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Topic 4.9

Wildlife as models for the study of how mixtures, low doses, and the embryonic environment modulate the action of endocrine-disrupting chemicals*

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Abstract: This paper will review briefly the use of wildlife as models in the study of how mixtures, low doses, and the embryonic environment modulate the action of endocrine active substances (EASs). In so doing, it will show how the issue of low dosages must be considered within the context of mixtures present in the environment and the endocrine background of the exposed individual. That is, in nature, EASs usually are found in mixtures in which the constituent parts are in concentrations well below their NOAEL (no observed adverse effect level) as determined in single compound studies in the laboratory. In addition, exposure always occurs on organisms in various endocrine states. Thus, the issue of mixtures and dosages must always be considered within the context of the endocrine background. Finally, the effects of exposure are passed down through the generations. The question of exposure then at the level of the individual becomes very complicated, as it must take into account that at every life stage, the naturally occurring endocrine milieu of the organism (or tissue), any EAS burden inherited from the mother or built up over the individual's life, and the social environment in which the individual develops and interacts as an adult, will influence the response to acute exposure.

INTRODUCTION

At any given time, organisms in nature are exposed to constantly varying mixtures of natural or man-made chemicals that can act as hormones or antihormones. Generally, these endocrine active substances (EASs) are present in the environment only at very low concentrations, a circumstance that along with the low hormonal activities most of them display has caused many researchers to discount such contamination as a present danger [1]. However, EAS activities tend to be assessed in laboratory experiments that almost exclusively investigate effects of single agents and not combinations. It is therefore

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conceivable that the potential of some of these EASs to produce adverse health effects when acting in unison may have gone unnoticed. Few studies attended to the mixture effect problem, and in almost all cases the question of agent interaction was reduced to the exogenous contaminants alone, while interactions with endogenous hormones were by and large neglected [2]. Confusion in the field is further heightened by the inconsistency with which possible agent interaction is conceptualized; only too often are terms like synergism, summation, potentiation, or additivity used variably. To understand the environmental endocrine-disruption problem, we need to shift our main focus from assessing single compounds to determining if, and how, EASs interact with each other, and classifying their interaction using a well-defined and generally accepted terminology. It is also essential to establish how they interact with the endogenous milieu present in the organism at the time of exposure. In particular for wildlife studies, identifying potential animal models in which such mixture effects could best be determined is a mandatory objective for the field.

Recent research has challenged some assumptions traditionally presupposed by risk assessment protocols in toxicology. For example, a variety of investigations have shown that EASs do not necessarily have to reach a concentration threshold before adverse effects can be observed [3–5]. Also, dose–response profiles of certain EASs are not always monotonic, but can have a nonmonotonic shape [3,6]. While tissue differences in sensitivity are appreciated [7,8], the equally important issue of differential genetic predisposition to sensitivity is another phenomenon that is normally not considered in traditional risk assessment studies [8,9]; since inbred laboratory rodent strains are roughly equivalent to individuals of a natural species, such findings are directly relevant to extrapolation to and from the wild. Together, effect threshold, dose–response curve shape, and species/individual/tissue sensitivity can vary not only with the chemical tested, but also with the endpoint used for the test [8]. All three parameters are also closely connected to the problem of combination effects, since the proper prediction of adverse health effects caused by exposure to a mixture of chemicals depends on an accurate analysis of the dose–response relationship of each individual agent to the investigated endpoint [10–12].

The main difficulty of assessing combination effects lies within the complexity and variability of the tested model system, which is particularly true for in vivo experiments and explains why most approaches to identifying mixture effects employ in vitro assays [12–15]. However, understanding the effects of EASs requires an appreciation of the complexity of biological organization and how the different levels might interact. This requires a research strategy that not only continually revises concepts within each level, but also informs studies at higher (or lower) levels.

Animal studies clearly show that in some cases contaminant interactions occur [2,16], yet the nature of these interactions remains elusive [10]. It is interesting to note that in these studies particular organisms are found to be better model species than others [16]. One major difficulty in studying combination effects has been the formulation of a null hypothesis that can be tested without knowing every mechanistic detail by which the effect occurs [10,17]. Once mechanisms are taken into consideration for a chemical mixture model, the degree of complexity of the system, and thus of the model itself, increases to unworkable proportions and the resulting model quickly becomes impractical. Further, deciding on the nature of an observed chemical interaction depends entirely on our current understanding of the underlying mechanisms and can change as this knowledge evolves [17]; for example, a "synergistic" interaction of two agents could thus quickly turn into zero-interaction of the two compounds [18]. Since the measured effect does not change with our knowledge of mechanisms, it is warranted to have empirical models available that can predict chemical interactions solely based on the measured effects. In such an "input/output" (i.e., dose of chemical/effect) approach that basically treats mechanism as a black box, the null-hypothesis is zero-interaction and any deviation from the prediction marks an interaction [19].

