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Dirty Turtles: Examining the Effects of Persistent Pollutants on Embryonic Turtle Development

Research Article

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Abstract

Background: In an anthropogenically-altered world, it is common to find pollutants such as plastics, pesticides, fertilizers, and heavy metals in waterways and soil. Many plastics, such as BPAs; organic chemicals that can absorb plastics, such as PCBs; and pesticides, such as atrazine, are known to be endocrine disrupting chemicals (EDCs). Many EDCs act as xenoestrogens and can affect the sexual development of numerous organisms, from mammals, such as humans; to reptiles, such as turtles. Turtles are long-living organisms that often have an omnivorous diet and demonstrate site fidelity, thus they are useful subjects in which to study the effect of EDCs on organismal development.

Methods: In this study, the effects of plastics, pesticides, flame retardants, and heavy metals on developing turtle embryos were examined across a variety of both freshwater and marine turtles. A search of existing relevant literature was carried out in November of 2016 using the database Web Of Science and Google Scholar by looking for the keywords "turtles" AND "sex-reversal" AND "endocrine disrupting chemicals (EDCs)", with no restrictions used on the years in which these studies were published. Web of Science returned 42 articles and Google scholar returned 3870 articles. Of these, 13 studies were deemed relevant and examined, encompassing 35 cases, and consisting of data from eight different species of turtles.

Summary: It was found that plastics and PCBs, even in low doses, had the greatest effects on sex reversal across turtle species, and could affect their behavior later in life as well. Pesticides showed an ability to alter the sex of the turtle, and also caused developmental defects. Flame-retardants and heavy metals were shown to be maternally transferred to offspring, but studies did not find obvious cases of sex reversal or developmental defects at low doses. Many turtle species are endangered, and thus understanding threats to their health and development is critical to their future survival.

Introduction

Habitat degradation, climate change, disease, and unsustainable collection for the pet trade has put significant stress on turtle populations. (1) As a result of these anthropogenically-driven factors, reptiles, including turtles, are some of the most threatened vertebrate taxa across the globe. (1) Another human-caused factor that is impacting turtle populations is the presence of persistent organic pollutants in the environment which, in tandem with other pollutants, are commonly found in the soil sediments where turtles lay their eggs. (2)

Many of these pollutants are endocrine disrupting chemicals (EDCs). (3) Polychlorinated biphenyls (PCBs), Bisphenol A (BPA), pesticides, nitrates and heavy metals have all been reported to be EDCs. (3) Adult turtles can be affected by these pollutants in their environment, but turtles can also be exposed to these chemicals during embryonic development, as these compounds can penetrate the eggshell in both their aqueous and gaseous phases through the soil. (4) Sex determination in many turtle species is temperature dependent (TSD), wherein sex is not determined by chromosomes, but instead by the temperature of the nest during the middle third of development. (5) Reptile and amphibian species where temperature or other environmental factors determine the sex of the embryo are highly susceptible to the effects of EDCs. (3)

EDCs can work either alone or in combination with other pollutants, and can affect the turtle as a developing embryo, hatchling, and later in life. (3) EDCs can affect sex determination, gonadal development, bone mass, immune response, testosterone levels, germ cell numbers, adult sexual behaviors—primarily those driven by testosterone, viability of offspring, fertility, and hatching rates. (2-3) Many EDCs are lipophilic, and can thus bioaccumulate in the turtle's tissues, resulting in important negative consequences for the rest of the food chain. (3)

Furthermore, there may be similarities in the way that turtles and humans respond to these EDCs. (6) The strong site fidelity, longevity, and omnivorous diet of turtles not only makes them important indicators of surrounding environmental health, but may also make them a good analog for how EDCs affect other long-living vertebrates, such as humans. (2,6) Furthermore, an important enzyme involved in sex determination is aromatase, which has been found to be conserved across most vertebrate taxa. (7) Similar pathways and structures in sex determining mechanisms may mean that all vertebrate taxa have a similar susceptibility to EDCs. (6) In humans, EDCs have been linked to reproductive disorders and reproductive cancers. (3) Thus, examining the effect of EDCs on turtles may be important for both environmental and human health.

Between 8 and 15 billion pounds of BPA are produced globally each year, and their high abundance makes it safe to assume it has a high incidence in the environment. (1) PCBs have been banned since 1970 in the United States, but are still present in the soil due to their long half-life. (2) Flame retardants were banned almost forty years ago, but can also still be found in soil today. (2) Many pesticides that are suspected to be EDCs are still in use. (4) Turtles are subject to a wide range of environmental and human-driven stressors, which can act together to bring greater stress to the species. For instance, increased temperature predicted from global climate change may drive TSD species to unsustainable sex ratios, (5) as well as increasing the ability of EDCs to have feminizing effects on the sex of the developing embryo, resulting in further skewing of the population's operational sex ratio. (3) It is critical to understand the full range of effects these environmental stressors produce in order to best conserve the many endangered turtle species.

