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Organochlorine pesticides: Agrochemicals with potent endocrine-disrupting properties in fish

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Abstract

Organochlorine pesticides (OCPs) are persistent environmental contaminants that act as endocrine disruptors and system toxicants. These pesticides (e.g. dichlorodiphenyltrichloroethane (DDT), dieldrin, toxaphene, among others) are ranked as some of the most concerning chemicals for human health. These pesticides (1) act as teratogens, (2) are neuroendocrine disruptors, (3) suppress the immune and reproductive systems, and (4) dysregulate lipids and metabolism. Using a computational approach, we revealed enriched endocrine-related pathways in the Comparative Toxicogenomics Database sensitive to this chemical class, and these included reproduction (gonadotropins, estradiol, androgen, steroid biosynthesis, oxytocin), thyroid hormone, and insulin. Insight from the Tox21 and ToxCast programs confirm that these agrochemicals activate estrogen receptors, androgen receptors, and retinoic acid receptors with relatively high affinity, although differences exist in their potency. We propose an adverse outcome pathway for OCPs toxicity in the fish testis as a novel contribution to further understanding of OCP-induced toxicity. Organochlorine pesticides, due to their persistence and high toxicity to aquatic and terrestrial wildlife as well as humans, remain significant agrochemicals of concern.

Keywords

pesticide; reproduction; metabolism; fish; computational toxicology; adverse outcome pathway

1. Introduction

Organochlorine pesticides (OCPs) were originally developed to remove insects and other pests from agricultural fields and thus improve crop yields. The annual loss of crops in the

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absence of pesticide use was significant and has been estimated roughly at one-third of the annual production (Aktar et al., 2009). OCPs were designed to interfere with and inhibit unique physiological pathways in the target organisms. In fact, the scientist who developed DDT (dichlorodiphenyltrichloroethane), Paul Hermann Müller, received the Nobel prize in 1949 for his work, as DDT also killed the mosquitos responsible for malaria and typhus (Smith, 1999). However, at the time of their release into commerce, there was little knowledge regarding the off-target effects in other organisms including invertebrates and vertebrates. As knowledge about off target effects, such as thinning of bird egg shells with exposure to DDT, became evident (Peakall, 1970), OCPs were replaced by other pesticides and removed from commerce during the 1970's.

OCPs encompass a rather large group of agrochemicals including DDT and its metabolites, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD); methoxychlor, dieldrin, toxaphene, lindane, endosulfan, among others (Figure 1). Their structures are quite distinct, with the only commonality being the presence of chlorine groups. OCPs are highly lipophilic (Kow's > 4) and are resistant to microbial degradation and thus are persistent in the environment. Even so, many of the OCPs activate common nuclear receptors and elicit similar toxicity outcomes. For example, p,p'-DDE and methoxychlor are both weak estrogens and weak anti-androgens (Balaguer et al., 1999, Kelce et al., 1995, Sonneveld et al., 2005, Waters et al., 2001). They have been found to accumulate in fatty tissues in non-target organisms and are associated with a myriad of diseases, including cancer (Cohn et al., 2019). As a consequence of this, OCPs are at the top of the list of toxic substances compiled by the Agency for Toxic Substances and Disease Registry (ATSDR) within the United States

Department of Health and Human Services. OCPs are frequently in the top 60 chemicals on the list, with DDT at # 13, dieldrin at #18, DDE at #21, chlordane at #22, aldrin at #25, toxaphene at #32, endosulfan at #44 and methoxychlor at #55.

Because of their persistence in the environment, it is difficult to remediate sites that were heavily contaminated with OCPs from the 1950's to 1980's. Some of these sites have been designated by the US EPA as Superfund Sites, which merit specific attention for cleanup. Superfund sites were established as a consequence of The Comprehensive Environmental Response, Compensation and Liability Act of 1980 (Bearden, 2012), which required the federal government to determine relative risks of contaminants in designated areas and to develop a risk mitigation strategy to protect human health. A superfund site is designated as such if there are priority contaminants above critical concentrations that are related to disease. Superfund sites are found throughout the USA and OCPs are found at many of these sites. As an example, we present a map of superfund sites that contain concerning levels of dieldrin and toxaphene (Figure 2).

In the past 20 years, research groups around the world have documented the health effects of exposure to OCPs in human health (for a comprehensive review, please see (Mrema et al., 2013)) and in the environment. It is clear now that OCPs function as endocrine disruptors, interfering with critical hormonal signaling events in vertebrates and invertebrates. In this review, we are limiting our discussion to OCP effects in fish, as they

live in polluted environments and as vertebrates, they have conserved molecular pathways that function similarly in other vertebrates including humans. Studies indicate that many OCPs function as weak estrogens (Das and Thomas, 1999, Garcia-Reyero et al., 2018, Shanle and Xu, 2011) or anti-androgens (Kelce et al., 1997) and may also perturb other hormonal systems including those produced by the thyroid (Brown et al., 2004, Langer, 2010). They have been implicated as neuroendocrine disruptors, interfering with brain function, and several, including dieldrin, have been linked to the incidence of Parkinson's disease. These compounds also interfere with normal metabolism (Olsvik and Softeland, 2018), mitochondrial oxidative respiration (Cowie et al., 2017, Cowie et al., 2017), and immune function (Martyniuk et al., 2016a, Martyniuk et al., 2016b). Thus, OCPs can directly or indirectly affect endocrine systems through perturbations in hormone synthesis and metabolism, and ATP production. We discuss each of these interferences in this review, starting from early development into adulthood.

2. Organochlorine pesticides as teratogens in developing fish

The endocrine system is shaped early in development, and exposure to toxicants can disrupt the developmentary trajectory of endocrine tissues. Fish embryos are used to screen chemicals for teratogenic effects, and the impacts of OCP exposure have been measured in early developmental stages of different species. Early studies reported the effects of OCPs on mortality and deformity, but more recent efforts have sought to understand how exposure to OCPs in early development affects neurotransmitter systems, which regulate hormone release, and brain development. Owens and Baer compared responses of different stages of development of the Japanese medaka exposed to DDT at concentrations ranging from 3 to 400 ng (per egg), revealing a dose dependent increase in lethality (Owens and Baer, 2000). The study showed there was little difference in survival among groups at the early stages of development, but rather toxicity was highest when the larvae began to absorb their yolk sacs (Owens and Baer, 2000). Thus, OCPs may be sequestered in lipids in developing fish and later released upon reabsorption of yolk, inducing toxicity.