Empirical approaches to assess combination effects require certain necessary conditions that have to be fulfilled or else the models fail. This is best exemplified in a paradox pointed out by

Berenbaum [18]. If a chemical A at a certain concentration d_i has an effect E_i, the effect it should have at twice the concentration, $2(d_i)$, should therefore also be twice as great, or $2(E_i)$. However, if the dose–response profile for chemical A is sigmoid, the effect at $2(d_i)$ will be greater than expected. Since a chemical cannot synergistically interact with itself, the observed effect is not summative and yet not synergistic, either. It was shown that effect summation requires linear dose–response relationships, which are rarely found or only at extremely low doses of a chemical [19]. This example shows how complex the problem of mixture effects is, and how difficult it can be to define the exact nature of an agent interaction.

This paper tries to conceptualize the current problem of studying mixture effects, in particular how to study it in wildlife species. We will show that low-dose effects, internal hormonal milieu, and the social context are subcomponents of this problem and how they could be taken into consideration in future experiments. In the following section, five animal groups will be reviewed as potential models for combination effects studies, followed by a discussion describing some newly emerging principles important for the field.

MODEL SYSTEMS

Teleosts

Some of the strongest evidence for endocrine disruption in wildlife has been obtained in fish populations exposed to effluents from sewage treatment plants and pulp mills containing xenoestrogens and antiandrogens, as well as in regions heavily contaminated with organochlorine pesticides such as the Great Lakes [20–23]. For example, measurement of estrogen-inducible proteins such as vitellogenin in male fish has been shown to be an especially sensitive biomarker of the presence of low concentrations of xenoestrogens in complex contaminant mixtures in freshwater and marine environments [20,24]. Van Der Kraak and coworkers demonstrated in white sucker (*Catostomus commersoni*) populations in Lake Superior that exposure to bleached kraft (pulp) mill effluent caused alterations in the reproductive fitness due to delayed age to sexual maturation, decreased gonadal size, a reduction in the expression of secondary sexual characteristics, reduced biosynthetic capacity of ovarian follicles, and elevated ovarian follicular apoptosis [25–29]. Thus, teleost fish have been used extensively as sentinels for detecting EASs in the environment and as models for investigating their mechanisms and sites of action on the hypothalamus-pituitary-gonadal (HPG) axis to disrupt reproduction. Knowledge gained from such field work along with substantial laboratory investigations on the underlying mechanisms by which EASs affect endocrine processes in teleosts provide an excellent basis from which the study of combination and low-dose effects can proceed.

A good example for how teleosts can play a pivotal role in elucidating EAS effects is an otherwise often ignored mechanism of endocrine disruption, that of neuroendocrine toxicity. Recent research in the Atlantic croaker (*Micropogonias undulatus*) by Peter Thomas and colleagues has shown that various EASs including organochlorine pesticides, PCB mixtures, and lead act at the hypothalamic level thereby impairing reproductive endocrine function [30,31]. In particular, it is changes in neurotransmitter concentrations in response to exposure to these neurotoxic chemicals that alters neuroendocrine function and ultimately disrupts reproduction. It could be demonstrated that the reproductive and neuroendocrine impairment in croaker after chronic exposure to environmentally relevant concentrations of the PCB mixture Aroclor 1254 (A1254) is associated with a reduction by 30–35 % of serotonin (5-HT) levels in hypothalamic regions involved in the neuroendocrine control of gonadotropin secretion [30]. Serotonin positively affects luteinizing hormone (LH) secretion in croaker by increasing gonadotropin releasing hormone (GnRH) production and synthesis of GnRH receptors in the pituitary. The decrease in hypothalamic 5-HT levels was due to inhibition of tryptophan hydroxylase, the rate-limiting enzyme in 5-HT synthesis, whereas the PCB mixture does not affect monoamine oxidase, the enzyme that converts 5-HT to its inactive metabolites. These studies suggest that the reproductive disruption occurs

through a reduction of GnRH and its receptors in response to the lowered 5-HT activity. This was supported by the observation that the loss of reproductive function could be restored with an implant of GnRH or by treatment with 5-hydroxytryptophan, which bypasses the biosynthetic step catalyzed by tryptophan hydroxylase and restores 5-HT levels [32]. Also, treatment with the specific tryptophan hydroxylase inhibitor *p*-chlorophenylalanine mimicked all the neurotransmitter and neuroendocrine effects of the PCB mixture, giving further credibility to this hypothesis.

Although this work with Aroclor 1254 addresses the effects of a combination of EASs (i.e., PCBs), no conclusions about possible interaction of the individual constituents can be drawn. In order to establish whether or not the individual PCB congeners interact to cause the overall effect and, if so, what kind of an interaction they display, it is necessary to determine the dose–response profile for each of the major PCB congeners alone. Nonetheless, the work on the Atlantic croaker establishes useable endpoints that, along with the developed methodology, could be employed to assay the individual PCB congeners of Aroclor 1254 and thus determine whether a combination effect exists. It would then also be possible to characterize the type of interaction found in this particular PCB-mixture.

