Embryotoxic effects of *in-ovo* triclosan injection to the yellow-legged gull

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Abstract

Triclosan (TCS) is an antimicrobial agent used in diverse personal care products that is considered as an emerging contaminant of both aquatic and terrestrial ecosystems. Although TCS aquatic ecotoxicity is well known, information on the presence and effects on terrestrial organisms is still scarce. This study was aimed at exploring the embryotoxicity of TCS to the yellow-legged gull (*Larus michahellis*) induced by the *in-ovo* injection of 150 ng TCS/g egg weight. Effects of TCS on embryo morphological traits (i.e. body mass, tarsus length and head size). Moreover, oxidative and genetic effects were assessed in the embryo liver, by measuring the amount of reactive oxygen species (ROS), the activity of antioxidant (superoxide dismutase and catalase) and detoxifying (glutathione *S*-transferase - GST) enzymes, the levels of lipid peroxidation and DNA fragmentation. After the injection, the concentration of TCS measured in the yolk of unincubated eggs (159 ± 35 ng/g wet weight, ww) was close to the expected concentration. Triclosan was found in residual yolk (2.9 ± 1.1 ng/g ww), liver (2.3 ± 1.1 ng/g ww) and brain (0.2 ± 0.1 ng/g ww) of embryos soon before hatching. Triclosan did not significantly affect embryo morphological traits, while it increased ROS levels and promoted GST activity, inducing the onset of both oxidative and genetic damage. This study demonstrated, for the first time in a wild euriecious bird species with mixed habits, that TCS can be maternally transferred to developing embryos, representing a potential threat for offspring.

Keywords: Personal care products; Triclosan; *in-ovo* manipulation; Embryotoxicity; Oxidative stress; Yellow-legged gull

Capsule: Triclosan induced oxidative stress in yellow-legged gull embryos

1. Introduction

In recent years, increasing evidence is accumulating for the chemical contamination of ecosystems by the so-called emerging pollutants, unregulated molecules whose fate and distribution in environment is often unknown (Ternes et al. 2015). Emerging pollutants mainly consist of human and veterinary pharmaceuticals, personal care products (PCPs) and various industrial additives that may induce toxic effects towards non-target organisms (Fent 2006). Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol; hereafter TCS) is a synthetic, broad-spectrum antimicrobial agent extensively used in household items, PCPs and medical devices for more than 40 years (Dann and Hontela 2011), resulting in a widespread contamination of natural ecosystems. TCS enters in the sewage system in its native form or as active metabolites. As the traditional wastewater treatment plants (WWTPs) are not specifically designed to remove emerging pollutants (e.g. Sturve et al. 2008), TCS enters surface waters, where it has been measured in concentrations ranging between 0.027 and 2.7 µg/L (Dann and Hontela, 2011; Bressy et al. 2017). Because of its hydrophobicity (log K_{ow} = 4.8), TCS is retained in municipal sewage sludge (Banihashemi and Droste, 2014) and can be transferred to agricultural soils (Fuchsman et al. 2010), contributing to terrestrial contamination. Moreover, several studies demonstrated that TCS can be accumulated in both aquatic and terrestrial organisms (Chalew and Halden 2009; Ricart et al. 2010). Considering its widespread occurrence and high biological activity, many studies have investigated TCS toxicity on diverse organisms. Although studies on mammals showed neither acute toxicity nor genotoxicity and developmental toxicity of TCS, a number of evidence demonstrated that non-target aquatic species, including algae, invertebrates and fish, are more sensitive to TCS than mammals (Dann and Hontela 2010). However, such investigations have revealed that acute effects occurred at high, unrealistic TCS concentrations (1.4 - 3,000 µg/L range), depending on the different sensitivity of the model species (reviewed by Dann and Hontela 2010). Avian studies of TCS acute toxicity performed on the bobwhite quail (*Colinus virginianus*) and the mallard duck (*Anas platyrhynchos*) have shown a No Observed Adverse Effect Level (NOAEL) of 210 mg/kg and > 2,150 mg/kg for bobwhite quail and mallard duck, respectively (Pedersen and Helsten 1993a,b), with a 50% lethal dose (LD50) of 860 mg/kg for the bobwhite quail (Pedersen and Helsten 1993c). In contrast, a variety of studies highlighted a variety of sub-lethal effects induced by environmental TCS concentrations to both invertebrate and vertebrate aquatic species, including negative effects on physiology (i.e. the onset of oxidative stress; Binelli et al., 2011; Liang et al., 2013), health status, survival, growth, development or reproduction (reviewed by Dann and Hontela 2010). Moreover, because of the structural similarity of TCS to diverse well-known endocrine disruptor chemicals, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and bisphenol A, as well as to thyroid hormones, several studies have

demonstrated that the exposure to this molecule can alter the endocrine functions in diverse species aquatic and terrestrial species, including humans (Dann and Hontela 2010 and references therein). Despite these findings, the information on the sub-lethal effects due to TCS exposure towards terrestrial organisms is still limited. Although a laboratory exposure of the tiger worm (*Eisenia fetida*) has shown that TCS can modulate the activity of antioxidant enzymes and cause genetic damage (Lin et al. 2010), to the best of our knowledge no study has investigated the sub-lethal effects of TCS on avian species.

