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Methodologies, bioindicators, and biomarkers for assessing gender-related differences in wildlife exposed to environmental chemicals

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Abstract

Male and female organisms may have significant differences in their exposure, toxicokinetics, and response to chemicals, but gender effects have received relatively little attention, often viewed as a confounder rather than of primary importance. In this paper, we examine some of the key issues and methodologies for incorporating gender in studies of the effects of chemicals on wildlife, and explore bioindicators and biomarkers of gender effects. Examining gender-related differences in response to chemicals is complicated in wildlife because of the vast array of species, and differences in niches, lifespans, reproductive cycles and modes, and population dynamics. Further, organisms are more at risk in some ecosystems than others, which may increase the magnitude of effects. Only by studying wild animals, especially native species, can we truly understand the potential impact of gender-specific effects of chemical exposure on populations. Several factors affect gender-related differences in responses to chemicals, including exposure, age, size, seasonality, and genetic and phenotypic variation. There are clear examples where gender-related differences have had significant effects on reproductive success and population stability, including destabilization of gamete release in invertebrates, and alterations of endocrine and neuroendocrine system functioning in vertebrates. A wide range of new technologies and methods are available for examining gender-related differences in responses to chemicals. We provide examples that show that there are gender-related differences in responses to chemicals that have significant biological effects, and these gender-related differences should be taken into account by scientists, regulators, and policy makers, as well as the public.

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1. Introduction

A wide range of chemicals can affect animals, including humans, at all levels of biological organization. How chemicals affect organisms depends on organismal characteristics and environmental variables. Environmental factors include exposure levels and characteristics of the media and the chemical bioavailability (Burger et al., 2003; Peakall and Burger, 2003).

While considerable attention has been devoted to age, size, and hormones in wildlife (Eskildsen and Grandjean, 1984; Furness and Lewis, 1990; Colborn, 1994; Becker et al., 2002; Burger and Gochfeld, 2004), and more recently to gender in humans (IOM, 2001; other papers in this volume), relatively little attention has been devoted to the role of gender in the fate and effects of chemicals in wildlife. While there are some studies on contaminant levels as a function of gender (Burger and Gochfeld, 1992a; Burger et al., 2002a; Komarnicki, 2000; Johnston et al., 2002), many studies ignore gender (i.e., do not distinguish the sexes), choose to examine only one gender, or report

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1 data by sex without commenting on similarities or differences.

3 The overall goal of this paper is to identify the variance
 5 in safety of exposure levels of chemicals as a function of
 7 gender. We examine the factors that interact with gender to
 9 influence how chemicals affect organisms, discuss meth-
 11 odologies to examine gender-related effects in wildlife, and
 13 make recommendations for future work to evaluate how
 15 gender interacts with chemicals to disrupt normal biolog-
 17 ical functioning of wildlife. One overarching question is
 19 whether there are causes for concern because of under-
 21 protection, due to a lack of understanding or the
 23 consideration of gender-effects of specific chemicals. In
 25 this paper, we define gender-effects as those examined
 27 when effects on males and females that are statistically
 29 compared, under similar conditions and age structures.
 31 Other papers in this issue discuss how gender affects gene
 33 expression, germ cells, physiology and behavior, with
 35 special reference to metals in vertebrates (Burger, 2006),
 37 and endocrine-active chemicals on large pelagic fish and
 39 marine mammals (Fossi et al., 2006), fish, amphibians and
 41 reptiles (Orlando and Guillette, 2006) and invertebrates
 43 (McClellan-Green et al., 2006). Together these papers
 45 provide a framework, approaches, and methodologies to
 47 assess the ecological effects of chemicals with respect to
 49 gender-related differences.

Understanding gender-related effects in wildlife is crucial
 for protecting and managing wildlife populations, for
 providing early warning of potential population effects, to
 act as sentinels and bioindicators of human effects, and to
 provide paradigms for understanding the mechanisms for
 disruption of human and ecological health. Both labora-
 tory and field studies of the interactions of gender, other
 factors, and chemicals are necessary to provide the full
 spectrum of understanding from exposure to fate and
 effects. While laboratory studies can elucidate the mechan-
 isms of action of chemicals, field studies are essential to
 understanding the full range of effects, both at the
 individual and population levels. It is, after all, wild
 populations of wildlife that we wish to protect and
 maintain in a healthy state.

43 2. Measurement endpoints and biological organization

45 There are several ways in which assessment of the risks
 47 from chemicals differ for humans and wildlife: (1)
 49 individual vs. population focus; (2) longevity vs. reproduc-
 51 tion; and (3) one receptor vs. hundreds or thousands,
 53 depending upon the ecosystem. While humans can be
 55 considered as one ecological receptor, they differ from
 57 other ecological receptors in one key point. With humans,
 we are interested in the health and well-being of
 individuals, but with wildlife we are interested in main-
 taining community integrity and healthy populations,
 rather than focusing on the individual (Burger and
 Gochfeld, 1992b, 1996a, 2004). Endangered or threatened
 species are an exception since every individual is important

to the overall gene pool and survival of the population. In
 this case, the individuals are important because they are
 crucial to the maintenance of a healthy population.

The difference between understanding gender-related
 influences to chemical susceptibility and effects in humans
 and wildlife also relates to scale. There can be thousands of
 species to consider within ecosystems, and many have very
 different life histories and life cycles, from unicellular
 organisms that live for only a few hours to some trees that
 live for centuries. In some species juveniles resemble adults,
 while in others the life stages are very different (e.g., a larva
 and a butterfly, a tadpole and a frog). This makes the task
 of understanding how gender relates to chemical suscept-
 ibility difficult, leading to the necessity for bioindicators
 and biomarkers (Fox, 1994; Wilson, 1994; Burger and
 Gochfeld, 2001, 2004; Burger, 2006; Bartell, 2006; Denslow
 and Larkin, 2006; Natesan and Slimak, 2006). The
 differential effects of chemicals as a function of gender
 can act on several levels of biological organization, from
 molecular to the population level. These include mutagen-
 esis, gene expression, biochemistry, physiology, morphol-
 ogy, development, reproduction, pathology, behavior, and
 social organization (Burger, 2006, this volume). Further, it
 should be remembered that some organisms start life with a
 chemical burden derived from their mothers. Approaches
 and methods to understand gender-related differences at
 each of these levels vary depending upon the species. Also,
 intraspecific differences (e.g., age, gender, and size) must be
 taken into account at every level of biological organization.

3. Species and ecosystems at risk

3.1. Species at risk

Wild species are vulnerable to the adverse effects of
 chemicals because of different methods of exposure,
 uptake, and metabolism. Species that are higher on the
 food chain can be exposed to high levels of contaminants
 (Burger, 1992, 2002). Similarly, males and females may be
 exposed to different levels of contaminants when they eat
 different foods, or eat prey of different sizes. It is well
 known that large fish generally have higher levels of
 contaminants. For example, mercury levels increase with
 size and age in fish (Braune, 1987; Lange et al., 1994;
 Lacerda et al., 1994; Burger et al., 2001). A similar
 relationship exists for other metals, such as selenium,
 arsenic and cadmium (Burger et al., 2002b). While the
 general world-wide decline in fish has been attributed to
 overfishing (Safina, 1997), contaminants may play a role,
 especially for large predatory species.

