RUNNING TITLE: HISTOPATHOLOGY AND FLAME RETARDANTS IN JAPANESE QUAILS

Japanese quail (*Coturnix japonica*) liver and thyroid gland histopathology as a result of *in ovo* exposure to the flame retardants tris(1,3-dichloro-2-propyl) phosphate and Dechlorane Plus

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Abstract

Japanese quails (*Coturnix japonica*) were exposed *in ovo* to tris(1,3-dichloro-2-propyl) phosphate (TDCIPP; 500 ng/µl), Dechlorane Plus (DP; 500 ng/µl), or a mixture of these two (500 ng/µl TDCIPP:500 ng/µl DP) to investigate effects on liver and thyroid gland morphology. Histological examination of 14-day old quails showed that exposure to TDCIPP or the mixture induced hepatic sinusoidal dilatation. No effects were seen for DP alone. In addition the mixture produced divergence of thyroid gland follicles and proliferation of follicular cells. Our study is the first demonstrating histopathological effects as a result of exposure during early development to the flame retardants TDCIPP or a TDCIPP-DP mixture suggesting the need for further research efforts to investigate adverse health effects associated with exposure to these environmental chemicals on wild birds.

Key words: Chlorinate flame retardants; Histopathology; mixture toxicity; Organohalogen compounds; TDCIPP; DP.

Introduction

Flame retardants (FR) are chemicals added to consumer products in order to prevent or delay combustion and the spread of fire (van der Veen and de Boer 2012). Due to continuous human and wildlife risk assessment of their toxic and/or endocrine disruptive effects, the production and use of certain FR, such as polybrominated diphenyl ethers (PBDE) and hexabromocyclododecane (HBCD) has been restricted worldwide (Stockholm Convention, 2016), and alternatives were consequently introduced. Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP; CAS No 13674-87-8) and Dechlorane Plus (DP; CAS No 13560-89-9) are two such currently available alternatives, substituting for the above-mentioned legacy FR.

Exposure to TDCIPP was previously found to compromise Japanese quail (*Coturnix japonica*) immune functions and to cause liver/biliary fibrosis as well as changes in lipid and steroid metabolism (Farhat et al. 2014). While information on the toxicity of these compounds in wildlife is still scarce, toxicity is suspected as these chemicals show structural similarities to other legacy chlorinated environmental contaminants. Most OHC (organohalogen contaminants) are widespread in the environment, bioaccumulate in biota and biomagnify through food chains due to their physical-chemical properties (Letcher et al. 2010). Further, OHC were reported to produce severe adverse health effects, including immune suppression, endocrine disruption, impaired reproduction and/or carcinogenic effects (Fairbrother et al. 2004; Grove et al. 2009; Sverko et al. 2011; van der Veen and de Boer 2012; Muusse et al. 2014; Sagerup et al. 2014). More specifically, previous observations in polar bear (*Ursus maritimus*), Arctic fox (*Vulpes lagopus*), glaucous gull (*Larus hyperboreus*), rock dove (*Columba livia*) and American kestrel (*Falco sparverius*) linked OHC exposure to histopathological lesions (Sonne et al. 2005; 2009; 2010; 2013; McKernan et al. 2009; Qiesar 2009). These studies have highlighted that liver and thyroid gland morphology can be affected by exposure to OHCs. Therefore, the present study aimed at investigating the

developmental histopathological effects of *in ovo* exposure to environmentally relevant concentrations of the currently available flame retardants TDCIPP and DP using Japanese quail as a bird model species.

Materials and Methods

Study species and design

The Japanese quail is a preferred species to use when studying ecotoxicology and developmental biology given its short generation time and different genetic strains. In addition, its limited spatial needs and husbandry costs make it a convenient avian model species. For these reasons, its physiology and neuro-endocrine biology is well-known (Huss et al., 2008; Jaspers 2015). Here; we conducted a controlled exposure study on Japanese quail eggs (n = 36) at the Norwegian University of Science and Technology (NTNU), Norway. The study was approved by the National Animal Research Authority of Norway (FOTS no. 7291). Fertilized eggs were obtained from a hatchery and injected *in ovo* at incubation day zero. An opening of 0.6 mm in diameter was made in the egg shell until visibility of the inner shell membrane, using a round shaped dentist drill bit mounted on an electrical drill (Robust 140W 9922 (GS), Town, Country). Then, the eggs were weighed and injected in the yolk sac with a Hamilton syringe mounted with a 25 G needle. A volume of 2 µl per gram egg was injected with an emulsion adjusted according to the egg mass following the equation

$$\frac{exposure\ concentration\ (ng\ g^{-1}\ egg)\ \times\ egg\ mass\ (g)}{emulsion\ concentration\ (ng\ \mu L^{-1})} = injection\ volume\ (\mu L)$$

