CHANGES IN THYROID PARAMETERS OF HATCHLING AMERICAN KESTRELS (*FALCO SPARVERIUS*) FOLLOWING EMBRYONIC EXPOSURE TO TECHNICAL SHORT CHAIN CHLORINATED PARAFFINS (SCCPS; C_{10-13}, 55.5% CL)

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Introduction

Chlorinated paraffins (CPs) are complex mixtures of polychlorinated *n*-alkanes categorized according to their carbon chain length: short chain (SCCPs, $C_{10} - C_{13}$), medium (C_{14} - C_{17}), and long chain ($C_{>17}$), chlorinated paraffins. SCCPs are primarily used in metalworking applications, as flame retardants, and in paints, adhesives, sealants, textiles, plastics and rubber (UNEP 2012). In 2012, the United Nations Environment Program (UNEP 2012) reported in the Revised Draft Risk Profile for SCCPs, that CPs were produced in the United States, the European Union (EU), Slovakia, Brazil, India, Japan and China. While annual global consumption of SCCPs is large (>25 tonnes/year), it has sharply declined over the past 20 years.

SCCPs are released through wastewater, landfills, and air emissions (UNEP 2012). Concentrations of SCCPs have been reported in fish and marine mammals in North and South America, Europe, Japan, Greenland and the Arctic (UNEP 2012 and references therein). Characterization of SCCP concentrations and exposure in terrestrial wildlife is limited. In 2010, SCCP concentrations were reported in the eggs of yellow-legged gulls (*Larus michahellis*) (4536 ± 40 pg/g wet weight (ww)) and Audouin's gulls (*Larus audouinii*) (6364 ± 20 pg/g ww) in Spain (Morales et al. 2012), and little auks (*Alle alle*) (5 - 88 ng/g ww) and kittiwakes (*Rissa tridactyla*) (5 - 44 ng/g ww) in the European Arctic (Reth et al. 2006). In Sweden, muscle of ospreys contained CPs of unspecified chain length (Jansson et al. 1993). Although the toxicity of SCCPs has been demonstrated in aquatic invertebrates, fish, frogs, and laboratory rats, there are limited avian studies and these reported no effects of SCCPs on egg parameters of domestic hens (*Gallus gallus domesticus*) and ducks (*Anas platyrhynchos*) (UNEP 2012). Despite reported accumulation of SCCPs in wild birds, to our knowledge, exposure-related toxicities and effects with respect to avian wildlife remain unknown.

Materials and Methods

Captive American kestrels (*Falco sparverius*) were used in the current study, given their sensitivity to several brominated and organophosphate flame retardants. Previous studies have reported greater sensitivity among kestrels than chickens, ducks, or other avian species, to polybrominated diphenyl ethers (PBDE) (e.g., Rattner et al. 2013). Animal handling procedures were approved by the USGS Patuxent Wildlife Research Center (PWRC) Animal Care and Use Committee. Eggs were obtained from 46 breeding pairs of American kestrels from a captive colony at the USGS PWRC in Laurel MD. Freshly laid eggs were collected daily, stored at 13°C for up to a week, and then artificially incubated at 37.5°C. Fertility was confirmed on embryonic day 4 or 5 (E4 or E5) (Pisenti et al. 2001). On E5, eggs of known fertility were either left un-injected or injected into the aircell with organic safflower oil (controls) or SCCP-TM mixed in safflower oil at concentrations encompassed within levels reported in wild avian eggs in the environment (10, 50 or 100 ng/g ww). Eggs were artificially incubated until hatching.

