Polychlorinated biphenyl toxicity in the thyroid gland of wild ungulates: an *in vitro* model

Ehdaa Eltayeb Eltigani Abdelsalam, Hana Bandouchova, Tomas Heger, Miroslava Kanova, Katerina Kobelkova, Monika Nemcova, Vladimir Piacek, Jana Sedlackova, Veronika Seidlova, Frantisek Vitula, Jiri Pikula

University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Ecology and Diseases of Zoo Animals, Game, Fish, and Bees, Brno, Czech Republic

Received March 26, 2020
Accepted May 26, 2020

Abstract

Polychlorinated biphenyls (PCBs) are carcinogens causing endocrine disruption. While production of PCBs is now banned, wildlife exposure still occurs due to environmental contamination. We investigated thyroid toxicity in wild ungulates using three-dimensional primary thyrocyte cultures exposed to PCB 138 for 24, 48, and 72 h at concentrations ranging within 0–3000 ng/ml. Thyrocyte viability ranged within 78.71–118.34%, 98.14–104.45%, and 84.16–106.70% in fallow deer-, mouflon-, and roe deer-derived cells, respectively. Viability decreased significantly in fallow deer ($P = 0.012$) and roe deer ($P = 0.002$) thyrocytes exposed for 48 h at 30 ng/ml. While cytotoxicity ranged within 2.36–16.37%, 3.19–9.85%, and 2.76–11.21% in fallow deer, mouflon, and roe deer, respectively, only roe deer displayed significantly higher cytotoxicity at a 3 ng/ml exposure ($P < 0.05$) and lower cytotoxicity at 30 ng/ml ($P < 0.01$). Exposure to 30 ng/ml for 24 and 48 h induced reactive oxygen species in fallow deer. Iodide uptake at 30 ng/ml exposure increased after 24 h in fallow and roe deer, but showed a significant drop after 48 and 72 h in fallow deer, mouflon, and roe deer. Thyroxine T_4 release at 30 ng/ml exposure decreased significantly after 48 and 72 h; 24, 48 and 72 h; and 48 h in fallow deer, mouflon, and roe deer, respectively. Our findings indicate time- and species-dependent effects of PCB on performance and thyrocyte function. Use of cell culture models reduces the number of experimental specimens, increases test species welfare and replaces whole organisms with specific target cells.

Environmental toxicants, thyroid disorders, iodine uptake, thyroxine, deer, mouflon

The thyroid is a major endocrine gland controlling metabolic, cardiovascular and developmental functions in vertebrates through secretion of the iodine-containing thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) (Aniolek 2019; Kendall 1919). The essential structure and dynamic unit of the thyroid gland are the thyroid follicles (Toda et al. 2011). These follicles have a spherical shape resulting from the organization of thyrocytes (80%) and C cells (20%) on the follicular stroma. Thyrocytes are polarized cells, with lateral microvilli facing the follicle lumen and the basal side facing the stroma. This unique structure allows the follicles to synthesize and release thyroid hormones via a mechanism that induces uptake of iodide I^- from circulation by regulating thyroid-stimulating hormone (TSH). Iodide then crosses into the follicular cellst hrough the transmembrane glycoprotein sodium/iodide symporter (NIS) located on the basolateral membranes of the thyrocytes. Iodide is later oxidized inside the follicular colloid through a rapid process controlled by thyroid peroxidase (TPO). Following oxidation, a biochemical process known as organification occurs, which incorporates iodine into thyroglobulin to produce thyroid hormones (van der Deure et al. 2010).

Primary thyroid cell cultures have been successfully obtained from several mammalian species, including humans, mice, pigs, and dogs (Dickson et al. 1981; Jeker et

Address for correspondence:
E.E.E. Abdelsalam, J. Pikula
Department of Ecology and Diseases of Zoo Animals, Game, Fish, and Bees
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackeho tr. 1946/1, 612 42 Brno, Czech Republic
E-mail: H15010@vfu.cz; pikulaj@vfu.cz
http://actavet.vfu.cz/

al. 1999). While thyroid primary cultures are considered analogous to the *in vivo* environment, their unique polarized structures mean that thyrocyte functioning cannot be obtained with conventional monolayer and suspension culture techniques (Dickson et al. 1981; Toda et al. 2011). More developed dimensional culture systems are necessary to allow thyrocytes to obtain semi-follicular structure and physiological polarity in a controlled environment that simulates their normal condition within the body. Three-dimensional cultures also facilitate a more efficient *in vitro* investigation of thyrocyte responses under stressful conditions (Toda et al. 2011).

One of the major stress factors contributing to thyroid disease is environmental exposure to toxicants. Several man-made substances, including polychlorinated biphenyls (PCBs), dioxins, and dioxin-like compounds, are thought to take part in thyroid disorders and autoimmune diseases (Miller et al. 2009; Brent 2010). Polychlorinated biphenyls are presumed to induce changes in levels of thyroid hormones and thyroid autoantibodies following occupational exposure (Langer et al. 1998; Longnecker et al. 2000). Extensive laboratory studies have been undertaken on animal models to investigate this issue, using both PCB mixtures and/or single congeners (Collins et al. 1977; Collins and Capen 1980; Chu et al. 1995). Physiological and histological findings in studies on rodents and non-human primates suggest that PCB interference on thyroid function may be dose- and duration-dependent (Collins et al. 1977; Collins and Capen 1980; van den Berg et al. 1988). Other typical findings include decreased serum T_3 and T_4 levels, though low PCB doses appear to have al esser effect on T_3 and T_4 levels (Collins et al. 1977; Collins and Capen 1980). Typical histopathological findings in rats exposed to either Aroclor mixture 1254 or single congeners include hyperplasia, hypertrophy, increased vacuolization of follicular cells and depletion of follicular colloid, reduced follicular size and thyroid enlargement (Collins et al. 1977; Collins and Capen 1980; Chu et al. 1995, 1996), while indicators PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180 contribute to anti-androgenic, anti-oestrogenic, anti-thyroidal, tumour-promoting and neurotoxic potencies, calculated for PCB mixtures based on hierarchical analysis (Hamers et al. 2011).

