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In ovo exposure to organophosphorous flame retardants: survival, development, neurochemical, and behavioral changes in white leghorn chickens

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ABSTRACT

Organophosphorous flame retardants (OPFRs) are contaminants of emerging concern. There is growing evidence of environmental contamination and exposures to both humans and wildlife. Here, the objective was to increase understanding of the potential neurodevelopmental effects of two relevant OPFRs, TMPP (tri (methylphenyl) phosphate; a non-halogen-containing OPFR) and TDCIPP (tris (1,3-dichloro-isopropyl) phosphate; a halogen-containing OPFR) in an avian embryo/chick model. We injected white leghorn chicken eggs with a range of TMPP (0, 10, 100, and 1000 ng/g) or TDCIPP (0, 10, 100, 1000, 50,000 ng/g) concentrations at incubation day 0 exposing embryos throughout the ~21-day *in ovo* period. Hatching success was unaffected by TMPP, but TDCIPP-exposed chicks had higher early-incubation mortality in 100 and 50,000 ng/g groups. On 7–9-day-old chicks, we assessed behavior *via* tests concerning righting reflex, angled balance beams, gait patterns, wing flap reflex, and open field movements. Chicks exposed to 100 ng/g TDCIPP achieved 40% lower maximum velocity in the open field test than vehicle-exposed controls, while those exposed to 1000 ng/g TDCIPP achieved 20% higher maximum velocity than vehicle-exposed controls. Chicks exposed to 50,000 ng/g TDCIPP showed reduced righting response success. There were no dose- or treatment-related differences in angled beam, gait analysis, or wing flap reflex tests. Cerebrum hemispheres from 10-day-old chicks were examined for neurochemistry (acetylcholinesterase [AChE] activity and both nicotinic [nACh] and muscarinic [mACh] acetylcholine receptor levels) and cerebellums were examined for histopathology. TDCIPP-exposed chicks had reduced number of degenerate Purkinje cells (TDCIPP, 1000 ng/g), possibly indicating disruption of neurodevelopment. No neurochemical effects were found in TMPP- or TDCIPP-exposed chicks. In general this study shows some possible neurodevelopmental effects in chicks exposed to TDCIPP when levels greatly exceeded those measured in wild bird eggs and no clear changes in TMPP-exposed chicks. This study builds upon previous *in vitro* studies as well as work on adult birds showing that toxic responses in avian models can vary among species and OPFRs.

1. Introduction

Flame-retardants are anthropogenic chemicals designed to prevent fires from starting and slow their progress, and are incorporated into a wide variety of products (van der Veen and de Boer, 2012). Due to their environmental persistence and potential toxicity, many jurisdictions such as the European Union are regulating the use of many brominated flame retardants (BFRs, *e.g.*, pentabrominated diphenyl ethers [pentaBDE], decabromodiphenyl ether [decaBDE]). As a result, replacement chemicals are coming into greater use, a majority of which likely have limited hazard data.

One important class of replacement for BFRs includes the organophosphorous flame retardants (OPFRs) which consist of at least 23 chemicals that contain a phosphorous functional group, are often triesters, and may be halogenated or non-halogenated (Bergman et al., 2012). There is growing evidence that OPFRs are accumulating throughout ecosystems, though more studies exist for aquatic organisms (Leonards et al., 2011; Sundkvist et al., 2010; Green et al., 2008) than for terrestrial wildlife. For example, tri (methylphenyl) phosphate (TMPP), a non-halogenated OPFR, has been found in soils and sediments (<0.05–288 ng/g; range of reported values; Leonards et al., 2011) and aquatic organisms (<0.04–137 ng/g Leonards et al., 2011; Sundkvist et al., 2010), though was below the limit of detection of 0.6 ng/g in Kittiwake (*Rissa tridactyla*) and common eider (*Somateria mollissima*) livers from the Svalbard archipelago in Norway (Evenset et al., 2009), as well as bird plasma and eggs (great black-backed gull (*Larus*

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marinus), common eider (*S. mollissima*)), white-tailed eagles (*Haliaeetus albicilla*), and shag (*Phalacrocorax aristotelis*) from various sites in Norway.

