses a higher degree of

variability that may be overlooked by measuring traditional endpoints (79). This can be the result of biological variation such as differences in season, inherent genetic variability within a population, or exposure to dynamic and complex mixtures of EDCs, for example. Technical variation associated with the collection and preparation of samples, sample size and analysis pipelines used can also contribute variability to generated data (79, 83). Thus, care should be taken with regard to experimental design and statistical analyses to reduce variability and subsequently improve the utility of *'-omics'* approaches.

Although each *'-omics'* approach has its own strengths and weaknesses as detailed above, their utility in wildlife studies is also dictated in part by the nature of the species being studied. Currently, the literature regarding the effects of EDCs via tissue specific transcriptome analyses is dominated by investigations in fish, presumably due to their relative small size, ease of capture and the fact that they can also be maintained in laboratory studies (78, 79). There are also a few reports in amphibians examining alterations in gene expression following exposure to pharmaceuticals that act as EDCs using RNA-seq or microarray methods. In male western clawed frogs (*Silurana tropicalis*), exposure to the 5α-reductase inhibitor, finasteride, results in increased testosterone levels and alterations in gene networks involved in meiosis, hormone production and GnRH function (84). In another study, treatment of American bullfrog (*Rana catesbeiana*) tadpoles with environmentally relevant concentrations of ibuprofen reprogrammed the liver transcriptome via alteration of the thyroid hormone signaling pathway, which the authors suggest may contribute to altered post-embryonic development (85). More recently, RNA-seq has been employed to examine the effects of developmental exposure to BPA and ethinyl estradiol (EE_2) on behavior and gene expression in the whole brain and the hypothalamus in painted turtles (*Chrysemys picta*) and California mice (*Peromyscus californicus*), respectively

(86, 87). Compared to transcriptomic studies, the number of EDC-focused studies on wildlife species that employ proteomic or metabolomics approaches are relatively few and primarily conducted in fish. However, some of these studies highlight an attractive aspect of proteomic and metabolic approaches, which is the utilization samples that can be easily collected in a minimally-invasive manner (78, 79). An excellent example of this is the recent study by Collette et al. (2016) (88) in which researchers identified changes in fatty acid composition in only 5-10 µL of urine collected from fathead minnows exposed to the anti-androgens vinclozolin or flutamide.

The application of genomic analyses to the study of threatened and endangered species is widespread, and has provided valuable insight into adaptation of populations to local environments, inbreeding depression, disease susceptibility and overall species fitness (89). In contrast, there are few examples of the application of transcriptomic, proteomic and metabolomics approaches to the study of endangered species, particularly with regard to the effects of EDC exposure. The studies above, however, demonstrate the versatility of *'-omics'* approaches and creatively applying them to the study of endangered species could prove extremely valuable. For example, RNA-seq studies could be performed on cryobanked fibroblast cell lines to provide useful gene sequence and expression information for a particular species. In previous studies, the expression of endocrine-related genes, including steroid hormone receptors, has been demonstrated (46). Thus, treatment of fibroblasts with suspected EDCs, followed by non-targeted RNA-seq may identify changes in gene expression and potentially could be used as species-specific experimental *in vitro* model of EDC action. The recent use of proteomic and metabolomics approaches in small amounts of easily collected biological samples such as saliva, urine and feces is also encouraging. For decades, endocrine research on endangered species has

relied almost exclusively on measuring hormones in these types of readily available samples. This provides an attractive opportunity to apply these techniques to threatened and endangered species to gain a better understanding of their physiology, particularly as it relates to response to EDC exposure.

Conclusions

There are undoubtedly a great number of challenges associated with studying the impacts of EDCs on reproduction of endangered species. However, as evidenced from the scant literature available on the subject, it is an area of research with excellent potential for growth. Furthermore, the growing human population, accelerated habitat loss and other factors are contributing to an increase in the number of species listed as threatened or endangered that will require human intervention to ensure their sustainability. The reliance upon non-invasive sample collection to study endocrine function of endangered species is not likely to change. Thus, the creative development of new technologies and integrative application of novel approaches will be needed to advance our knowledge in this area of research. If this is accomplished, we stand to gain clearer insight into the mechanisms by which EDCs act on endangered species, and ultimately may begin to mitigate the reproductive impacts of EDCs on endangered animal species through better-informed policy decisions.

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Figure 1. Activational effects and mechanisms of exposure to endocrine disrupting chemicals (EDCs). Endogenous hormone synthesis, activity, and excretion are regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Each of these tissues is a potential target of EDC exposure. Through a variety of potential mechanisms (blue), EDCs can disrupt the coordination of enzymes, binding proteins, receptors and gene expression involved in the production and regulation of hormones including gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P_4), and estrogen (E_2). The full range of phenotypic impacts of EDC exposure through these mechanisms is largely uncharacterized, nevertheless some established reproductive abnormalities are highlighted in green.

Figure 2. Organizational effects of exposure to endocrine disrupting chemicals. EDC exposure can be particularly impactful during development. These organizational effects can influence the sex-specific development of reproductive and neuroendocrine tissues, and result in permanent abnormalities in hormone production and reproductive function, which are shown in green.

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