Birds can be exposed to TCS by trophic transfer along the food chain or direct uptake, although both these hypotheses have not been adequately documented (Dann and Hontela, 2011). Because of its lipophilic nature, TCS owns a great propensity for bioaccumulation in aquatic species, including mussels and fish (Dann and Hontela 2010 and references therein). Similarly, TCS may transfer from soil to biota. For instance, Kinney and co-authors (2008) showed a bioaccumulation factor of 27 in earthworms from amended soils. Thus, the predation of aquatic species and earthworms by birds could result in TCS trophic transfer up the food chain, although this has not yet been documented. Seabirds could be especially exposed to TCS, and in general to antropogenic contaminants, because of their high position in the food webs, specific ecological habits (they are often omnivorous species) and relative long life-span (van der Schyff et al. 2016). Specifically, bird eggs have been individuated as a non-invasive tool to monitor the levels of priority (Muñoz-Arnanz et al. 2014) and emerging contaminants (Custer et al. 2012; Vicente et al. 2015). However, the information on the presence and the toxicity of TCS towards birds and its potential maternal transfer to the eggs is still limited. Only one study has detected TCS in the eggs of the European starling (*Sturnus vulgaris*; concentration range: 9.4-37.9 ng/g wet weight, ww) and the American kestrel (*Falcus sparverius*; concentration range: 4.2-13.4 ng/g ww; Sherburne et al. 2016). However, the information on potential toxic effects on embryo development and offspring is still scarce (Sherburne et al. 2016). To date, no previous study has assessed the presence and the potential toxicity of TCS in a wild bird species. Thus, the present study was aimed at exploring the embryotoxicity of TCS in the yellow-legged gull (*Larus michahellis*), in the wild. One hundred-fifty ng/g egg weight of TCS was injected into the yolk of yellow-legged gull eggs at the time of laying and the adverse effects on embryo morphological traits (i.e. body mass, liver and brain mass, tarsus length and head size) were investigated. In addition, as previous studies revealed that TCS exposure can induce oxidative and genetic damage in several species, a suite of oxidative and genetic biomarkers was applied on embryo liver. In detail, the amount of reactive oxygen species (ROS), the activity of antioxidant (superoxide dismutase, SOD and catalase, CAT), detoxifying enzymes (glutathione *S*-transferase, GST) and the levels of lipid peroxidation (LPO) were assessed as biomarkers of oxidative stress, while DNA fragmentation was

measured as a genetic damage endpoint. As previous studies demonstrated that TCS exposure induced an oxidative stress situation in diverse model species (e.g. Binelli et al. 2011; Matozzo et al. 2011; Wang et al. 2014), we expect that TCS injection induces an imbalance of the oxidative status (i.e. ROS overproduction and modulation of antioxidant enzyme activity), leading to oxidative and genetic damage. Moreover, as the amount of maternally-transferred non-enzymatic antioxidants decreases with the position in the laying sequence (Rubolini et al. 2011), whereby the third-laid eggs show a lower amount of antioxidants compared to the first- and second-laid eggs, we may expect that embryos from the third-laid eggs are more prone to oxidative stress with respect to their siblings.

2. Materials and Methods

2.1 Study species

The yellow-legged gull (*Larus michahellis*) is a large, monogamous gull species inhabiting Mediterranean coasts and inland habitats, where it often breeds colonially. *L. michahellis* is an omnivorous species feeding on both aquatic and terrestrial animals, as well as on wastes found in landfill. As the yellow-legged gull has a widespread distribution and is evaluated as Least Concern by the BirdLife International, it is considered an excellent non-conventional biological model for ecotoxicological studies (Parolini et al. 2017a). Females lay 1-3 eggs (1-4 days interval). Eggs from the same clutch hatch asynchronously (1-4 days of hatching spread) 27-31 days after laying (Cramp 1998). Eggs, which usually weigh from 80 to 100 g, are suitable for experimental manipulation.

2.2 In-ovo *TCS manipulation*

The study was carried out in the Comacchio lagoon (44° 20’ N - 12° 11’ E, Northeastern Italy) where a large colony of yellow-legged gull breeds. The study area was visited every single day to mark new nests and newly laid eggs to monitor the progress of laying sequence. When a new egg from a clutch was found, it was temporarily removed for experimental manipulation and replaced with a ‘dummy’ egg in order to avoid interference with incubation behavior.

Triclosan powder (CAS number - Sigma Aldrich, Italy) was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution (100 µg/L) that was then seeded in sterile vials. DMSO was used as carrier solvent because of its excellent solvent properties and low toxicity (Gad 2005). In addition, a previous manipulative study performed on the yellow-legged gull eggs from the same colony showed that the injection of DMSO into the eggs was not embryotoxic (Parolini et al. 2016). One hundred fifty (150)

