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Effects of beta-benzene hexachloride *in-ovo* on the embryonic growth and development of domestic mallard ducks (*Anas platyrhynchos domesticus* L.)

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Abstract

This was the first study conducted to test if varying levels of ß-Benzene Hexachloride affects the embryonic growth and development of Mallard using *in-ovo* procedure. In a sanitized environment, 200 fertile eggs at day 0 (n=200) were exposed to varying concentrations of β-Benzehexacholorohexane (β-BHC). The five treatments with four replications (10 eggs rep^{-1}) were the sham-injected control (T1), positive control (T2), 150 ppb (T3), 300 ppb (T4) and 600 ppb (T5) of β-BHC. All treatments except T1 were administered by *in-ovo* using sterilized olive oil as vehicle. The embryonic weight and body length were determined on days 7, 14, 21 and 28. On the day of hatching (day 28), movement and feeding behaviors were documented using DSLR camera. The results revealed that the significant decrease in embryonic weight and length were observed at 600 ppb ß-BHC. Likewise, eggs treated with 300 and 600 ppb β-BHC manifested splay leg incidence. The reduced total hatchability of 300 and 600 ppb β-BHC may be attributed to leg weakness. Therefore, 300 ppb β-BHC present in duck eggs is critical.

Keywords: β-Benzehexachloride, in-ovo, mallard, embryonic

Introduction

Lake shellfish feed resources were found having 0.05 ppm heavy metal Cadmium but negative for organochlorine pesticides (OCPs) (Laguna Lake, Philippines), however golden apple snail (*kuhol*) from rice paddies were found having 0.03–0.08 ppm Cadmium and 0.01–0.02 ppm Endosulfan [1]. Surprisingly duck commercial feeds were also found having OCPs, like α-, β- and γ- Benzenehexachloride (BHC) ranging from 0.02 to 0.150 ppm [2]. Likewise, they reported that low levels of α- and β- BHC enhances the growth (steroidal effect) while γ-BHC suppressed the growth of growing Mallard ducks. It was also reported that these organochlorine pesticides (OCPs) present in feeds accumulate in the liver, ovary and fat depots of Mallard ducks [1].

The combined effects of low levels of Cd and organochlorine pesticide residues present in feed of sexually mature Mallard ducks through liver histopathology were hydrophic degeneration, local mononuclear infiltration, centrilobular necrosis, hemorrhage and vacuolar degeneration, hence these pathological changes is suggestive of reduced egg production [3]. Similarly, low levels of organochlorine pesticide residues in combination with Cd in feed results in significant decrease in liver size and 35 days earlier age of first egg lay [2].

Although OCPs are not acceptable to be found in human food or animal feed, apparently these residues can not be avoided, hence several international agencies are studying the tolerable daily intake (TDI), low adverse effect level (LOAEL), no effect level (NOEL), no observed adverse effect level (NOAEL) and maximum residue limit (MRL) of OCPs. The procedure sometimes varies and the values differ greatly between several organizations. The LOAEL, NOEL, NOAEL and MRL of hexachlorobenzene for example are 0.17 ppb d^{-1} (WHO) [4], 160 ppb d^{-1} [5], 50 ppb d^{-1} [6] and 1000 ppb (FAO/WHO) [7], respectively.

Testing of chemicals using an *in–ovo* treatment assay in Japanese quails was done to establish the sensitive embryonic endpoint and a test system for detecting androgenic and anti-androgenic potential of chemicals [8]. The *in–ovo* technology application is used for vaccination against Marek's disease, infectious bursal disease virus (IBDV), injection of plasmid DNA encoding β-galactosidase *in–ovo* and several other substances were accomplished to improve survival and health of the avian species [9]. This study however, was the first to confirm using *in–ovo* procedure if the tolerable limits of certain OCP, i.e., β-BHC is within the no-observable-adverse-effect level (NOAEL) from embryonic growth until hatching.

Since, β-BHC and OCPs [1,2] have been reported to be present in commercial feeds and in eggs in this study, therefore varying levels of β-BHC, an organochlorine residue was used in this study to determine the no-observed-adverse-effect-

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level (NOAEL). Cadmium was absent in eggs in this study, supportive of other findings that there is restricted transfer of Cd to eggs at high doses [10]. This study also aims to optimize the *in-ovo* administration protocol for assessing the effect of specific unwanted residues on the embryonic growth and development of the Mallard duck focusing on β-BHC.