An array of teratogenic effects has been reported in developing fish with OCP exposure. In a study by Ton et al., developing zebrafish larvae were exposed to the organochlorine DDT and dieldrin, and several endpoints related to neuronal development were assessed at 48 and 96 hpf (Ton et al., 2006). A teratogenicity index was calculated as the ratio of dose that causes 50% mortality (LC50) over the dose that causes 50% developmental malformations (EC50), i.e. the ratio of LC50/EC50 (Ton et al., 2006). Scoring for malformations included, heart rate, rate of circulation of blood cells, number of red blood cells, edema, hemorrhage, ventricle swelling, brain necrosis, jaw formation, caudal embryo morphology, and motility. A teratogenicity index >1 was considered teratogenic. In this study, DDT had a relative teratogenic index of 3.5 at 96 hours post fertilization, whereas dieldrin had a value of 0.1 (Ton et al., 2006). These values were compared to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a highly teratogenic chemical which was given a score of 15.3.

DDT and dieldrin were also reported to slow the heart rates and induce tremors in early-stage zebrafish, as well as deplete cholinergic and dopaminergic neurons. This is relevant as acetylcholine and dopamine can stimulate or inhibit hormone releasing factors in the

central nervous system (CNS). In a more recent study investigating the effects of dieldrin on early life stages, Sarty et al. (Sarty et al., 2017) exposed both chorionated and dechorionated zebrafish embryos for 48 hours to μM concentrations of dieldrin, followed by a 6-day depuration period. Zebrafish did not exhibit significant mortality until they hatched, and as expected, dechorionated fish showed a higher rate of mortality compared to fish that retained the chorion early on. Moreover, the dechorionated fish showed reduced expression of dopamine active transporter and specific dopamine receptors. Similar to the study by Ton et al., cardiac hemorrhaging, skeletal deformities, and tremors were observed with increasing concentrations of dieldrin (Sarty et al., 2017, Ton et al., 2006). It is clear from these studies that early exposure to OCPs can affect neurodevelopment, and more specifically, may impact the ontogeny of the dopaminergic system.

Other OCPs have been studied for their effects in developing fish embryos. Kapp and colleagues investigated the potential for malformations and deformity in response to different chemical structures of the pesticide toxaphene (Kapp et al., 2006). Toxaphene is a complex mixture of compounds that are created by reacting chlorine gas with camphene. Exposures in the range of 15 to 20 mg toxaphene/L induced deformities that included yolk sack edema, loss of pigmentation, and spinal malformation toxicity. The study also noted that toxaphene prevented circulation of the blood through the heart, even though there was a heartbeat; however, the mechanisms as to why this occurred remains unknown. In a follow up study, Perez-Rodrigues and colleagues exposed zebrafish embryos to toxaphene from 6 to ~126 hours post- fertilization (5 day exposure) (Perez-Rodriguez et al., 2019). Morphological and molecular endpoints were assessed, and the study revealed an increase in deformity score (encompassing pericardial edema, skeletal defects, and yolk sack edema) with higher concentrations of toxaphene. Oxidative phosphorylation rates were also assessed in ~30 hpf embryos after a 24-hour exposure to toxaphene, and it was observed that non-mitochondrial respiration was significantly decreased with 11.1 and 111 μg toxaphene/mL. While these concentrations are not environmentally relevant, it is possible that under chronic exposure paradigms effects on respiration occur at lower concentrations. Transcripts involved in oxidative stress were measured and heat shock protein 70 was induced in larvae exposed to 1.11 μg toxaphene/mL (Perez-Rodriguez et al., 2019). It was hypothesized that reduced oxidative phosphorylation was associated with the higher deformity rates in the larvae, and that the induction of heat shock protein 70 may be a protective mechanism to mitigate toxicity during development.

The mechanism of action of OCPs in embryos can be complex, affecting respiration and neural development as noted above. However, OCPs are complex molecules with the potential to act as endocrine disruptors. Toxicity assessments in zebrafish embryos and larvae to different organochlorine pesticides demonstrated that, even in early staged embryos, OCPs can regulate vitellogenin (vtg), the egg yolk precursor protein (Chow et al., 2013). In the study, OCPs such as endosulfan, methoxychlor, and hepatachlor increased *vtg1* expression in either embryos or larval zebrafish. These data suggest that some OCPs act as estrogenic chemicals when fish are undergoing critical stages of sex determination. Indeed, microinjection studies with o,p'-DDT revealed complete male to female sex reversal in Japanese medaka 10 weeks after a single pulse of the chemical into the egg (Edmunds et al., 2000). Despite this understanding, there are little data in fish linking early OCP exposure

to long-lasting effects later in life. There are several studies that link embryonic exposures to adult effects. For example, embryonic oxidative stress results in reproductive effects for adult zebrafish (Newman et al., 2015); exposure to azoxystrobin, an aryloxypyrimidine fungicide, delays sexual development and results in reproductive effects (Cao et al., 2019) and embryonic exposure to atrazine alters neuroendocrine function in adult zebrafish (Wirbisky et al., 2016). Clearly more research is required to link embryonic exposures to OCPs to population level effects in fish.

In summary, OCPs are teratogens and alter developmental processes of fish causing malformations. That they can act as estrogens or anti-androgens during this sensitive period, suggests that they may also alter neuroendocrine pathways during sensitive developmental endpoints that help form the brain. These endpoints are discussed further below.