While several *in vivo* studies have indicated an association between environmental pollutant exposure and thyroid disruption, the potential for local thyroid disruption is difficult to track down based on circulating hormone levels alone (Brent 2010). Moreover, for many environmental toxicants, cytotoxicity studies evaluating thyroid toxicity have yet to be undertaken, whether in humans or animals (Brent 2010).

Based on *in vivo* experiments, PCBs have been found to interfere with thyroid function on several levels, implicating the hypothalamus-pituitary-thyroid (HPT) axis. Nevertheless, the impacts of direct PCB toxicity on thyrocytes, and its contribution to thyroid disorders, has been poorly investigated. To address this, we isolated follicles from the thyroid glands of three wild even-toed ungulates, fallow deer (*Dama dama*), mouflon (*Ovis orientalis*), and roe deer (*Capreolus capreolus*), for cultivation using a three-dimensional collagen system. Using this *in vitro* model, we undertook a series of experiments to examine the toxic effects of PCB 138, one of the most frequently occurring congeners in wildlife of economic importance (Nesvadbová et al. 2019). We predicted that PCB 138 would show dose- and exposure duration-dependent cytotoxicity resulting in disruption of iodide uptake and thyroid hormone production by thyrocytes.

Materials and Methods

We performed our experiments using a two-step procedure. First, we designed a concentration range for the PCB 138 congener centred lower and higher than environmentally relevant exposure levels for wild ungulates (muscles, adipose tissue and liver ofred and roe deer, wild boar) in both the Czech Republic and other European regions (Bachour et al. 1998; Warenik-Bany et al. 2016), i.e., 0.3, 3, 30, 300, and 3000 ng/ml. Using thistoxic exposure range, we then tested cells for viability and cytotoxicity. Second, we assessed the concentration that

induced significant differences in fallow-, roe deer-, and mouflon-derived thyrocytes for adverse effects on thyroid function using toxic exposures of 24, 48, and 72h.

Isolation of thyroid follicles for cultivation using the three-dimensional collagen system

Single follicles were isolated from thyroid gland tissue fragments dissected from game ungulates as described by Jeker et al. (1999), with minor modifications to fit our experimental conditions. Animals used for thyroid sampling included 1 mouflon female (2.5–3 years old), 2 fallow deer females (8 month and 1.5 years old), and one roe deer female (1 year old). Dual enzymatic digestion was conducted using a culture media containing 1mg/ml collagenase I (Life Technologies Corporation, Grand Island, NY, USA) and 1unit/ml dispase (STEMCELL™ Technologies Canada Inc. Vancouver, British Columbia, Canada) at 37 °C, shaken for 45 min. The cells were then washed with phosphate buffer saline (PBS) (Sigma-Aldrich Co., Saint Louis, MO, USA) and centrifuged at 1,340 *g* for 4 min. The supernatant was then discharged and the follicles allowed to grow on collagen pre-coated culture vessels in rich media supplemented with 25% NuSerum, Corning® Nu-Serum™ IV (Discovery Labware, Inc., Bedford, MA, USA) and 10 ng/ml somatostatin (Sigma-Aldrich Co.) in a controlled environment set at 37°C and 5% CO_2. The follicular cells were then processed with a collagen I hydrogel 3D Collagen Cell Culture System (EMD Millipore Corporation, Billerica, MA, USA) before being transferred to multiwall plates.

PCB 138 preparation

The PCB 138 congener (Sigma-Aldrich Co.) was first dissolved in dimethyl sulphoxide DMSO (Sigma-Aldrich Co.). Serial dilutions were then made in culture media suitable for each test (DMEM/F-12Gibco® Invitrogen (Life Technologies Corporation) or DMEM w/o sodium pyruvate (Biosera, Nuaille, France) using five concentrations (0, 0.3, 3, 30, 300, and 3000 ng/ml). In addition, two controls were prepared using DMSO solvent only and culture medium only. Considering the short duration of cell exposure experiments, there was no analytical check of prepared PCB 138 concentrations stability.

Cell viability testing

Thyroid follicular cells were cultivated in white 96-well plates (SPL Lifesciences Co. Ltd, Pocheon-city, Gyeonggi-do, Korea) at amounts ranging from 2.5 to 3 $\times 10^5$/ml in collagen hydrogel.The cells were then cultured with PCB 138 at concentrations of 0, 0.3, 3, 30, 300, and 3000 ng/ml alongside both controls. Viability was assessed based on the reductive ability of metabolically active cells using the RealTime-Glo™ MT Cell Viability Assay (Promega Corporation, Madison, WI, USA) and a NanoLuc® luciferase luminescent probe.Viability was measured at 24, 48, and 72 h post exposure using a Cytation™ 3 Imaging Reader (BioTek® Instruments, Inc. Winooski, Vermont, USA) to record luminescence signals.

Cytotoxicity testing

Thyroid follicle cells were cultivated in black Costar® 96-well plates (Corning Incorporated, Corning, NY, USA) at amounts ranging from 2.5 to 3 $\times 10^5$/ml in collagen hydrogel. The cells were then cultured with PCB 138 at concentrations of 0, 0.3, 3, 30, 300, and 3000 ng/ml alongside both controls. Cytotoxicity was assessed based on membrane damage associated cell death using the CellTox™ Green Cytotoxicity Assay (Promega Corporation). Cytotoxicity was assessed using fluorescent cyanine dye, which preferentially stains DNA released from bursting cells, using a Cytation™ 3 Imaging Reader (BioTek® Instruments) to record fluorescence at 24, 48, and 72 h.

Reactive oxygen species detection assay

Follicular cells were cultivated in black Costar® 96-well plates (Corning Incorporated) at amountsranging from 2.5 to 3 $\times 10^5$/ml in collagen hydrogel. Three groups of cell culture plates were prepared simultaneously under the same conditions and allowed to grow within the hydrogel matrix. On day 0 of the experiment, the cultures were exposed to the culture medium only control (0 ng/ml), the DMSO solvent control, and 30 ng/ml of PCB 138. Generation of reactive oxygen species (ROS) was determined based on oxidation of a dihydroethidium fluorescent probe using the ROS Detection Cell-Based Assay Kit (DHE) (Cayman Chemical, Ann Arbor, MI, USA). The assay was carried out after 24, 48, and 72 h of cell culture treatment, following the manufacturer's instructions. The plates were read using the Cytation™3 Imaging Reader (BioTek® Instruments, Inc.) to record fluorescence.