Tris (1,3-dichloro-isopropyl) phosphate (TDCIPP), a halogenated OPFR, is persistent in the environment, and is poorly removed during the sewage treatment processes of municipal wastewater treatment plants (~70% of TDCIPP in influent remained in effluent from Swedish plants; Marklund et al., 2005). TDCIPP has been found in sediments (<0.09–8800 ng/g; range of reported values; Leonards et al., 2011; Green et al., 2008), aquatic organisms (<0.025–140 ng/g; Leonards et al., 2011; Sundkvist et al., 2010), and in wild bird eggs (1.9 ng/g; 3 eggs from great black-backed gull (*L. marinus*), 1 egg from common eider (*S. mollissima*)) and blood (0.16 ng/g; plasma from 2 white-tailed eagles (*H. albicilla*) and 1 shag (*P. aristotelis*)) at various sites in Norway (Leonards et al., 2011). Chen et al. (2012) found TDCIPP up to 0.17 ng/g in herring gull (*Larus argentatus*) eggs from the Great Lakes, though many samples were below the method limit of quantification. Additionally, TDCIPP was found in yolk (mean 0.93 ng/g) and albumen (mean 0.47) of 16 herring gull eggs, as well as adult herring gull tissues (mean concentrations of 4.43 ng/g, 5.04 ng/g, and 1 ng/g in fat, muscle, and red blood cells, respectively; and from non-detect up to 0.1, 0.37, and 1.28 ng/g in blood plasma, liver, and brain, respectively) from the Great Lakes (Greaves and Letcher, 2014).

Studies on mammalian models have shown various OPFR congeners to be neurotoxic (Craig and Barth, 1999; Jortner et al., 2005; Flaskos et al., 1994; Dishaw et al., 2011). However, despite potential for exposures in the real world, it is not well known if OPFRs are neurotoxic to birds. An early study documented ortho isoforms of TMPP to induce ataxia and reduce plasma cholinesterase levels in chickens (Bondy et al., 1960). There was 100% mortality among adult chickens fed 4.8 g/kg TDCIPP per day for 5 days, with lower doses causing weakness in legs and wings (Ulsamer et al., 1980). *In vitro* studies on primary cultures of embryonic chicken neuronal cells found that TDCIPP caused cytotoxicity, though no molecular effects were found (Crump et al., 2012). In egg-injection studies involving embryonic chickens: embryos exposed to 261,400 ng/g TMPP exhibited developmental malformations, reduced tarsus length, increased liver-somatic index, up-regulation of hepatic enzymes, and increased plasma bile acid concentrations, but not reduced pipping success or altered plasma thyroid hormone (T4) status (Crump et al., 2014); while TDCIPP reduced growth and plasma thyroid hormone levels, and induced hepatic xenobiotic metabolism gene expression (Farhat et al., 2013). Given connections between thyroid hormone and Purkinje cell development (Heuer and Mason, 2003; Kimura-Kuroda et al., 2002) and the neurological and hormonal effects of TMPP and TDCIPP outlined above, investigating the effects of TMPP and TDCIPP on Purkinje cell development and behavioral outcomes may be warranted. Indeed, other organophosphate esters have been demonstrated to affect Purkinje cell development (Abou-Donia et al., 2006). In addition, from an avian *in vitro* study of 16 flame retardant chemicals, both TDCIPP and TMPP were identified as priorities for further investigation based on hepatotoxicity (TMPP LC_{50} not found in chickens, but ~31 μM in herring gull (*L. argentatus*); TDCIPP LC_{50} 60.3 μM in chicken and 38.9 μM in herring gull) and hepatic mRNA expression change in genes related to xenobiotic metabolism, thyroid hormone, lipid metabolism, and growth (Porter et al., 2014).

