The Environmental Contaminant DDE Fails to Influence the Outcome of Sexual Differentiation in the Marine Turtle Chelonia mydas

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In many turtles, the temperature experienced during the middle of egg incubation determines the sex of the offspring. The implication of steroid sex hormones as the proximate trigger for sex determination opens the possibility that endocrine-disrupting contaminants may also influence the outcome of sexual differentiation. In this study we investigate the potential effects of DDE (a common DDT metabolite) on sexual differentiation of Chelonia mydas (green sea turtle). Four clutches of eggs collected from Heron Island, Queensland, Australia, were treated with DDE at the beginning of the thermosensitive period for sexual determination. An incubation temperature of 28°C or less produces male hatchlings in this species, whereas 30°C or more produces female hatchlings. Dosed eggs were consequently incubated at two temperatures (27.6°C and 30.4°C) on the upper and lower boundaries of the sex determination threshold for this species. DDE, ranging from 3.3 to 66.5 pg, was dissolved in 5, 10, and 25 µl ethanol and applied to eggshells above the embryo. Less than 2.5 ng/g DDE was present in eggs prior to dosing. Approximately 34% of the applied DDE was absorbed in the eggs, but only approximately 8% of applied DDE was found in embryos. Thus, treated eggs, corrected for background DDE, had up to 543 ng/g DDE. The sex ratio at these doses did not differ from what would be expected on consideration of temperature alone. Incubation time, hatching success, incidence of body deformities, hatching size, and weight were also within the limits of healthy developed hatchlings. Approximately 34% of the applied DDE was absorbed in the eggs, but only approximately 8% of applied DDE was found in embryos. Thus, treated eggs, corrected for background DDE, had up to 543 ng/g DDE. The sex ratio at these doses did not differ from what would be expected on consideration of temperature alone. Incubation time, hatching success, incidence of body deformities, hatching size, and weight were also within the limits of healthy developed hatchlings. This indicates that the eggs of C. mydas in the wild with concentrations of DDE less than 543 ng/g should produce hatchlings with relatively high hatching success, survival rate, and normally differentiated gonads. Key words: Chelonia, DDT, endocrine-disrupting contaminant, temperature-dependent sex determination. Environ Health Perspect 106:185-188 (1998). [Online 26 February 1998] http://ehpnet1.niehs.nih.gov/docs/1998/106p185-188podreka/abstract.html

An estimated rate of over 12,000 tons of DDE per year ended up in the oceans in the 1970s (23,24). While some is adsorbed to sediments of the ocean floor (25), most of it enters the marine food chain (23,24). Once in an organism, DDE can affect the endocrine system, resulting in effects on growth, development, and reproduction (10,26,27). The embryo stage appears to be the developmental stage most vulnerable to the effects of endocrine-disrupting contaminants (12). Abnormal development, embryonic deformities, increased mortality, and lower hatching success have been shown to occur because of DDE contamination in a range of organisms including reptiles (28,29).

In sea turtles, DDE accumulates in adipose tissue (15) and is eventually passed to eggs (30,31). DDE concentrations occurring in turtle and other reptile eggs in their natural environment have been measured in many studies (Table 1), but the effects of this pollutant on embryos are mentioned in only a few (28,29,32,33). In this paper we examine the effects of DDE contamination on offspring sex ratios and embryonic development in the marine turtle Chelonia mydas. Background DDE levels in eggs collected in the wild were also measured and compared to published DDE values for reptile eggs from other studies, and the effectiveness of topical administration of DDE in solution was assessed.

**Materials and Methods**

**Source of eggs.** Freshly laid eggs from the clutches of four female Chelonia mydas (Table 2) were collected from Heron Island, Queensland, Australia, on 12 January 1995, chilled to 12°C, and transported by air to the University of Canberra, Australian Capital Territory. All eggs were assigned to experimental incubators within 72 hr. Initially, all eggs were placed in incubators set at 27.5°C to await an assessment of their viability. They were placed in 5-liter circular plastic food containers and covered by moist vermiculite (three parts vermiculite to four parts water by weight). After 15 days of incubation, eggs were checked for shell whitening (34), which indicates that development had commenced. Extreme care was exercised when inspecting the eggs so as not to jolt or rotate them, as cases of movement-induced mortality are well documented (35,36). Eggs that were not turgid or that lacked the white patch were removed, and the remaining eggs were consolidated into the minimum number of containers in preparation for allocation to experiments.

**DDE background levels.** Fifteen eggs from the four clutches (Table 2) were used for determination of background levels of DDE. They were incubated at 27.5°C until stage 21 (day 15 of incubation (34,37)) and then dissected. Stage of the embryo was determined by comparison with a standard series (34). Albumin, clear fluids, yolk, and...
embryo were separated from shell and shell membranes and stored frozen.