Materials and methods

Treatment preparations

Fertile eggs (n=200) having almost similar weight of 50 grams were acquired from a single commercial farm in Victoria, Laguna. The experiment was divided into five groups. The varying concentrations of β-BHC considered were based on the level present in feed as reported earlier [2]. Likewise, Endosulfan was also present in feed (90 ppb) and bioaccumulated in duck eggs at 150 ppb in this study, however it was not used because purity of this chemical is commercially unavailable. The stock solution of 150 (T3), 300 (T4) and 600 (T5) ppb of lindane (100% β-BHC powder; SIGMA-ALDRICH, Supelco, Indonesia) were prepared in a double distilled water. Then the injectable solutions were prepared using sterile olive oil. The olive oil was placed in a glass cylinder covered with a foil, then it was placed inside a pressure cooker gauged for 20 psi for a period of 20 minutes. Only the vehicle (olive oil) was applied in the positive control (T2), while the sham-treated control (T1) none was administered.

Commercial feeds

Fresh stocks of commercial feeds being used by the different duck raisers for their animals were sampled and were subjected to analysis of pesticide residues and Cd contents. Two sets of 500g feed samples were washed with 500 ml distilled water each and the washings were collected in a similar borosilicate containers. The collected washings were acidified to pH 2.0 and were prepared for wet digestion in acid under the hood for at least eight hours for Cd analysis and the other set was preserved in ice or kept frozen prior to pesticide residue analysis. Samples were then transported to University of the Philippines–Natural Science Research Institute–Research Analytical Service Laboratory (UP-NSRI-RASL with PNS ISO/IEC 17025:2005) for further processing.

Sample preparation and analysis

Continuous liquid-liquid extraction (LLE)

Using frozen or fresh washings of the samples, organic solvents were added in a continuous liquid-liquid extractor, dried and concentrated and were exchanged, when necessary, into a compatible solvent designed for semi-volatile organic compounds.

Acid digestion

The biological samples (500g) were acid digested using analytical grade reagents or concentrated and fuming HNO3, hydrogen peroxide 50 volume, and concentrated HCL on an aluminum hot plate in pre-weighed beaker as digestion vessel with watch glass cover for efficient refluxing. Refluxing of samples during acid digestion was allowed until the digestates appeared clear or until salt crystals formed at the bottom. All digestions were accompanied with blank samples containing ultrapure water only. The digestates were reconstituted using ultrapure water upon filtration through 1 Whatman ashless paper and were made to volume in 50 ml volumetric flask with glass stopper. The samples were then ready for injection into Flame-Atomic Absorption Analyzer (Flame-AAS).

Analytical procedures

Different types of analyses were conducted by the University of the Philippines, Natural Sciences Research Institute–Research and Analytical Services Laboratory in the biological samples that were submitted. These include the moisture content, cadmium and organochlorine pesticide content analyses in the samples which employed different methods of analysis such as the Gravimetric, Atomic Absorption Spectrophotometry and Gas Chromatography/Mass Spectrometry. Gravimetric analysis is a method of quantitative chemical analysis in which the constituent sought is converted into a substance (of known composition) that can be separated from the sample and weighed (Encyclopædia Britannica) while GC/MS is a method which combines the features of gas-liquid chromatography and mass spectrometry. The gas chromatography (GC) portion separates the chemical mixture into pulses of pure chemicals and the mass spectrometer (MS) identifies and quantifies the chemicals. Atomic Absorption Spectrophotometry, on the other hand, analyzes the concentration of elements in a sample based on the energy absorbed from certain wavelengths of light (usually 190 to 900nm). It is designed to determine the amount (concentration) of an object element in a sample, utilizing the phenomenon that the atoms in the ground state absorb the light of characteristic wavelength passing through an atomic vapor layer of the element. These processes were conducted at the UP-NSRI-RASL and results were taken as soon as the analyses have been carried out.

Administration of treatments

The eggs were injected at day 0 before the start of incubation period. The cohort of eggs was placed in a vertical position with the air sac upwards for 30 min before the treatment administration. The outer air sac location was cleaned and disinfected with 70% isopropyl alcohol. A heat-sterilized pin was utilized to prick a hole just above the air sac to facilitate the entry of the treatment solution. Using 1 ml syringe, 50 µl of the treatment solutions was injected within the air cell. The punctured portion of the egg was immediately sealed using glue (Elmer's Glue, USA) accomplished in a sterile environment.

Incubation and data gathering

After labeling, the eggs were grouped according to replications, placed inside metal trays set in an automatic turning incubator

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at 37.5–37.72^{0}C. The daily temperature and relative humidity were checked until hatching. The incubated eggs were sampled in the afternoon at day 7, 14, 21 and 28 (expected date of hatching). One egg was sacrificed in all treatments from each replicate.