Measurement of iodide uptake

Follicular cell iodide uptake was assessed using the non-radioactive method developed by Waltz et al. (2010), which incorporates the use of colorimetric determination of intracellular iodide concentrations utilizing the Sandell-Kolthoff reaction. Follicular cells were cultivated in clear 96-well plates at amounts ranging from 2.5 to 3 $\times 10^5$/ml in collagen hydrogel containing bovine TSH 1mU/ml (EMD Millipore Corporation). Three groups of cell culture plates were prepared simultaneously under the same conditions and allowed to grow within the hydrogel matrix. On day 0 of the experiment, the cultures were exposed to the culture medium only control (0 ng/ml), the DMSO solvent control, and 30 ng/ml of PCB 138. Assessment was conducted after 24, 48, and 72 h of cell culture treatment. In parallel, solutions of ammonium cerium (IV) sulphate, sodium arsenite (III), and sodium iodide (EMD Millipore Corporation and Sigma-Aldrich Co.) were prepared for the Sandell-Kolthoff reaction and preserved as described by Waltz et al. (2010). On each assessment day, sodium iodide at

a concentration of 10 µM in Hank's Balanced Salt Solution (HBSS; Life Technologies Corporation) supplemented with 10 mM HEPES buffer (Sigma-Aldrich Co) was added to each group for 60 min at the end of each exposure time. All steps of the Sandell-Kolthoff reaction were performed and assay absorbance read using Cytation™3 Imaging Reader (BioTek® Instruments, Inc.) set at 420 nm. Iodide uptake in real samples was then determined based on reading from an iodide standard curve.

Measurement of thyroxin T4 release

Release of thyroxin from thyroid follicular cells was assessed using the Thyroxin (T_4) Competitive ELISA kit (Life Technologies Corporation), the assays being conducted strictly according to the manufacturer's instructions. Follicular cells were cultivated under the same conditions as the previous tests, with minor modifications to achieve integral conditions for the *in vitro* functional bioassay of thyroid follicular cells (Dickson et al. 1981; Marshall and Ealey 1986). Thyrocytes were cultivated in 24-well plates at amounts ranging from 2.5 to 3 $\times 10^5$/ml in collagen hydrogel. On day 0 of the experiment, the cultures were exposed to the DMSO solvent control,culture medium only control (0 ng/ml), and 30 ng/ml of PCB 138. The cells were also stimulated with bovine TSH 1mU/ml (EMD Millipore Corporation) at the beginning of each time interval. On each assessment day, sodium iodide was added at a concentration of 10 µM. Samples for ELISA were collected at the end of each time interval and quickly preserved at -20 °C before performing measurements. Absorbance was read at 450 nm using an EL×808 multi-plates reader (BioTek® Instruments, Inc.). Thyroxine levels in the samples were determined based on a fitting curve prepared by measuring a range of thyroxine standards.

Statisical analysis

Statistica for Windows®10 (StatSoft, Inc., Tulsa, OK, USA) was used to compare different experimental groups using procedures such as one-way analysis of variance (ANOVA), *posthoc* analysis of means by LSD test, Tukey Honest Significant Difference (HSD) test, Levene's method for testing homogeneity of variances, log-transformation of non-homogenous parameters prior to analysis, and comparisons with non-parametric Kruskal-Wallis test. Levels of significance were set at $P < 0.05$ or $P < 0.01$.

Results

Cell viability and cytotoxicity

Thyrocyte viability in primary cell cultures and cytotoxicity of 24, 48, and 72 h exposure to PCB 138 was evaluated by comparing the data against the non-exposed controls and DMSO-treated controls. As there were no significant differences between control thyrocytes grown in the standard culture medium and the DMSO solvent-treated cells, we combined these two controls for comparative purposes. Thyrocyte viability ranged from 78.71 to 118.34%, 98.14 to 104.45%, and 84.16 to 106.70% in fallow deer-, mouflon-, and roe deer-derived cells, respectively. Significantly lower viability was observed in cells exposed for 48 h to 30 ng of PCB 138 per 1ml of culture medium (fallow deer: P= 0.012; roe deer: P = 0.002). Cytotoxicity ranged from 2.36 to 16.37%, 3.19 to 9.85%, and 2.76 to 11.21% in fallow deer, mouflon, and roe deer, respectively. Only roe deer-derived cells showed significantly higher cytotoxicity when exposed to 3 ng of PCB 138 per 1ml of culture medium ($P < 0.05$), with cytotoxicity lower at 30 ng exposure ($P < 0.01$).No other significant differences in cell viability and cytotoxicity were detected.Based on the these results (i.e., significant effects induced by exposure to 30 ng of PCB 138), all subsequent functional experiments were performed atthis exposure level.

Induction of reactive oxygen species

Exposure to 30 ng/ml PCB 138 for 24 and 48 h induced ROS generation in fallow deer-derived primary cell culture thyrocytes (Figs 1A and 1B). Compared with the combined controls, there were no significant cell responses in mouflon- and roe deer-derived thyrocytes following 24, 48, and 72 h exposure (Figs 1A and 1B),or in fallow deer cells at 72 h exposure.

Iodide uptake and thyroxineT4 release

Compared with the controls (culture medium only and DMSO solvent), iodide uptake by 30 ng/ml PCB 138-exposed thyrocytes increased 24 h after exposure in fallow and roe

deer. On the other hand, fallow deer, mouflon and roe deer cells cultured for 48 and 72 h were characterized by a significant drop in iodide uptake (Fig. 2).Thyrocytes displayed a significant decrease in thyroxine T_4 release after 48 and 72 h; 24, 48 and 72 h; and 48 h of exposure in fallow deer, mouflon, and roe deer, respectively (Fig. 3).