There is now convincing evidence that, in addition to the classic genomic mechanism of steroid action via binding and activation of nuclear steroid receptors, steroids also act at the cell surfaces of target tissues to initiate rapid, nongenomic responses, and that these actions are mediated by steroid membrane receptors. Several recent studies in teleost models have provided the first clear evidence that nongenomic steroid actions, like genomic ones, are susceptible to interference by xenoestrogens [33], and that this involves binding of these compounds to steroid membrane receptors [34]. Competition studies showed that the xenoestrogens Kepone and *o,p′*-DDD cause concentration-dependent displacement of progestin binding to the oocyte progestin membrane receptor over the range of 10^{-4} to 10^{-6} or 10^{-7} M, and also cause inhibition of progestin-induced oocyte maturation in vitro over the same concentration range (equivalent to 20–40 ppb, a tissue concentration frequently reported in fish from contaminated environments). Xenoestrogens can also interfere with the nongenomic actions of progestins to stimulate sperm motility as well as estrogens to inhibit testicular steroidogenesis in teleosts by binding to the membrane receptors thought to mediate these effects [35]. Taken together, these studies indicate that nongenomic steroid actions may be as susceptible to endocrine disruption by chemicals as are genomic actions and warrant further study. Possible interactive effects of individual chemicals in mixtures on this mechanism of endocrine disruption have not been investigated, although a complex mixture of polycylic aromatic hydrocarbons present in a water-soluble fraction of No. 2 fuel oil, has been shown to interfere with the nongenomic action of progestins to induce oocyte maturation in croaker [36].

Although there is now substantial evidence that a broad range of EASs can impair processes critical for the recruitment of fishes such as reproductive output and larval survival, comprehensive and reliable predictions of the impacts of environmental exposure to EASs on the size of fish populations are currently lacking. Density-independent mechanisms such as pollution often determine recruitment success at low spawning stock levels. In these cases, even small changes in fecundity and mortality rates of eggs and larvae may result in an eventual decline in population size. Currently, an interdisciplinary effort is underway to predict the population consequences of exposing croaker to environmentally realistic, low sublethal concentrations of EASs, both as mixtures and individual compounds. Integrated assessments of endpoints of critical stages of the reproductive cycle including sexual differentiation, production and maturation of the gametes, fertilization, and larval survival and ecological performance, are inputs into individual-based and matrix projection population models. Physiological models are being developed to integrate endocrinological, biochemical, and morphometric assessments of reproductive function at each critical adult stage. For example, plasma gonadotropin, estradiol-17β, vitellogenin, hepatic estrogen receptor concentrations, ovarian size, oocyte size distribution, and fecundity can be measured during the period of gamete production and gonadal growth in females to obtain an overall assessment of reproductive and endocrine functions at this stage. The endocrine and reproductive effects of EASs are often subtle and complex, involving multiple components of the reproductive endocrine

system. This complicates the interpretation of both laboratory and field studies on EASs with the result that the potential long-term population consequences of environmental exposure to these compounds are difficult to predict. A variety of approaches, such as the modeling one described above, will be required to obtain a comprehensive understanding of risk of environmental contamination with EASs to fish populations and aquatic communities.

Amphibians

In light of current global declines in amphibian populations and their potential link with environmental contaminants, it is astonishing that effects of EASs on members of this taxon have not been examined more extensively. This lack of basic information is particularly surprising since their large clutch sizes, well-studied embryonic development, partially aquatic life style and complex life cycle of amphibians make them ideal model organisms for screening EASs, both singly and in combination.

In a comparative approach using multiple endpoints in a variety of species, Tyrone Hayes has found that exposure of amphibians to naturally occurring steroids like corticoids, estrogens, androgens and thyroid hormones causes dose-dependent differential responses that vary between species or even between developmental stages within a species [37–39]. For example, exogenous estradiol causes all young to develop as females in the South African clawed frog (*Xenopus laevis*), has no effect in the boreal toad (*Bufo boreas*) and, depending upon the dose, produces all males or all females in the leopard frog (*Rana pipiens*). Further, while it induces vitellogenin production in adult individuals, it does not affect vitellogenin synthesis in larval South African clawed frogs. In another species, the African reedfrog (*Hyperolius argus*), exogenous estrogens induce a color change (from a green dorsum to a reddish background with white spots), within androgens induce vocal sac development, and thyroid hormones induce tail reabsorption [40]. All of these changes can be produced by exposure over a six-day period. A survey of various compounds revealed that several steroidal estrogens induce color change as well as several synthetic estrogens (e.g., ethynyl estradiol, DES) and that tamoxifen blocks the color change when coadministered with estradiol. In addition, several phytoestrogens and various pesticides have estrogenic activity [41]. A number of androgens have also been examined for their ability to induce vocal sac development and the goitrogen, thiourea, has been shown to inhibit tail reabsorption in this species. In the South African clawed frog and the leopard frog, gonadal differentiation/sex ratio, laryngeal size, and time to metamorphosis are used as a measure of estrogen, androgen, and thyroid hormone activity, respectively [42].

In addition to documenting sexual chimeras in nature and relating their incidence with levels of the herbicide atrazine, Hayes has studied the effects of atrazine in the laboratory, finding that in the South African clawed frog it produces demasculinized and feminized gonads, hermaphrodites, and demasculinized secondary sex characters [42]. There is evidence suggesting that these effects are mediated by induction of the aromatase enzyme and not by direct interference of atrazine with the estrogen receptors [42]. Interestingly, the effects on the gonads in the South African clawed frog were produced at a dosage of 0.1 ppb, or more than 600 times lower than effective doses in in vitro mammalian studies and more than 30 million times lower than doses effective in in vivo mammalian studies. Similar effects are observed in the leopard frog, where larvae exposed to 0.1 or 25 ppb of atrazine result in hermaphrodites as well as males with testicular oocytes.