The growth and development of growing embryo of samples for every treatment was determined by weighing the embryo after separating the liquid fraction and wiping the embryo carefully using a commercially available paper folded tissue then weighed using top balance weighing scale (mg sensitive up to 100 decimal places). The head diameter and body length were measured after capturing the image with standard ruler.

After adjusting the image pixels with the standard marker in ImageJ® (NIH, USA), the head diameter was determined by getting the longest horizontal diameter, while the body length was done by measuring from the tip of the shoulder to the base of the tail feather marked during documentation using macrolens of EOS Digital Camera (Canon, Japan).

The percent hatchability was recorded during the expected date of hatching. The behavior of the exposed ducklings was documented. The chicks were observed for mortality and gross visible abnormality like lethargy, movement, and bone disorder. The observations were documented using EOS® DSLR (Canon, Japan) and the images were processed using ImageJ® (NIH, USA) software.

Statistical analysis

The data was analyzed using Statistical Analysis System (version 9.1). Single factor experiment was employed to observe the main effects of varying treatments in day 7, 14, 21 and 28 separately for every period. The measured parameters were embryonic weight, body length and head diameter. The mean comparison used was Duncan Multiple Range Test (DMRT), after the analysis of variance (ANOVA) declared significance at $P<0.05$ level.

Results and discussions

As shown in (**Table 1**), cadmium was detected in feeds (0.049 ppb) but the levels in duck eggs were below the estimated method detection limit (EMDL), apparently Cd is not transferred in eggs at low levels. This is supported by the report [**10**] that Cd transfer to eggs is restricted in laying hens exposed to 7.5mg Cd/kg body weight. Endosulfan, an organochlorine residue (OCPs) found present in commercial feed at 90 ppb indicates bioaccumulation in egg at 150 ppb. Since pure Endosulfan is not available, β-BHC was used in this *in-ovo* study of determining the No Observable Adverse Effect Level (NOAEL) for embryonic growth and development of ducks. Other OCPs like Heptachlor, Chlordane, and Nonachlor were absent in feed but were present in eggs, implying the need for validating the hypothesis that these isoforms of OCPs may also be present in drinking water and/or in the air and/or litter floors (rice hulls).

Table 1. Organochlorine Pesticides (OCPs) and heavy metal cadmium levels in feeds and duck eggs of female domestic Mallard, randomly selected and pooled sample (n=8) from the University Animal Farm on 18 September 2009. The OCPs was analyzed using Gas Chromatography/Mass Spectrometry with the corresponding EMDL, while cadmium was determined using Atomic Absorption Spectrophotometry by the U.P. Natural Science Research Institute (PNS ISO/IEC 17025:2005).

Organochlorine Pesticide ppb or ng/g (as received)	**Duck Eggs**	**Duck Feeds**	**EMDL**
alpha–BHC	**<EMDL**	**<EMDL**	**0.6**
beta–BHC	**<EMDL**	**<EMDL**	**0.5**
Hexachlorobenzene	**<EMDL**	**<EMDL**	**0.3**
Heptachlor	**1.3**	**<EMDL**	**0.5**
Aldrin	**<EMDL**	**<EMDL**	**0.5**
Heptachlor Epoxide	**<EMDL**	**<EMDL**	**0.4**
Gamma–Chlordane	**3.0**	**<EMDL**	**0.6**
o,p'–DDE	**<EMDL**	**<EMDL**	**0.5**
Endosulfan I	**<EMDL**	**<EMDL**	**0.8**
alpha–Chlordane	**2.0**	**<EMDL**	**0.8**
trans–Nonachlor	**1.0**	**<EMDL**	**0.7**
Dieldrin	**<EMDL**	**<EMDL**	**2.0**
p,p'–DDE	**<EMDL**	**<EMDL**	**0.7**
o,p'–DDD	**<EMDL**	**<EMDL**	**0.7**
Endrin	**<EMDL**	**<EMDL**	**2.0**
Endosulfan II	**151**	**90**	**2.0**
p,p'–DDD+*o,p*'–DDT	**<EMDL**	**<EMDL**	**0.5**
cis–Nonachlor	**<EMDL**	**<EMDL**	**0.9**
Endosulfan Sulfate	**<EMDL**	**<EMDL**	**0.5**
p,p'–DDT	**<EMDL**	**5**	**0.9**
Endrin Ketone	**<EMDL**	**<EMDL**	**2.0**
Methoxychlor	**<EMDL**	**<EMDL**	**0.9**
Mirex	**<EMDL**	**<EMDL**	**0.7**
Heavy Metal Cadmium (ppb or ng/g)			
Cadmium	**<EMDL**	**0.049**	**0.01**