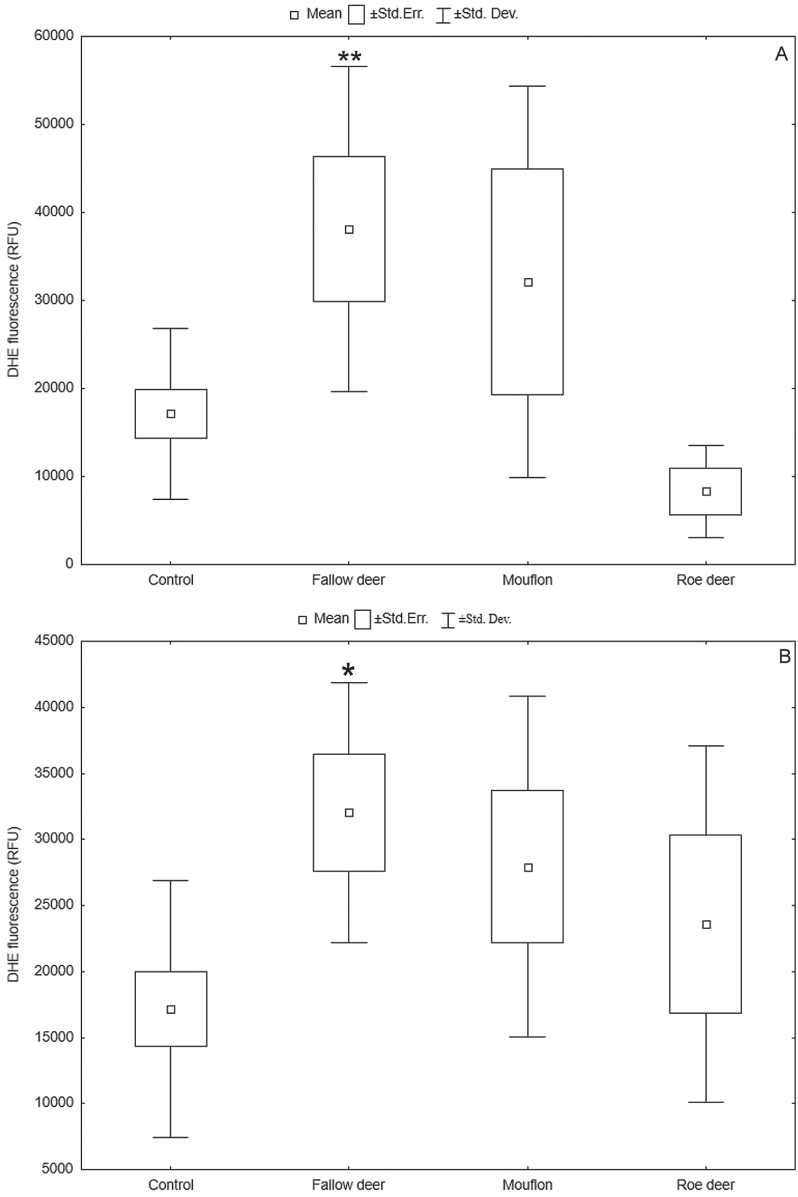

Fig. 1. Total dihydroethidium fluorescence representing ROS generation in thyrocytes
Game animal-derived cells exposed to 30 ng/ml PCB 138 for 24 h (A) and 48 h (B). PCB = polychlorinated biphenyl; RFU = relative fluorescence unit; ROS = reactive oxygen species;* = $P < 0.05$, ** = $P < 0.01$ when compared against culture medium only and dimethyl sulphoxide solvent controls combined for all three game species.

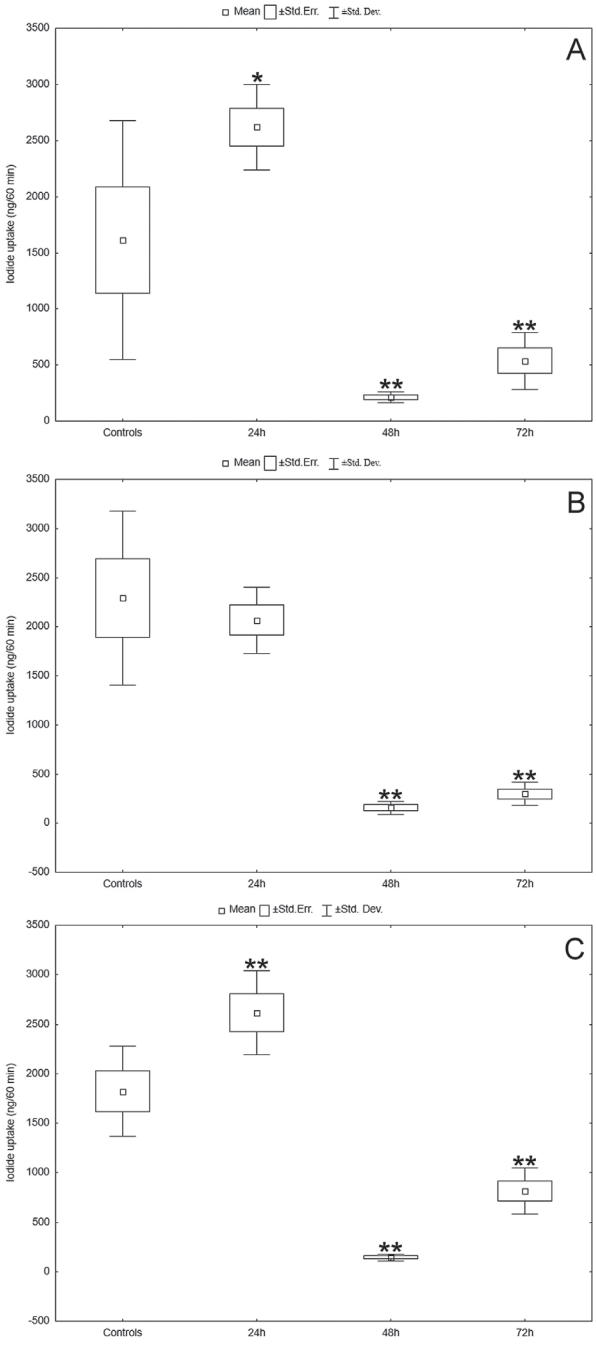

Fig. 2. Iodide uptake by thyrocytes following exposure to PCB 138 (30 ng/ml)
(A) Fallow deer-, (B) mouflon-, and (C) roe deer-derived cells exposed for 24, 48, and 72 h. PCB = polychlorinated biphenyl; * = $P < 0.05$, ** = $P < 0.01$ when compared against combined culture medium only and dimethyl sulphoxide solvent controls.