3. Organochlorine pesticides as system toxicants and endocrine disruptors in adult fish

3.1 Neuroendocrine disruption

It is well documented that OCPs are readily taken up by lipid rich tissues including the brain. Studies conducted in the laboratory and investigations of fish collected from polluted sites over the years have demonstrated that the CNS is susceptible to OCP bioaccumulation. For example, a study conducted in Argentina reported that mature male silversides (*Odontesthes bonariensis*) collected from river watersheds showed a mean level of 5.4 ng/g wet weight of total OCPs in the male brain; whereas, in the female brain, it was reported to be four times higher at 20.6 ng/g wet weight (Barni et al., 2014). Noteworthy was that, in the pre-spawning period, the total concentration of OCPs in the male and female brain was higher than that at maturation. The exact reason for this discrepancy was not elucidated in the study but may be due to differences in biotransformation capacities of males and females throughout the year. Indeed, cycling hormones will have a pronounced effect on enzyme activity and metabolism in general. The difference between males and females could also be due to the blood brain barrier, although we are not aware of studies that have compared male and female blood brain barriers for permeability in fish. The blood brain barrier changes in mammals with age (Erdo et al., 2017) and it would be expected to do so in other vertebrates as well. Thus, sex differences can exist for deposition of OCPs in the CNS, as well as differences based on the reproductive maturity of the individual.

In largemouth bass (*Micropterus salmoides*), Dang et al. demonstrated that the brain can rapidly accumulate both p,p' DDE and dieldrin during oral dosing experiments (Dang et al., 2016). In wild caught largemouth bass from Lake Apopka, Florida, brain burden for total Drins (dieldrin, aldrin, endrin) and DDXs (DDT, DDE, DDD) were comparable to other tissues such as the kidney and liver. It was suggested by the authors that the brain be considered a potential organ for monitoring long-term accumulation of OCPs and other lipophilic contaminants in the environment. Significant levels of OCPs in the brain have also been reported for other teleost fishes such as the female European eel (*Anguilla anguilla*) (Bonnineau et al., 2016). Animals collected from Belgian rivers were analyzed for an array of DDT metabolites; the concentration of p,p'-DDE, p,p' DDD, and p,p' DDT ranged from

4.7 to 16.2 ng/g wet weight in the brain of wild caught eels. Similarly, Wu et al. (Wu et al., 2013) collected four species of commonly consumed freshwater fish including crucian carp, grass carp, snake head fish, and silverfish from Lake Baiyangdian, a body of water on the East Coast of China in close proximity to Beijing. DDT related compounds such as o,p' DDE, p,p' DDT, and others were present in the brain in the low ng/g wet weight range. It is clear that OCPs accumulate in the CNS of fish over time in contaminated areas.

Once OCPs cross the blood-brain barrier, there is the potential for neurotoxicity. In developing zebrafish, Ton et al. showed that 100 μM DDT and ~20 μM dieldrin induced tremors and increased motility 96 hours post fertilization (Ton et al., 2006). The study showed that DDT at 10 and 20 μM induced apoptosis in the CNS based upon acridine orange staining. There was increased cell death, loss of catecholaminergic and dopaminergic neurons, frequent tremors and disorganized motor neurons in the caudal region of the embryo following exposure. The accumulation of these agrochemicals in the CNS is problematic, not only from the point of view of neurotoxicity, but also from the point of view of neuroendocrine disruption (Leon-Olea et al., 2014); OCPs in the brain can disrupt hormonal and neural connections between the hypothalamus, pituitary, and tissue targets leading to inappropriate endocrine signaling. This may be subtle over time compared to more overt processes such as apoptosis and neuronal cell death. For example, dieldrin can alter the expression of neurotransmitter systems in the teleost brain, such as gamma-aminobutyric acid (GABA) and dopamine signaling (Martyniuk et al., 2010, Martyniuk et al., 2012). GABA is a major stimulator of luteinizing hormone (LH) release from the pituitary while dopamine is a major inhibitor of LH release in fish (Trudeau et al., 1993). Injection of a single dose at 10 mg diledrin/kg into male and female largemouth bass altered neurotransmitter levels in the brain after 7 days in females (Martyniuk et al., 2010). GABA was significantly increased in the cerebellum and the hypothalamus of female largemouth bass but not in male bass. 3,4-dihydroxyphenylacetic acid (DOPAC) and dopamine levels were not changed in any brain region (hypothalamus, telencephalon, cerebellum) of male or female fish. A transcriptome analysis was also conducted in the female hypothalamus and a number of pathways were affected by dieldrin. These included DNA repair, inflammatory pathways, steroid signaling, base excision repair, nucleotide excision repair, and apoptosis. In a follow-up study, largemouth bass were fed 3 mg dieldrin/kg over a two-month feeding exposure (rather than chemical injection) to mimic environmentally relevant exposure scenarios. Microarray analysis in the male and female hypothalamus revealed that gene networks related to retinoic acid receptors, gonadotropin releasing hormone, and follicle-stimulating hormone were affected in females whereas in males, activin and androgen receptor signaling pathways were altered in expression (Martyniuk et al., 2013). Disruption in these hypothalamic neuroendocrine networks were hypothesized to lead to downstream effects on reproduction.

Other mechanistic studies into the neurotoxicity of OCPs have been conducted at the protein level. Proteomic investigations into the largemouth bass hypothalamus revealed that proteins responsive to dieldrin are associated with neurodegenerative diseases such as microtubule associate protein tau, myelin basic protein, ATP synthase subunit proteins, and lactate dehydrogenase (Martyniuk et al., 2010). Thus, OCPs such as dieldrin may alter cell structure in the teleostean CNS, impairing neuronal function.

There is other direct evidence for negative effects of OCPs in the CNS, Mortenson and Arukwe investigated the thyroid hormone system response in juvenile immature Atlantic salmon with waterborne exposure to 10 μg DDE/L for 5 days (Mortensen and Arukwe, 2006). The study revealed that in the brain of salmon, DDE acted to blunt the expression of thyroid receptor alpha mRNA in response to T4. DDE had no effect on thyroid receptor beta mRNA. The study revealed that, within the brain, OCPs like DDE can disrupt the expression of thyroid dependent genes.