Body weight and length

In all treatments gradual increase in embryonic weight occurs from day 7–14, while linear trend follows from day 14–28, data not shown. The growth curve follows a curvilinear

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pattern. (**Table 2**) shows the mean weekly body weight of all treatments. The eggs exposed to 600 ppb (T5) obtained the lowest weekly gain in embryonic weight, i.e., 15 vs 18 g. During the course of development from day 7 to 28, the eggs that were exposed to 600 ppb obtained the lowest average body weight significantly. It must be noted that during the third week of incubation (day 21), the other treatments have rapidly gained in weight. However, 600 ppb treatment gained in weight was the slowest (13.7 g only) compared to the sham-injected eggs, positive control, 150 ppb and 300 ppb β-BHC (23.9, 21.4, 22.1 and 22.0 g respectively). During hatching (day 28) the chicks exposed to 600 ppb β-BHC obtained a mean body weight of 42.9 g, significantly lower than sham-injected (46.8 g), positive control (46.6 g), 150 ppb ss-BHC (47.5 g), and 300 ppb β-BHC (45.60 g). The increasing concentration of β-BHC treatment of fertile duck eggs significantly reduced body weight gain of the developing embryo proportionally with β-BHC higher than 150 ppb. The decrease in body weight can be an indicator of toxicity which is at the level of 300 ppb or higher. Body weight was identified as one of the endpoints in subchronic toxicity study on Wistar rats [**11**]. There was a significant reduction in body weight in males at 22.5 ppm d^{-1} and females at 25 ppm d^{-1} of β-HCH [**11**]. Similar pattern of significant decrease in the body length of the embryo that were exposed to β-BHC ($P<0.05$) was noted. The mean body length (cm) of 600 ppb exposed duck embryo (4.4 cm) obtained significant differences among other treatments. The eggs exposed to β-BHC were observed to have a slowed growth not just in weight but also on body length. Significantly shorter body length was observed in 600 ppb β-BHC (**Table 3**). Chronic exposure to noxious chemicals manifest slowed growth and immune suppression [**12**]. Significant depression in embryonic weight, body length and head diameter is consistent with 600 ppb ss-BHC (**Tables 2**, **3** and **4** respectively). The level of 600 ppb β-Benzene hexachlorohexane (β-BHC) is clearly detrimental to embryonic growth and development. A tendency to decrease in embryonic weight is observed with 300 ppb (β-BHC) at day 14 and 28, however because of high individual variance, 300 ppb ss-BHC treatment was not significantly detected.

Table 2. Mean weekly embryonic weight of duck eggs treated with sham-injected, positive, 150 ppb, 300 ppb and 600 ppb β-BHC.

Treatment	Mean Weight (g)
T1 (sham-injected)	18.74±0.84[a]
T2 (positive control)	18.13±0.90[a]
T3 (150 ppb)	18.46±1.14[a]
T4 (300 pb)	17.77±0.77[a]
T5 (600 ppb)	15.18±1.71[b]

a,b mean values within a column with different superscripts differ significantly ($P<0.05$).

Hatchability and feeding responses

The percent hatchability of T1 (sham-injected), T2 (positive control), T3 (150 ppb ss-BHC), T4 (300 ppb ss-BHC) and T5 (600 ppb ss-BHC) were observed to be proportionally declining with increase in level of β-BHC residues. Splay leg incidences were observed in T4 and T5 (more in T5), which is indicative of neurotoxicity as the major effect of β-BHC (**Figure 1**). **Table 5** shows the reduction in the percent hatchability of ducks at day 28 which can be attributed to difficult mobility of the developed embryo due to splay leg condition. Lindane is teratogenic to mallard embryo at doses greater than five times the field level of application [**13**]. At higher dosage, this was supported by an increase in fetal death within the first five days due to the β-HCH that has been administered to rat dams at 20 ppm d^{-1} during gestation period [**14**]. In our experiment, embryos exposed to low doses of lindane (600 ppb) were observed to be lethargic and less responsive compared to sham-injected control group. Ruffled feathers and splay leg were observed in 300 ppb and 600 ppb β-BHC hatched chicks. The level of β-BHC that manifests neurotoxicity in Mallard was 300 ppb and can be the critical level for duck eggs. After hatching the ducklings were provided with light, water and *ad libitum* diet. It was observed that the 600 ppb exposed embryos were less responsive to feeding compared to other treatments. A study in humans that were exposed to food contaminated with γ-HCH have experienced decreased

Table 3. Mean weekly body length (cm) of duck eggs treated with sham-injected, positive, 150 ppb, 300 ppb and 600 ppb β-BHC.