Many aquatic mammal species, as top predators, can
 exercise a dramatic regulatory control at the community
 level. Their decrease or disappearance in some areas can
 drastically alter community structure through direct and
 indirect effects on their prey and competitors. In the last
 few decades various studies have shown that several
 aquatic mammal species are sensitive to the toxicological

effects of certain xenobiotic compounds (Helle et al., 1976; DeLong et al., 1979; Fuller and Hobson, 1986; Reijnders, 1986; Brouwer et al., 1989; De Swart et al., 1996; Bernhoft et al., 2000), including the large class of endocrine disrupting chemicals (EDCs). Some environmental chemicals, including agricultural runoff, industrial effluent, plasticizers, detergent components, organochlorines (OCs), and metals are known to alter normal endocrine and neuroendocrine system function and are called EDCs (Colborn, 1994; Knobil et al., 1999; Guillette and Crain, 2000).

High concentrations of PCBs and DDTs, known to be potential EDCs, have been detected in a variety of aquatic organisms. Top predators often acquire large burdens of persistent pollutants through biomagnification of compounds received from contaminated prey (Colborn, 1998; Tanabe et al., 1988). Various marine mammals, particularly top predators such as pinnipeds, odontocete cetaceans (Tanabe et al., 1988; United Nations Environment Programme (UNEP), 2001) and polar bears (*Ursus maritimus*), are potentially “at risk” due to contamination (Fig. 1). Indeed, both reproductive and nonreproductive toxicities have been found in many fish-eating mammals that live in riverine or coastal areas where contaminant burdens are generally higher than in the open ocean.

Some reptiles, which have environmental sex determination and differentiation, are particularly susceptible to EDCs and perhaps other chemicals. Depending on chemical and dose, exposed organisms can be sex-reversed, or can exhibit intersex or imposex. Alligators exposed to pesticides in Lake Apopka, Florida (USA), and red-eared sliders (*Trachemys scripta*) and snapping turtles (*Chelydra serpentina*) exposed to OC compounds in the wild, exhibit decreased populations that are correlated with altered endocrine system, gonad, and liver function and altered anatomical structure (Guillette et al., 1995, 1996, 1999; de Solla et al., 1998).

Birds provided the classic case of endocrine disruption resulting in eggshell thinning and reproductive failure (Risebrough, 1986). In the early 1960s, many studies showed an inverse relationship between concentrations of DDE or DDD in eggs and the thickness of the shell. The eggshells became so thin that when the birds sat on them to incubate, the eggs broke. Brown pelicans (*Pelecanus occidentalis*) in California and Florida showed significant shell thinning, leading to population declines (Anderson and Hickey, 1972; Anderson et al., 1975). In some years, the pelicans and other fish-eating birds raised no young.

Here we summarize some mammalian examples that provide cause for concern of different gender susceptibility to EDCs and reproductive alterations potentially related to these contaminants:

- Some cetaceans, particularly odontocetes, have detectable and sometimes extremely high levels of substances known or suspected to be EDCs such as PCBs, DDTs, chlorinated pesticides, brominated flame retardants and tributyl tin (TBT). There are several examples suggesting that exposure to OC insecticides and PCBs affect endocrine function and reproduction in marine mammals. For example, transformation of epididymal and testicular tissue has been observed in North Pacific minke whales (*Balaenoptera acutorostrata*, De Guise et al., 1995). De Guise et al. (1994) reported the identification of a true hermaphrodite in bowhead whales (*Delphinapterus leucas*).
- Another example is the endangered beluga whales (*D. leucas*) of the St. Lawrence estuary, who exhibit tumors and reproductive problems (De Guise et al., 1994; Martineau et al., 2002). Beluga whales from the St. Lawrence have high levels of PCBs and OC pesticides in their blubber (Hobbs et al., 2003; Gouteux et al., 2003), showing some of the highest burdens among marine mammals. These whales have also exhibited compromised immune systems, and impaired reproduction

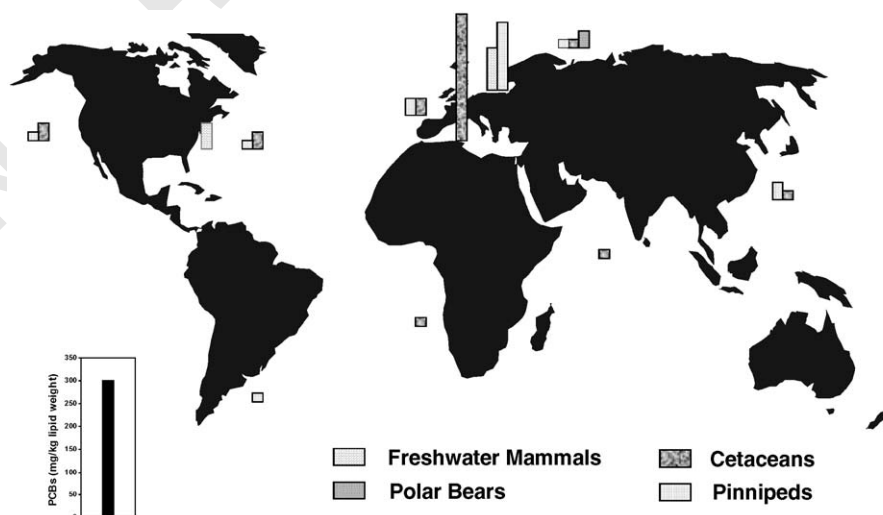


Fig. 1. Concentration range of total PCB levels ($\mu\text{g/g}$ lipid weight) in marine fish-eating mammals from various part of the world (by Fossi et al., in press).

(Beland et al., 1993). Laboratory mice fed diets containing beluga whale blubber from this region also showed reproductive impairment (Mendoza et al., 2003).

- Remarkable differences were found in OC levels between male and female Mediterranean cetacean species (Marsili and Focardi, 1996). OC levels are higher in males than females because females lose up to 90% of their total body burden of these xenobiotics during pregnancy and lactation.
- The polar bear is a top predator of the Arctic marine ecosystem. Pseudo-hermaphroditism observed in polar bears (*U. maritimus*) is thought to be an effect of EDCs. Two female polar bears at Svalbard (Norway) had both female and male genitals (Wiig et al., 1998). The two pseudo-hermaphrodites were genetically females but also had a small penis in front of their vagina. The observed rate of female pseudo-hermaphroditism in the Svalbard area was 1.5% (4/269). The authors believe that pseudo-hermaphroditism could be a result of endocrine disruption by OCs, especially PCBs, which concentrate to very high levels in fat.
- Populations of harbor seals (*Phoca vitulina*) from the Dutch Wadden Sea had low reproductive success and declining population numbers that were attributed to the impact of PCBs (Reijnders, 1980). Other studies showed that female harbor seals fed fish from the polluted Wadden Sea had a lower reproductive success (50%) than seals fed less contaminated fish. Implantation failure was found to be associated with lower levels of 17B-estradiol (Reijnders, 1990), induced by OCs.