A stock dosing solution of SCCP-TM was prepared using a commercial SCCP-TM stock solution, Chloroparaffin® (C_{10-13}, 55.5% Cl, 100 µg/mL in cyclohexane; Dr. Ehrenstorfer GmbH, Augsburg, Germany). The cyclohexane solvent was then volatilized and removed to dryness. A solution of the SCCP-TM (300 µg of SCCP-TM in 100 µL of hexane) was prepared and added to 3.0 mL of safflower oil (President's Choice Organics, 100% pure safflower oil). Out-gassing the residual hexane resulted in a100 ng/µl solution of SCCP-TM in safflower oil. Preparation of the stock control solution (3.0 mL safflower oil) followed the same protocol

without addition of the SCCP-TM. The 10 and 50 ng/µl SCCP-TM safflower oil solutions were prepared serially from the stock 100 ng/µl dosing solution, and in sufficient volume for all the necessary kestrel egg injections. A volume of both the control and SCCP-TM dosing safflower oil solutions was taken for subsequent chemical analysis to confirm the analytical measured concentration versus the calculated nominal concentration.

Blood was collected from the right jugular vein of each hatchling, centrifuged, and the plasma frozen until subsequent thyroid hormone determination. Organs were removed and weighed. The left thyroid was fixed in buffered formalin for histopathology, and the right thyroid gland and plasma frozen for analysis of total thyroxine (TT_4) and total triiodothyronine (TT_3) concentrations. Genetic sex was determined by real-time PCR following the methods of Brubaker et al (2011) with slight modification. To assess concentrations of TT_4 and TT_3, thyroid glands were digested following the methods of McNabb et al. (2004). For each individual hatchling, TT_3 levels were analyzed in the gland digestate and plasma using the AccuBind® ELISA T3 Kit (Monobind Inc., Lake Forest, CA, USA), and TT_4 levels were analyzed using the Accu-Bind Neo-Natal Total T_4 kit (Monobind Inc.). Each sample was analyzed in duplicate. Duplicate precision (%CV) averaged 3.95, 3.72, 8.23, and 17.44% for glandular TT3, glandular TT4, plasma TT3, and plasma TT4, respectively.

Parametric and non-parametric statistical tests were used to identify differences in endpoints related to the SCCP-TM (55.5% Cl) exposure. Unless noted, comparisons were made to the vehicle-exposed control kestrels. Chi-square contingency tables identified statistically significant treatment differences concerning the presence/absence of edema and deformities. Glandular TT_4 and TT_3 concentrations were log-transformed. Residuals were tested for homogeneity of variance (Levene's test) and normality (Kolmogorov-Smirnov D). A one-way analysis of variance (ANOVA) was used to test for significant sex then treatment differences followed by least squares means (LSM) *post-hoc t*-tests for all pairwise comparisons without adjustments. Spearman's correlation coefficient analysis was performed to determine statistically significant correlations between TT_4 and TT_3 concentrations with hatchling morphometrics and somatic organ indices. Somatic indices, calculated as organ mass/embryo mass, were analyzed using Kruskal-Wallis non-parametric ANOVAs. Statistical significance was considered to be $p \leq 0.05$ and all statistical analysis was conducted using SAS® Version 9.3.

Results and Discussion:

There were no significant effects of the SCCP-TM exposure on hatching success, deformities (beak/feet), or the presence of edema in the kestrels (p-values ≥ 0.33). In contrast, developmental malformations reportedly occurred in embryonic African clawed frogs (*Xenopus laevis*) exposed to a commercial mixture of SCCPs (C_{12} 56% Cl) at 500-fold higher concentrations (≥ 5 mg/L) (Burýšková et al. 2006).

Endocrine, physiological, and morphometric measures were only assessed in kestrels that successfully hatched independently. There was no effect of the SCCP-TM exposure on the somatic index of the thyroid glands in contrast to that observed in embryonic kestrels exposed to DE-71 (Rattner et al. 2013). However, McNabb et al. (2004) reported that changes in thyroid gland size may not be a sensitive indicator in birds of hypothyroidism because of intermittency of feedback signals and hypothalamus-pituitary-thyroid axis responses.