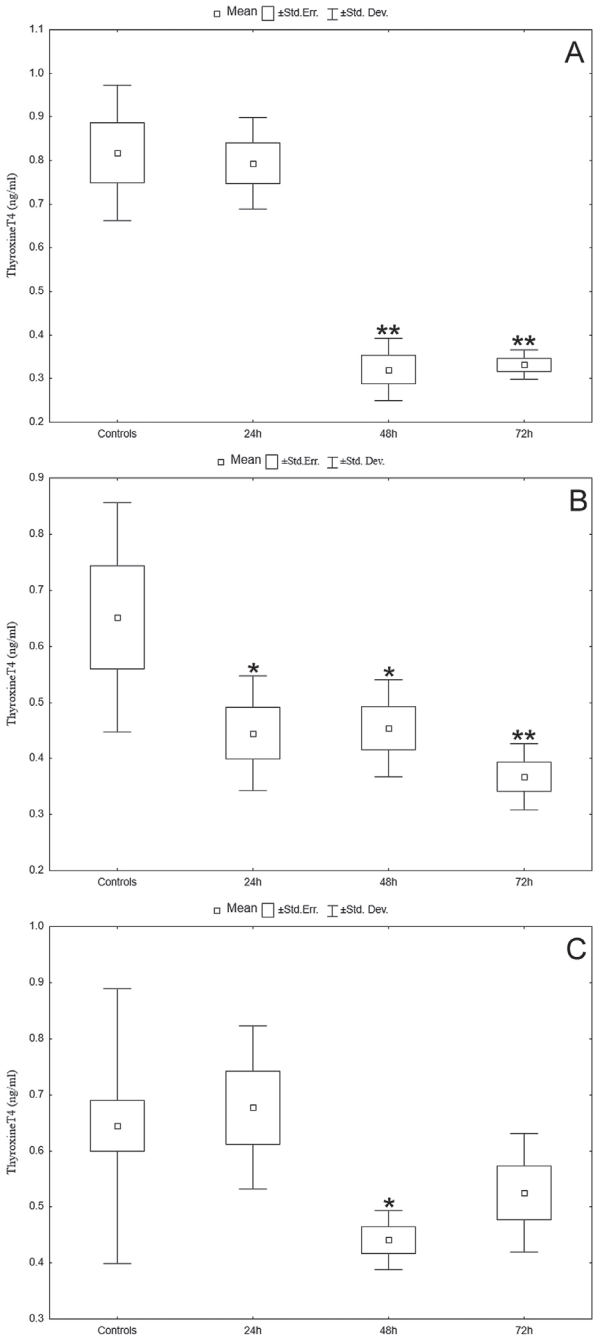

Fig. 3. Thyroxine T4 release by thyrocytes following exposure to PCB 138 (30ng/ml)
(A) Fallow deer-, (B) mouflon-, and (C) roe deer-derived cells exposed for 24, 48 and 72 h. PCB = polychlorinated biphenyl; * = $P < 0.05$, ** = $P < 0.01$ when compared against combined culture medium only and dimethyl sulphoxide solvent controls.

Discussion

Using an *in vitro* cell culture model, we have demonstrated that PCBs at environmentally relevant levels exert adverse effects on both thyrocyte performance and function in wild ungulates. In addition to lower magnitude effects related to cell viability, cytotoxicity, and ROS induction, our findings suggest functional disruption manifesting as altered iodide uptake and decreased thyroxine release by thyrocytes. Importantly, the observed effects characterize responses of specific thyroid follicle cells (thyrocytes) to toxic exposure disconnected from interaction with the HPT axis regulating thyroid function through release of thyrotropin at the organism level. In this case, the challenge is to determine whether different PCB compounds can interrupt thyroid function via possible autonomous interactions with thyrocyte-associated molecules that organize their function as presumed in previous observations (Katarzy et al. 2015). The present study focused mainly on the effects of a single toxicant, i.e., an indicator PCB 138 congener, using a concentration range corresponding to levels found in tissues of terrestrial wildlife (Bachour et al. 1998; Warenik-Bany et al. 2016). However, under environmental conditions, exposure to PCB mixtures tends to be more common. Multiple stressors in wildlife elicit physiological costs and trade-off responses when acting at sub-lethal levels, with multiple environmental stressors and pollutants often resulting in synergistic effects (Pikula et al. 2010; Bandouchova et al. 2011; Osičková et al. 2014). Indeed, such effects are a highly probable mechanism for the development of thyroid disease resulting from environmental pollution (Brent 2010).

Based on structural criteria, the biological performance of PCB congeners may differ considerably when exposure is to a single congener or to a mixture of congeners. Polychlorinated biphenyls occur as organic molecules that contain chlorine atoms in their hydrocarbon rings. Despite similarities in features and toxicity mechanisms, abundance and position of the chlorine atom in each congener determines its characteristics and biological transformation within living organisms. Polychlorinated biphenyls' metabolization pace, lipid solubility level, and metabolite formation are all chemical structure-dependent (Hansen 2001), making their toxic effect congener-specific.

Our observations on the effect of PCB 138 on cell viability indicate only slight changes in cell metabolic capacity in respect to exposure duration. In this study, there appeared to be no major alteration in cell growth proliferative capacity; however, such effects have been observed in previous studies on different cell lines treated with a variety of congeners, including PCBs 138, 153, 101, and 118, either singly or as a mixture (Bonefeld-Jørgensen et al. 2001; Vondrácek et al. 2005; DeCastro et al. 2006; Radice et al. 2008). Effects on proliferation were more pronounced after prolonged exposure; i.e., starting from 48 h on up to 6 days in the case of PCB 138 (Radice et al. 2008). At the same time, cytotoxicity, which is marked by cell membrane damage, appeared to be proportional to the cell viability pattern, followed by induction of ROS (represented by superoxide generation). This suggests that the main pathway of metabolic biotransformation in PCBs utilizes an oxidation step, characterized by conversion of parent PCB compounds into their daughter metabolites via enzymatic reactions regulated by cytochrome CYP450 enzymes, and catalytic coupling to specific CYP 450 enzymes, resulting in production of hydroxylated PCBs (OH-PCBs), known as mono and dihydroxylate PCBs. Thus, PCB toxicity may occur as a consequence of unfavourable CYP 450 coupling or inhibition of cytochromes, leading to ROS production. Additionally, PCB metabolites may undergo further oxidization into quinones and semiquinones, which may then result in generation of superoxide anion radicals (Srinivasan et al. 2001). Although differing in structure, both dioxin- and non-dioxin-like PCBs share the same biological fate. This same metabolic mechanism has previously been described regarding toxicity of dioxin-like PCB 77,