3.2. Ecosystems at risk

Some ecosystems are more at risk from chemical exposure than are others. For example, species in tundra biomes are more at risk than those in temperate regions (Burger, 1997), and these may translate into even greater gender differences in susceptibility. Differences are due mainly to the shorter food chains in tundra regions, compared to other regions, and the lower species diversity within different species groups. Here we report some case studies of differential gender susceptibility in ecosystems heavily exposed to environmental contaminants.

3.2.1. The Mediterranean Sea

Man-made chemicals range across all continents and oceans. Some geographic areas are potentially more threatened than others (e.g., the Mediterranean, Fig. 1). Levels of some xenobiotics and heavy metals are much higher in the Mediterranean Sea than in other seas and oceans (Marsili and Focardi, 1996; Fossi et al., 2003). In this environment top predators (large pelagic fish and marine mammals) exhibit altered reproduction and development associated with high concentrations of polyhalo-

genated aromatic hydrocarbons (PHAHs) and toxic metals (Fossi et al., 2002).

For example, dramatic induction of typically female proteins, vitellogenin (Vtg) and zona radiata proteins (Zrp), were detected in several males of Mediterranean swordfish (*Xiphias gladius*). The data show values of Zrp and Vtg averaged higher in males or were in the same range as those of reproductive females, which suggests that this species is being exposed to xenoestrogens in the Mediterranean Sea (Fossi et al., 2001, 2002). These data, and those published by De Metrio et al. (2003) demonstrate a high percentage of intersex in Mediterranean swordfish, sound a warning about potential reproductive alterations in this large pelagic fish.

In Mediterranean cetaceans, a linear correlation between OCs known as endocrine disruptors and bioaccumulation processes and mixed-function oxidase (BPMO) activity (CYP1A1) was detected in striped (*Stenella coeruleoalba*) and common (*Delphinus delphis*) dolphin skin biopsies (Fossi et al., 2003). The main effect in these species was noninduction of BPMO in females with increasing levels of contaminants. A similar result was obtained in fin whales sampled in the Ligurian Sea (Marsili et al., 1998). This difference in the inductive capacity of skin BPMO activity between males and females of these species can represent a sign of a different gender-susceptibility to OCs but more research is required to elucidate the mechanisms.

3.2.2. The Great Lakes

The Great Lakes provides another example where a mixture of chemicals resulted in a range of physiological and behavioral abnormalities in several species of fish-eating birds. Gilbertson et al. (1991) summarized episodes of apparent PCB or polychlorinated dibenzo-*p*-dioxin (PCDD) effects on fish-eating birds in the Great Lakes. These included reports of embryo mortality, subcutaneous and pericardial edema, growth retardation, liver damage, aberrant breeding behavior, and developmental defects in gulls, terns and cormorants (Fox et al., 1991).

4. Factors influencing how chemical effects vary by gender

4.1. Exposure

Exposure assessment provides data on contact and uptake of substances (such as contaminants) in air, water, soil and food. Such data are essential not only for risk assessment, but for understanding pathways that can be altered or modified to reduce risk or adverse effects. Determining the potential pathways of exposure is the initial step in exposure assessment, and involves identifying the sources, pathways, and receptors of concern. The typical endpoint of exposure assessment is the dose to an organism, which for large animals (e.g., vertebrates) is expressed on a body weight or per kilogram basis (e.g., mg/kg/day). The latter has implications for gender because often males are heavier than females, and may eat different

types or sizes of prey (often with higher contaminant loads themselves; Burger et al., 2002a). Further, males and females may feed in different habitats, encountering different food types, and thus different contaminant loads.

Whether a particular pathway is applicable requires information on the contact of target organisms, including sensitive subpopulations (either males or females). Gender is another factor in exposure, just as young animals may have higher exposure because they are often restricted to nesting sites or brooding sites, adults may roam far from the point source (Burger and Gochfeld, 2001). Gender may influence exposure if one sex spends less time in a contaminated medium, or if one sex is less susceptible because they are protected genetically (physically or biochemically). Much less information is available on gender susceptibility than about many other aspects of exposure assessment (Burger et al., 2003).

The fact that an organism contacts a contaminated medium (e.g., ingestion of contaminated soil or prey) does not automatically result in transfer of 100% of the contaminant to the organism. The efficiency of transfer depends on two phenomena: intestinal absorption and bioavailability. The intestinal tract varies in the percent of a material (nutrient or contaminant) that it can pass through the intestinal wall into the blood stream. The absorptive capacity, difficult to measure, is influenced by age (young versus adult), transit time (rapid transit reduces absorption efficiency), and co-occurring materials (e.g., calcium inhibits lead uptake). Further, a chemical may be bound more or less tightly in different matrices, and the ease with which the matrix releases the substance determines its bioavailability. A variety of procedures exist for directly estimating bioavailability (Ellickson et al., 2001).

Some species are sedentary, others are migratory; thus exposure may vary in different places, and both the timing and the location of migratory paths must be known to understand exposure. For example, migratory terns (*Sterna* spp.) may spend the breeding season in the northern hemisphere, and migrate to the southern hemisphere for the winter. Since they molt on the wintering ground, breast feathers collected during the breeding season were most likely grown (and thus reflect exposure) during the winter (Burger et al., 1992).

4.2. Age and gender

In the wild, males and females may be exposed to different levels of contaminants during their lifetime. Bioaccumulation of chemicals and gender-susceptibility can depend on several factors, such as changes in metabolic rate, hormonal state, reproductive state and size variations. Here we summarize some studies of differences in gender bioaccumulation and susceptibility related to age of the organisms:

- Level of total mercury determined in the white muscle

tissues of swordfish (*X. gladius*) caught in the Azores ranged from 0.06 to 4.91 µg/g. Mercury concentrations were studied in relation to length, weight, age and sex and significant sex-based differences were found. The rate of mercury accumulation in males is significantly faster than in females and for medium-large sized fish, mean mercury levels were higher in males (Monteiro and Lopes, 1990).

- The role of OCs in the induction phenomenon of typically female proteins (Vtg and Zrp) in adult males of the Mediterranean swordfish (Fossi et al., 2001a,b, 2002) was supported by the correlations between Zrp levels in plasma and PCB concentrations in muscle and Vtg levels in plasma and PCB concentrations in liver of male specimens. PCB levels in liver were also correlated with total length of male specimens. These results suggest that high biomarker responses (Vtg and Zrp) in Mediterranean male specimens, increasing with the age of the fish, may be due to bioaccumulation of EDCs, such as OC compounds, during growth.
- Marsili and Focardi (1996) determined the age of 62 striped dolphins by counting dentine growth layer groups in the teeth and a growth curve was plotted. Correlations were detected between levels of OCs and age and sex of the dolphins. Old male specimens showed higher levels of OC than old female specimens.