Large numbers of newly metamorphosed leopard frogs with malformed hind limbs discovered by middle school students on a field trip in rural Minnesota started a controversy about the possible link of such aberrations to EAS exposure. The frogs exhibited a variety of malformations of the hind limbs, including supernumery of bones, bone bridging and rotations, hypertrophy and hyperplasia of bones, but the majority of animals had truncated limbs, reduced bone segments and/or elements [43]. Based on their observations in a large integrative study, Loeffler et al. [44] concluded that "taken together […] the data suggest that multiple causal agents converge in varying combinations, proportions, exposure times, and sequences upon different developmental stages of the limb bud to produce the range of ob-

served malformation phenotypes". Such a synergism between trematode infection and pesticide exposure as cause of limb deformities has recently been suggested by Kiesecker [45]. In field and laboratory experiments, Kiesecker observed that parasite infection with the trematodes *Ribeiroia sp.* and *Telorchis sp.* was necessary for limb deformation in the wood frog (*Rana sylvatica*), but that previous exposure of tadpoles to low doses (based on EPA maximum contaminant levels for drinking water) of herbicides or insecticides increased the rate and degree of deformities. Kiesecker concluded that EAS exposure may decrease immunocompetency of exposed animals, thereby making them more susceptible to parasite infections, which may also explain why deformities have been reported for almost three centuries [46], and yet only recently dramatic increases in their occurrence have been observed.

On the other hand, the predominance of limb truncations has also been discussed as a possible effect of exposure to EASs that act similar to retinoic acid (RA). A natural derivative of vitamin A, RA is known to cause skeletal reduction most likely via the induction and maintenance of such genes as sonic hedgehog (Shh) and *Hox* genes in the developing or regenerating limb. Hence it has been suggested that certain EASs may cause limb truncations either by acting like RA binding to its receptors (RARs and RXRs) or by heterodimeric activation of the RA receptors. Bruce Blumberg (Topic 4.6) has isolated a compound from water of a Minnesota lake as a lead suspect potentially responsible for such an RA-like activity. Other possible causes that have been discussed (e.g., predation of limb buds in tadpoles, UV radiation) seem not to explain the complexity of the observed effects sufficiently.

Reptiles

Reptiles are particularly suitable models for studying the effects of environmental contaminants. A wide geographic distribution, presence in a variety of habitats, longevity, and carnivore lifestyle makes them reliable biomonitors. Furthermore, reptiles exhibit similar sensitivity to contaminants as have been reported for birds and mammals [47]. There is a substantial amount of information on the effects of EASs in reptiles available today, due largely to the seminal work by Louis Guillette and coworkers on the American alligator (*Alligator mississippiensis*) of Lake Apopka in Florida (Topic 4.7). The red-eared slider turtle (*Trachemys scripta elegans*) has also served as an excellent model system for endocrine disruption on several levels. Not only has it allowed for organismal (sex determination) and physiological (circulating steroid hormone titers) studies, it has also proven an invaluable model for studying EASs singly, in mixtures, and in low doses. A certain advantage of this species and the alligator over more traditional animal models is the plasticity of the sex determination process. In the red-eared slider incubation temperature, not sex chromosomes, determines gonadal sex during the midtrimester of embryonic development, a process known as temperature-dependent sex-determination (TSD). In the red-eared slider, eggs incubated at constant temperatures below 28.6 °C develop as males, eggs incubated at or above 29.6 °C develop as females [48].

The current working hypothesis concerning the mechanism of TSD in the red-eared slider is that sex steroid hormones are the physiological equivalent of incubation temperature with incubation temperature affecting the expression of genes involved in sex steroid synthesis and the sex steroid hormone receptors. A variety of evidence supports this interpretation. For example, incubation temperature effects can be overridden (i.e., an individual's putative gonadal sex can be reversed) by applying exogenous steroids to the egg during the thermosensitive period of sexual development. Importantly, the gonadal characteristics of these hormone-determined females are indistinguishable from those of temperature-determined females. Incubation at higher temperatures produces female hatchlings, but administration of aromatase inhibitor—which effectively halts the production of estrogens—to the eggshell will result in 100 % males. In sum, application of steroid hormones, steroidogenic enzyme inhibitors, and other chemicals onto the eggshell during incubation can redirect the effects of incubation temperature exogenous estrogens induce female sex determination, while nonaromatizable androgens induce male sex determination [48,49]. Further, both categories of hormones have strong dosage effects and also synergize with incubation temperature to induce gonadal differentiation.