The purpose of this study is to examine plastics and PCBs, pesticides and fertilizers, flame retardants and heavy metals, and to determine which of these could be considered the most detrimental to turtles based on their

effects on sex determination, embryonic development, and traits and behaviors later in life. It is predicted that the EDCs which are known to be xenoestrogens—synthetic compounds that imitate estrogen in the body, such as PCBs, organic compounds associated with plastic; and atrazine, a pesticide, would have the greatest effect on turtles overall. It is expected that turtles subjected to known xenoestrogens will show particularly high rates of sex reversal, as estrogen is necessary for sex determination in turtles with TSD and can have a feminizing effect. (8)

Methods

13 articles were identified and deemed to be useful for this study, and some of these articles were comprised of multiple cases. As such, the results of 35 different cases of turtles being subjected to EDC exposure were examined. Across these 35 cases, eight species of turtles were examined including *Trachemys scripta elegans*, *Malaclemys terrapin*, *Chrysemys picta*, *Chelydra serpentina*, *Graptemys pseudogeographica*, *Graptemys ouachitensis*, and *Chelonia mydas*. All turtles were freshwater species except Chelonia mydas, a marine species. Studies were grouped into three sections: "Plastics, PCBs and 17β-estradiol", "Pesticides" and "Other", consisting of flame retardants and heavy metals. Plastics and PCBs were grouped together as PCBs are often associated with plastics in the environment. Plastics examined included Bisphenol A, organic chemicals examined included PCB, as well as one hormone, 17β-estradiol. Pesticides and fertilizers examined included atrazine, simazine, metolachlor, azinphos-methyl, dimethoate, chlorpyrifos, carbaryl, endosulfan I, endosulfan II, captan, chlorothalonil, dimethenamid, glyphosate, tefluthrin, ammonia, and DDE. Flame retardants examined included BDE-47, BDE-48, BDE-99, and BDE-100. Heavy metals examined included manganese, copper, zinc, selenium, and arsenic. All data was recorded in a table. The effects of the pollutants on the turtle eggs were separated into three categories: "effect on sex", "effect on development", and "effect later in life". Data for the categories of "effect on sex" and "effect on development" were recorded as "Yes", "No", or "NA" for when no information was given in a paper. The "effect later in life" category had more descriptive entries, such as "maternally transferred" or "reduced fertility". For the "effect later in life" category, any entry of a consequence of the chemical was recorded as a "Yes". The frequency of "No" or "NA" entries was also recorded for the "effect later in life" category.

Results

Plastics and PCBs, and 17β-estradiol

Of the seven studies (1,2,6,7,10,11,14) where the effects of plastics and PCBs on developing turtle embryos were examined, all chemicals examined: PCB, 17β-estradiol, and Bisphenol A had feminizing effects on the sex of the turtle. One study found that PCB caused deformities such as malformed scutes in hatchlings of *Malaclemys terrapin*. (2) The other six studies (1,6,7,10,11,14) either reported that there were no deformities, or did not examine the hatchlings for any gross abnormalities. Five studies (1,2,6,7,10) reported an effect later in life on the turtles. These effects included reduced fertility, altered mating behavior, and the ability of the toxin to be maternally transferred to offspring. PCB, 17β-estradiol, and Bisphenol A were all reported to be capable of producing one or more of these effects.

Pesticides and Fertilizers

The majority of studies involving the effects of pesticides and fertilizers on turtle embryos and hatchlings did not examine the turtles for abnormalities in sexual development. Fourteen of the nineteen cases examined did not look for any evidence of sex reversal in turtles subjected to pesticides or fertilizers. (4,9) Of the cases that did look for an effect on sex, three (9,13) of the five (4,9,13,20) cases reported either full or partial sex reversal of the hatchlings. In the studies, three species across two genera were examined for the effects of atrazine exposure: *Chrysemys picta, Graptemys pseudogeographica*, and *Graptemys ouachitensis*. All cases where atrazine was applied to the eggs reported some degree of sex reversal upon hatching.