ng TCS/g egg weight were arbitrarily injected into the yolk of gull eggs because i) no data of its presence in the yellow-legged gull eggs are currently available and ii) this concentration is similar to the levels of other emerging pollutants found in the egg yolk of our model species (i.e. PFOS, Parolini et al. 2016). Moreover, the concentration of TCS we injected into the yolk was in the same order of magnitude compared to the concentration of other organochlorine compounds measured in the eggs of the yellow-legged gull sampled in coastal Mediterranean ecosystems (e.g. Zapata et al. 2018). The manipulation of TCS level into egg yolk was performed according to a within-clutch design, whereby both control and TCS-injected eggs were established within each clutch. This design allowed to minimize the consequences of environmental and parental effects. As no evidence demonstrated that the concentration of TCS in yellow-legged gull eggs varies according to the egg size or laying order, the same amount of TCS was injected in all the eggs, disregarding these two factors. The clutches were assigned sequentially to the following treatment schemes, according to the order in which the first egg was found (nest: laying order, treatment): nest 1: egg 1, TCS injection (TCS); egg 2, carrier solvent (C); egg 3, TCS; nest 2: C-TCS-C; nest 3: TCS-C-C; nest 4: C-TCS-TCS and so forth with the following nests. Injections were performed in the yolk following a previously validated procedure (Possenti et al. 2016; see Fig. S1 in Supporting Information). Treated eggs were injected with 30 µL of the TCS solution, while 30 µL of DMSO only were injected in control eggs. Before being injected, the egg was weighed (to the nearest g) and placed with the longitudinal axis vertical. After 15 min, the eggshell was disinfected, and a hole was drilled close to the acute pole using a sterile pin. The injection was performed using a 1 mL sterile syringe mounting a 0.6 × 30 mm needle. Immediately after extracting the needle from the egg, the hole was sealed with a drop of epoxydic glue and a small piece of eggshell was superimposed to the hole. After the injection procedure, the egg was returned to their original nests to be incubated by parents. Eggs from 26 nests were injected. All the injected eggs, including eggs that did not develop, were collected to prevent any dispersion of TCS in the wild. In addition, the eggs from 5 nests were injected and collected immediately after the injection to verify the reliability of the manipulative procedure by measuring the amount of TCS into the yolk. After sampling and separating from the albumen, the yolk was weighed (to the nearest g) and stored at -20 °C until analysis of TCS concentration. Starting 5 days before the earliest expected hatching date (ca. 24 days after laying), the other experimental nests were visited every day to check for any sign of imminent hatching of the eggs. When eggshell fractures were observed (i.e. 'cracking stage'), eggs were collected and quickly frozen at -20 °C until the embryo dissection. In the lab, the eggshell was removed, and the residual yolk sac isolated from the embryo. Before the dissection, the embryo was weighed (to the nearest g), and tarsus and head size (i.e. occipital-beak length) were measured by a calliper. The liver and the brain were detached from the embryo, weighed and quickly frozen at

-80 °C pending oxidative and genetic biomarker analyses. Morphometric measurements were performed blind of treatment, embryo sex and position in the laying sequence by the same operator to ensure consistency. Sex of each embryo was assessed by the amplification of the CHD gene as reported by Parolini et al. (2016).

This study was performed under the permission of the Parco Regionale del Delta del Po (Protocol #388, 20 January 2016), which allowed both the manipulation of the biochemical quality of the eggs by injecting TCS and the subsequent sampling of manipulated eggs at the 'cracking stage'.

2.3 Triclosan determination in the yolk and embryo organs

Standard solutions of triclosan (native TCS) and labelled internal standard (IS) ($^{13}C_{12}$-TCS) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Acetonitrile (ACN) was supplied by Fisher scientific, hexane (Hex) by Acros Organics, dichloromethane (DCM), iso-octane, sodium chloride (NaCl), potassium hydroxide (KOH), and sodium sulphate (Na_2SO_4) by Merck, pentafluorobenzoyl chloride (PFBCl) by Sigma Aldrich, milliQ water (MQ) by ELGA LabWater. Clean-up empty polypropylene (PP) cartridges of 6 mL were purchased from Agilent Technologies.

The concentration of TCS in the yolk from unincubated eggs was measured to verify that the injection procedure has been correctly performed, while TCS transfer to the embryos was validated by measuring its concentration in the residual yolk sac that was not absorbed by the embryo (at the cracking stage it accounts for ca. 70% of its total amount at laying; Parolini et al., 2017a), as well as in embryo liver and brain.

The extraction of TCS from yolk, liver and brain samples was performed according to Geens et al. (2012), with slight modifications. After homogenizing and before mixing the samples with anhydrous sodium sulphate (Na_2SO_4) to obtain a dry powder, the samples were weighted (about 200 mg) in a 15 mL polypropylene (PP) tube and spiked with 50 µL of labelled internal standard (IS) (containing 200 pg/µL $^{13}C_{12}$-TCS in methanol). Two mL of acetonitrile (ACN) were added to sample homogenates, which were vortexed for 1 min, ultra-sonicated for 10 min and centrifuged for 3 min at 3,500 rpm. The supernatant was then transferred to a pre-cleaned glass tube. The extraction was carried out twice and the extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted in 1 mL milliQ water (MQ) vortexing for 20 s. For the extractive derivatization, 50 µL of potassium hydroxide solution (KOH, 2 M), 50 µL of pentafluorobenzoyl chloride solution (PFBCl, 5%) and few crystals of sodium chloride (NaCl) were added to the extract solutions and vortexed for 20 s. After adding 3 mL of hexane and vortexing the solutions for 2 min, the extracts were centrifuged for 5 min at 3,500 rpm and the hexane layer was loaded onto 6 mL PP cartridge filled with 1 g acidified silica (AS, 10%,