Eggs were injected with DP (70:30 anti:syn; n = 9), TDCIPP (n = 8) or their mixture (n = 10). The administered dose was 500 ng/µl for both the DP and TDCIPP exposure group while the mixture

group contained both 500 ng/µl of DP and 500 ng/µl of TDCIPP. A control group consisted of eggs that remained non-injected (CTRL-NON; n = 9). Eggs were incubated at 37.5 °C and 50–70 % relative humidity during a period of 17–18 days, using three incubators (type 180, America A/S, Thisted, Denmark and J. Hemel, Verl, Germany) modified to hold quail eggs. The 36 quail chicks were held in a controlled animal facility at the Department of Biology, NTNU, in small enclosures (45 cm × 45 cm) with no more than 4 chicks in the same experimental group. Sawdust was used as floor material (24-25 °C, 30-40 % humidity). All chicks were exposed to 12 hr light/dark cycle, access to an infrared heat lamp (75 W), and offered feed and water *ad libitum.* For the first 18 days after hatching of the first eggs, the chicks were given a corn-based feed (feed A: "Oppdrett Fjør 1 Mais"; Felleskjøpet A/S, Lillestrøm, Norway; Table 1) and during the last 8 days the original feed was substituted with an oat meal-based feed (feed B: "Oppdrett Fjør Kraft 6"; Felleskjøpet A/S, Lillestrøm, Norway; Table 1). The chicks were euthanized at 14 days of age by decapitation, and were investigated for hepatic contaminant residues and histopathology of liver and thyroid gland, stored in 10 % buffered formaldehyde (1:10 tissue:formalin ratio) (Table 2).

Histopathological investigations

The histology tissue preparation was performed at the Department of Veterinary Disease Biology, University of Copenhagen, Denmark. Tissues were embedded in paraffin, sectioned at 2-3 µm, and haematoxylin-eosin stained. This staining method renders the nuclei blue/black while the cytoplasm is stained in various shades of pink (Bancroft et al. 1996). The microscope used to examine the slides was a Leica Microsystems Ltd. mounted with a Leica Microsystems Ltd. camera DFC295. All slides were initially examined using 100× and 400× magnifications. Histological examination of liver tissue focused on lymphoid cell aggregates (granulomas and infiltrates), necrosis, lipid vacuoles and sinusoidal dilatation while the thyroid examination focused on

115 proliferation of follicular cells and size and number of thyroid follicles. Histological data were
116 collected as “present” and “not present” for each histological alteration per individual. The number
117 of thyroid follicles was counted in three random and independent fields at 200× magnification. The
118 first field was selected by locating the approximated center of the organ and then moving vertically
119 up, such that the edge of the organ was just outside the reading field. The second field was chosen
120 by locating the approximated center of the organ and then moving the field vertically down, such
121 that the edge of the organ was just outside the reading field. The third field was selected by again
122 locating the approximated center of the organ and then moving the field horizontally to the right
123 such that the edge of the organ was just outside the reading field. For each location; if there were
124 any artefacts, the reading field was moved to the nearest region without artefacts. Only clearly and
125 demarcated follicles were counted.
126
127 *Chemical analyses of feed and liver tissue*
128 Chemical analysis was performed at the Toxicological Centre at the University of Antwerp,
129 Belgium. Feed and quail liver samples were weighed, homogenized and spiked with isotopically-
130 labelled DP (^{13}C-DP) or TDCIPP (TDCIPP-d15) for the respective analyses. Samples were
131 extracted with a hexane:dichloromethane mixture (HEX:DCM; 1:1, *v*:*v*) for DP analysis and with a
132 mixture of acetonitrile and 1% acetic acid for TDCIPP analysis. Samples were extracted by
133 successively using vortexing (1 min), ultra-sonicating (5 min) and centrifugation (3 min). This
134 extraction step was performed twice and each time the supernatant was transferred to a clean tube.
135 The extract for DP analysis was then cleaned-up using a polypropylene cartridge (Supelco)
136 containing silica (Merck) acidified with 44% H_2SO_4 and topped with anhydrous Na_2SO_4 (Merck).
137 DP was eluted with hexane, then evaporated to dryness and reconstituted in 100 µL iso-octane
138 containing the recovery standard CB-207. The extract for TDCIPP was cleaned-up on an Oasis®

139 WAX cartridge (Waters) and TDCIPP with 5% NH_4OH in methanol, then evaporated to dryness
140 and reconstituted in 50 µL TPHP-d15 (1 ng/µL in MeOH, recovery standard) and 50 µL of MilliQ
141 water, respectively.