All of the studies examined searched for an effect on the development of the turtle embryo after being exposed to pesticides or fertilizers. Of the 19 cases examined, four (9,13) reported gross abnormalities and 15 (4,9,20) reported that there were no gross abnormalities. Two of the reported cases of developmental defects were concluded to have been again caused by atrazine, and were once again reported in *Graptemys pseudogeographica* and *Graptemys ouachitensis*. (13) The other two reported cases were caused by tefluthrin and ammonia and developmental defects were observed in *Chelydra serpentina*. (9)

Fifteen of the nineteen cases did not extend their studies to look for an effect later in life of pesticides and fertilizers on the turtles. Of the four (9,13,20) cases that did, three (13,20) reported either a lower first year survival in the turtle, as for atrazine in *Graptemys pseudogeographica* and *Graptemys ouachitensis*, or reported the ability of the compound to be maternally transferred, as for DDE in *Chelonia mydas*.

Only one pesticide, chlorothalonil, was found to not have an effect on either the sex, development, or had an effect later in life. (9) Chlorothalonil was applied to the eggs of *Chelydra serpentina*.

Other

None of the nine cases that examined the effects of flame retardants or heavy metals on turtles studied the embryos or hatchlings for evidence of sex reversal. Moreover, none of the studies on flame retardants examined the turtle embryos for gross abnormalities. All heavy metals tested were found to have no effect on the development of the turtle. However, all flame-retardants and heavy metals tested were found to be maternally transferrable from the affected mother to her eggs. (15, 17)

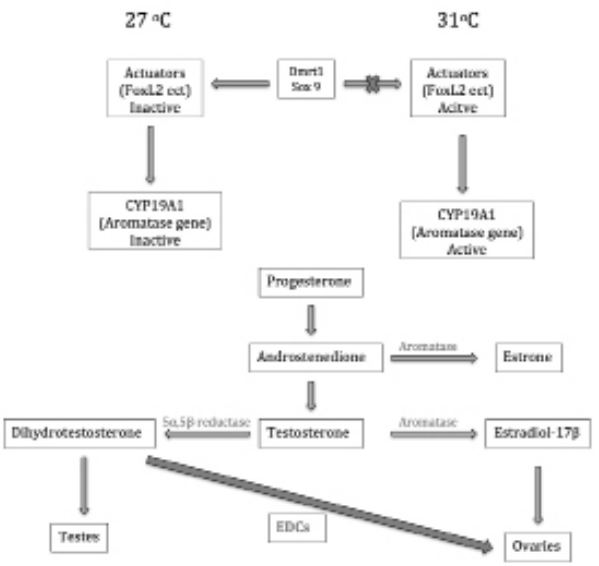

Figure 1: Simplified mechanism of EDCs on the TSD pathway. Modified from Jeyasuria and Place, 1998 and Mizoguchi and Valenzuela, 2016.

Discussion

These results suggest that the most potent EDCs are PCBs and the pesticide atrazine, as both these compounds have the ability to induce complete sex reversal and cause gross abnormalities in the embryos, as well as affect the turtle later in life (Table 1, 2, 3). EDCs are capable of interfering with the sex determination process of animals (Figure 1) and can cause feminization and demasculinization of the animals affected. (1) Furthermore, both PCB and atrazine are known xenoestrogens, so exposure to these compounds during embryonic development can thus cause complete sex reversal during the development process. (3) Additionally, due to their prevalence in soils, these two compounds are also the most like

ly for turtles to encounter while still in the egg—the time when they are most susceptible to the effects of EDCs. (1, 7, 9) These results support the prediction that known xenoestrogens would not only affect the sex of embryos, but other traits throughout the growth and development of turtles, even after hatching.

Pollutant	Species of Turtle	Effect on Sex	Effect on Development	Effect later in life	Source
PCB	*Trachemys scripta elegans*	Yes	N/A	Reduced fertility	(10)
PCB	*Malaclemys terrapin*	Yes	Yes	Yes	(2, 12)
PCB	*Trachemys scripta elegans*	Yes	N/A	N/A	(11)
PCB	*Trachemys scripta elegans*	Yes	No	Malformed müllerian ducts	(7)
17β-estradiol	*Trachemys scripta elegans*	Yes	N/A	Yes (Mating behavioral)	(6)
Bisphenol A	*Trachemys scripta elegans*	More estradiol	N/A	N/A	(14)
Bisphenol A	*Chrysemys picta*	Yes	No	Yes (Maternal transfer)	(1)

Table 1: Summary of the effects of plastics and associated organic pollutants on various turtle species from literature review.