DDE concentrations in albumin, fluids, yolk, and embryo were determined using methods modified from those recommended for chicken eggs (38). The samples were homogenized and duplicate subsamples of approximately 5 g were weighed and placed in glass Kimax tubes (Kimble Kontes, Vineland, NJ). Acetonitrile (nanograde, 25 ml; Mallinckrodt, Phillipsburg, NJ) was added and the sealed tubes were shaken manually for 2 min and centrifuged at 2,500 rpm for 5 min. Supernatant (25 ml) containing any DDE was decanted and diluted to 100 ml with distilled deionized water. This aqueous acetonitrile solution (50 ml) was purified using tandem C18 (Alltech Associates, Inc., Deerfield, IL) and 100% hexane (HPLC, Gibbstown, NJ) and 100% acetonitrile (nanograde; Mallinckrodt) at a flow rate of 1 ml/min. The collected eluate, evaporated to 2 ml (using a warm bath and a stream of high purity nitrogen), was analyzed for DDE using a gas chromatograph fitted with an electron capture detector (HP-5890 Series II GC fitted with HP-1 and BP-10 capillary columns; HP7673 auto-injector; Hewlett-Packard Corporation, North Ryde, Australia). Matching duplicate determinations for each sample were averaged to obtain estimates of DDE in the egg tissues. Recoveries of DDE from spiked samples were high (98 ± 23% for whole egg and 101 ± 6% for embryo).

**Table 1.** Range of values of DDE measured in eggs of reptiles with temperature-dependent sex determination compared to values obtained in the present study for dosed eggs and undosed eggs collected from Heron Island, Queensland, Australia

| Species name                     | DDE (ng/g wet wt) | Locality                          | Reference  |
|----------------------------------|-------------------|-----------------------------------|------------|
| Alligator mississippiensis       | 890–29,000        | Lake Apopka, Florida              | (32)       |
| Crocodylus acutus                | 370–2,900         | Everglades National Park, Florida | (37)       |
| Chelydra serpentina              | 877 ± 481*        | Cootees Paradise, Lake Ontario,   | (29)       |
| Chelonia mydas                   | Up to 543         | Lab dosing, University of Canada  | Present study |
| Chelydra serpentina              | 150–430           | Hamilton Harbour, Lake Ontario    | (16)       |
| Caretta caretta                  | 56–150            | Merritt Island, Florida           | (14)       |
| Chelonia mydas                   | ND–5              | Ascension Island                  | (33)       |
| Chelonia mydas                   | 1.3–2.4           | Heron Island, Queensland, Australia | Present study |

ND, level not detectable.

*Mean ± standard deviation.

DDE concentrations are mean ± one standard deviation. Because only one egg was used from clutch C, no standard deviation can be reported.

**Table 2.** Details of four turtles and their clutches and the concentrations of DDE measured in a sample of eggs from each clutch

| Turtle tag no. | Clutch no. | Clutch size | DDE concentration (ng/g) |
|----------------|------------|-------------|--------------------------|
| G2-T112791     | A          | 156         | 2.0 ± 0.3 (n=5)          |
| G1-T28866      | B          | 88          | 1.5 ± 0.0 (n=5)          |
| O4-T6287      | C          | 73          | 1.7 ± 0.3 (n=1)          |
| G2-T85154      | D          | 139         | 1.5 ± 0.1 (n=4)          |

DDE concentrations are mean ± one standard deviation. Because only one egg was used from clutch C, no standard deviation can be reported.

**Table 3.** Quantities of DDE administered to eggs of *Chelonia mydas* in solutions of 5, 10, and 25 μl absolute ethanol (EtOH).

| DDE (μl) | DDE (μl)/5 μl EtOH | DDE (μl)/10 μl EtOH | DDE (μl)/25 μl EtOH |
|----------|--------------------|--------------------|--------------------|
| 0        | 0                  | 0                  | 0                  |
| 3.3      | 6.7*               | 13.3*              | 33.3*              |
| 10.0     | 20.0               | 40.0               | 90.0               |
| 13.3*    | 26.6               | 66.5               |                    |

*One set of control eggs received neither ethanol nor DDE, and there were 12 DDE treatments and three ethanol-only controls.

*One sample of eggs from each clutch*.
Hatchling success and any hatchling deformities were recorded. The right gonad, kidney complex, and associated ducts of each hatchling were removed, embedded in wax, sectioned, and stained with hematoxylin and eosin. The sex of each gonad was assessed by examination under a light microscope according to criteria established by Miller and Limpus (37). Where an assessment was not possible, the second gonad was examined.

Results

DDE background levels. Untreated eggs had between 1.3 ng/g and 2.4 ng/g DDE (Table 2) (mean ± standard error, 1.7 ± 0.3, n = 15). There was no significant added variance component due to differences among clutches in DDE concentrations (F = 5.96; df = 3, 11; p < 0.05). Eggs in clutch A had significantly higher concentrations of DDE (2.0 ng/g) than eggs in clutch B (1.5 ng/g) or D (1.5 ng/g) (Tukey Multiple Comparison Test; p < 0.05) (Table 2). There were no significant differences between clutches A and C (1.7 ng/g), or B, D, and C (Table 2).