PCB 126, and PCB 169 to fish, birds, and reptiles (Schlezinger et al. 2000, 2006). Furthermore, the mechanism has also been investigated regarding potential PCB-induced carcinogenic effects brought about by generation of ROS in association with human CYP1B1 uncoupling (Green et al. 2008).

Considering that extra hepatic CYP1B1, which plays a role in dioxin-like PCB metabolism, is expressed in tissues associated with tumour-formation organs such as ovaries, prostate, colon, and thyroid, the above mechanism would appear more consistent with the carcinogenic traits of highly toxic PCBs. In the case of PCB 138, which is classified as non-dioxin-like, metabolism controlled by CYP2B enzymes, while its metabolites may generate reactive hydroxylated PCBs, which may then produce other hydroquinones via CYP induction. Such products are relevant to the toxicological promotion of oxidative stress (James 2001; Grimm et al. 2015). Moreover, OH-PCBs can biologically persist like their parent compounds, exerting a range of toxic effects. A number of studies have addressed accumulation of OH-PCBs and other metabolites in blood and tissues of wild birds and mammals, and have identified links to adverse thyroidal and developmental influences (Fängström et al. 2005; Gutleb et al. 2010).

The ROS induction mechanisms of PCBs or their daughter compounds can occur through activation of protein complex neutrophilic oxidase (NADPH) (Fonnum et al. 2006; Myhre et al. 2009), which plays an important role as a regulator for endogenous ROS production and intra-thyroid oxidation. Perturbation of molecules in this complex can result in thyroid deficits, which could be linked to PCB 138 toxicity observed in fallow deer-derived thyrocytes in the present study, which showed that superoxide generation was followed by fluctuations in iodide uptake and T_4 release (Figs 1, 2 and 3). In thyrocytes, NADPH oxidase comprises the thyroid dual oxidases DUOX1&2 and NOX4. Thyroid dual oxidases DUOX1&2 are the essential enzymes responsible for peroxide H_2O_2 generation required in T_3 and T_4 synthesis. During thyroid hormone biosynthesis, H_2O_2 is utilized by TPO for iodide oxidation and organification in the thyroid follicular colloid. Mechanisms for H_2O_2 production involve the joint action of DUOX2 and its maturation factor DUOXA2 (Yoshihara et al. 2012; Sugawara 2014). In addition to H_2O_2, superoxide anions O^{-2} can also be generated as an initial product of both DUOX2 and NOX4 (Carvalho 2013; Sugawara 2014). Modulation in DUOX expression can result in modificationsto iodine regulation inside the thyroid follicles. Under natural conditions, suppression of DUOX2 genes automatically shuts down the iodide organification process and iodide uptake and transportation (Yoshihara et al. 2012). Disparity in DUOX2 and NOX4 regulation has previously been observed in cases of congenital hypothyroidism and thyroid cancers raised by DUOX2 and NOX4 mutations (Moreno et al. 2002; Weyemi et al. 2009; Grasberger 2010; Yoshihara et al. 2012). Development of such cases is assumed to be based on the genotoxic effect induced by ROS generation associated with increased H_2O_2 production.

Peaks in iodide levels seen only shortly after exposure (24 h) may have been aroused reciprocally by ROS generation, while the excessive iodide uptake could inversely trigger ROS production (Suagawara 2014), while PCB 138 interaction with intracellular iodide uptake at extended exposure durations (Fig. 2) may also be attributable to its metabolic pathways. Polychlorinated biphenyls are also involved in formation of protein-adducts, which is a presumed mechanism in the toxicity of dioxin-like and highly chlorinated (e.g., penta- and hexa-chlorinated) congeners, including PCB 138 (Grimm et al. 2015). Protein-adducting is suspected to induce macromolecular disruption in cellsas a result of complex formation with biomolecules such as DNA, RNA and amino acids, and the inner mitochondrial membranous particles containing phosphorus. This may also explain the slight perturbation in cell metabolic efficiency observed in our viability test, indicating the potential interference of PCB 138 with basal or apical membranous binding or transporting molecules, such as cell surface-TSH receptors. Such interference will

subsequently affect the thyrocyte transporting complexes responsible for iodide uptake from circulation due to TSH stimulation blockage. Alternatively, direct interaction with transporting molecules that control iodide uptake and diffusion into the follicular lumen, such as NIS and pendrin, may be involved, either through complex formation or via changes in their expression rate related to DNA/RNA adducting. Such an effect has been observed in previous studies on porcine thyroid tissue and chicken-derived thyrocytes. Interestingly, in these cases, exposure to different highly chlorinated PCBs exerted a spectrum of downregulation with no effect on NIS expression (Pocar et al. 2006; Katarzy et al. 2015).

The decrease in T_4 levels released throughout exposure duration used among our groups (Fig. 3) is an indication of the ability of PCB 138 to interrupt thyroxine synthesis and release as a consequence of iodide perturbation. Hence, together with the proposed initial interaction with TSH, NIS, and pendrin, PCB 138 may be further engaged with other cellular molecules and organelles important for T_3 and T_4 synthesis and availability (van der Deure et al. 2010). Many other compounds are believed to intervene in the expression of molecules such as TPO and TG (Pocar et al. 2006; Katarzy et al. 2015), which are both key organizers for the synthesis process. Indeed, histopathological changes in follicular colloids and thyrocyte lysosomes have been linked to PCB toxicity in previous studies (e.g., Collins et al. 1977). Potential injuries to the colloidal fluid constituent of TG and thyroidal lysosomes, which ultimately control T_4 release via hydrolysis of T_4-containing thyroglobulin, may also be secondarily involved in the depletion of thyroxine levels.