Pollutant	Species of Turtle	Effect on sex	Effect on development	Effect later in life	Source
Atrazine	*Chelydra serpentina*	No	No	N/A	(5)
Atrazine	*Chelydra serpentina*	N/A	No	N/A	(4)
Atrazine	*Graptemys pseudogeographica*	No (but [illegible] ovary growth in males)	Yes	Yes (Low 1st year survival)	(11)
Atrazine	*Graptemys ouachitensis*	No (but [illegible] ovary growth in males)	Yes	Yes (Low 1st year survival)	(11)
Simazine	*Chelydra serpentina*	N/A	No	N/A	(5)
Metolachlor	*Chelydra serpentina*	N/A	No	N/A	(4)
[illegible]	*Chelydra serpentina*	N/A	No	N/A	(4)
Dimethenamid	*Chelydra serpentina*	N/A	No	N/A	(5)
Chlorpyrifos	*Chelydra serpentina*	N/A	No	N/A	(4)
Carbaryl	*Chelydra serpentina*	N/A	No	N/A	(4)
Endosulfan I	*Chelydra serpentina*	N/A	No	N/A	(4)
Endosulfan II	*Chelydra serpentina*	N/A	No	N/A	(4)
Captan	*Chelydra serpentina*	N/A	No	N/A	(4)
Chlorothalonil	*Chelydra serpentina*	No	No	No	(4)
[illegible]	*Chelydra serpentina*	N/A	No	N/A	(5)
[illegible]	*Chelydra serpentina*	N/A	No	N/A	(5)
[illegible]	*Chelydra serpentina*	N/A	Yes	N/A	(5)
Ammonia	*Chelydra serpentina*	N/A	Yes	N/A	(5)
DDE	*[illegible]*	No	No	Yes (Maternal Transfer)	(20)

Table 2: Summary of the effects of pesticides on various turtle species from literature review.

Pollutant	Species of Turtle	Effect on Sex	Effect of Development	Effect Later in Life	Source
BDE-47	*Chelydra serpentina*	N/A	N/A	Yes (Maternal Transfer)	(15)
BDE-48	*Trachemys scripta elegans*	N/A	N/A	Yes (Maternal Transfer)	(15)
BDE-99	*Chelydra serpentina*	N/A	N/A	Yes (Maternal Transfer)	(15)
BDE-100	*Trachemys scripta elegans*	N/A	N/A	Yes (Maternal Transfer)	(15)
Mn	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
Cu	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
Zn	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
Se	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)
As	*Chelonia mydas*	N/A	No	Yes (Maternal Transfer)	(17)

Table 3: Summary of the effects of flame retardants and heavy metals on various turtle species from literature review.

In reference to TSD, the presence of EDCs can produce females at otherwise male producing temperatures (Figure 1). (6) EDCs are also more likely to affect the development of animals with TSD, such as turtles, which could further explain the cases of complete sex reversal, gross abnormalities, lower first year survival, reduced fertility and maternal transfer found when turtle eggs were subjected to PCB or atrazine. (1,2,10- 13)

Regardless of whether they exhibit genotypic sex determination (GSD) or TSD, all turtles also use hormones to determine sex. TSD pathways differ from GSD pathways in that the sex-determining hormones that are activated depend on environmental cues, not genetic markers. (3) EDCs can infiltrate and disrupt this hormonal pathway. Unlike mammals, reptiles do not have an Sry gene, but do have several downstream genes that are components of the male sex determining pathway, such as Dmrt1 and Sox9. (3) Normally, in turtles, at low temperatures Dmrt1 and Sox9 are active. This results in actuators such as FoxL2 to be inactive, which in turn results in CYP19A1, the gene that codes for aromatase, to be inactive as well (Figure 1). (3) Aromatase is an enzyme important to the female sex determining pathway across vertebrates, and converts testosterone into 17β-estradiol, which causes embryonic ovary development. (3) Without aromatase, testosterone is instead converted to dihydrotestosterone by 5α,5β-reductase (Figure 1). (3) Dihydrotestosterone is a hormone that leads to the development of testes in the embryo. (3)

There are several ways in which EDCs can disrupt the normal pathway of sexual development. EDCs can act as aromatase inhibitors, hormone mimics, hormone antagonists blocking the action of the hormones or alter when a hormone is produced. (3) PCBs, for instance, can mimic estrogen; BPAs can decrease steroid binding affinities; atrazine can inhibit 5α,5β-reductase; nitrates often found in fertilizers can alter steroid levels; and heavy metals can interfere with estrogen action. (3) As the pathways of sexual development in turtles normally operate in a dosage dependent manner, the introduction of an EDC can upset the balance of hormones and alter the sex of the embryo. (3) EDCs can also affect hormones that drive phenotype during development, which may explain the gross abnormalities observed by some studies when the turtle embryos had been subjected to EDCs.