4.3. Seasonality (periodicity)

The age-old question of nature vs. nurture has been raised many times throughout science in a wide variety of fields. However, it has never been more important than when considering the growth and development of wildlife. Physical parameters of the environment affect all pathways of life, including gene expression and metabolism. These events allow individuals to reproduce at the correct times, utilize nutritional resources during the proper season, and facilitate movement through the environment. Everything from the rutting of male deer, to the upstream migration of salmon, to the formation of antifreeze proteins in fish and the maturation or growth of accessory sex organs (ASO) in teleosts and gastropods, are controlled by physical signals and cues from the environment. Each of these events is regulated by environmental cues that elicit a cascade of signaling molecules that precipitate a set of physiological and behavioral changes. These physiological processes are complementary between the sexes with each gender (i.e., male and female), possessing identical sexually specific components. They allow for the synchronization of mating, growth and maturation of juveniles and metabolism of critically important endogenous and exogenous compounds.

Environmental factors such as photoperiod, temperature, tidal cycle and pheromones/kairomones (scents that attract or repel heterospecifics) influence the response of an

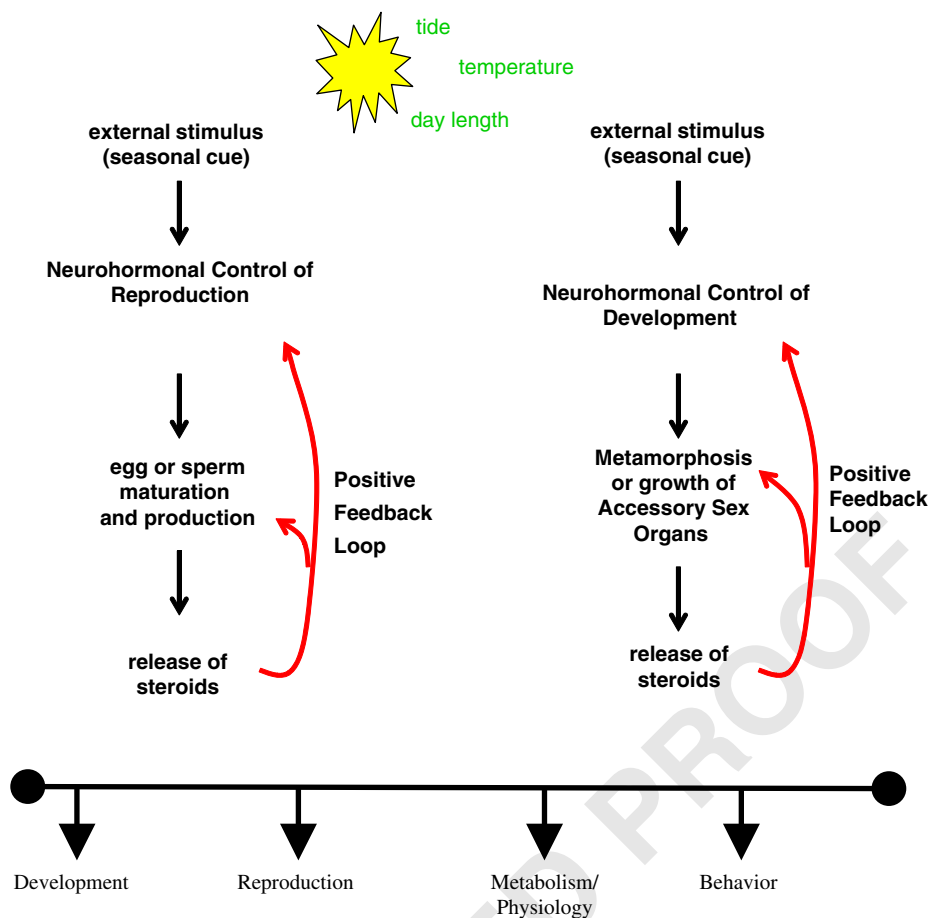


Fig. 2. Role of environmental cues on development and reproduction in wildlife.

organism to endogenous and exogenous compounds (Fig. 2). A range of signal responses and biosynthetic processes are specific for each gender type. Exposure to contaminants may alter the timing of these events through the delay or accelerated expression of a single gene or metabolic activity. For example, Chandler et al. (2003) showed that exposure to the pesticide fipronil causes a delay in molting and reproduction of male amphipods, yet similar effects were not observed in females. While this may appear to be of minor consequence, the reproductive cycle of these individuals is rather short, lasting approximately 5 days. In the case of amphipods, a slight shift in male maturation by 2 days during the reproductive period of the organism effectively eliminates 40% of the reproductive cycle. For organisms possessing a short life span, this has grave consequences for the stability and survival of the population.

Oberdörster et al. (2000) observed similar delays in molting and reproduction in grass shrimp (*Palaemonetes pugio*) exposed to pyrene. Male grass shrimp exhibited a lag in their molting periods and a significant delay in their mating activity. As a result, by the time males entered the breeding phase, the females were well into their breeding cycle. The end result was a decrease in the number of offspring produced by exposed males and a decrease in the

overall population number. Transfer of this gender-specific response to the field implies a drastic reduction in the local population and disruption of the ecosystem.

Furthermore, the seasonality of exposure of an organism to contaminants may also affect the metabolic response or clearance of the contaminant from the individual. Female mud snails (*Ilyanassa obsoleta*) exposed to the antifouling compound tributyltin during the regenerative phase of their reproductive organs exhibited a decrease in cytochrome P450 aromatase activity compared to nonexposed females (Oberdörster and McClellan-Green, 2002). However, when females were exposed during the breeding season (development of reproductive organs was complete), no detectable difference were evident.

The breeding season for these animals exhibit a yearly cycle with the reproductive phase occurring January–early April, followed by the regressive phase (late April–August) and lastly by the regenerative phase (September–December), during which the gonadal structures and ASO are reformed (Fretter and Graham, 1964). Both males and females follow similar patterns of sexual development and growth, yet females exposed to organotin compounds during the nonbreeding season alter their morphological development through the growth of a penis (Fig. 3), and in many instances, are not able to reproduce. The proposed

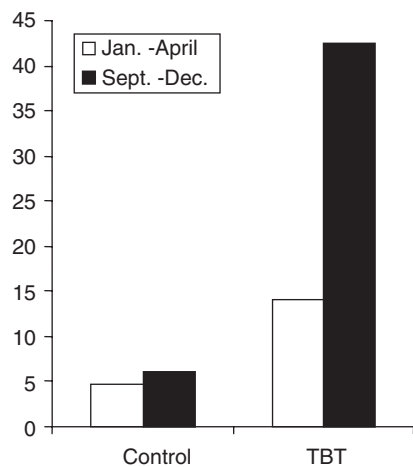


Fig. 3. Y-axis is percent of female mud snails (*Ilyanassa obsoleta*) that become imposex (the development of male sexual characteristics (i.e., a penis and a sperm duct) in female prosobranch gastropods) following a 45-day exposure to tributyltin during the ASO regenerative phase (September–December).

pathway responsible for this abnormal morphological growth is a disruption of the critical neurohormonal peptide produced in the ganglia of the female snail in response to the tributyltin exposure. Meanwhile, the males of this species, while experiencing increased penial development in response to organotins, do not appear to be affected in their reproductive capacity by the presence of the contaminants.