The red-eared slider has proven to be an exceptional in vivo model system for mixtures and low doses of EAS exposure. Mixtures have been tested in two studies of the red-eared slider. Initially, Bergeron et al. [50] examined the effects of different PCB compounds on sexual differentiation and identified PCBs that alone cause a significant increase of female hatchlings at an all-male producing incubation temperature (26 ºC). In combination, two of them synergized and increased ovarian development, whereas treatment with the individual PCBs required 10-fold higher concentrations. In a second study, eight compounds identified in the yolk of alligator eggs from Lake Apopka, Florida [51] were administered to red-eared slider eggs in the ecologically relevant concentrations identified in the alligator yolk. When all eight compounds were applied in a single-dose mixture, they significantly increased the ratio of females to males [51]. Results from single-compound exposures at the same dosages indicate that these compounds behave differently in combination than they do singly, again emphasizing the need for further studies using chemical mixtures reflecting proportions found in nature. Five of the compounds—the PCB mixture A1242, *trans*-nonachlor, *cis*-nonachlor, *p,p′*-DDE, and chlordane—altered sex ratio outcomes when applied to eggshells during development [51]. Aroclor 1242 produced the most powerful effects, shifting the ratio of females almost twofold, while chlordane had the greatest effect when combined with estradiol.

Research with the red-eared slider has challenged a central concept of traditional toxicology, namely the notion that a threshold (no observed adverse effect level, NOAEL) has to be reached below which adverse effects will not occur. Gaylor and coworkers [52] proposed the concept that a threshold dose may not exist when an exogenous molecule mimics an endogenous one by acting through the same mechanism. Given that a large number of turtle eggs can be manipulated at one time, it became possible to test this concept. First, in a retrospective analysis of published data on the effects of varying doses of estradiol at three different incubation temperatures on sex determination in the red-eared slider, Sheehan et al. [4] found that in each case the results fit the Michaelis–Menten model of a single protein-molecule interaction driving a reversible process. This in turn led to a larger study that determined that the Michaelis–Menten provided an ED_{50} of 5.0 with a 95 % confidence limit of ±2.0 ng (endogenous dose = 1.7 ± 1.3 ng; exogenous dose = 3.3 ± 1.7 ng) and an $r^2 = 0.90$ for fit of the modified equation. The lowest dose applied, 0.4 ng/10 g egg, increased the female fraction by 11.4 % beyond the temperature control. Considering that only 0.2 % of the estradiol applied to the eggshell ends up in the embryo [53], it becomes apparent that even very low dosages of steroid hormones or their mimics can have profound biological effects.

Other studies have determined the effects of low doses of xenobiotic compounds during development and after hatch [54]. Chlordane, a suspected antiandrogen in this species, does not affect aromatase activity in either the brain or the adrenal-kidney-gonad (AKG) complex of red-eared sliders. However, A1242 significantly increases aromatase activity levels in the red-eared slider brain—but not in the AKG—during a crucial developmental period. After this crucial period, A1242 causes an increase in aromatase activity in the AKG just prior to hatch. In complementary studies, basal steroid levels and steroid levels in response to follicle-stimulating hormone administration have been examined in hatchling males and females treated during embryogenesis with A1242, chlordane, or *trans*-nonachlor. Males treated with the A1242 or chlordane exhibit significantly lower testosterone levels than controls, while chlordane-treated females have significantly lower progesterone, testosterone, and 5α-dihydrotestosterone levels relative to controls. These results are similar to those reported by Guillette et al. [55] for juvenile alligators from Lake Apopka, Florida. Males treated with A1242 or *trans*-nonachlor display an elevated estradiol response to FSH administration vs. control males. Taken together, these results suggest that the effects EASs exert during embryonic development extend beyond birth. They also suggest the alterations in sex steroid hormone levels observed in animals from contaminated areas may result from EAS-induced alterations in the neuroendocrine axis controlling gonadal sex steroid hormone production. Whether the strong effects observed with A1242 are due to the interaction between the mixture's various PCB congeners remains unclear. However, as with the A1254 studies in fish, the results of the A1242 experiments with the red-eared slider provide a good starting point from which to further

investigate possible effects of the individual mixture constituents, which then allows evaluating the nature of the interaction.

Recently, we extended these studies to the polyhalogenated aromatic hydrocarbons (PHHs) PCB-126 and dioxin (TCDD) [56]. While there was not a significant sex reversal at the dosages used (PCB-126 50, 500, and 5000 ng; TCDD 5, 50, 500 ng), it was possible to evaluate the delivery of these compounds to different compartments over time. Using the spotting method, eggs were treated at the middle of the sensitive period and analyzed 16 days after application. Results indicate that 90 % of PCB-126 and 96 % of TCDD was retained in the shell. For PCB-126, that portion of the total amount transferred across the shell (10 %, 705 ng) was distributed as follows: albumin - 14 %; yolk - 70 %; and embryo - 16 %. The total amount of TCDD transferred across the shell (4 %, 16 ng) was similarly distributed: albumin - 20 %; yolk - 55 %; and embryo - 25 %. Finally, this research revealed that the distributions of PCB-126 and TCDD in the interior of the egg were not proportional to lipid content. Rather, there was evidence of greater accumulation in both the albumin and the embryo relative to the yolk, suggestive of selective binding.