Endosulfan, which is a broad spectrum OCP, has been shown to affect acetylcholinesterase activity in adult zebrafish (Pereira et al., 2012). The specific activity of acetylcholinesterase was decreased at a concentration of 2.4 μg endosulfan/L over 96 hours. This also corresponded to impaired swimming performance and there was a decrease in distance travelled and mean speed of the zebrafish following this treatment.

As a final example, OCPs can directly affect GnRH and gonadotropin cell populations as demonstrated in the cichlid fish *Cichlasoma dimerus* (Piazza et al., 2011). Larval fish exposed to 0.1 μg endosulfan/L showed decreased signal for GnRH (nucleus/cytoplasm ratio) and an increase in nuclear area of FSH beta positive cells, supporting the idea that some OCPs can affect neuroendocrine cells directly.

Taken together, an array of neurotransmitter and neuroendocrine systems appear to be impacted by OCPs in fish. This is important as wild populations have been chronically exposed to these legacy agrochemicals for many years; the long-term impact of such exposures on neuroendocrine systems, neurological function, and behavior in these animals have yet to be determined.

3.2 Reproductive Axis

OCPs are known endocrine disrupting chemicals that can bioaccumulate in reproductive organs and alter various aspects of reproductive physiology and sexual behavior (Barni et al., 2014, Feist et al., 2005, Johnson et al., 2007, Ree and Payne, 1997). For example, endosulfan did affect the behavior of cichlid fish as well as their liver and testicular morphology (Da Cuna et al., 2011).

One of the most common adverse outcomes associated with OCPs exposure is the abnormal production of sex steroid hormones. For example, testosterone has been widely associated with male courting and reproductive behavior (Mangiamele and Thompson, 2012). Both laboratory-and field-based studies have shown that OCPs can suppress the levels of testosterone in juveniles and reproductive adult fish. For example, ray-finned fish exposed to endosulfan for 30 days exhibited significantly lower levels of testosterone in testicular tissues compared to unexposed animals (Islam et al., 2017). In a field survey of immature white sturgeon, Feist et al. (Feist et al., 2005) reported a correlation between reduced levels of testosterone and high concentration of DDE and other OCPs in gonadal and liver tissues. Similarly, wild male catfish and tilapia caught in basins polluted with endosulfan, heptachlor and DDT exhibited reduced levels of testosterone in the plasma (Agbohessi et al., 2015). These findings correspond to mammalian studies that describe the anti-androgenic effects of

OCPs and their impact on male sex hormones (Du et al., 2014, Fowler et al., 2007, Murono et al., 2006).

OPCs can also act as weak estrogens and alter the production of female-specific hormones and proteins. The increase of vtg in the plasma of reproductive male fish has been associated with exposure to methoxychlor, dieldrin, p,p'-DDE, o,p'-DDT and β-endosulfan (Han et al., 2011, Hemmer et al., 2001, Larkin et al., 2002, Versonnen et al., 2004). This precursor of yolk proteins is synthesized in the liver of reproductive females. Following exposure to OCPs such as dieldrin and p,p'-DDE, increased gene expression of vtg has been observed in livers of male largemouth bass and male zebrafish (Garcia-Reyero et al., 2006, Sun et al., 2016). In another study, juveniles exposed to p,p'-DDE during gonadal development expressed abnormally high levels of vtg in the plasma (Monteiro et al., 2015). These results provide further evidence of the estrogenic potency of OCPs.

Other established markers of estrogenicity such as 17β-estradiol (E2), can be perturbed by these chemicals in both males and females. However, data available suggest that the effects on E2 production may vary depending on the fish species, pesticides, and exposure duration. For example, reports of suppressed E2 levels were found in both sexes of the largemouth bass chronically exposed to dieldrin or DDE under laboratory conditions (Garcia-Reyero et al., 2006, Martyniuk et al., 2013). In contrast, wild male and female tilapia and catfish caught in an OCP-polluted river exhibited high E2 levels (Agbohessi et al., 2015). These discrepancies suggest that differences in OCP mixture composition and exposure duration can have different effects on the estrogen biosynthesis pathway. Additional studies are needed to better understand OCPs modes of actions and nonmonotonic responses in fish.

The regulation of estrogenic and androgenic pathways is tightly linked to gonadal tissue development, gamete production, fecundity and fertility. Alterations of these biological processes have been documented in OCP-contaminated organisms. In male fish, spermatogenesis appears to be sensitive to OCPs exposure. Dutta et al. (Dutta et al., 2006) reported damages to Sertoli cells and reduction in the number of Leydig cells in bluegill fish exposed to endosulfan for 2 weeks. These two types of cells play an important role in testosterone synthesis. Hence, structural and functional damages to these cells could explain the reduction in plasma testosterone levels frequently observed in OCP-exposed male fish. The decrease in circulating testosterone can in turn reduce the maturation and proliferation of sperm. This hypothesis is supported by Agbohessi et al. who measured low numbers of mature spermatozoa in the testes of wild catfish and tilapia exposed to environmental mixtures of OCPs (Agbohessi et al., 2015). However, Da Cuña et al. treated *C. dimerus* with low levels of endosulfan and found a build-up of spermatozoa in the gonads (Da Cuna et al., 2011). This study was conducted over 4 days and it is possible that reduced maturation and number of sperm occur over longer exposure periods. Acute exposure has led to poor quality of spermatozoa as evidenced in a study conducted by Das Neves et al. in which African catfish exposed to methoxychlor for 4 days showed increased vacuolization of the male gametes (Das Neves et al., 2018). Some laboratory studies have also reported the development of ova-testes in male fish subjected to prolonged exposure to pesticides such as p,p' DDE and endosulfan (Han et al., 2011, Sun et al., 2016). It should be noted that the incidence and severity of testicular lesions were often dose dependent.