prewashed twice with 2.5 mL of dichloromethane (DCM)). The extraction was repeated with 2.5 mL hexane and the organic layer was transferred to the same cartridge. The PP cartridges were then eluted with 8 mL DCM. The final extract was concentrated to near dryness, reconstituted in 200 µL of iso-octane, and after being vortexed for 30 s, it was transferred to amber injection vials. The quantitative analyses of TCS in all biological samples were performed using gas chromatography (GC) (Agilent Technologies, 6890N Network GC systems) coupled to mass spectrometry (MS) (Agilent Technologies, 5973 Network Mass Selective Detector) in electron capture negative ionization mode (ECNI) (Geens et al. 2009, 2012).

2.4 Quality assurance and quality control

Two procedural blanks (Na_2SO_4 with addition of IS) were included in each batch of samples to evaluate possible interfering effects and/or any contaminations during the sample preparation and following GC/MS analyses. Levels of TCS in the procedural blank were very low (<10 pg). Moreover, fresh chicken yolk and liver samples (*Gallus gallus*; purchased in a supermarket from Antwerp, Belgium) were used to assess possible matrix effects. Levels of TCS in chicken eggs and liver were very low (<10 pg) and comparable to procedural blank values.

The linearity of the method was assessed through the average response factor of the whole calibration range. Seven matrix-matched calibration points for egg yolk analysis were prepared, while one additional point was added in the lower range for liver and brain calibration curve. Calibration curves were best fitted to a linear model and were constructed by plotting the TCS/IS ratio against the ratio of mass of TCS/IS. The IS% recoveries were within the range of acceptability. The percentage of recovery was 107 ± 9 % (98 - 116 %) for Std 1 and 99 ± 10 % (89 - 109 %) for Std 2.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3 × standard deviations (SD) and 10 × SD of the blank concentrations, respectively, returning a LOD of 120 pg/g and a LOQ of 400 pg/g.

2.5 Oxidative stress and genetic biomarkers

All the reagents and solvents used for biomarker analyses were purchased by Sigma Aldrich (Italy). Biomarker analyses (i.e. the amount of reactive oxygen species (ROS), the activity of SOD, CAT, and GST enzymes, the levels of lipid peroxidation (LPO) and DNA fragmentation) were performed on embryo liver because it is the main organ for detoxification and excretion of lipophilic chemicals and some studies demonstrated that TCS induced adverse effects on the liver of avian species (e.g.

Guo et al., 2018). Biomarkers were not performed on the brain because measured TCS concentration was negligible (Table 1).

Liver samples were homogenized by an automatic homogenizer in 100 mM phosphate buffer (pH 7.4), with the addition of 100 mM KCl and 1 mM EDTA and. The measurement of ROS was performed according to Deng et al. (2009), using a dichlorofluorescein-diacetate (DCFH-DA) method. The homogenates were centrifuged at 16,500 rpm for 20 min at 4 °C. Then, 20 µL of the homogenate was seeded to a 96-well plate and incubated for 5 min at room temperature. One hundred µL of phosphate buffer saline (PBS; pH 7.4) and 8.3 µL of DCFH-DA (10 mg/mL dissolved in DMSO) were added to each sample. The plate was incubated at 37 °C for 30 min. Fluorescence was measured at $\lambda = 485$ nm (excitation) and $\lambda = 536$ nm (emission) and the amount of ROS was expressed as arbitrary units (AU) of DCF-H/ mg protein.

The activity of SOD, CAT and GST was assessed in liver homogenates according to Parolini et al. (2010). The homogenate was centrifuged at 16,500 rpm for 1 h at 4 °C. The sample was held in ice and quickly processed for the determination of total protein content according to Bradford (1976), and enzymatic activities. The CAT activity was assessed monitoring the consumption of H_2O_2 (50 mM) at $\lambda = 240$ nm. The SOD activity was measured through the assessment of the degree of inhibition of the reduction of the cytochrome c (10 µM) at $\lambda = 550$ nm performed by the superoxide anion generated by the reaction occurring between xanthine oxidase (1.87 mU/mL) and hypoxanthine (50 µM). The GST activity was determined by monitoring for 1 min the reaction between the sample, reduced glutathione (1 mM) and 1-chloro-2,4 dinitrobenzene at $\lambda = 340$ nm.

Lipid peroxidation was measured according to Ohkawa et al. (1979). Two hundred µL of liver homogenate was added to a trichloroacetic acid (TCA; 12%), thiobarbituric acid (TBA, 0.37%) and Tris-HCl (0.6 M) solution, which was boiled for 1 hour. After 20 min cooling at 4 °C and 15 min centrifugation at 11,500 rpm, the absorbance of the obtained supernatant was measured at $\lambda = 535$ nm. The amount of thiobarbituric acid reactive substances (TBARS) was expressed as nmol TBARS/g ww.