142 Quantification of DP was performed using an Agilent 6890-5973 gas chromatography mass
143 spectrometry system, equipped with a 15 m x 0.25 mm x 0.10 µm DB-5 capillary column (J&W
144 Scientific, USA) and operated in electron capture negative ionization mode. Quantification of
145 TDCPP was performed with liquid chromatography – tandem mass spectrometry (LC-MS/MS) on
146 an Agilent 1100 series LC coupled to an Agilent 6410 triple quadrupole MS detector. The LC was
147 equipped with a Kinetex® Biphenyl column (50 x 2.1 mm, Phenomenex). Procedural blanks,
148 analysed simultaneously with every batch of 7 samples, were consistent (RSD < 30 %) and
149 therefore mean value was subtracted from the sample values. The limit of quantification (LOQ) was
150 established based on a signal to noise ratio of 10 since both compounds were not detected in the
151 procedural blanks. Mean recovery of the internal standards TDCIPP-d15 and ^{13}C-DP were 88 ± 3%
152 and 103 ± 2%, respectively.

153 .

154 *Statistical analyses*

155 General linear models (GLM) and multiple regressions were applied to determine effects of
156 contaminant exposure, feed change and interactions on liver and thyroid histopathology. The
157 variable feed was expressed as the ratio of the numbers of days on feed B to total number of days,
158 and was arcsine transformed, as data occurred close to 0 and 100 % (Zar 1974), according to

$$\text{asin}\left(\sqrt{\frac{days\ on\ food\ B + \frac{3}{8}}{total\ days + \frac{3}{4}}}\right)$$

159 Preliminary analyses using a binomial error structure and a logit link function showed that data
160 were often overdispersed as indicated by a higher residual deviance than degrees of freedom, and

161 therefore a quasi-binomial error structure was selected (Crawley 2007). All statistical analyses were
162 performed using R version 2.12.1 (R Core Team 2015). The criterion for significance was set at
163 $p<0.05$.
164
165 **Results and Discussion**
166 Previous studies showed that changes in feed may affect metabolism and protein diet
167 requirements (Thrall et al. 2004), which subsequently may also affect liver morphology. In the
168 present study, feed change did not significantly influence liver or thyroid morphology. Liver
169 samples were examined for hepatic cell infiltration, granulomas, focal necrosis, lipid vacuoles and
170 sinusoidal dilatation. The morphology of the liver parenchyma was difficult to evaluate as this
171 tissue lacks distinct interlobular septa in avian species (Hodges 1974). Hepatic cell infiltration and
172 granulomas could not be distinguished; therefore these were categorized as aggregations of
173 lymphoid cells. The lymphoid aggregations were present in both control and exposed groups with
174 no marked difference among these (Table 2). No hepatic necrosis was observed in any of the slides.
175 It is worthwhile noting that a large amount of adipose vacuoles were observed within hepatocytes in
176 all exposed and control chicks. Due to the adipose storage complex in the liver of avian species, it
177 was not possible to determine the nature of the lipid vacuoles (Sato and Kamada 2011). Hepatic
178 sinusoidal dilatation was found in 4 individuals, all from the exposed groups (2 from TDCIPP and 2
179 from MIX; Table 2, Figure 1) with a significant difference among the groups. *In ovo* TDCIPP
180 exposure and accumulated tissue residues of TDCIPP led to development of hepatic sinusoidal
181 dilatation (Table 2). DP alone did not markedly affect liver morphology. However DP in the
182 presence of TDCIPP induced chemical accumulation in liver to induce sinusoidal dilatation. Thus
183 TCDIPP alone or in a mixture was needed to induce this effect.