DDE rates of uptake. The amount of DDE taken up by the whole egg contents (excluding shell and shell membranes) and the embryo alone was regressed against the amount of DDE applied and the volume of ethanol used to apply the DDE. Once the amount of DDE applied was included in the model, no further variation in DDE taken up could be explained by the volume of ethanol used. DDE uptake could be predicted from DDE applied using the following equations:

\[ DDE_{\text{uptake by the whole egg}} = 0.3387 \times DDE_{\text{applied}} - 0.2339 \]  

(1)

\[ DDE_{\text{uptake by the embryo}} = 0.0757 \times DDE_{\text{applied}} - 0.0545 \]  

(2)

where DDE is in micromgrams and DDE applied ranged from 6.7 µg to 66.5 µg (Fig. 1). The slopes of both relationships were significant (Equation 1: F = 206.6; df = 1, 12; p < 0.01; \( R^2 = 0.99 \); and Equation 2: F = 179.7; df = 1, 12; p < 0.01; \( R^2 = 0.99 \)), but the intercepts were not. Hence, about one-third of DDE applied penetrated the eggs (Fig. 1), and about 8% of DDE applied was absorbed by the embryo (Fig. 1); therefore, levels up to 543 ng/g DDE were predicted in eggs exposed to up to 66.5 µg DDE dissolved in 25 µl of ethanol.

DDE effects on hatchlings. Stepwise multiple logistic regression of hatching sex against the predicted amount of DDE taken up by the whole egg, the amount of ethanol applied, and incubation temperature indicated that temperature alone exerted an influence on offspring sex (\( x^2 = 204.0; df = 1; p < 0.0005 \)). The probability of an egg yielding a female hatching at 27.6°C was 99%, compared to 94% at 30.4°C. Predicted outcomes of sexual differentiation were consistent, with expectation based on the effect of temperature alone (males at low temperatures and females at high temperatures). Hence, we could not demonstrate that either DDE or ethanol affected hatching sex at either temperature.

Multiple logistic regression was also used to explore any relationships between embryo survival and the amount of DDE taken up by the whole egg, the amount of ethanol applied, and incubation temperature. Only incubation temperature was found to have a significant effect on embryo survival (\( x^2 = 21.7; df = 1; p < 0.0005 \)). The probability of an egg hatching at 27.6°C was 94% compared to 72% at 30.4°C.

The incubation time to pipping, deformity rate, and hatching size and weight were regressed against predicted total DDE in the egg and volume of ethanol applied for each temperature. Duration of incubation, hatching weight, head width, or carapace length were not significantly related to the amount of DDE or ethanol applied. However, eggs incubated at the lower temperature of 27.6°C took longer to develop (62.6 vs. 52.3 days; F = 2.437; df = 1, 283; p < 0.0005) and yielded hatchlings that were heavier (25.0 vs. 24.2 g; F = 17.0; df = 1, 272; p < 0.0005) and had significantly wider heads (12.5 vs. 12.3 mm; \( F = 7.99; df = 1, 282; p < 0.01 \)) than hatchlings that emerged from the 30.4°C incubation. Carapace length was unaffected by all treatments (46.2 mm).

One female hatching incubated at 30.4°C and exposed to 42.8 ng/g DDE in the egg (predicted by Equation 1) showed a saddleback condition of extreme celosomia (body deformity) (34). No other cases of body deformities were observed.

Discussion

DDE concentrations in Chelonia mydas eggs collected from Heron Island were much lower than the levels reported in other studies, i.e., in the eggs from Florida beaches in 1976 [up to about four times lower (13)] and from the Ascension Islands in 1972 [up to about 7 times lower (39)] (Table 1). Some DDE in C. mydas eggs from Heron Island was expected because of the global contamination of oceans with DDT (23,24). Concentrations of DDE are even higher in other reptile species with temperature-dependent sex determination from other areas (Table 1). The foraging grounds of the four turtles whose eggs were collected on Heron Island must therefore be relatively uncontaminated by DDE. Differences in DDE concentration between clutches were statistically significant but small (e.g., 2 vs. 1.5 ng/g; Table 2). These differences probably reflect variances in the background levels of DDE in the foraging area of the female turtles (23), differences in time since last nesting (40), or natural variation in DDE uptake and retention time (41) of each individual.

Topical administration of DDE dissolved in ethanol was found to be an effective method of contaminating eggs in the laboratory because the relationship between the concentration and amount applied and the amount taken up by the egg and embryo was statistically significant and suitable for prediction (Fig. 1). However, only 34% of the initial dose found its way into the egg, and only about 8% of applied DDE found its way into the stage 28 embryo (34) (Fig. 1). The remainder presumably remains on the surface and within the pores of the eggshell and shell membranes or is lost by volatilization. Guilleto (29) reported an uptake of up to 62% when Alligator mississippiensis eggs were dosed with estradiol in oil. It is likely that the penetration of solutes varies from species to species depending on the eggshell structure (42,43) and on the solvents used. Nevertheless, there is a limit to the amount of ethanol that can be applied to an egg before risking its survival (thought to be about 25 µl); because about one-third passes into the egg of Chelonia mydas and only 8% is taken up by the embryo, there is a limit to the amount of DDE that can be administered to the developing embryo by topical administration using ethanol.

Within these limitations, we could demonstrate no effects of contamination on incubation time to pipping, hatching success, sexual differentiation, or hatching deformities. While hatching success decreased and
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