Finally, while there has been much attention paid to different forms of the estrogen receptor, it is equally important to keep in mind that there are multiple forms of estrogens and that each may have different binding affinities. For example, in the red-eared slider, estriol (E_3) is ten times more potent than is estrone (E_1) or estradiol (E_2) in overcoming the effects of a male-producing incubation temperature [16,49]. This difference is clearly evident when comparing either the dosage at which 50 % of the embryos are sex-reversed or the magnitude of the regression coefficients from statistical models. However, while E_3 is more potent than is E_2 and E_1 at reversing gonadal sex in the red-eared slider, it is less likely to synergize with temperature to reverse gonadal sex. These variations in dosage effects and synergy of different natural estrogens may be caused by differential affinity of the estrogen receptor for different ligands (i.e., higher affinity for more polar estrogens), cooperative binding in response to certain ligands (i.e., cooperative binding with more polar estrogens), and/or differential transactivation of downstream genes in the ovary determining cascade by certain ligands (i.e., more polar estrogens induce greater transcriptional activity). If any of these events are occurring, they could result in a synergistic increase of response at lower doses. These results have implications for the developmental mechanisms underlying sex determination and its sensitivity to the physical and chemical environment.

Birds

Some of the earliest observations on the endocrine impact of manmade environmental contaminants were made on birds, as may be best exemplified by Rachel Carson's milestone work *Silent Spring* [57]. In it, Carson suggested that the insecticide DDT might have caused the decline in passerine populations in the United States, a hypothesis that was soon thereafter confirmed. While Ratcliffe [58] in the late 1960s could only show that eggshell thinning in birds in England correlated with the onset of the local commercial use of DDT, others subsequently demonstrated that *o,p′*-DDT was indeed estrogenic and that it was responsible for the decline in shell thickness in many species [59]. It was also observed that in birds, exposure to *o,p′*-DDT caused feminization of embryos, alterations of sex ratios and breeding patterns, malformations, and abnormal sexual behavior [60,61]. Shortly thereafter, studies at the Great Lakes identified yet another source for endocrine disruption in piscivorous birds, PCBs. As apex predators, many waterfowls consume and accumulate PCBs that have made their way through the food chain into the adipose tissue of lake teleosts. The degree of bioaccumulation in birds is directly dependent on factors such as congener content and composition of prey, sex and age, and residence time of individual animals in PCB-contaminated areas [62]. With its long half-life and its presence in egg yolk [63], the PCB congeners were cause for great concern, which unfortunately was confirmed when studies showed that PCB exposures in birds caused the Great Lakes Embryo Mortality, Edema, and Deformity Syndrome (GLEMEDS) [64], as well as other effects such as altered liver enzyme activity [65].

Interestingly, PCB contamination has been demonstrated to cause aberrant behavior in birds, including egg-destroying behavior in captive mallards (*Anas platyrhynchos*) and gray herons (*Ardea cinerea*), decrease in nest defense behavior and nest attentiveness in herring gulls (*Larus argentatus*) and glaucous gulls (*L. hyperboreus*), merlins (*Falcon columbarius*), and prairie falcons (*F. mexicanus*), prolonged and increased aggressive behavior in male ring doves (*Streptopelia risoria*), and increased frequency of male courtship behavior leading to a delayed clutch initiation in the kestrel (*F. sparverius*) [64,66–72]. In a study testing the effects of in ovo exposure to PCBs in kestrels, Fernie et al. [73] reported that embryonic exposure suppressed ovipositioning in females, delayed clutch initiation, and reduced clutch sizes and fledgling success in both males and females. Post-hatch oral exposure of zebra finch chicks with estradiol benzoate (EB), but not 4-octylphenol (OP), was shown to cause sex-specific impairments of adult reproductive performance, which in some instances were additive when both sexes were treated [74]. These impairments included reduced egg production and increased egg breakage, reduced fertility, and reduced number of hatched chicks in pairs treated with 100 nmol EB/g body mass. In another study, Quaglino et al. [75] demonstrated that posthatch estrogen exposure increased the volume of brain nuclei involved in controlling singing, thus masculinizing the brain of female zebra finches. Interestingly, even high doses of OP, methoxychlor, or dicofol (100 nmol/g body mass) did not cause similar effects.

In spite of the important seminal work, research on the effects of EASs in birds has comparatively fewer current contributions to the field of EAS research than do teleost, amphibian, reptile, or mammalian studies.

Mammals

Mammals will continue to be the models of choice for application to human health issues. Rodent studies were instrumental in elucidating how DES results in severe endocrine disruption. There now have been a number of studies demonstrating how exposure early in life to environmentally relevant doses of EASs can cause alterations of reproductive organs. While one cannot consider laboratory rodents as wildlife, they are useful in illuminating an important problem that must be considered in endocrine disruption studies with wildlife. That is, it is important to understand that the internal hormonal milieu serves as a background against which exogenous hormones will act. Recent studies with laboratory rodents indicate that the endogenous hormonal milieu prior to or at the time of exposure can markedly alter the effect of EASs. For example, differences among individuals in responsiveness to estradiol have been associated with minute variations of hormone levels during critical periods in fetal development as a function of the individual's position in the uterus. This effect of fetal position within a uterine horn relative to the sex of neighboring fetuses is referred to as the intrauterine position effect. Research indicates that differences in background levels of estradiol among adjacent embryos during embryogenesis play a pivotal role in how EASs alter development.