Our study indicates that PCB 138 may adversely influence thyroid function in a sub-lethal manner independently of the HPT axis, primarily through perturbation of intracellular iodide uptake and disruption of T_4 synthesis and release. This effect appears to be both time- and species-dependent. Further, our methodology highlights a primary advantage of using *in vitro* experiments, i.e., the ability to perform tests or measurements that would otherwise not be possible or ethical in living subjects. Use of cell culture models reduces the number of experimental specimens, increases tested species welfare, and replaces the whole organism with specific target cells. As such, the results of the present study were obtained in line with the 'Three Rs' concept of animal research.

Acknowledgements

This study was supported through Grant No. FVHE/Pikula/ITA2020 of the University of Veterinary and Pharmaceutical Sciences Brno. We are grateful to Dr. Kevin Roche for correction and improvement of the English text.

References

Aniołek O 2019: The effect of thyroid hormone deficiency on erythropoiesis in dogs. Acta Vet Brno **88**: 257-264

Bachour G, Failing K, Georgii S, Elmadfa I, Brunn H 1998: Species and organ dependence of PCB contamination in fish, foxes, roe deer, and humans. Arch Environ Con Tox **35**: 666-673

Bandouchova H, Pohanka M, Kral J, Ondracek K, Osickova J, Damkova V, Vitula F, Treml F, Pikula J 2011: Effects of sublethal exposure of European brown hares to paraoxon on the course of tularemia. Neuroendocrinol Lett **32**: 77-83

Bonefeld-Jørgensen EC, Raun H, Høj T, Marie A 2001: Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. Toxicology **158**: 141-153

Brent GA 2010: Environmental exposures and autoimmune thyroid disease. Thyroid **20**: 755-761

Carvalho DP 2013: Role of the NADPH oxidases DUOX and NOX4 in thyroid oxidative stress. Eur Thyroid J **2**: 160-167

Chu I, Villeneuve D, Yagminas A, Lecavalier P, Håkansson H, Ahlborg U, Valli V, Kennedy S, Bergman A, Seegal R, Feeley M 1995: Toxicity of PCB 77 (3,3',4,4'-tetrachlorobiphenyl) and PCB 118 (2,3',4,4'5-pentachlorobiphenyl) in the rat following subchronic dietary exposure. Fund Appl Toxicol **26**: 288-292

Chu I, Villeneuve D, Yagminas A, Lecavalier P, Poon R, Feeley M, Kennedy S, Seegal R, Håkansson H, Ahlborg U, Valli V, Bergman A 1996: Toxicity of 2,2′,4,4′,5,5′-Hexachlorobiphenyl in rats: Effects following 90-day oral exposure. J Appl Toxicol **16**: 121-128

Collins WT, Capen CC 1980: Fine structural lesions and hormonal alterations in thyroid glands of perinatal rats exposed *in utero* and by the milk to polychlorinated biphenyls. Am J Pathol **99**: 125-142
Collins WT, Capen CC, Kasza L, Carter C, Dailey RE 1977: Effect of polychlorinated biphenyl (PCB) on the thyroid gland of rats. Ultrastructural and biochemical investigations. Am J Pathol **89**: 119-136
DeCastro B, Korrick S, Spengler J, Soto A 2006: Estrogenic activity of polychlorinated biphenyls present in human tissue and the environment. Environ Sci Technol **40**: 2819-2825
Dickson JG, Hovsepian S, Fayet G, Lissitzky S 1981: Follicle formation and iodide metabolism in cultures of human thyroid cells. J Endocrinol **90**: 113-124
Fängström B, Athanasiadou M, Athanassiadis I, Weihe P, Bergman Å 2005: Hydroxylated PCB metabolites in nonhatched fulmar eggs from the Faroe Islands. Ambio **34**: 184-187
Fonnum F, Mariussen E, Reistad T 2006: Molecular mechanisms involved in the toxic effects of polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs). J Toxicol Environ Heal A **69**: 21-35
Grasberger H 2010: Molecular and cellular endocrinology defects of thyroidal hydrogen peroxide generation in congenital hypothyroidism. Mol Cell Endocrinol **322**: 99-106
Green RM, Hodges NJ, Chipman JK, Donovan MRO, Graham M 2008: Reactive oxygen species from the uncoupling of human cytochrome P450 1B1 may contribute to the carcinogenicity of dioxin-like polychlorinated biphenyls. Mutagenesis **23**: 457-463
Grimm FA, Hu D, Kania-Korwel I, Lehmler HJ, Ludewig G, Hornbuckle KC, Duffel MW, Bergman A, Robertson LW, Robertson LW 2015: Metabolism and metabolites of polychlorinated biphenyls (PCBs). Crit Rev Toxicol **45**: 245-272
Gutleb AC, Cenijn P, Velzen MV, Lie E, Ropstad E, Skaare JU, Malmberg T, Bergman Å, Gabrielsen GW, Legler J 2010: *In vitro* assay shows that PCB metabolites completely saturate thyroid hormone transport capacity in blood of wild polar bears (*Ursus maritimus*). Environ Sci Technol **44**: 3149-3154
Hamers T, Kamstra JH, Cenijn PH, Pencikova K, Palkova L, Simeckova P, Vondracek J, Andersson PL, Stenberg M, Machala M 2011: *In vitro* toxicity profiling of ultrapure non–dioxin-like polychlorinated biphenyl congeners and their relative toxic contribution to PCB mixtures in humans. Toxicol Sci **121**: 88-100
Hansen LG 2001: Identifcation of steady state and episodic PCB congeners from multiple pathway exposure. In: Robertson LW, Hansen LG (Eds): PCBs: Recent advances in environmental toxicology and health effects. The University Press of Kentucky, pp. 47-56
James MO 2001: Polychlorinated biphenyls: Metabolism and metabolites. In: Robertson LW, Hansen LG (Eds): PCBs: Recent advances in environmental toxicology and health effects. The University Press of Kentucky, pp. 35-46
Jeker LT, Hejazi M, Burek CL, Rose NR, Caturegli P 1999: Mouse thyroid primary culture. Biochem Biophys Res Commun **257**: 511-515
Katarzy D, Hrabia A, Kowalik K, Sechman A 2015: Comparison of the *in vitro* effects of TCDD, PCB 126 and PCB 153 on thyroid-restricted gene expression and thyroid hormone secretion by the chicken. Environ Toxicol Pharmacol **9**: 496-503
Kendall EC 1919: The physiologic action of thyroxin. Endocrinology **3**: 156-163
Langer P, Tajtáková M, Fodor G, Kocan A, Bohov P, Michálek J, Kreze A 1998: Increased thyroid volume and prevalence of thyroid disorders in an area heavily polluted by polychlorinated biphenyls. Eur J Endocrinol **139**: 402-409
Longnecker MP, Gladen BC, Patterson DG Jr, Rogan WJ 2000: Polychlorinated biphenyl (PCB) exposure in relation to thyroid hormone levels in neonates. Epidemiology **11**: 249-254
Marshall NJ, Ealey PA 1986: Recent developments in the *in vitro* bioassay of TSH and thyroid-stimulating antibodies. In: McGregor AM (Ed.): Immunology of endocrine diseases. Immunology and medicine, vol. 3. Springer, Dordrecht, pp. 25–49
Miller MD, Crofton KM, Rice DC, Zoeller RT 2009: Thyroid-disrupting chemicals: Interpreting upstream biomarkers of adverse outcomes. Environ Health Persp **117**: 1033-1041
Moreno JC, Bikker H, Kempers MJ, van Trotsenburg A, Baas F, de Vijlder JJ, Vulsma T, Ris-Stalpers C 2002: Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. N Engl J Med **347**: 95-102
Myhre O, Mariussen E, Reistad T, Albert Ø, Aarnes H, Fonnum F 2009: Effects of polychlorinated biphenyls on the neutrophil NADPH oxidase system. Toxicol Lett **187**: 144-148
Nesvadbová M, Bořilová G, Hulánková R 2019: PCR-RFLP identification of meat from red deer, sika deer, roe deer, fallow deer, mouflon, wild boar, hare and cattle. Acta Vet Brno **88**: 103-111
Osičková J, Banďouchová H, Kováčová V, Král J, Novotný L, Ondráček K, Pohanka M, Sedláčková J, Škochová H, Vitula F, Pikula J 2014: Oxidative stress and liver damage in birds exposed to diclofenac and lead. Acta Vet Brno **83**: 299-304
Pikula J, Bandouchova H, Hilscherova K, Paskova V, Sedlackova J, Adamovsky O, Knotkova Z, Lany P, Machat J, Marsalek B, Novotny L, Pohanka M, Vitula F 2010: Combined exposure to cyanobacterial biomass, lead and the Newcastle virus enhances avian toxicity. Sci Total Environ **408**: 4984-4992
Pocar P, Klonisch T, Brandsch C, Eder K, Fro C 2006: AhR-agonist-induced transcriptional changes of genes involved in thyroid function in primary porcine thyrocytes. Toxicol Sci **89**: 408-414