While it is obvious that many factors may affect the gender-specific action of contaminants on organisms in the wild, we have only presented a few select examples in this discussion. Each species or selective population has adapted to the specifics of their environment to utilize the resources available to them. The introduction of anthropogenic compounds into these systems can upset the balance of critical biological processes such as disrupting the reception or processing of environmental seasonal cues. These cascading mechanisms may be overcome through adaptive evolution or genetic variability.

4.4. Genetic and phenotypic variation

The genetic variation underlying phenotypic differences observed between males and females is rarely considered in the studies of environmental chemical exposure, uptake, effect, and excretion. One of the qualities of a healthy population is variation in the genotype of the individuals which comprise that population (Ricklefs, 1990). The female or male phenotype develops through an interaction between its genotype and environmental factors. Female and male organisms are adapted to their environment and are able to survive and reproduce within a certain range of environmental variables (Randall et al., 1997). We think of environmental factors as naturally occurring physical and chemical characteristics (e.g., photoperiod, temperature,

and pH), but today we must also include the presence of environmental chemicals (e.g., EDCs).

It is also important to consider the effects of EDCs on sex determination and sexual differentiation of normal gender differences (Orlando and Guillette, 2006, this volume). Sex determination of all vertebrate organisms appears to have a genetic component. In endotherms (birds and mammals), there are sex chromosomes which contain gene(s) that control the observed sexual dimorphism of the sexes. In fishes, amphibians, and reptiles, there are either sex chromosomes or a polygenic system of sex determination. Many of the genes involved with sex determination in mammals are similarly expressed in nonmammalian vertebrates. Fishes, amphibians, and some reptiles are closely connected to their environment, which plays a critical role in sex determination and sexual differentiation of some of these organisms. Interestingly, the teleost fishes, being the most numerous and most widely distributed vertebrate taxon, are also the most labile regarding their sex determination and differentiation.

The environment has a strong influence on the development of the female or male phenotype in most ectotherms. Temperature, in particular, determines the sex in some reptiles and fishes (Conover and Heins, 1987; Crews et al., 1994). What is of interest is the plasticity with which sex determination and differentiation in ectotherms functions. That is, external environmental factors can dramatically alter the fate of the ectotherm bipotential gonad, a phenomenon not observed in mammals, where genotype is the controlling factor. This may be an adaptation to the contact that ectotherms have with the environment that birds and mammals left behind in the adoption of endothermy from their Archosaurian ancestors (Price, 1996).

Gender differences in mammals and birds are affected by exposure during embryogenesis of organ anlagen to sex-specific hormones (Gilbert, 1997). We know that there are sexually dimorphic differences in behavior, gametogenesis, and sex steroid synthesis in the gonad, and degradation in the liver (Short and Balaban, 1994). Brain-regulated behavior is affected differentially. Female mosquitofish (*Gambusia holbrooki*) exposed to androgenic paper mill effluent placed in aquaria with nonexposed females display male-like reproductive behavior and exposed males display decreased reproductive behavior (Orlando and Guillette, 2006, this volume). Gonadogenesis in mosquitofish exposed to paper mill effluent and alligators (*Alligator mississippiensis*) exposed to pesticides show differential gender effects (Orlando and Guillette, 2006, this volume). Lastly, hepatic metabolism in some fishes (i.e., female and male fathead minnows, *Pimephales promelas*) and in some reptiles (i.e., female and male alligators) exposed to environmental chemicals exhibit gender-specific differences (Parks and LeBlanc, 1998; Gunderson et al., 2001).

The observed gender difference appears to depend on the nature of the EDC (i.e., whether the chemical is androgenic, estrogenic). For example, if the EDC is androgenic,

1 it generally seems to masculinize/defeminize females and
 2 depending on the concentration and organ, can either
 3 hypermasculinize males or demasculinize males, possibly
 4 through negative feedback to the brain and/or pituitary
 5 (Orlando et al., 2003). Also, gender differences in response
 6 to chemical exposure can alter genetic variation, thus
 7 affecting phenotypic variation by differential mutation or
 8 epigenetic mechanisms at the germ cell level (Swales and
 9 Spears, 2005; Anway et al., 2005).

10 We know that there are gender differences in the
 11 spontaneous mutation rates of gametes in humans, with
 12 aneuploidy being more common in oocytes and point
 13 mutations in spermatocytes (Crowe, 1999; Adler et al., this
 14 volume). Also, in mouse gametes exposed to chemicals, the
 15 mutation rates are lower in oocytes compared to sperma-
 16 tocytes. Importantly, however, in the zygote the maternal
 17 genome appears to be more susceptible to congenital
 18 malformations compared to the male genome. We know
 19 that most epigenetic regulation of gene expression (mater-
 20 nal vs. paternal) occurs during embryogenesis. Normally,
 21 epigenetic imprinting contributes to phenotypic variation
 22 observed in the offspring of the same parents, however
 23 little is known about the effects of exposure to environ-
 24 mental chemicals and imprinting. Gender differences from
 25 chemical exposure could result from differential imprinting
 26 rates of the female or male genome during gametogenesis.
 27 In a recent study, germ cells in male rats exposed in utero
 28 to a fungicide (vinclozolin) and an insecticide (methoxy-
 29 chlor) were affected by an epigenetic mechanism (methyla-
 30 tion). This transgenerational effect induced by known
 31 endocrine disruptors caused decreased spermatogenesis
 32 and infertility in the males, whereas there was no major
 33 effect on female rats from the same pregnancies (Anway et
 34 al., 2005).

35 Collectively, differential effects on the brain, gonad,
 36 liver, and gametes discussed in this section illustrate some
 37 of the differences in how female and male organisms
 38 respond to exposure to environmental chemicals. Exposure
 39 to environmental chemicals is often thought to decrease
 40 genetic variation, as the more sensitive individuals perish or
 41 are rendered nonreproductive from the exposure. However,
 42 there is a body of literature which shows that environ-
 43 mental perturbations or stressors which are transient and
 44 variable often result in actual increases in phenotypic
 45 variation (Forbes and Depledge, 1996; Holloway et al.,
 46 1997; Orlando and Guillette, 2006). It has been hypothe-
 47 sized that this variation in response may be an early stage
 48 in the evolution of a population (Holloway et al., 1990a, b).
 49 This increased phenotypic variation within a population
 50 may be the result of differential gene expression and
 51 represents plasticity in the biological response to environ-
 52 mental stressors. Reduction in genetic variation increases
 53 the vulnerability of wildlife populations. The examples
 54 given in this section suggest that there are gender
 55 differences in the contribution of this variation within
 56 populations and underscores the need for more research in
 57 this area.