The intrauterine position phenomenon has been described in mice, rats, gerbils, and pigs, as well as in twin fetus pregnancies in lambs and humans [76–79] and is due to the diffusion of sex steroid hormones (testosterone and estradiol) across the fetal amniotic and chorionic membranes of adjacent fetuses [80]. In mice, male fetuses developing between two females (2F fetuses) are exposed to an approximately 30 % higher concentration of estradiol than males developing between two male fetuses (2M fetuses). 2F female fetuses are exposed to approximately 35 % higher concentration of estradiol than are 2M fetuses of the same sex [78]. Fetuses positioned beside only one male fetus (1M fetuses) have more intermediate steroid hormone concentrations. These small differences in estradiol result in significant differences in the course of development and subsequent morphological, physiological, and behavioral characteristics [3,81–84].

The extreme sensitivity of the fetus to small differences in endogenous estradiol strongly suggested that humans and wildlife could be influenced by endocrine-disrupting chemicals even at the relatively low exposure levels typically encountered in most environments [85]. For example, prenatal ex-

posure of male mice to environmentally relevant levels of bisphenol A [86–88] significantly alters the structure and function of the reproductive organs. Adult male offspring of CF-1 mice fed bisphenol A during their pregnancy have, relative to control males, significantly larger prostate and preputial glands, decreased weights of the seminal vesicles, epididymides and testes, as well as decreased sperm production [89,90]. Other reports of low-dose effects of bisphenol A in rodents include an accelerated rate of embryonic development, changes in the mammary gland, vagina, prostate, sperm production, epididymis, and pituitary response to estradiol (reviewed in Palanza et al. [91]).

Female young of pregnant mice fed 0 or 2.4 mg/kg bisphenol A in corn oil vehicle during days 11–17 of gestation are significantly heavier at weaning than control females [92]. Interestingly, the bisphenol A-exposed females with the highest background levels of estradiol in utero (2F females) are significantly heavier than the 2F control females, while bisphenol A-treated or control females exposed to the lowest levels of background estradiol in utero (2M females) show no difference in body weight at weaning. The body weight at weaning of females exposed to intermediate levels of background estradiol in utero (1M females) is also significantly heavier than their control 1M counterparts. Similarly, the body weight at weaning for bisphenol A-treated, 2F and 1M males are also significantly greater than control male pups from the same intrauterine positions [93].

Prenatal exposure to bisphenol A significantly accelerates the timing of puberty in female mice by decreasing the number of days between vaginal opening and first vaginal estrus [92]. An analysis according to intrauterine position indicates that 0M females show the greatest response to prenatal bisphenol A exposure, with the interval between vaginal opening and first vaginal estrus shortened by approximately 5 days relative to 0M controls. In contrast, bisphenol A-treated 2M females showed a similar timing of puberty as the 2M controls. The age at vaginal opening does not differ based on prenatal treatment or intrauterine position, which is expected since vaginal opening is not a marker of puberty in laboratory strains of mice [94]. The influence of background levels of sex steroid hormones due to intrauterine position has also been observed with regard to the effect of dioxin on prostate development in male rat fetuses. A single injection of dioxin (1 mg/kg) to pregnant rats on gestation day 15 resulted in a significant disruption of prostate development in 0M male fetuses, but no effect on prostate development in 2M males was observed [95].

GUIDING PRINCIPLES

From the discussion so far there emerge some principles that could guide future research on endocrine disruption in particular in wildlife populations. They can be divided into theoretical, experimental, and philosophical principles.

Theoretical principles

Combination effects have been characterized in various ways depending on different concepts. We believe that the vagueness with which terms like “synergism”, “additivity”, or “antagonism” have been used demands clarification and general agreement on definitions that could be used to guide future studies on mixtures. Obviously, definitions of different interaction types depend greatly on what type of model is used to determine agent interaction to begin with. As discussed above, empirical models that ignore underlying mechanisms have been suggested by some [18], while others give mechanistic approaches priority. Which of the two approaches is more promising and practical is a matter for debate; here we only want to emphasize the fact that without proper modeling of chemical interaction, combination effects cannot be determined reliably.

The complexity encountered in studies of EAS effects on the organismal level is reflected also on the cellular level, where actions via mechanisms other than nuclear steroid receptor binding of EASs can cause disruptions. For example, there is now convincing evidence that steroids also act at the cell surface of target tissues to initiate rapid, nongenomic responses and, further, that these actions are me-

diated by steroid membrane receptors. Such new findings have to be taken into consideration when EAS actions are being modeled and experimental data analyzed.

Although genetic predisposition, internal milieu, and molecular processes have been shown to be essential for an organism's responsiveness to EASs, epigenetic factors are equally demonstrable and need to be considered. This would include the aforementioned intrauterine position phenomenon, where very small increases in background estradiol, early in development, substantially increase the sensitivity of the female mouse to bisphenol A. It is likely that similar phenomena occur in other mammals, including humans. Even in species that characteristically have singleton births the fetus may experience similar epigenetic effects, as studies with humans have indicated. Endogenous levels of sex steroid hormones also vary among individual human fetuses due to a variety of factors such as the first vs. subsequent pregnancy [96], twin vs. singleton birth, [97,98], and race [99]. As a result, individuals with increased levels of background estradiol during development may form a sensitive subpopulation particularly susceptible to the effects of EASs on postnatal growth rate and puberty.