Treatments	Day 7	Day 14	Day 21	Day 28	Mean ± SEM
T1 (sham-injected)	1.30	4.20	7.40	10.34	**5.81±0.24[a]**
T2 (positive control)	1.27	4.30	7.34	11.00	**5.96±0.13[a]**
T3 (150 ppb)	1.08	3.97	7.15	10.03	**5.56±0.24[a]**
T4 (300 pb)	1.16	3.53	7.09	9.06	**5.21±0.34[a]**
T5 (600 ppb)	1.02	3.35	5.52	7.86	**4.44±0.39[b]**
Means±SEM	**1.17±0.10[d]**	**3.87±0.184[c]**	**6.90±0.30[b]**	**9.66±0.48[a]**	

a,b mean values in the same row/column with different superscripts differ significantly ($P<0.05$).

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Table 4. Mean weekly head diameter (cm) of duck eggs treated with sham-injected, positive, 150 ppb, 300 ppb and 600 ppb β-BHC.

Treatments	Day 7	Day 14	Day 21	Day 28	Mean ± SEM
T1 (sham-injected)	0.69	1.50	2.33	4.48	**2.25±0.08[a]**
T2 (positive control)	0.69	1.28	2.16	3.78	**1.98±0.16[a]**
T3 (150 ppb)	0.71	1.53	2.20	4.07	**2.13±0.08[a]**
T4 (300 pb)	0.66	1.44	2.33	3.37	**1.95±0.09[a]**
T5 (600 ppb)	0.76	1.27	1.80	3.52	**1.84±0.19[b]**
Means ± SEM	**0.70±0.074[d]**	**1.40±0.124[c]**	**2.16±0.09[b]**	**3.84±0.196[a]**	

a,b mean values within a row/column with different superscripts differ significantly (P<0.05).

Figure 1. Some newly hatched ducklings exposed to sham-injected (**T1**), positive control (**T2**), 150 ppb β-BCH (**T3**), 300 ppb β-BCH (**T4**) and 600 ppb β-BCH (**T5**) showing normal appearances, splay leg incidence and ruffled feathers.

Table 5. Numbers of eggs set and hatched of sham-injected and positive controls and varying levels of β-Benzenehexachloride (150, 300 and 600 ppb).

Treatment	Incubated Eggs	Hatched Ducklings	% Hatchability
T1 (sham-injected)	40	38	95.0
T2 (positive control)	40	33	82.5
T3 (150 ppb)	40	30	75.0
T4 (300 pb)	40	26	65.0
T5 (600 ppb)	40	24	60.0
TOTAL	200	93	75.5

appetite, although difficulty in movement was the visible sign of weakness [15]. Other studies concluded that low doses of lindane were found to induce neurochemical, behavioral, metabolic and even structural changes in developing mammals [16,17]. The current observation also corroborated with another study [18] even though their levels were twice or more than our duck egg study. According to their study, there was a reduced auditory startle response habituation in 11-day-old offspring of maternal rats that were exposed to ≥5.6 ppm d^{-1} doses of γ-HCH in the diet from day 6 of gestation through day 10 of lactation. Apparently, based on available literatures and the result of our experiment, avian species may have lower tolerance to lindane (β-BHC) residues. The neurotoxic effects of β-HCH were observed in six female B6C3F1 mice for immunotoxicity study [18]. The β-BHC at estimated doses of 0, 19, 57 or 190 ppm d^{-1} was included in the diet of female B6C3F1 mice for 30 days. Clinical signs of ataxia or loss of full control of bodily movement were immediately observed in the 57 or 190 ppm d^{-1} group, although 57 to 190 ppm is way beyond our level which is only within 0.60 ppm, administered only once. However, it was observed that 80% of the 190 ppm d^{-1} mice became laterally recumbent and moribund or lack of vigor being in terminal decline prior to stimuli [19].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	RSV	DSR	CAL	JRO	MMI	SSC
Research concept and design	✓	--	--	--	--	--
Collection and/or assembly of data	--	✓	--	✓	✓	--
Data analysis and interpretation	--	✓	--	--	--	--
Writing the article	✓	✓	✓	✓	✓	--
Critical revision of the article	✓	--	✓	--	--	✓
Final approval of article	✓	--	--	--	--	--
Statistical analysis	✓	✓	--	--	--	--

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