5. Methods to examine gender-related differences

59 Traditional methods of examining gender differences
 60 involved primarily distinguishing males from females and
 61 examining organismal and organ effects. While this
 62 approach resulted in descriptions of effects on individuals
 63 and populations, it did not elucidate mechanisms. Re-
 64 cently, many new approaches and techniques have been
 65 developed to examine gender-related differences from the
 66 molecular to the population level. The methods to examine
 67 gender-related differences as a function of endpoints are
 68 shown in Table 1, and species-specific examples follow in
 69 Table 2. In both tables, references to the approaches or
 70 methodologies are provided.

5.1. Wildlife and risk assessment

71 Risk assessment is a formalized method of examining the
 72 potential harmful effects to organisms of stressors, includ-
 73 ing chemicals (National Research Council (NRC), 1983,
 74 1993), rather than the less formal approach to under-
 75 standing ecosystem damage (NRC, 1986). Ecological risk
 76 assessment (ERA), with a particular emphasis on exposure
 77 assessment, is a critical tool for understanding the risks to
 78 receptors within contaminated and disrupted ecosystems.
 79 There are numerous books and articles on ERA (Sheehan
 80 et al., 1984; NRC, 1986; Bartell et al., 1992; Cairns et al.,
 81 1992; Suter, 1993; Linthurst et al., 1995), but gender effects
 82 are generally lacking.

83 Partly the need for information on how chemical effects
 84 differ by gender comes from the necessity of including
 85 gender as a variable in risk assessments. Understanding the
 86 factors that make some individuals or sub-populations
 87 more vulnerable to a particular chemical (or other stressor)
 88 is critical to protecting and maintaining healthy wildlife
 89 populations. Although ERAs initially concentrated on
 90 simplifying assumptions regarding age and gender, it is
 91 increasingly clear that such differences dramatically affect
 92 uptake, biokinetics, and functional effects. Therefore, risk
 93 assessment models must take into account this variation.

94 Further, inclusion of gender in the selection of bioindi-
 95 cators may be critical for some monitoring programs. A
 96 bioindicator can be defined as any organism that responds
 97 predictably to contamination in ways that are easily
 98 observable and quantifiable. While ecologists should
 99 clearly select bioindicators that are appropriate for under-
 100 standing ecosystem health (Cairns, 1990), selecting indica-
 101 tors that are indicative of human health will make such
 102 indicators more biologically, technically, and societally
 103 relevant (DiGiulio and Monosson, 1996; Burger and
 104 Gochfeld, 1996a, 2001, 2004; Carignan and Villard, 2001;
 105 Burger, 2006). Bioindicators are equally important in
 106 assessing ecosystem (or individual species) recovery (Har-
 107 well and Kelly, 1990), the efficacy of management and
 108 remediation practices, and for long-term stewardship
 109 monitoring, as well as for monitoring degradation.

Table 1 Methods to examine gender-related differences as a function of endpoints				
Biological level	Measured endpoint	Test	Gender differences	References
Organism—behavioral	Sex specific behavior	Observation, photography, videography	Sexually dimorphic sex characteristics: size, sex-specific behavior, color, etc.	Kendall et al. (1998)
Organism—morphological	Length, mass	Micrometers, digitizing hard-/software	Different levels in males and females	Matson et al. (1993), Lloyd (2001)
Organism	Brain, gonad, liver/indices Contaminant levels	Chemical analyses		Burger et al. (1994, 2000a, b, 2001, 2002a, 2004a, b) Paez-Osuna et al. (1995) and Temara et al. (1997) Lloyd (2001)
Tissue/cell—morphological	Quantifying cell type	FACS	Ovarian, testicular germ and somatic cells	
	Tissue/cell physical measurement	Ocular micrometer, digitizing hard-/software	Sex-specific duct system	
Tissue/cell—biochemical	Cell/tissue abnormalities [Signaling molecules and receptors]	Histopathology Radioimmunoassays, radioreceptor assays, enzyme immunoassays HPLC-GCMS	Female E ₂ , P ₄ and 17,20-BP Male T, DHT, 11-KT	Humason (1997) Guillette et al. (1999) Nash (1999), Knobil and Neill (1999) Humason (1997)
	Signaling molecules and receptors localization	Immunohistochemistry	Female/male protein of interest	Lloyd (2001)
	Vitellogenin	Immunohistochemistry	Female/male protein of interest	
	Choriogenins		Male liver P450	Denslow et al. (2000) (Arukwe et al. (1998) Guo et al. (1993)
	Enzyme activity			
Tissue/cell—molecular	Single gene expression	Substrate degradation <i>In situ hybridization</i>	Female/male mRNA of interest	Rapley and Walker (1998), Lloyd (2001)
	Gene expression (1–4)	Real time reverse transcription polymerase chain reaction (rtRT-PCR)	Female/male mRNAs of interest	Bustin (2000)
Transcriptomics, proteomics, metabolomics	All or specific expressed genes or proteins (within whole cell, tissue, or organism)	Differential display mRNA Microarrays for DNA or proteins	Potentially powerful technologies to globally examine normal environmental chemical exposure and altered gender differences at the genomic scale	Denslow et al. (1999); Larkin et al. (2003a, b) Burgess and Hazelton (2000); Murphy (2002); van Steensel and Henikoff (2003); Brouwer et al. (2004) Michaud and Snyder (2002)
		Microarrays for proteins Laser-capture microdissection		(Burgess and Hazelton (2000); Ellsworth et al. (2003)

The importance of understanding gender-related differences in uptake, toxicodynamics, toxicokinetics, and all aspects of sexual dysfunction is clear from all the recent research with endocrine active substances (NRC, 1999; Damstra et al., 2002; Miyamoto and Burger, 2003). While the study of endocrine active substances began with the exposure of individuals to relatively high levels of estrogen, it has spread to include nearly all forms of life, natural and anthropogenic substances, and a variety of mechanisms of endocrine control. It will take many years to sort out the varied effects in different groups of organisms, and the differential effects of these chemicals on males and females, on developing fetuses, and older individuals. Because of the complexity of the current research findings with endocrine