DNA precipitation assay was performed to quantify DNA strand breaks in liver homogenates by using a fluorescence method (Olive 1988). Seventy-five µL of liver homogenate was mixed with 600 µL of a SDS (2%), EDTA (10 mM), tris-base (10mM) and NaOH (40 mM) buffer. The solution was mixed with 600 µL KCl (0.12 M) and heated at 60 °C for 10 min, mixed by inversion and cooled at 4 °C for 30 min. Hoechst 33258 dye solution (1 µg/mL diluted in a solution 0.1 M Tris-acetate buffer; 4 mM sodium cholate and 0.4 M NaCl, pH 8.5) was added to the supernatant and mixed for 5 min.

Fluorescence was measured at λ = 360 nm (excitation) and λ = 450 nm (emission). The method was calibrated using salmon sperm genomic DNA as a standard and the DNA stand breaks were expressed as µg DNA/mg protein.

2.6 Statistical analyses

The effect of TCS injection on embryo morphology, oxidative stress and genetic biomarkers was analysed by linear mixed models (LMMs), including egg treatment, embryo sex and egg the position in the laying sequence as fixed factors, as well as their two-way interaction terms. Clutch identity was included in the models as a random effect. Non-significant ($P > 0.05$) interaction terms were removed from the final model all in a single step. In the model of embryo morphological traits, the mass of the egg at the time of laying was included as a covariate. After running the Grubbs' test (extreme studentized deviate method), significant outliers (0-2 data points, depending on considered biomarker) were removed. False Discovery Rate (FDR) correction for multiple comparisons was performed *a posteriori* to consider multiple-testing and to correct for multiple comparisons. All statistical analyses were performed using SAS 9.3 PROC MIXED.

3. Results

3.1 Triclosan concentration in yolk and organs of embryos

TCS levels in the yolk of the 15 unincubated eggs (7 control eggs and 8 TCS-injected eggs) were quantified to check for the reliability of the injection method. Mean TCS concentration (± standard deviation) in the yolk of TCS-injected eggs was 159 ± 35 ng/g ww, while it was always below the limit of detection in the yolk of control eggs (Table 1).

Since lipophilic contaminants are known to be transferred from yolk to developing embryos (Bertolero et al. 2015), TCS concentrations were measured in the residual yolk sac and in embryo liver and brain (8 embryos from control eggs and 10 embryos from TCS-injected eggs). We performed these analyses on the embryos from complete clutches only (6 nest with 3 eggs at the eggshell cracking stage). TCS was still present in the residual yolk at the late stages of embryonic development, although its mean concentration (2.9 ± 1.1 ng/g ww) was notably lower than the one measured in the yolk from unincubated eggs, confirming the transfer of this compound to the embryo. Accordingly, TCS was detected in the liver (2.3 ± 1.1 ng/g ww) and limitedly in the brain (0.2 ± 0.1 ng/g ww) of embryos from TCS-injected eggs, while levels in control eggs were undetectable (Table 1).

301 *3.2 Effect of TCS on embryo morphology*

302 The analysis of the proportion of eggs showing imminent sign of hatching (i.e. cracking stage) was
303 carried out considering all the injected-eggs (n = 78 eggs from 26 nests). No significant difference in
304 the proportion of eggs that reached the cracking stage was observed between TCS-treated eggs (22/40
305 = 0.550; $\chi^2_1 = 0.089$; P = 0.864) and the control (proportion of eggs at cracking; 25/38 = 0.657; P =
306 0.864). The sex ratio (proportion of males) did not significantly differ between the experimental
307 groups (control embryos: 12/25 = 0.480 and TCS-treated embryos: 9/22 = 0.454; $\chi^2_1 = 0.001$; P =
308 0.969). Morphological analyses were performed on 44 embryos (22 from control eggs and 22 from
309 TCS-treated eggs) from 20 nests. Embryo mass did not differ between the experimental groups, but
310 significantly covaried with the mass of egg at the time of laying (Table 2). In addition, the mass of
311 embryos depended on laying order (Table 2), with embryos from second-laid eggs being significantly
312 larger than siblings from first- (P = 0.033) and third-laid eggs (P = 0.024). Linear mixed models of
313 tarsus length, brain and liver mass did not show significant effect of treatment, embryo sex, laying
314 order and their two-way interactions, except for a marginally non-significant effect of TCS treatment
315 on the head size (Table 2). Finally, head size significantly covaried with the mass of original egg and
316 differed between sexes, with males having larger head size compared to that of female embryos
317 (estimated marginal means (± standard error) of males 434 (3.5) and females 418 (3.2)).

318 *3.3 Effect of TCS on oxidative stress and genetic biomarkers*

319 Triclosan injection significantly increased the amount of hepatic pro-oxidants (Table 3; Fig. 1a),
320 which varied according to the position in the laying sequence, independently of TCS treatment.
321 Embryos from third-laid eggs showed larger amount of oxidant species than those from first- (P =
322 0.005) and second-laid eggs (P = 0.002). In addition, a significant effect of treatment by laying order
323 interaction was found (Table 3; Fig. 2a), whereby ROS levels measured in embryos from third-laid
324 eggs were significantly higher in TCS-treated embryos compared to controls (P < 0.001; Fig. 2a).
325 Linear mixed models of CAT and SOD enzymes did not show a significant effect of fixed factors and
326 their two-way interactions (Table 3; Fig. 1b,c). However, TCS injection significantly increased GST
327 activity compared to controls (Table 3; Fig. 1d). Moreover, hepatic GST activity differed according
328 to the laying order, whereby embryos from first-laid eggs had higher values than those from third-
329 laid eggs (P = 0.014). Triclosan injection caused a marginally non-significant increase on lipid
330 peroxidation in embryo liver (Table 3; Fig. 1e), while no significant effect of sex or laying order was
331 observed (Table 3). However, a significant effect of treatment by laying order interaction was noted,
332 with statistically significant differences between experimental groups observed in embryos from the
333 second-laid eggs (Table 3; Fig. 2b). Triclosan treatment and sex did not affect DNA fragmentation in