That *in ovo* TDCIPP exposure affects hepatic sinusoidal dilatation is supported by the cholestasis of liver/biliary fibrosis and disrupted lipid and steroid metabolism reported by Farhat et al. (2014). The finding of TCDIPP-mediated sinus dilatation indicates a circulatory disturbance resulting in increased blood volume and stasis within the liver resulting in turgor, a condition that may lead to reduced hepatic metabolic capacity (Thrall et al. 2004). The hepatic sinusoidal dilatation was also found in a previous study on turbots (*Psetta maxima*) exposed to the FR compound BDE-47 that produced circulatory disturbances (Barja-Fernández et al 2013).

The thyroid glands were examined for proliferation of thyroid follicular cells, diverging size of thyroid follicles and number of follicles (Figure 2). Proliferations of thyroid follicular cells and large variation in follicle size were only found in one individual from the MIX group (Table 2). The difference between the MIX and non-injected CTRL group was significant; however, this difference should be interpreted with great caution due to small sample size. Yet, other studies noted similar histological changes in wild birds related to exposure to organochlorines and flame retardants (Sonne et al. 2010; 2011; 2013). No marked difference was found in mean follicle number among the exposure groups (Table 2).

Exposure to TDCIPP and a mixture of TDCIPP and DP may lead to morphological changes in liver tissue and thyroid glands. The alterations were similar to those found in studies of captive birds experimentally exposed to legacy persistent organic pollutants (Hoffman et al. 1996; McKernan et al. 2009; Qiesar 2009). Other investigations of wild birds exposed to both legacy and new organic environmental contaminants reported similar morphological changes reflecting adverse biological effects on thyroid hormone system (Letcher et al. 2010; McNabb and Fox 2003; Moccia et al. 1986; Saita et al. 2004; Sonne 2010; Sonne et al. 2010, 2013). In order to compare with life-long exposure in wild birds it is recommended for future research to conduct a follow-up period where exposure is extended to also include oral feeding for 1-2 months following hatching allowing

208 comparison with empirical field data and to supplement molecular and biochemical analyses
209 (Letcher et al. 2010).
210
211 **Conclusions**
212 Data indicate that currently available flame retardants TDCIPP and DP may induce
213 pathological changes in bird liver and thyroid glands following *in ovo* exposure. The
214 histopathological changes in liver may be attributed to increased blood volume due to TDCIPP (and
215 DP) exposure and subsequent tissue residue accumulation. Further research is needed to determine
216 the biological effects from these flame retardant chemicals on birds and other wildlife.
217
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Table 1. The composition of the different feed types (feed A and feed B) given to each quail until 14 days of age. The main protein source for feed A was corn, beans and sunflower, and for feed B it was oats, wheat and fishmeal. The presented difference (%) compares feed B to feed A. The concentration of TDCIPP and DP present in the two feed types is also given.

	Food A	**Food B**	**Difference**
Analytic contents (g per 100 g)			
Crude protein	21.9	23	↑5%
Fibre	7.3	4.8	↓34%
Fat	7.4	7.8	↑5%
Crude ash	6.5	4.9	↓25%
Selenium	0.4	0.4	0%
Calcium	1	0.73	↓27%
Phosphor	0.81	0.55	↓32%
Sodium	0.16	0.17	↑6%
Lysine	1.07	1.24	↑16%
Methionine	0.34	0.54	↑59%
Additives (per kg)			
E672 Vitamin A (IE)	10000	10000	0%
E671 Vitamin D3 (IE)	4500	4950	↑10%
vitamin E (mg)	80	120	↑50%
Ion (II) (mg)	53	53	0%
Iodine (mg)	1.1	1.1	0%
Cobber (mg)	15	15	0%
Manganese (mg)	128	128	0%
Zink (mg)	83	83	0%
Selenium (mg)	0.36	0.22	↓39%
Enzymes			
4a1640 6-fytase EC 3.1.3.26		500 FTU	
E1641 Endo-1,4-betaxylanase EC 3.2.1.8		70 AXC	
E1634 Endo-1,3 (4)-betaglucanase EC 3.2.1.6		100 AGL	
E1602 Endo-1,4-betaglucanase EC 3.2.1.4	800 U		
E1602 Endo-1,3 (4)-betaglucanase EC 3.2.1.6	1800 U		
E1602 Endo-1,4-betaglucanase EC 3.2.1.8	2600U		
Flame retardant concentration (ng g^{-1})			
DP	0.01	0.01	0%
TDCIPP	<2.00	3.09	↑155%