While most of the research on OPC-induced reproductive toxicity has been conducted in male fish, there is some evidence that OCPs may have similar effects on fish ovarian tissues. For example, female African catfish exposed to dieldrin for 30 days led to a delay in gonad maturation, and elevated the number of immature oocytes (Lamai et al., 1999). Other researchers have reported follicular atresia, disorganization of ovarian tissues and reduced number of viable eggs laid (Agbohessi et al., 2015, Gormley and Teather, 2003, Ree and Payne, 1997). The impact of OPCs on egg quality is not surprising. These lipophilic pesticides are known to accumulate in the gonads and may be transferred to the embryos, thus affecting their development. This mechanism may also explain some of the teratogenic effects of OCPs on early life stages that were discussed above in section 2.

Based on the data discussed in this review, we propose an adverse outcome pathway to describe the impact of OCPs on male reproduction (Figure 3). The available data on molecular and apical endpoints suggest that prolonged exposure to environmental levels of OCPs (in low to mid ng/L range) is likely to disrupt sex hormone production, including a reduction of testosterone. At the same time there is an increase in vitellogenin, suggesting agonism of the the estrogen receptor. These changes lead to impaired sperm cell maturation and release, and reduced fecundity. Current experimental data are less complete for female reproduction compared to males, making it difficult to construct a similar AOP for females; thus we focus only on males.

3.3 Immune Dysfunction

There are a few studies with species of high ecological and economic relevance, such as alligators (Rooney et al., 2003) and largemouth bass (Martyniuk et al., 2016b) that show a connection between OCP exposure and immune dysfunction. A 4-month mesocosm exposure of largemouth bass to OCP mixtures present in the muck farms of central Florida north of Lake Apopka had significant effects on the immune system, in addition to the expected alterations in the reproductive axis (Martyniuk et al., 2016b). In this study, transcriptomics analysis via microarray indicated significant changes in transcripts involved in inflammatory response, platelet function, complement activation, macrophage activation and response, lymphocyte proliferation, eosinophil chemotaxis, T-cell tolerance, agglutination and hemagglutination, all of which relate to the immune system. Subsequent laboratory-based dietary exposures using food pellets spiked with p,p’DDE and methoxychlor also led to similar findings (Martyniuk et al., 2016a). Fish treated with OPC-contaminated diet for 3 months exhibited significant alterations of transcripts involved in immune function including acute phase reaction, platelet function and inhibition and these transcript changes were related to immune system diseases such as Lupus Nephritis.

Several studies have documented the decrease of leukocytes in the head kidney of fish treated with OCPs and decrease of apoptosis of granulocytes (Cuesta et al., 2008, Milston et al., 2003, Misumi et al., 2005). Increased plasma cortisol suppresses immune function in fish (Ainsworthet al., 1991, Pickering and Pottinger, 1989, Thomas et al., 1987) by depressing circulating lymphocytes and decreasing lymphocyte proliferation (Narnaware and Baker, 1996, Weyts et al., 1997). Other effects reported include decreased phagocytosis and lymphocyte mitogenesis, as well as increased apoptosis of B cells (Pulsford et al.,

1994, Weyts et al., 1998, Weyts et al., 1997). Other studies suggest that cortisol synthesis in response to a stressor may be depressed by exposure to OCPs (Benguira et al., 2002).

Potential targets of endocrine disruption in the hypothalamus-pituitary-adrenal (HPA) axis includes adrenocorticotropic hormone (ACTH), which acts on sensitive tissues to release cortisol and corticosterone (Bonga, 1997). Although there is sparse information on mechanisms by which OCPs alter the immune response, o,p-DDD and p,p'-DDE suppressed the response to ACTH by interrenal tissues in the cichlid fish, *Sarotherodon aureus* (Ilan and Yaron, 1980), interfering with cortisol secretion. This in turn could interfered with glucocorticoid signaling since cortisol in a primary agonist of the glucocorticoid receptor. While zebrafish have only one glucocorticoid receptor (Alsop and Vijayan, 2008), other fish possess two instead of one (Meyer and Van de Peer, 2005, Prunet et al., 2006), each featuring alternative splice variants (Schaaf et al., 2008) making generalizations across fish species difficult. The glucocorticoid receptor(s) regulate genes involved with the immune system, as well as glucose metabolism, stress response, blood pressure and osmoregulation (Aluru and Vijayan, 2009, Burnstein and Cidlowski, 1989, Veillette et al., 2007, Weyts et al., 1999).

It should be noted that immunotoxicity of OCPs is well documented in mammalian models and this aligns with what we and others have reported in fish. For example, exposure to three different OCPs, chlordecone, methoxychlor and o,p'-DDT were shown to accelerate the development of systemic lupus erythematosus in ovariectomized mice (Sobel et al., 2005). The mice used in the study were female (NZB X NZW), a hybrid strain of New Zealand mice bred specifically for their susceptibility to lupus erythematosus. OCPs were administered via implantable pellets for a period of 8 weeks. The mice developed renal disease that was confirmed to be immune complex glomerulonephritis, a hallmark for lupus. The disease acceleration was most striking for chlordecone, in this study, making the link of OCP exposure of farmworkers and lupus, more tractable.

It is evident that more mechanistic research is needed to elucidate the effects of OCPs on immune system in fish and other aquatic organisms. Evidence collected to date suggests that fish respond to OCPs in ways that are similar to higher vertebrates including reduced leukocytes and lymphocytes, and lupus like symptoms. More in-depth studies are needed using aquatic organisms to identify the molecular initiating and key events involved in immune suppression and autoimmune disorders that are typically associated with OCPs exposure in mammals.

3.4 Lipids and metabolism

The transcriptomics studies conducted with largemouth bass in the field and the laboratory have indicated major alterations in lipid biosynthesis, lipid transport and lipid metabolism pathways (Martyniuk et al., 2016a, Martyniuk et al., 2016b). While lipids are mainly known for their role in membrane composition and as a reservoir for energy, there are many other roles for lipids as well in which they function as signaling molecules made on site and which are important for reproduction and immune function. Thus, many of the adverse effects noted above can also be a result of disrupted lipid metabolism. Lipids include a wide array of molecules (in the thousands) that classify based on their structures into various groups

with phospholipids making an important mechanistic group. The advent of lipidomics as another OMIC method has enabled researchers to study changes in lipid moieties in relation to physiological competence of organisms (Drier et al., in review). That OCP exposure can change transcripts involved in lipid metabolism suggested that this cellular compartment is also important to consider.