334 embryo liver (Fig. 1f), while a significant effect of laying order (Table 3) was noted. Embryos from
335 second-laid eggs showed low genetic damage compared to siblings from first- ($P = 0.009$) and third-
336 laid eggs ($P < 0.001$).

337

338 **4. Discussion**

339 The results from the present, manipulative study showed that TCS was not detected in the egg yolk
340 of yellow-legged gulls (used as control eggs of the manipulation experiment) breeding in the
341 Comacchio lagoon. A previous investigation showed that TCS was detected in the eggs of secondary
342 (the European starling) and tertiary (the American kestrel) bird consumers in the wild demonstrating
343 its potential to be transferred and accumulated in avian eggs (Sherburne et al. 2016). However, the
344 negligible TCS concentrations found in the eggs of yellow-legged gulls from the colony we studied
345 might be due to its low environmental levels in such geographical area. Alternatively, we may
346 speculate that TCS is accumulated in maternal tissues, but it is then biotransformed and/or
347 metabolized by mothers and thus it is not or only limitedly transferred to the eggs and thus to the
348 offspring. However, we cannot exclude that birds can accumulate TCS and transfer it to their eggs,
349 leading to potentially harmful consequences to the developing embryos.

350 For this reason, we tested the effects of the injection into the yolk of a TCS dose (150 ng/g egg weight)
351 on the phenotype of gull embryos shortly before hatching. Although previous studies of birds showed
352 that the concentration of maternally-transferred TCS was about three-fold lower than 50 ng/g ww
353 eggs (Sherburne et al. 2016), we injected a higher concentration similar to the levels of other lipophilic
354 chemicals measured in the eggs of our model species, to test the toxicity and the mechanism of action
355 of this chemical during embryo development. At the same time, the concentration we injected was
356 1,000-fold lower than the dose administered to adults of the bobwhite quail that caused a significant
357 decrease of body weight (Pedersen and Helsten, Report 102–024–03) to investigate sub-lethal toxicity
358 and to prevent acute effects towards embryos, as early developmental stages are considered the most
359 sensitive to contaminant exposure (Ottinger et al. 2008).

360 Our findings showed that TCS was transferred to the embryos during development, as demonstrated
361 by the limited TCS amount remained in the residual yolk sac, as well as by its presence in the liver
362 and partially in the brain, at the cracking stage. However, the TCS concentration measured in the
363 focal organs, as well as in the residual yolk, were lower than an order of magnitude compared to that
364 injected and measured into the yolk of unincubated eggs. These results suggest a differential transfer
365 of TCS to the different somatic tissues of embryos during development. We may speculate that the
366 most of the injected TCS dose was transferred and accumulated in lipids of embryo muscles.

Alternatively, the low amount of TCS found in residual yolk and embryo organs might be due to degradation of this chemical during development. However, a companion analysis of TCS byproducts in residual yolk sac and focal embryo organs did not revealed measurable levels of these byproducts.

However, embryo morphological traits (i.e. body mass, tarsus length and mass of focal organs) were not affected by TCS treatment, with the only exception of a marginally non-significant effect on head size. These results are different from those found in the previous studies investigating the adverse effects due to TCS exposure on embryonic development and/or morphology of diverse animal species. For example, TCS has been found to reduce biomass and shell growth in juvenile snail *Achatina fulica* (1-day post-fertilization at 40 mg/kg; Wang et al. 2014), to induce malformations in zebrafish (96 hpf at 0.5 mg/L; Oliveira et al. 2009) and medaka fish embryos (concentration $\geq$ 5 ng TCS/egg; Nassef et al. 2010), as well as to decrease body weight in the bobwhite quail (up to 147 mg/kg body weight; Pedersen and Helsten, Report 102–024–03). These discrepancies may depend on the tested doses, the duration of exposure, the differences in experimental approach and the sensitivity of organisms to TCS. Therefore, the toxicity of this chemical to either terrestrial or aquatic wildlife species remains to be elucidated.