Table 2. Liver concentrations [mean ± SD (min-max)] of unrestricted flame retardants and number of chicks with liver and thyroid gland histopathology concentrations in 36 Japanese quail chicks exposed *in ovo* to tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), Dechlorane Plus (DP), a 1:1 TDCIPP:DP mixture (MIX) or unexposed (CTRL, non-injected). <LOQ: not detected above the limit of quantification; HSD: hepatic sinusoidal dilatation; LC: lymphoid cell infiltrations; NE: necrosis; LV: lipid vacuoles; PTF: proliferations of thyroid follicular cells and high variation in follicle size; FN: follicle number [mean±SD (min-max)].

Exposure to	***n***	**Chemical analyses**			**Liver histopathology**				**Thyroid gland histopathology**	
		TDCIPP	**DP**	**MIX**	**HSD**	**LC**	**NE**	**LV**	**PTF**	**FN**
TDCIPP	8	3.5±0.16 (3.4-3.6)	<LOQ	<LOQ	2*	5	0	8	0	80±21 (46-110)
DP	9	<LOQ	27±13 (5.1-54)	<LOQ	0	6	0	9	0	100±36 (57-170)
MIX	10	<LOQ	24±12 (10-52)	2.5±0.36 (2.3-2.9)	2*	5	0	10	1*	85±45 (40-160)
CTRL	9	<LOQ	0.20±0.10 (0.10-0.20)	<LOQ	0	4	0	9	0	97.3±45 (46-170)

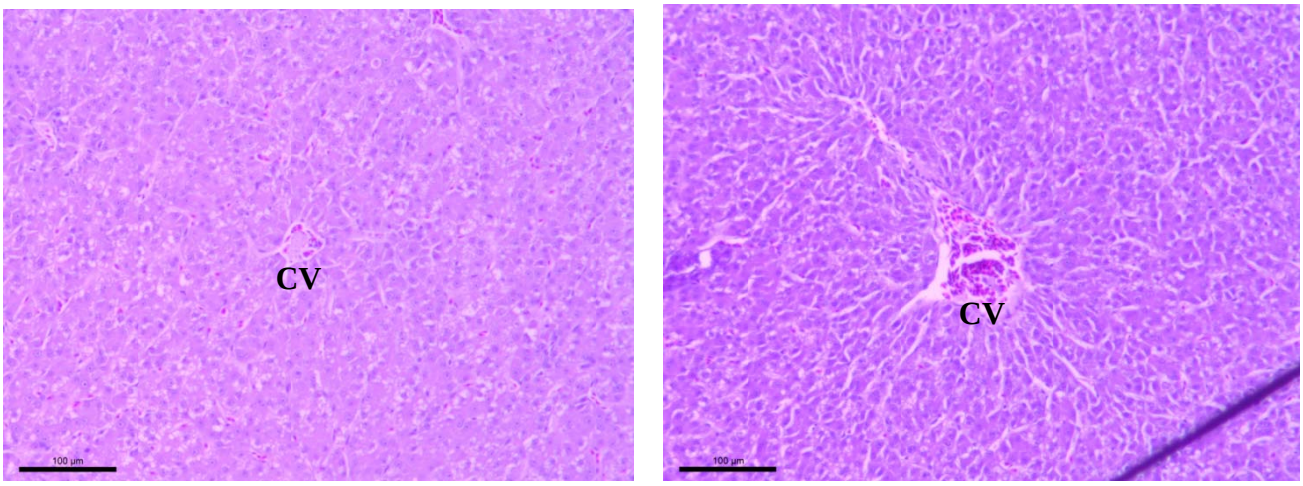
*: Significant higher prevalence compared to the CTRL group (GLM: $3.06 \leq F \leq 3.10$; both $p = 0.05$).