Table 2 Survey of gender-related effects in various organisms by type of exposure or contaminant					59
Taxa	Organism	Environmental chemical/mixture	Gender effect	References	61
Cnidaria	Coral	Sewage treatment plant effluent			63
Arthropods		Environmental	[Cu] in female is greater than that in male	Zodle and Wittman (2003)	65
		Environmental	[Pb] in male is greater than that in female	Rabitsch (1995)	67
		Fipronil	Delayed molting, reproduction and development in males	Oberdörster et al. (1999)	69
Mollusks		Pyrene	Delayed molting and reproduction in males	Oberdörster et al. (2000)	71
	Mud snail	Tributyl tin	Morphological changes in females (penal formation), decreased egg production	Oberdörster and McClellan-Green (2002); Horiguchi et al. (2000)	73
	<i>Ilyanassa obsoleta</i>			Gooding et al. (2003)	75
	Mud snail	Tributyl tin	Altered T degradation and excretion	Gibbs et al. (1991); Oehlmann et al. (1996)	77
	Whelk <i>Nucella lapillus</i>	Tributyl tin	Phallus development in male affected		81
	Giant Abalone <i>Haliotis madaka</i>	Tin	Morphological changes in females (vas deferens growth)	Horiguchi et al. (2000)	83
Fishes	Florida gar <i>Lepisosteus platyrhincus</i>	Environmental	Female gonads had higher levels of manganese and selenium, and female livers had higher levels of manganese. Males had higher levels of mercury in gonads	Burger et al. (2004b)	85
	Walleye <i>Stizostedion vitreum</i>	Environmental	[DDE] in male is greater than that in female	Johnston et al. (2002)	87
	White sucker <i>Catostomus commersoni</i>	Paper mill effluent	↓ Plasma and synthesis of sex steroids, altered pituitary–gonad axis, ↓ stress response in both sexes. ↓ fecundity in males. ↑ MFO activity	Van Der Kraak et al. (1992); Munkittrick et al. (1994)	89
	Eelpout <i>Zoarces vivipraous</i>	Paper mill effluent	Male skewed sex ratios in embryos and altered plasma sex steroids	Larsson and Förlin (2002)	91
	Mosquitofish <i>Gambusia affinis</i> , <i>Gambusia holbrooki</i>	Paper mill effluent	Masculinized anal fin morphology, sex behavior, ↓ embryos, and ↓ size in females	Howell et al. (1980); Orlando et al. (2003)	93
			hypermasculinized anal fin morphology, ↑ testis mass in males		95
	Rainbow trout <i>Oncorhynchus mykiss</i> and	Sewage treatment plant effluent	Vitellogenesis and decreased testis mass in males	Harries et al. (1996); Jobling et al. (1996); Jobling et al. (2002)	97
Roach <i>Rutilus rutilus</i>	Sewage treatment plant effluent	Vitellogenesis and decreased plasma T in males	Folmar et al. (1996)	99	
Carp <i>Cyprinus carpio</i>	Sewage treatment plant effluent	Vitellogenesis and decreased plasma T in males	Folmar et al. (1996)	101	
Walleye			Folmar et al. (2001)	103	

Table 2 (continued)

Taxa	Organism	Environmental chemical/mixture	Gender effect	References	
		Sewage treatment plant effluent	Vitellogenesis and decreased plasma T in males		59
	Fathead minnow <i>Pimephales promelas</i>	Cattle feedlot effluent	Altered head morphometrics and decreased T synthesis in males	Orlando et al. (2004)	61
			↓ E ₂ /T in females		63
	Atlantic croaker <i>Micropogon undulates</i>	Pb	Altered H–P–G-axis	Thomas (1989); Thomas (1999)	65
			↓ Plasma and synthesis of sex steroids		67
		PCB mixture (Alachlor 1254)			69
			↑ Plasma and synthesis of E ₂		71
	Kelp bass <i>Paralabrax clathratus</i>	Cd PCBs, Cd, DDT	Decreased induced spawn, decreased GtH-II, decreased plasma estradiol	Spies and Thomas (1997)	73
	Swordfish <i>Xiphias gladius</i>	PCBs, DDTs	Induction of Vtg and Zrp in males	Fossi et al. (2001a, b, 2002)	75
	Bluefin tuna <i>Thunnus thynnus</i>	PCBs, DDTs	PCBs, DDTs	Fossi et al. (2002)	77
Amphibians	Reed frog <i>Hyperolius argus</i>	DDT	Demasculinization of larynx, skin coloration, ↓ plasma T and B in males	Hayes et al. (1997); Noriega and Hayes (2000)	79
	African clawed <i>Xenopus laevis</i> and leopard frogs <i>Rana pipiens</i>	Atrazine	Demasculinization of larynx, skin coloration, ↓ plasma T in males	Hayes et al. (2003)	81
	Cricket frog <i>Rana rugosa</i>	Dibutyl phalate	Ovarian development in males	Ohtani et al. (2000)	83
	African clawed frog	DES, EE ₂	Vitellogenesis and ↓ plasma T in males	Palmer et al. (1998)	85
	Red-spotted newt <i>Notophthalmus viridescens</i>	Endosulfan	Demasculinized pheromonal communication and behavior in males	Park and Propper (2002)	87
Reptiles	American alligator <i>Alligator mississippiensis</i>	Environmental	[Hg] higher in females than in males (in fat)	Burger et al. (2000b)	89
	Pine snake (hatchling)	From egg	[Mn] higher in females than in males	Burger (1992)	91
	<i>Pituophis melanoleucus</i>	Environmental	[As, Pb, and Mn] higher in females than in males	Burger et al. (2005)	93
	Water snake <i>Nerodia sipedon</i>	Environmental	[Pb] higher in females than in males (whole body)	Burger and Gochfeld (2004)	95
	Brown anole lizard <i>Anolis sagrei</i>	Environmental			97
	Red-eared slider turtle <i>Trachemys scripta</i>	PCB mixture	Sex reversal males to females	Willingham and Crews (1999)	99
	Snapping turtle <i>Chelydra serpentina</i>	PCB mixture	Feminization of precloacal length to posterior lobe of plastron in males	de Solla et al. (1998)	101
	American alligator	Dicofol, DDE, toxaphene,	Demasculinized phallus development and ↓ plasma and synthesis of T and sex steroid degradation in males	Guillette et al. (1994); Guillette et al. (1995); Guillette et al. (1996); Gunderson et al. (2001)	103
			↑ Plasma and synthesis of E ₂ /T and polynuclear		105

1 Table 2 (continued)

3	Taxa	Organism	Environmental chemical/mixture	Gender effect	References	59
5				oocytes/polyovular follicles in females		61
7				Sex reversal males to females		63
9			DES, EE ₂			65
11	Birds	Several species of sea birds	Environmental	Females higher than males for 13 metals and tissues. Males higher only for 6	Burger, 1995 this volume (1995). Burger and Gochfeld (1992a b)	67
13		Quail			Gochfeld and Burger (1987)	69
15		Herring Gull <i>Larus argentatus</i> , Cormorant, <i>Phalacrocorax auritus</i>	PHASh, PCBs in Great Lakes	Poor reproductive success, developmental abnormalities, behavioral deficits, enlarged thyroid glands and depressed thyroid gland hormone stores	Ottinger et al. (1999); Ottinger et al. (2002) McNabb and Fox (2003); Fox et al. (1978, 1991)	71
17						73
19						75
21	Mammals	Opossum <i>Didelphis</i> spp.	Environmental	[Pb] higher in females than in males	Burger et al. (1994)	77
23		Polar bear <i>Ersus maritimus</i>	PCBs, heavy metals	Pseudo-hermaphroditism in females	Norheim et al. (1992); Zhu and Norstrom (1993); Wiig et al. (1998) (CF)	79
25		Striped Dolphin <i>Stenella coeruleoalba</i> and common dolphin <i>Delphinus delphis</i>	PCBs, DDTs, HCB	BPMO (CYP1A1) induction in male skin biopsies—no induction in female	Fossi et al. (2003) (CF)	81
27		Fin whale <i>Balaenoptera physalus</i>	PCBs, DDTs, HCB	BPMO (CYP1A1) induction in male skin biopsies	Marsili et al. (1998) (CF)	83
29		Beluga whale <i>Delphinapterus leucas</i>	PCBs	True hermaphroditism	De Guise et al. (1994)	85
31		Bowhead whale <i>Balaena mysticetus</i>	PCBs	Pseudo-hermaphroditism in males	Tarpley et al. (1995)	87
33						89

35 active substances, and the differential effects on males and
 37 females, risk assessments must be completed for each
 39 species, in each situation. With more knowledge about how
 41 gender interacts with endocrine active substances on classes
 or groups of species, generalizations may be possible.