Although no negative effects on morphology were observed, TCS induced an oxidative stress situation in treated embryos with respect to controls (Fig. 1). In fact, TCS treatment promoted the activity of glutathione *S*-transferase (GST), a phase II enzyme involved in the biotransformation of several lipophilic xenobiotics that catalyse the conjugation of glutathione with both endogenous and exogenous substrates (Lin et al. 2010). The increase of hepatic GST activity observed in TCS-treated embryos compared to controls may indicate its involvement in TCS detoxification processes. Previous studies of zebrafish, zebra mussel and earthworm have suggested that the enhancement of GST activity may be caused by the employment of TCS as a substrate that is biotransformed and then excreted from the target organ (Lin et al. 2010; Binelli et al. 2011). Because of the intense metabolic activity produced by TCS detoxification processes, the hepatic ROS levels in embryos from TCS-injected eggs were significantly increased relative to controls. These findings were consistent with those from a previous study carried out on an invertebrate species (the monogonont rotifer *Brachionus koreanus*), whereby an increase in intracellular ROS content has been observed with a simultaneous increase in GST activity (Han et al. 2016). As ROS overproduction due to chemical exposure can lead to negative long-lasting consequences for the organisms, a complex antioxidant system evolved to prevent oxidative stress and damage. Among endogenous antioxidants, the enzymes superoxide dismutase (SOD) and catalase (CAT) play the major role in defending against accumulation of reactive oxygen species such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), respectively, modulating their activities. According to previous studies, the activity of SOD and CAT

401 was not affected by TCS treatment, suggesting an inhibition caused by this chemical (Canesi et al.
402 2007; Matozzo et al. 2012). However, other investigations have shown that TCS exposure enhance
403 the activity of both these enzymes, which showed a decreasing trend after prolonged exposure or at
404 high exposure concentrations (Binelli et al. 2011; Lin et al. 2010; Wang et al. 2014). We may
405 speculate that the amount of TCS-induced ROS exceed the capacity of scavenging them, inhibiting
406 the activity of SOD and CAT, leading to an impairment of the antioxidant defense system (Atli and
407 Canli 2010). Alternatively, the lack of activation of SOD and CAT may depend on the early life stage
408 when they were measured. In fact, a previous study of chicken embryos has shown that the activity
409 of antioxidant enzymes is very low (Surai 1999) and could not be efficient in preventing ROS toxicity.
410 Thus, we suggest that the maternally-transferred non-enzymatic yolk antioxidants play the most
411 important role in the antioxidant defense during early developmental stages (Surai 2002). However,
412 our findings suggest that embryos did not efficiently counterbalance the increase of ROS caused by
413 TCS exposure and could experience an oxidative stress situation. In fact, previous experiments
414 performed on earthworms, snails and blue mussels showed that TCS-induced ROS overproduction
415 caused a dose-dependent increase of lipid peroxidation and genetic damage (Matozzo et al. 2011;
416 Wang et al. 2014). However, this hypothesis was only partially supported by our data because we
417 found a marginally non-significant increase of both lipid peroxidation and DNA fragmentation in the
418 liver from embryos developed in treated-eggs compared to controls.

419 Interestingly, TCS treatment affected the amount of ROS and LPO levels according to the laying
420 order (Fig. 2). Considering embryos from the third-laid eggs only, controls had lower levels of hepatic
421 ROS compared to their TCS-treated siblings. Such excess of ROS may be due to the lower amount
422 of maternally-transferred exogenous antioxidants (e.g. carotenoids and vitamin E) in the third-laid
423 eggs compared to first- and second-laid eggs (Rubolini et al. 2011). This pattern of variation of
424 exogenous antioxidants may establish a disadvantage for the third-laid egg embryos, which suffer the
425 great amount of ROS that cannot be efficiently counteracted. However, the levels of lipid
426 peroxidation in embryos from the third-laid eggs did not significantly differ from those of controls.
427 In contrast, LPO levels were more severe in TCS-treated embryos compared to controls both from
428 second-laid eggs only, which has an intermediate biochemical composition in terms of antioxidants
429 between the first- and the third-laid eggs (Rubolini et al. 2011). We may speculate that maternally-
430 transferred antioxidants do not mechanistically prevent oxidative damage, as suggested by a previous
431 study of the yellow-legged gull showing contrasting oxidative stress results in embryos from the
432 second-laid eggs after the injection of a putative pro-oxidant molecule (Parolini et al., 2017b).

433 In conclusion, the present study first demonstrated that TCS can be transferred from eggs to
434 developing embryos of a wild seabird species and negatively affect the phenotype of the offspring,

inducing an imbalance of the oxidative status in the embryo liver. Considering the variability in maternal allocation of yolk antioxidants in the yellow-legged gull eggs, it should be interesting to explore the differential effects of TCS on embryos from the second- and third-laid eggs, which receive a lower amount of antioxidants and could be more prone to the oxidative effects induced by TCS. . Our findings suggest that TCS might be a threat for omnivorous and top-consumer birds feeding of both aquatic and terrestrial preys that can accumulate a great amount of TCS in their tissues, because they might transfer to their eggs, and consequently to their offspring, a great amount of this chemical. Moreover, considering that early-life effects can result in serious consequences to diverse life-history traits at adulthood, further ecotoxicological studies should be needed to shed light on the toxicity and long-term consequences due to TCS exposure in birds.

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589 Table 1. Concentration of TCS (mean ± standard deviation; ng/g ww) measured in the yolk of
590 unincubated eggs and of eggs at cracking stage, and in the liver and brain of yellow-legged gull
591 embryos. Sample sizes are reported for each treatment (C = control; TCS = triclosan-injected eggs)
592 and position in the laying sequence (first, second and third laid eggs).