There also appears to be a tendency to regard the individual as separate from the social environment in which it develops. This is a mistake. For example, Adkins–Regan [100] has found that the hormones an individual is exposed to early in development as well as the social context in which it is reared are critical in the development of an individual's preference of a sex partner when adult. As others before her, she finds that if zebra finch nestling or embryos are treated with estradiol or fadrazole, an aromatase inhibitor, as adults they will be masculinized in their behavior and will prefer to pair with other females even if potential male partners are available. However, the zebra finch is a highly social species. Both parents rear the young, and they grow up in a mixed sex society. If adult males are removed from the breeding cages, so that young females are not exposed to male birds or to other male-female pairs during development, they also will no longer prefer males as sexual partners. What this suggests is that the developmental context can accentuate the effects of early endocrine disruption. Skewed sex ratios and feminized males thus can have impacts beyond the actual endocrine disruption of the individual.

Similarly, it is well known that the "society of the litter" can profoundly affect the development of adult sexuality and its underlying neural substrates in rodents. There is a long history of research in developmental psychobiology showing that adult sexual behavior can be altered either by manipulating hormones early in life or by stressing the mother during her pregnancy. Meaney [101] has shown that it is the quality and amount of care a pup receives from its mother that mediates these effects. Not only is the stress reactivity of the pup affected by the maternal care it receives but, as on reaching adulthood, these pups exhibit altered maternal behavior toward their own young, thereby perpetuating the effect. Another consideration is how the mother may interact with young that have been feminized or demasculinized by EAS exposure in utero. Moore [102] has demonstrated that mother rats lick the anogenital region of male pups more than they do female pups and, further, that this difference accentuates the copulatory behavior of the pup when it reaches adulthood. Thus, the alteration of the sexual differentiation process by early endocrine disruption might be magnified by the quality of care the young receive from mother.

Experimental principles

Although otherwise not congruent, most models of agent interaction agree that evaluation of the consequences of exposure to a mixture requires an adequate dose–response analysis of each component chemical [10,19]. But what constitutes an adequate dose–response study seems controversial. It has been suggested that at least three different concentrations have to be tested to get a sufficient profile [103]. However, the number of concentrations analyzed is only one aspect of the dose–response analysis; its dose range is another. It may be advisable to not only test EASs with the traditional standard safety margin of one or two orders of magnitude below the concentration that causes observable effects, but instead to go far below and above it. Testing over 6- to 7-log dose ranges seems sufficient to estab-

lish an adequate dose–response profile. It is worth mentioning that the concentration range to be tested may vary with the specific endpoint monitored.

Besides addressing the problem of how to define agent interactions properly, future mixture effect studies will also have to test environmentally relevant rather than experimentally convenient EAS combinations. Testing compounds that may interact with the same molecular site can help to test the accuracy with which empirical models predict EAS interaction [13], but in order to assess the risk posed by EAS contamination it is important to emulate the real-world situation in laboratory studies. Ideally, of course, mixture effects should be approached in wildlife studies, but these will have to go hand-in-hand with laboratory studies. The latter are indispensable since they alone can provide data with which the possible interaction of different EASs can be characterized.

One objective of this paper is to suggest what qualities are needed in good models for assessing endocrine disruption primarily in the wild. In general, a good model species for assessing combination effects, low-dose effects and the interaction of endogenous and exogenous hormones is characterized by (1) having one or more physiological endpoint(s) highly susceptible to EAS exposure, (2) having an endocrine physiology at different stages of their life history that is well studied, (3) have a general biology that makes them susceptible to environmental contamination with EASs, (4) occurring in sufficient abundance to enable replicate studies, and (5) have a sufficiently short generation time to make transgenerational studies feasible. The species discussed here are examples of models that fulfill all five requirements.

Philosophical principles

We know only what we study and, as a result, we tend to study only that which we know. Until the field biologist understands what is involved with laboratory studies, and the laboratory scientist has familiarity with the uncertainty of the field, research on endocrine disruption will continue to be dominated by subdisciplines that ignore one another.

The priority assigned to mechanistic studies is not only theoretically a problem, but also philosophically. Assuming that the existence of low-dose effects or EAS interactions can only be established reliably by identifying their molecular mechanisms presupposes a vertical cause-effect relationship where one can extrapolate from one level of organization to the next. However, as much as molecular mechanisms are crucial, reliance on this view is overly simplistic, for it ignores the phenomenon of emergence, where the properties of the whole cannot be predicted based on the properties of its parts. Even if more were known about cellular events underlying endocrine disruption, one would still have to study the effect on an organismal level. This is manifest in what we term the "Common Sense" Principle. Namely, if one sees an effect on the organismal level but fails to find molecular correlates, or simply can't think of a mechanism to explain it, that does not mean the effect is not real.

Finally, the notion of mechanistic studies as the ultimate judge of the ongoing controversies in the field also bears the philosophical dilemma that it is an inaccurate test of the hypothesis. The null-hypothesis, that low-dose exposure does not cause measurable effects, can only be falsified by finding low-dose effects. But since exactly that has been confirmed, mechanistic studies cannot do away with it. What they can do is to further our understanding of how low-dose effects and EAS interactions work on the cellular level. Put into context of the organism, this information is of immeasurable value and importance.

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