43 7. Conclusions

45 Numerous approaches and methods have been used in
 47 wildlife studies to examine how effects of chemicals differ
 in relation to gender. We consider that gender-related
 49 effects have been examined only when males and females
 have been directly compared under similar conditions.
 51 Analytical tools exist for examining mutagenesis, gene
 expression, biochemistry, physiology, development, pathol-
 53 ogy and behavior, all of which have consequences for
 organism well-being, reproductive success, and population
 55 stability. Some examples of the tools can be found in Table
 1. While there are many mechanistic studies being
 57 conducted in laboratories on a variety of wildlife, the
 diversity of wildlife make the tasks daunting. The most
 useful studies will be those that have both laboratory and
 field components to identify both the real-world effects on
 reproduction and populations, as well as mechanisms.
 There are some glaring holes in our information about
 gender-related differences in wildlife, including: (1) gender
 differences in exposure and fate of chemicals in fish in the
 wild; (2) higher accumulation of contaminants by female
 vertebrates despite additional excretion methods (through
 eggs, fetuses, and breast milk); (3) seasonal variation in
 gender-related differences in exposure, uptake and effects;
 (4) data on gender-related effects in response to endpoints
 in addition to endocrine disruption; and (5) a lack of
 information on the effects of chemicals on females,
 especially with endocrine active chemicals.
 More importantly, there are some clear examples where
 chemicals affect males and females differently, resulting in
 significant consequences for both individuals and popula-
 tion stability. For example, females seem to have higher
 levels of some heavy metals in tissues than males, leading to
 potentially greater effects. The mechanisms and effects of
 these differences need to be examined in laboratory
 experiments. When grass shrimp are exposed to pyrene in
 the laboratory, males exhibit a delay in mating activity,

effectively desynchronizing reproduction, thereby decreasing offspring production and increasing population instability. In species such as marine invertebrates, that may spawn only one or two nights in a year, consequences of destabilization could be significant. Such effects may be common in invertebrates, but require extensive study across a broad range of species, including higher level organisms, and chemicals.

The classic case of gender-related difference in response to chemicals comes from endocrine-active substances. More than 30 years ago the production of thin egg-shells in birds with high DDT levels led to low or zero reproductive success in fish-eating birds and some raptors, and consequently caused significant population declines. This ultimately led to a banning of DDT in many countries. Research over the last 10 years with endocrine active substances in a wide range of wildlife species has demonstrated disruption of secondary sex characteristics, reproductive cycles, parental behavior, and reproductive success.

There are other examples of gender differences in response to chemicals in other vertebrate groups. For example, fishes exposed to paper mill effluent (common sucker, eelpout, or mosquitofish) exhibit strong gender differences in effects including: decreased embryo or oocyte production, masculinized embryo sex ratio, decreased growth, and development of a "phallus-like" morphology and reproductive behavior in females; increased testis mass, hypermasculinized phallus morphology, and demasculinized reproductive behavior in males. Reptiles (alligators) exposed to pesticides in the field and laboratory exhibit strong gender differences including decreased estrogen/androgen steroid hormone circulating concentration and synthesis, polynuclear oocytes and polyovular follicles in females, and decreased circulating concentration, synthesis, and altered degradation of testosterone in males. These few examples indicate that there are gender-related differences in responses to chemicals that have significant biological effects, and should be taken into account by scientists, regulators, and policy makers, as well as the public.

8. Recommendations for bioindicators and biomarkers for chemical/radiological susceptibility by gender

1. While traditionally there have been numerous studies of contaminant levels in a wide range of wildlife species, relatively few have included gender as a variable. All studies of contaminant levels in organisms should specify gender differences where possible.
2. Some laboratory studies of wildlife do not distinguish or report sex. All laboratory studies with wildlife should include gender in the experimental design (it should be so stated if only one gender is used). To elucidate gender-related differences, future studies should include both sexes, in the same experiment, or in companion experiments.
3. There is a clear need to connect tissue contaminant

- levels, doses, and effects in both field and laboratory studies in both males and females. This will allow interpretation of the biological significance of field levels of contaminants.
4. There is a paucity of information on gender-related differences on fish and amphibians, and in a wide variety of invertebrates. Thus, significantly more attention should be directed at gender-related fate and effects studies with these groups.
5. While there are numerous studies of gender-related effects of endocrine active substances, far less attention has been devoted to the gender-specific effects of other chemicals in wildlife, particularly metals. All studies should consider the range of chemicals wildlife are exposed to, and not just one class of contaminants.
6. When comparing contaminant effects by gender, it is critical to examine the reproductive stage of the gonads, and the life stage of the organisms, because different life stages have different metabolic, genomic and hormonal profiles.
7. Phenotypic variation should be examined within a context of gender-related differences in response to chemicals.
8. The few studies of gender-based differences in invertebrates in response to chemicals show that some chemicals can cause destabilization of breeding cycles, underscoring a need for studies on a broader range of invertebrates specifically examining male and female differences.
9. Methodological tools that need to be developed include: (a) nondestructive biomarkers for endangered and threatened species; (b) cell culture models for study of gender-related susceptibility to chemicals; (c) easy and inexpensive techniques for gender-identification in the field; and (d) new molecular technologies for the analysis of gender-differences in global gene and protein expression, and metabolic processes.
10. There needs to be more focus on those wildlife species where gender-based differences in response to chemicals lead to significant biological effects. Such examples should be clearly brought to the attention of regulators and policy-makers.

In conclusion, our understanding of how gender alters the effects of chemicals on morphology, physiology, behavior, reproductive success, and ultimately on population dynamics, is in its infancy. There are data for only a few vertebrate species, and far fewer invertebrates, that clearly demonstrate the modes, actions and effects of gender. Gender studies will prove not only to be a fruitful area of research in the future, but is absolutely essential for understanding the mechanisms of chemical effects, and the well-being of wildlife populations.

9. Uncited references

Arukwe et al., 1998; Atkinson et al., 2003; Burger, 1993; Burger and Gochfeld, 1996b; Colborn et al., 1993; Folmar et al., 2001; Fossi et al., 1999; Fujise et al., 1998; Ruby et al., 2003.

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