Recent published studies suggest the immune system is linked to specific cellular lipid profiles (Koberlin et al., 2015), with key changes in ceramides and sphingolipids playing central roles in the cellular effects that occur after the recognition of viruses and bacteria through the toll-like receptors. Other lipids such as diacylglycerols and phospholipids can be converted into eicosanoids, which are also involved in inflammation, immune suppression and CNS function (Balazy, 2004). Lysophospholipids have also been defined as having important biological action in a number of conditions including immune function, pain and cancer (Wepy et al., 2019). More research is needed to identify how lipid metabolism fits into both immune and reproductive dysfunction for fish. This has not been studied adequately in fish to date.

Many agrochemicals affect metabolic processes, altering key processes such as glycolysis, oxidative phosphorylation, fatty acid utilization, and other biochemical pathways. Organochlorine pesticides are also reported to affect metabolic capacity and oxidative respiration in a diverse range of fish species. Endosulfan for example, alters the activity of malate dehydrogenase (MDH) in the skeletal muscle of freshwater catfish *Clarias batrachus* (Mishra and Shukla, 2003). The authors proposed that a decrease in the activity of MDH suggests a decline in the efficiency of aerobic energy metabolism, which can affect skeletal muscle function. A loss of activity of MDH was also reported in the liver of channel catfish exposed to levels of 2.5 nM - 7.4 nM endosulfan (1 to 3 ug endosulfan/L, respectively) for seven days (Mishra and Shukla, 1997). In another experiment, freshwater catfish exposed to 0.06 mg endosulfan/L for 21 days resulted in decreased activity of enzymes associated with glycolysis (Tripathi and Verma, 2004). More specifically, endosulfan decreased the activity of citrate synthase (CS) and glucose 6-phosphate dehydrogenase (G6-PDH) in the brain, liver and skeletal muscle as well as brain lactate dehydrogenase (LDH).

In the fathead minnow, p,p’-DDT (ranging from 0.5 to 2 ug p,p’-DDT/L) affected ATPase activity in the brain of fish and there was a decline of 43% in oligomycin-sensitive mitochondrial magnesium 2+ ATPase (Desaiah et al., 1975). Inhibition of brain ATPase by DDT was noted at 118 and 266 days in fish chronically exposed to DDT in water and food in the ppb range (ug/L). Negative effects of OCPs on energy production in the central nervous system are supported by transcriptome studies in zebrafish, which point to the mitochondria as a significant target for organochlorine pesticides such as dieldrin (Cowie et al., 2017). Taken together, reduced ATP capacity and impaired metabolism are expected to impact endocrine function and overall health of fish.

4. Biomarkers of exposure: Focus on hormone signaling pathways

4.1 Comparative toxicogenomics database

Bioinformatics can uncover novel molecular signaling pathways related to organochlorine pesticide susceptibility. To determine if there were novel and conserved pathways affected by select OCPs, we retrieved publicly available data from the Comparative Toxicogenomics Database (Davis et al., 2019) (downloaded 2019-06-24). Pesticides queried included DDT, p,p' DDE, dieldrin, methoxychlor, and toxaphene and pathway data were compiled (Supplemental Data 1). Using such an approach is useful for identifying new endocrine disruptors (Basili et al., 2018) and can unite biomarker molecules by shared factors and functional classification. We identified a number of enriched endocrine-related pathways ($P<0.001$) in the CTD that are affected by organochlorine pesticides at the gene level (Supplemental Table S1). These included signaling pathways related to reproduction (gonadotropins, estradiol, androgen, steroid biosynthesis, oxytocin), thyroid hormone, and insulin. These pathways were significantly enriched for all five OCPs, and clearly suggests that reproductive processes are sensitive to OCP exposure along the hypothalamic-pituitary-gonadal axis. Noteworthy is that thyroid hormone signaling was identified; however, unlike reproduction, there is less known about OCP disruption in thyroid system. Transcripts involved in estrogens signaling included estrogen receptors, heat shock proteins, and MAP kinases to name but a few. Hormones such as estrogens and progestins are intimately related to immune function, and OCPs may have affects in immune system at the gene level via estrogenic and androgenic signaling. This will be an exciting avenue of research in the future, the role of agrichemicals in modulating immune function through hormone signaling.

4.2 Insight from the Tox21 and ToxCast Screening Program

Additional evidence for organochlorine pesticides as endocrine disruptors comes from computational efforts and the Tox21 and ToxCast chemical screening programs. Using the EPA CompTox Chemicals Dashboard (version 3.0.8, released May 10th, 2019), we further elucidated potential mechanisms for select OCPs, including dieldrin, DDT, methoxychlor, and toxaphene. These high-throughput cell-based reporter assays are used to determine the potential of a chemical for endocrine disruption, as well as cytotoxicity, aryl hydrocarbon receptor activation, mitochondrial dysfunction, and oxidative stress among other modes of action. A number of organochlorine pesticides have been tested in these cell-based screening assays. Based upon the ToxCast and Chemical Activity Summaries, the data indicate that these agrochemicals activate or inhibit estrogen and androgen receptor activity (Table 1), and OCPs activate or inhibit nuclear receptors below the AC50 for cytotoxicity (Figure 4).

Currently, little is known about the impact of organochlorine pesticides on retinoic acid signaling cascades in teleost fishes, which is important for thyroid hormone signaling and gene expression activation/inhibition. This is a knowledge gap that should be addressed more completely as thyroid hormone is critical for normal development and metabolism. In fact, exposure to organochlorine pesticides during early fish development can lead to adverse effects that involve metabolism and neuroendocrine signaling. For example, zebrafish exposed to toxaphene during development exhibit reduced oxygen consumption rates for non-mitochondrial respiration, a process that can lead to energy deficits with the

embryo (Perez-Rodriguez et al., 2019). The study also reported developmental defects that included pericardial edema and skeletal deformities, hypothesized to be linked changes in oxygen consumption rate. Further studies should examine the effect to the organochlorine pesticides on developing eggs; this is relevant as OCPs settle into sediment, bind organic matter, and remain bound for many years, resistant to degradation. Sediment in many cases offers the ideal substrate for fish embryonic development and can lead to aberrant development trajectories in sensitive fish species. This has been observed with dieldrin exposure and zebrafish (Sarty et al., 2017).