The aforementioned studies document building evidence that birds exposed to OPFRs may suffer from adverse neurological effects. However, the evidence has been obtained mainly from studies focused on adult birds and *in vitro* methods, and little is known of early-life exposures despite this life stage usually being particularly sensitive to insults. While adverse outcomes related to survival and neurological function have been studied *in ovo* and *in vitro*, respectively, whether or not these observations translate into live hatchlings remains to be investigated. Given the potential for neurotoxicity and gross motor ability such as ataxia in adult birds, it is worth investigating potential impacts on behavior and motor ability of the more sensitive hatchlings. Here, the objective was to increase understanding of the potential neurodevelopmental effects of these chemicals towards avian models by using air cell egg injections to expose developing embryos. Two representative OPFRs, including a non-halogenated (TMPP) and halogenated (TDCIPP) chemical, were studied. Structural and functional outcomes were studied at multiple levels of neurological organization, including studies on brain neurochemistry, neuropathology, and chick behavior, as well as general developmental outcomes related to hatching. The focus here was on characterizing neurological components that were previously shown to be affected by OPFRs and other organophosphate esters, including cholinergic signaling and Purkinje cell development.

2. Materials and methods

2.1. Animal exposures

Two independent egg-injection studies were conducted in early 2013 using methods described elsewhere (Rutkiewicz and Basu, 2013). Fertilized white leghorn chicken (*Gallus domesticus*) eggs were obtained from the Michigan State University Poultry Farm. On day 0 of incubation, eggs were weighed and randomly injected into the air cell with 50 μl of either TMPP (CAS# 1330-78-5, Sigma-Aldrich, ≥89.0% purity) or TDCIPP (CAS# 13674-87-8, Sigma-Aldrich, ≤100% purity) dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, ≥99.7% purity), resulting in nominal doses of 0, 10, 100, or 1000 ng/g egg (for TMPP study) or 0, 10, 100, 1000, or 50,000 ng/g egg (for TDCIPP study). Non-injected and vehicle-injected controls were included. Previous studies have indicated that TDCIPP injected in this manner results in embryonic exposure (Farhat et al., 2013). In both studies, control groups had 15 individuals each. In the TMPP study, dosed groups had 20 individuals each. In the TDCIPP study, dosed groups had 21 individuals each, with the exception of the 50,000 ng/g group ($n = 15$) which was viewed *a priori* as an exploratory dose. Eggs were incubated at 37 °C and 60–70% humidity. Throughout each study, researchers were blind to treatment groups. All aspects of this study were approved by University of Michigan's University Committee on the Use and Care of Animals.

2.2. Survival, hatching, and euthanasia

Embryonic survival was assessed every 2–3 days by a combination of candling and the Buddy Digital Egg Monitor (Avian Biotech International, Tallahassee, FL). On day 19 of incubation, incubator humidity was raised to 70–80% and eggs were transferred to hatching trays. Pipping and hatching were monitored every 3–5 h during the 4-day hatch period. Post-hatch, chicks were housed in a brooder (up to 22 chicks/brooder; brooder size 92 cm × 60 cm × 28 cm, l × w × h) at 30–37 °C and 30–50% relative humidity on a 12 h light/12 h dark schedule. At 10 days post-hatch, chicks were euthanized by decapitation. For most chicks, the cerebrum was divided into left and right halves and immediately frozen on dry ice; the left cerebrum was used for cholinergic analyses, while the right was archived at −80 °C. For a subset of 12 chicks (TMPP; 6 each of vehicle-injected controls and 1000 ng/g-treated) or 16 chicks (TDCIPP; 8 each of vehicle-injected controls and 1000 ng/g-treated), whole brains were immersed in formalin for histological assessments.

2.3. Behavior

Chicks aged 7–9 days-post hatch were subjected to behavioral testing. Chicks were removed from the brooder and tested individually for open field, righting reflex, and angled balance beam. For gait analyses and wing flap reflex, birds from the control (both non-injected and vehicle-injected) and 1000 n/g-treated groups were removed from the brooder in groups of four, and tested in groups of four or two, respectively. All tests were video-recorded for ease of scoring and quality assurance. Following testing, all chicks were promptly returned to the