593

		TCS concentration (ng/g ww)		
		first-egg	*second-egg*	*third-egg*
Unincubated eggs		(C=3; TCS=2)	(C=2; TCS=3)	(C=2; TCS=3)
Yolk	C	< LOD	< LOD	< LOD
	TCS	162 ± 11	189 ± 5	127 ± 37
Eggs at cracking stage		(C=4; TCS=2)	(C=2; TCS=4)	(C=2; TCS=4)
Yolk	C	< LOD	< LOD	< LOD
	TCS	2.4 ± 1.9	3.3 ± 1.2	2.8 ± 0.5
Embryo liver	C	< LOD	< LOD	< LOD
	TCS	2.6 ± 1.0	2.3 ± 0.8	2.1 ± 1.5
Embryo brain	C	< LOD	< LOD	< LOD
	TCS	0.4 ± 0.1	0.2 ± 01	0.2 ± 0.2

594 < LOD = below limit of detection

595

Table 2. Linear mixed model (LMM) of embryo morphological traits in relation to the main and two-way interaction effects of egg TCS treatment, sex and laying order. Mass of the original egg was included as covariate while clutch identity as a random effect. The non-significant effects of the two-way interactions between fixed factors were excluded from the final model. (N = 44 embryos). Significant effects are reported in bold. False Discovery Rate correction for multiple comparisons was performed and results are reported in the footnote of the Table.

	F	Degrees of freedom	*P*
Embryo mass			
Treatment	0.76	1,24	0.391
Sex	1.25	1,30	0.273
Laying order	4.43	2,30	**0.021**[a]
Egg mass	9.61	1,29	**0.004**[c]
Tarsus length			
Treatment	0.96	1,24	0.337
Sex	2.09	1,31	0.158
Laying order	0.66	2,29	0.526
Egg mass	1.39	1,28	0.248
Brain mass			
Treatment	0.04	1,27	0.835
Sex	1.17	1,35	0.287
Laying order	1.04	2,30	0.367
Egg mass	0.93	1,26	0.345
Liver mass			
Treatment	2.19	1,24	0.152
Sex	0.01	1,32	0.930
Laying order	2.14	2,29	0.137
Egg mass	0.57	1,26	0.458
Head size			
Treatment	4.15	1,23	0.053
Sex	19.10	1,28	<**0.001**[b]
Laying order	1.54	2,29	0.232
Egg mass	10.36	1,31	**0.003**[c]

[a] P = 0.063 at False discovery rate test
[b] P = 0.003 at False discovery rate test
[c] P = 0.006 at False discovery rate test

Table 3. Linear mixed model (LMM) of markers of oxidative status in embryo liver in relation to the main and two-way interaction effects of egg TCS treatment, sex and laying order. Clutch identity was included as a random effect. The non-significant effects of the two-way interactions between fixed factors were excluded from the final model. The number (N) of embryos for each analysis is shown. Significant effects are reported in bold. False Discovery Rate correction for multiple comparisons was performed and results are reported in the footnote of the Table.

	N	F	Degrees of freedom	P
Amount of oxidant species	44			
Treatment		10.50	1,23	**0.004**[a]
Sex		0.40	1,30	0.532
Laying order		6.79	2,21	**0.005**[d]
Laying order × Treatment		6.70	2,32	**0.004**
Lipid peroxidation	46			
Treatment		4.03	1,31	0.053[b]
Sex		1.86	1,32	0.182
Laying order		2.34	2,28	0.115
Laying order × Treatment		4.59	2,33	**0.017**
DNA fragmentation	45			
Treatment		3.73	1,24	0.066[b]
Sex		0.88	1,30	0.354
Laying order		9.33	2,24	**0.001**[e]
Sex × Treatment		5.33	1,28	**0.028**
SOD	44			
Treatment		0.65	1,26	0.428
Sex		0.25	1,36	0.620
Laying order		2.10	2,26	0.143
CAT	47			
Treatment		0.93	1,42	0.342
Sex		1.91	1,42	0.174
Laying order		0.75	2,42	0.481
GST	47			
Treatment		6.04	1,25	**0.021**[c]
Sex		1.57	1,31	0.220
Laying order		3.15	2,22	**0.047**[f]

[a] P = 0.020 at False discovery rate test
[b] P = 0.082 at False discovery rate test
[c] P = 0.050 at False discovery rate test
[d] P = 0.012 at False discovery rate test
[e] P = 0.005 at False discovery rate test
[f] P = 0.078 at False discovery rate test

Figure captions:

Fig. 1 Boxplot graphs of: a) the amount of reactive oxygen species (ROS), b)CAT activity , c) SOD activity, d) GST activity, e) levels of lipid peroxidation (LPO) and f) levels of DNA fragmentation in embryo liver. Sample sizes are reported above histograms. Significant differences between TCS-treated (grey boxplot) and control (white boxplot) embryos are indicated by the asterisk (*$P < 0.05$).

Fig. 2 Estimated marginal means (+ SE) of the two-way interaction between treatment and laying order of: a) the amount of reactive oxygen species (ROS) and b) levels of lipid peroxidation (LPO) in embryo liver. Sample sizes are reported above histograms. White histograms represent the control group, while black histograms represent TCS-treated group. Significant differences between TCS-treated and control embryos are indicated by the asterisk (**$P \leq 0.001$).

Fig.S1 graphical representation of the injection method use to manipulate the levels of Triclosan in the yolk of yellow-legged gull.