FIGURE LEGENDS

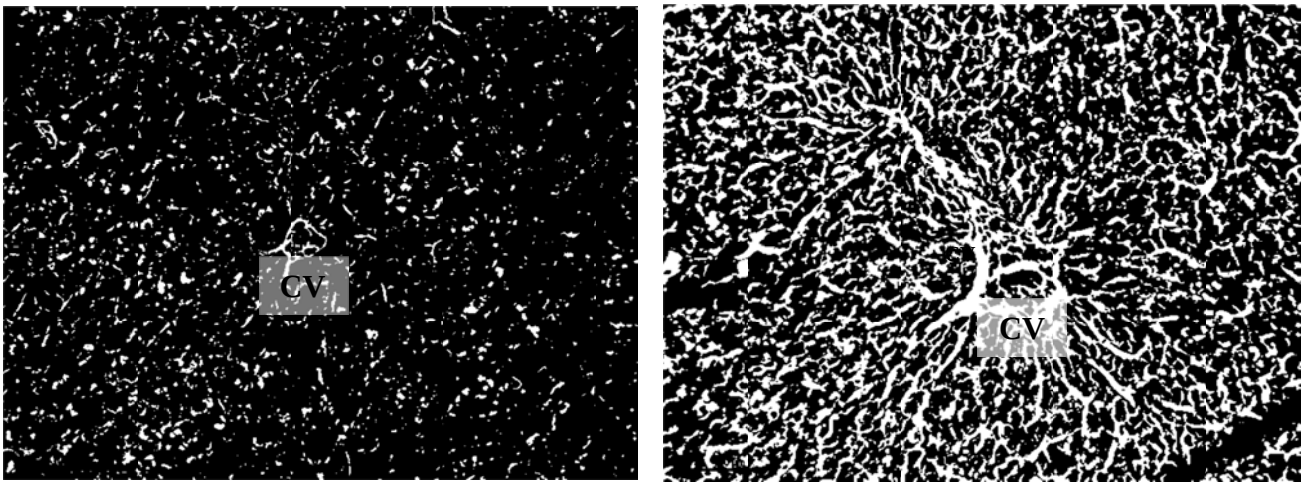
Figure 1. Liver micrographs showing dilated central vein (CV) and dilated sinusoids. A: CTRL. B: TDCIPP exposed. C and D are contrast micrographs of A and B, respectively, emphasizing sinusoids (white) and liver parenchyma (black). HE ×100. Bar: 100 µm.

Figure 2. Thyroid micrographs showing proliferations of thyroid follicular cells and high variation in follicle size. A: CTRL. B: MIX exposed. F: Follicle, C: Colloid, FC: follicle cells. HE ×200. Bar: 100 µm.

FIGURES

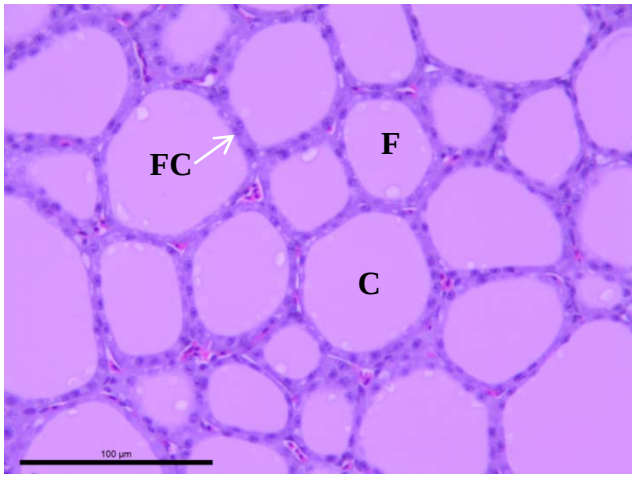


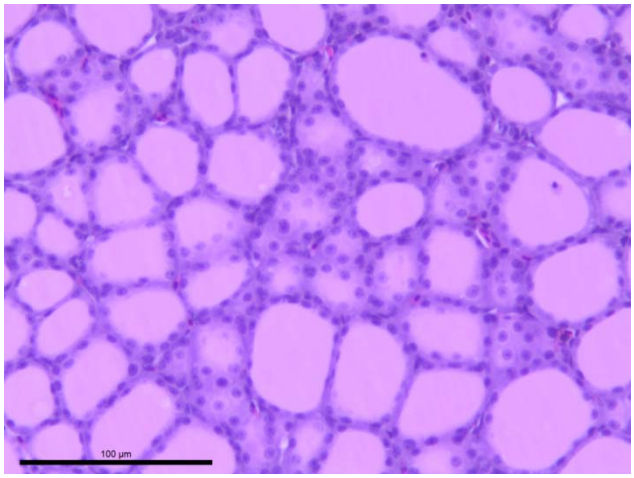
A B



C **D**

FIGURE 1.





A **B**

FIGURE 2