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Assay optimisation and age-related baseline variation in biochemical markers in Lesser Black-backed gulls

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ABSTRACT

Free-ranging animals are often used as bioindicators of both short- and long-term changes in ecosystem health, mainly to detect the presence and effects of contaminants. Birds, and gulls in particular, have been used as bioindicators over a broad range of marine and terrestrial ecosystems. In this study, we standardise the conditions for the use of a suite of biochemical markers in non-destructive matrices of Lesser Black-backed Gull (*Larus fuscus*) to facilitate future biomonitoring of marine and terrestrial contaminants. We characterized cholinesterase (ChE) in plasma and optimized assay conditions for ChE activity as a marker of neurotoxic damage. Moreover, we quantified variation in activity of ChE, lactate dehydrogenase (LDH), glutathione-*S*-transferase (GST) and catalase (CAT) as well as variation ranges of lipid peroxidation (LPO), in free-ranging adults and captive chicks. The main ChE form present in plasma of both adults and chicks was butyrylcholinesterase (BChE) followed by acetylcholinesterase (AChE), whose relative proportion in plasma tended to decrease with increased chick age. LPO levels and GST activity in blood cells (BCs) decreased significantly with increasing chick age, while BChE and LDH activity in plasma were not age-dependent. CAT in BCs tended to decline non-significantly in older chicks. Results of this study underscore the importance of standardising assay conditions and assessing intrinsic baseline variation in biochemical markers, before biochemical quantification. Data presented here provide a foundation for future use of BChE and LDH activity in plasma, as well as oxidative stress markers (LPO, CAT and GST) in BCs, to monitor environmental stress effects in Lesser Black-backed gulls.

1. Introduction

Widespread environmental contamination and pollution are amongst the main factors contributing to the fast decline in marine biodiversity and degradation of coastal ecosystems (Johnston et al., 2015; Johnston and Roberts, 2009; McCauley et al., 2015; Ogden et al., 2014). Therefore, monitoring the presence and consequences of contaminants comprises a critical component of ecosystem health and integrity assessments. Based on the assumption that cumulative effects of environmental changes are likely to be reflected in local organisms (e.g. through changes in diversity, abundance, reproductive success and/ or growth rate), wildlife species have been increasingly used as bioindicators of both short- and long-term changes in ecosystem health (Siddig et al., 2016).

Birds are important functional components of ecosystems, and their abundance and sensitivity to both direct and indirect environmental effects, such as contamination or shifts in prey availability, make them highly appropriate sentinels for ecosystem change (Breininger et al., 2004; Mistry et al., 2008; Smits and Fernie, 2013). Approaches commonly used to assess the effects of contaminants on birds include the use of biomarkers, measures that correspond to biochemical or physiological parameters for which stress-mediated changes in baseline values are detectable before direct components of fitness are compromised (Domingues et al., 2015; Schettino et al., 2012). Common examples in birds include the assessment of cholinesterase activity (Horowitz et al., 2016), oxidative stress (Henry et al., 2015; Tkachenko and Kurhaluk, 2012) and lactate dehydrogenase activity (Barata et al., 2010; Katavolos et al., 2007). However, proper use of biomarkers, and in particular of biochemical markers, requires prior knowledge of species-specific and age-related baseline and threshold values to

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discriminate stress-mediated responses from natural background variation and variation due to developmental stage (Domingues et al., 2015; Mayack and Martin, 2003).

Cholinesterases (ChE) comprise two forms: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). ChE activity is often used in environmental toxicology as a proxy for exposure to organophosphate and carbamate pesticides, both used to control disease vectors and insect pests (Parsons et al., 2000; Santos et al., 2016; Strum et al., 2010). The toxicity of OP and CB pesticides mainly results from inhibition of ChE activity and accumulation of acetylcholine at the nerve synapses and neuro-effector junctions, causing impaired nerve function and, in extreme cases, death by paralysis (Sultatos, 2006). In birds, AChE is primarily found in the brain whereas BChE is mainly present in plasma. However, several studies showed that AChE and other non-specific esterases (e.g. carboxylesterases – CbE) might also occur in avian plasma, in varying concentrations and proportions depending on species and developmental stage (Santos et al., 2012; Strum et al., 2008). In order to use ChE activity as a marker of exposure to ChE-inhibiting contaminants, it is necessary to characterize the enzyme form(s) present in the tissue assayed, based on substrate specificity and susceptibility towards selective AChE and BChE inhibitors (Gard and Hooper, 1993; Monteiro et al., 2005; Radic and Taylor, 2006; Santos et al., 2012).

Excessive production of reactive oxygen species (ROS) that overwhelms normal antioxidant defences is another common effect of metal and pesticide exposure which may eventually damage cell contents such as lipids and DNA (Ferreira et al., 2010; Henry et al., 2015; Livingstone, 2001). These effects can be assessed through the study of lipid peroxidation (LPO), catalase (CAT) and glutathione-*S*-transferase (GST) activities (Barata et al., 2010). Finally, lactate dehydrogenase (LDH), an enzyme involved in carbohydrate metabolism, is known to catalyse the oxidation of lactate into pyruvate (Diamantino et al., 2001). LDH activity measured in plasma provides valuable indication of the occurrence of tissue and/or organ damage, in particular of the liver (Barata et al., 2010).