5. Conclusions

Organochlorine pesticides remain problematic in contaminated environments and discussion on how to best remove the chemicals or reduce exposure to these chemicals is vigorously debated. Research by many over several years has revealed that these pesticides (1) affect early development of fish as teratogenic compounds, (2) act as neuroendocrine disruptors, (3) suppress male and female reproductive systems, (4) dysregulate immune functions and lipid biosynthesis, and (5) alter metabolic function. At the molecular level, these chemicals disrupt signaling pathways related to reproduction (gonadotropins, estradiol, androgen, steroid biosynthesis, oxytocin), thyroid hormone, and insulin. Weight of evidence from laboratory and field experiments, as well as insights from the Tox21 and ToxCast Screening Program indicate that these agrochemicals activate and antagonize estrogen receptors, androgen receptors, and retinoic acid receptors with relatively high affinity, although there are differences in potency of activation among organochlorine pesticides. Despite being banned, it is important to remain diligent on assessing risks for OCP exposure as these chemicals are long-lasting, persistent, and bioaccumulate to toxic levels. Future studies should evaluate multi-generational effects of such exposure, to understand how these chemicals impact molecular pathways and organismal health in the long term.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ACTH	Adrenocorticotropic hormone
ATSDR	Agency for Toxic Substances and Disease Registry
CNS	Central nervous system
CS	Citrate synthase
DDD	Dichlorodiphenyldichloroethane

DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DDXs	Combination of DDT, DDE, DDD
DOPAC	3,4-dihydroxyphenylacetic acid
Drins	Combination of dieldrin, aldrin, endrin
E2	17β-estradiol
G6-PDH	Glucose 6-phosphate dehydrogenase
GABA	Gamma-aminobutyric acid
HPI	Hypothalamus-pituitary-interrenal
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
MDH	Malate dehydrogenase
OCP	Organochlorine pesticides
StAR	Steroidogenic acute regulatory protein
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
Vtg	Vitellogenin

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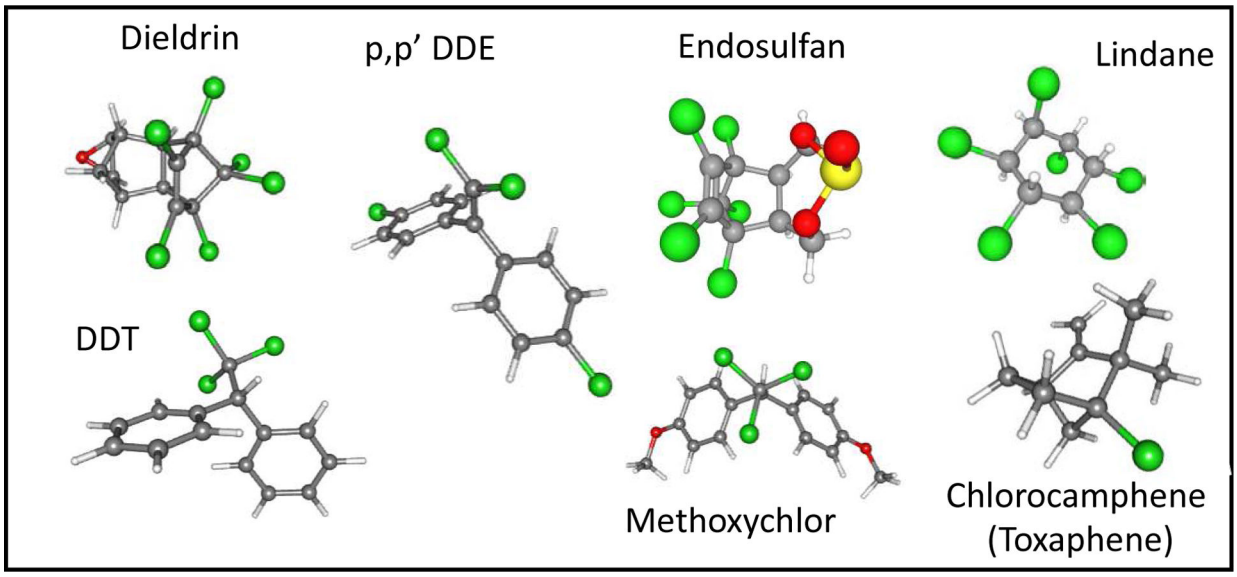

Figure 1.

The 3D-structures of some of the major organochlorine agrochemicals. Green indicates positions of the chlorines, red indicates an oxygen, and yellow indicates the position of the sulfur group. Toxaphene is a mixture of chlorinated compounds, where chlorine can be found bonded with carbons at different positions in the molecule. Images extracted from NCBI PubChem.

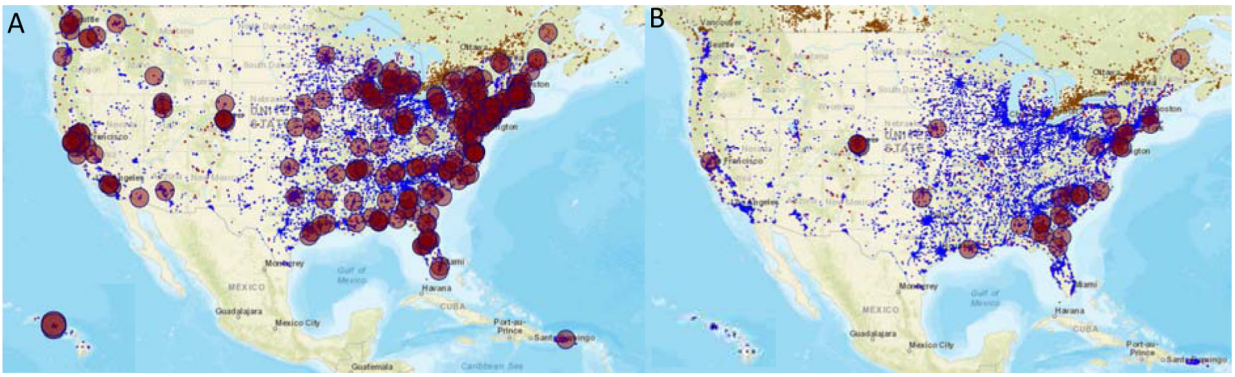

Figure 2:

Two examples of Superfund sites (Red circles) containing organochlorine pesticides in the United States. (A) Dieldrin (B) Toxaphene. Inset to the bottom left is Hawaii. Alaska does not have any Superfund site designation for these agrochemicals. Blue indicates locations for chemical contaminants in the Toxic Release Inventory (TRI) Program, for example contaminated sites that contain cancer causing agents. Red indicates Superfund Site locations. Generated from TOXMAP (US Department of Health and Human Services)

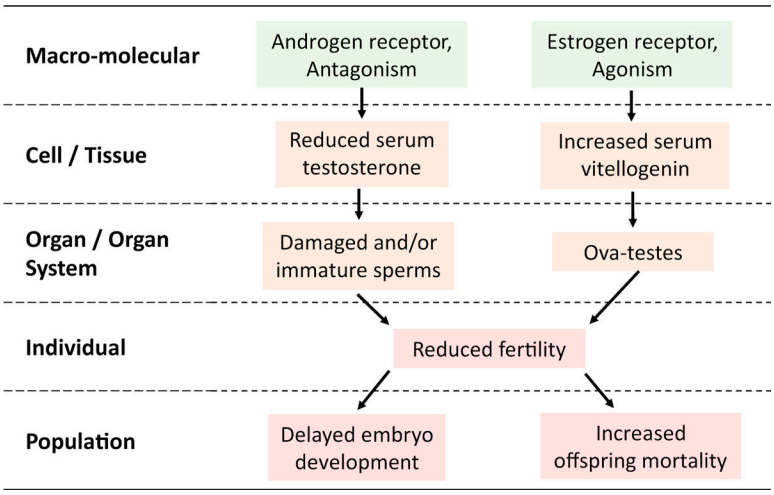

Figure 3.

Proposed adverse outcome pathway based on the disruption of the androgen and estrogen receptor activities in fish testes by organochlorine pesticides.

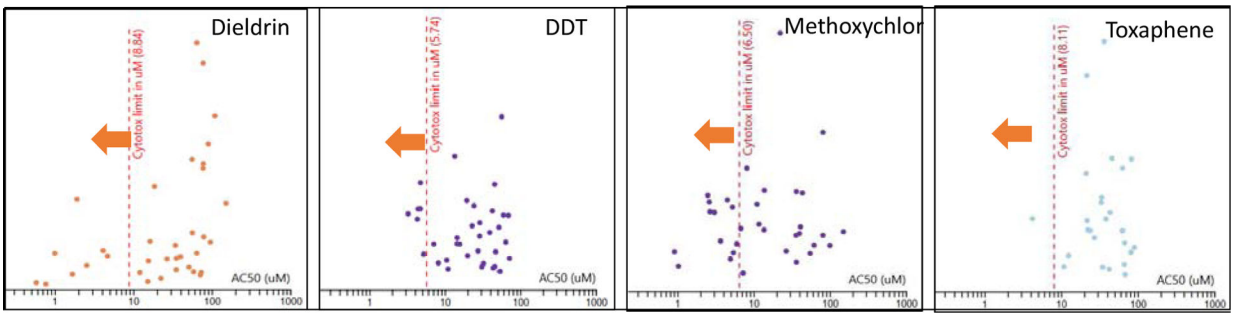

Figure 4.

ToxCast and Chemical Activity Summary for selected organochlorine pesticides. Depicted are the ‘active” calls for assays designed to test nuclear receptor activity. All assays on the left side of the dotted line indicated by the orange arrow are active at concentrations below observed cytotoxicity. EPA CompTox Chemicals Dashboard (version 3.0.8, released May 10th, 2019).

Table 1.

ToxCast and Chemical Activity Summary for OCPs. Organochlorine pesticides depicted in the table have significant estrogenic and anti-androgenic effects.

Chemical	MODEL	RECEPTOR	AGONIST	ANTAGONIST	BINDING
Dieldrin	ToxCast Pathway Model (AUC)	Androgen	0.00	4.15e-2	-
	ToxCast Pathway Model (AUC)	Estrogen	1.81e-2	0.00	-
	COMPARA (Consensus)	Androgen	Inactive	Active	Inactive
	CERAPP Potency Level (From Literature)	Estrogen	-	Inactive (Inactive)	Active (NaN)
	CERAPP Potency Level (Consensus)	Estrogen	Inactive (Inactive)	Inactive (Inactive)	Inactive (Inactive)
DDT	ToxCast Pathway Model (AUC)	Androgen	0.00	6.42e-2	-
	ToxCast Pathway Model (AUC)	Estrogen	0.190	0.00	-
	COMPARA (Consensus)	Androgen	Inactive	Active	Active
	CERAPP Potency Level (From Literature)	Estrogen	Active (Weak)	-	Active (Weak)
	CERAPP Potency Level (Consensus)	Estrogen	Active (VeryWeak)	Active (Moderate)	Active (VeryWeak)
Methoxychlor	ToxCast Pathway Model (AUC)	Androgen	0.00	4.29e-2	-
	ToxCast Pathway Model (AUC)	Estrogen	0.254	0.00	-
	COMPARA (Consensus)	Androgen	Inactive	Active	Active
	CERAPP Potency Level (From Literature)	Estrogen	Active (Weak)	-	Active (Weak)
	CERAPP Potency Level (Consensus)	Estrogen	Active (VeryWeak)	Active (Weak)	Active (VeryWeak)