There were no effects of the SCCP-TM concentrations on circulating TT_4 (♀♂) ($p \geq 0.36$) or TT_3 concentrations (♀♂) ($p \geq 0.11$). However, the *post-hoc* results for the plasma TT_3 concentrations are noteworthy in relation to future studies: male hatchlings demonstrated a significant suppression of plasma TT_3 when exposed to 10 (0.70 ± 0.14 ng/ml) or 50 ng/g ww (0.62 ± 0.13 ng/ml) SCCP-TM compared to control males (1.11 ± 0.5 ng/ml) (*post-hoc* $p \leq 0.05$). In addition, there was a marginally significant (*post-hoc* $p = 0.06$) doubling of plasma TT_3 in the females exposed to 100 ng/g SCCP-TM (0.88 ± 0.17 ng/ml) versus control females (0.45 ± 0.13 ng/ml). Comparatively different patterns were observed in male rats exposed to other technical mixtures of SCCPs: plasma free T_3 (FT_3) and TT_3 concentrations were unaltered, but plasma FT_4 and TT_4 concentrations suppressed, when exposed to 1000 mg/kg of Chlorowax 500C (58% Cl), Cereclor 56L (56% Cl), or Chlorparaffin 40G (40% Cl) (Wyatt et al. 1993). Concentrations of circulating thyroid hormones are highly regulated necessitating further investigations of the thyroid gland (hormone concentrations, histology) and hepatic deiodinase activity to fully understand chemical alterations of thyroid function.

Embryonic exposure of kestrels to SCCP-TM significantly altered glandular TT_4 concentrations (♂ only) ($F_{3,28}$ = 4.26 p = 0.02) but not glandular TT_3 (♀♂). Consistent with the pattern of effects on circulating TT_3 concentrations in the same male hatchlings, those exposed to 10 or 50 ng/g ww of SCCP-TM had significantly reduced glandular TT_4 concentrations (*post-hoc p*-values ≤ 0.05), with a directional reduction in glandular TT_4 for those males exposed to 100 ng/g ww of SCCP-TM (*post-hoc p* = 0.06); relative to control males, glandular TT_4 levels were suppressed by 33%, 22% and 20%, respectively. The suppression of glandular TT_4 levels suggests alterations in thyroid gland function.

The thyroid hormone concentrations of these hatchling kestrels were statistically correlated with other endpoints. In male hatchlings, glandular TT_4 concentrations were negatively correlated with yolk sac mass (N = 18; r = -0.52 p = 0.03), and plasma TT_4 was positively correlated with body length (N = 23 r = 0.52 p = 0.01). For female kestrels, glandular TT_4 concentrations were positively associated with the somatic indices of the spleen (N = 29; r = 0.54 p = 0.003) and heart (N = 21; r = 0.65 p = 0.001); plasma TT_4 concentrations were also weakly associated with the heart (N = 15; r = 0.50 p = 0.06) and negatively associated with the length of incubation (N = 24 r = -0.64 p = 0.0007). The biological significance of these relationships is unknown for kestrels, but thyroid hormones are involved in the avian immune system (e.g., spleen), and in birds, both are sensitive to contaminants (e.g., Smits et al. 2002; Mora et al. 2006). There is a close relationship between thyroid hormone status and cardiac size and function (Ojamma 2010). Exposure to thyroxine resulted in hypertrophied hearts in laboratory rabbits at 16 days of exposure (Talafih et al. 1983), whereas rats experienced a 30% increase in total heart weight (Klein and Hong 1986).

Preliminary conclusions include that the *in ovo* exposure of male kestrels to the SCCP-TM (55.5% Cl) at 10, 50 and 100 ng/g ww, suppressed circulating TT_3 concentrations and glandular TT_4 concentrations, while females demonstrated a doubling of plasma TT_3 concentrations at the highest SCCP concentration only. Plasma TT_4, glandular TT_3 concentrations, and the somatic index of the thyroid gland, were unaffected by the exposure to SCCP-TM (55.5% Cl). The biological associations between thyroid hormones and various endpoints warrant further investigation. Assessment of additional endpoints relating to thyroid function (i.e., thyroid gland histology, hepatic deiodinase enzyme activity), hepatic oxidative status, and anticipated hepatic concentrations of SCCP-TM, is ongoing.

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