Adult turtles can also be affected by EDCs. Since EDCs have the ability to bioaccumulate, (3) turtles living in contaminated environments may absorb them through their diets. In females, high levels of EDCs in the tissues may lead to maternal transfer. In turtle eggs, the yolk can be maternally loaded with hormones, and these maternally loaded hormones have been found to be able to override the sex determining pathways in the embryo should the concentration be high enough. (3) EDCs that mimic hormones may be loaded into the yolks in the same way through maternal transfer, subjecting the developing turtle embryo to significant EDC exposure even if the environment in which the egg was laid is not contaminated. In males, adult exposure to EDCs may result in "demasculinizing" effects, lowering their testosterone levels and perhaps altering mating behavior. (6) For instance, male painted turtles use their long, sexually dimorphic nails to stroke the face of a potential mate, a behavior that is noted to be driven by testosterone levels. (6) Lower testosterone levels could alter this behavior and result in lower mating success, even without visible abnormalities in the turtle. (6)

Another important anthropogenic source of estrogen is 17β-estradiol (often mentioned in articles alongside BPAs). 17β-estradiol is the main estrogen found in birth control pills. (6) On average, 10μg of 17β-estradiol is excreted daily in the urine of humans who take oral contraceptives, and though there exists technology to completely remove 17β-estradiol in waste treatment plants, it has not been used. (6) Consequently, traces of 17β-estradiol have been found contaminating the water and groundwater in areas where turtles could be exposed, and these areas are often also contaminated by other EDCs. (6) The abundant amounts of feminizing chemicals could cause increasingly more female turtles to hatch, skewing the sex ratio towards unsustainable levels and heavily impacting the future of turtle populations.

A limitation of this study is that many of the studies examined only looked at the effect of EDCs on either the hatchling or the adult, and rarely followed a turtle throughout its life. Some potential and serious effects of

EDCs are high deformity rates in juveniles, increased mortality, and slower growth, (2) but these results are unlikely to be seen in studies looking only at hatchlings and adults. Additionally, many studies euthanized and dissected hatchlings to examine them for signs of sex reversal, making it impossible to see how the sex reversal affected the behavior or reproductive success of those turtles later in life. These premature analyses of the turtles may create an underestimate of the potential effects of EDCs.
Moreover, there were no standard protocols used across all studies. Some studies were conducted in a laboratory setting, (1,2,4,6,7,9,10,11,13,14) while others focused on observations in the field. (15,17,20) Some studies in the lab only applied the contaminant once to the turtle eggs, whereas others applied it continuously throughout the experiment. The dosage of the EDCs applied was also not consistent across the studies. These inconsistencies may have created false positives in my collected results if one technique was more successful at inducing abnormalities than another.
It is also important to note that there may be a publication bias towards papers that have significant or interesting results. This may result in papers focusing on contaminants known to cause abnormalities in developing turtles, and consequently cause an overestimate of the effects of EDCs on turtles.

It is important to examine the effects of EDCs across the life of the turtle, and to look at a wider range of EDCs across varying concentrations in future studies in order to determine the full range and severity of the effects of EDCs on turtle development.

Conclusion

Although many EDCs known to be particularly potent, such as PCBs, are now banned from use, they can still be found in the soil and water. (1,2,4,7) Certain pesticides that are known to be xenoestrogens, however, such as atrazine, are still widely in use. (9) As turtles live in the water and lay their eggs in the soil, they can be exposed to these EDCs at varying stages of embryonic development and throughout their lives. Turtles are subject to a wide range of stressors surrounding habitat loss, climate change, and human activities. These results show that EDCs could cause further population declines in already stressed turtle populations by reducing first year survival, reducing mating success, decreasing immune response, causing gross abnormalities, and skewing the operational sex ratio to unsustainable levels. (2,3) As the hormone levels in the egg yolk can also affect sex determination, EDCs absorbed into the egg could mimic maternally loaded hormones and override genetic factors and/or the temperature-influenced and temperature-activated pathway of sexual development in the embryo. (3) It is thus suspected that EDCs with such characteristics would result in the highest incidence of sex reversal in turtles.

Turtles are already heavily endangered, and in order to properly and thoroughly protect these animals we must understand the full range of stressors that they are subjected to, of which EDCs are a major stressor. It is important for both the health of aquatic ecosystems and the organisms that rely on these ecosystems to understand and mitigate the detrimental effects of EDCs and to determine why and how these substances should be regulated and limited.

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