Radice S, Chiesara E, Fucile S, Marabini L 2008: Different effects of PCB101 , PCB118 , PCB138 and PCB153 alone or mixed in MCF-7 breast cancer cells. Food Chem Toxicol **46**: 2561-2567

Schlezinger JJ, Keller J, Verbrugge LA, Stegeman JJ 2000: 3,3',4,4'-Tetrachlorobiphenyl oxidation in fish, bird and reptile species: relationship to cytochrome P450 1A inactivation and reactive oxygen production. Comp Biochem Physiol C **125**: 273-286

Schlezinger JJ, Struntz WDJ, Goldstone JV, Stegeman JJ 2006: Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners. Aquat Toxicol **77**: 422-432

Srinivasan A, Lehmler H, Robertson LW, Ludewig G 2001: Production of DNA strand breaks *in vitro* and reactive oxygen species *in vitro* and in HL-60 cells by PCB metabolites. Toxicol Sci **102**: 92-102

Sugawara M 2014: Reactive oxygen species and thyroid diseases. In: Laher I (Ed.): Systems biology of free radicals and antioxidants. Springer, Heidelberg, pp. 3521-3538

Toda S, Aoki S, Uchihashi K, Matsunobu A, Yamamoto M, Ootani A, Yamasaki F, Koike E, Sugihara H 2011: Culture models for studying thyroid biology and disorders. ISRN Endocrinol **2011**: 275782

van den Berg KJ, Zrucher C, Brouwer A 1988: Effects of 3,4,3',4'-tetrachlorobiphenyl on thyroid function and histology in marmoset monkeys. Toxicol Lett **41**: 77-86

van der Deure W, Medici M, Peeters R, Visser T 2010: Genetic influences on thyroid function tests. In: Brent GA (Ed.): Thyroid function testing. Springer, Boston, MA, pp. 21-43

Vondrácek J, Machala M, Bryja V, Chramostová K, Krcmár P, Dietrich C, Hampl A, Kozubík A 2005: Aryl hydrocarbon receptor-activating polychlorinated biphenyls and their hydroxylated metabolites induce cell proliferation in contact-inhibited rat liver epithelial cells. Toxicol Sci **83**: 53-63

Waltz F, Pillette L, Ambroise Y 2010: A nonradioactive iodide uptake assay for sodium iodide symporter function. Anal Biochem **396**: 91-95

Warenik-Bany M, Strucinski P, Piskorska-Pliszczynska J 2016: Dioxins and PCBs in game animals: Interspecies comparison and related consumer exposure. Environ Int **89-90**: 21-29

Weyemi S, Caillou B, Talbot M, Ameziane-El-Hassani R, Lacroix L, Lagent-Chevallier O, Ghuzlan AA, Roos D, Bidart JM, Virion A, Schlumberger M, Dupuy C 2009: Intracellular expression of reactive oxygen species-generating NADPH oxidase NOX4 in normal and cancer thyroid tissues. EndocrRelat Cancer **17**: 27-37

Yoshihara A, Hara T, Kawashima A, Akama T, Tanigawa K, Wu H 2012: Regulation of dual oxidase expression. Thyroid **22**: 1054-1062