Our study species, the Lesser Black-backed Gull (*Larus fuscus*), is a generalist bird that exploits a wide range of food sources in both marine and terrestrial habitats (Camphuysen et al., 2015; Kubetzki and Garthe, 2003), and thus may be exposed to a wide range of contaminants such as heavy metals and pesticides. This study aimed to standardise the conditions for the use of a suite of biochemical markers of effects in non-destructive matrices[1] of chicks of different ages (12, 21 and 30 days-old respectively) and adults. In order to achieve that, we (i) characterize ChE form(s) present in plasma of chicks and adults, (ii) establish the appropriate assay conditions for the use of plasma ChE activity as marker of contaminant exposure and (iii) determine the level of variation in ChE, LDH, GST and CAT activities and LPO levels in chicks raised under environmentally controlled conditions and in free-living adults. While we expect BChE to be the main ChE form present in plasma of chicks and adults, we also expect AChE to be present in plasma of chicks and adults too. Their proportions, as well as the levels of all other markers assessed, will likely vary, reflecting age-related changes in physiology.

2. Material and methods

2.1. Chemicals

2,6-Di-tert-butyl-4-methylphenol (BHT), acetylthiocholine iodide (AcSCh), S-butyrylthiocholine iodide (BuSCh), propionylthiocholine iodide (PrSCh), eserine hemisulphate, tetraisopropyl pyrophosphoramide (iso-OMPA), 1,5-bis(4-allyldimethylammonimphenyl)-pentan-3-one dibromide (BW284C51) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich Europe (Germany). Bradford reagent was purchased from Bio-Rad (Germany), while all other chemicals were purchased from Merck (Germany).

2.2. Bird rearing and sampling procedures

A total of 54 first or second laid eggs (third laid egg was excluded as it is expected to present reduced maternal investment) were collected from nests located at the outer port of Zeebrugge, Belgium (51°21′N, 03°11′E) during the last week of May 2014, and taken to aviary facilities located within the Wildlife Rescue Centre Ostend (VOC Ostend), where all experimental procedures of this study were carried out. Eggs were then incubated until hatching (temperature 37.5 °C, humidity 62%), upon which chicks were housed together under a heat lamp up to the age of 12 days, when they were transferred to an outdoor flying cage equipped with tunnel-shaped shelters (to provide protection against wind and rain) until the age of 30 days. After this period, chicks were mixed with wild gulls under rehabilitation to remove any potential effect of human imprinting before release (see further). Blood samples (0.5 mL) were drawn from the branchial vein at 12, 21 and 30 days of age. During the entire experiment, chicks were fed *ad libitum* on a mixture of water-soaked pellets (Dry Cat Food; BONI SELECTION ®) and fish (Smelt, *Osmerus sp.*), supplemented with vitamin C and B1.

Adult samples were collected during mid/late May of 2014 and 2015 in the Outer Port of Zeebrugge. Before clutch completion, a total of 11 adult males, 10 adult females and 3 adults of unknown gender were trapped in their nest using walk-in cages. Upon capture, a blood sample (1 mL) was collected from the brachial vein using sterile 1 mL Terumo syringes with 25-G needles and transferred to capillary tubes with EDTA (Microvette® CB 300, Sarstedt), to allow further separation of plasma and blood cells. Volume of blood collected per chick was always calculated to be below 1% of their body weight, to meet maximum, safe amount of blood to withdraw criteria. All blood samples were kept cold (4 °C) and transferred to the lab. Samples were then centrifuged in an Eppendorf 5417 R centrifuge (Rotor radius: 9.5 cm; 6000 rpm, 10 min, 4 °C) and plasma and blood cells (BC) were separated into different Eppendorf aliquots. All aliquots were stored at −80 °C until analysis.

2.3. Sample processing for biochemical analysis

Plasma aliquots were defrosted on ice and diluted in the appropriate buffer solution. Samples used for ChE characterization and determination were prepared by diluting plasma aliquots in the proportion of 1:500 in phosphate buffer (0.1 M, pH = 7.2). For lactate dehydrogenase (LDH) determinations, plasma aliquots were diluted in the proportion of 1:100 in Tris/NaCl buffer (0.1 M, pH = 7.2). The plasma samples used for ChE characterization were prepared from plasma aliquots previously pooled (chicks and adults: N = 4, pools of 3 individuals each), before dilution in phosphate buffer, to obtain the necessary volume of sample to carry characterization and retain higher interspecific variability. To determine lipid peroxidation (LPO), catalase (CAT) and glutathione-S-transferase (GST) levels, BC samples were defrosted on ice, 1 mL of phosphate buffer (0.1 M, pH = 7.4) was added to each BC pellet (both chicks and adults) and homogenised using a sonicator while kept on ice. Following homogenisation, a 150 μL aliquot of homogenate was separated, and 2.5 μLof 2,6-Di-tert-butyl-4-methylphenol dissolved in ethanol (BHT 4%) was added to the sample. Samples were then stored at −80 °C until LPO determination (within 24 h). A second 20 μL aliquot of homogenate was separated for posterior homogenate protein determination and stored at −80 °C. The remaining homogenate was then centrifuged in a Heraeus Megafuge 8 centrifuge (Rotor radius: 8.5 cm; 10,000*g*, 20 min, 4 °C). Two 100 μL aliquots of the post-mitochondrial supernatant (PMS) were separated and stored at −80 °C until CAT and GST determination (within 7 days).

[1] Biological samples for which no animal sacrifice is necessary (i.e. blood).

2.4. Cholinesterase characterization

ChE presence in plasma of chicks and adults was characterized by testing substrate preferences and sensitivity to selective inhibitors in separate assays. To evaluate substrate preferences of the ChE form(s) present in plasma, increasing concentrations (0.005–20.48 mM) of Acetylthiocholine iodide (AcSCh), S-butyrylthiocholine iodide (BuSCh) and propionylthiocholine iodide (PrSCh) were selected as substrates. Non-specific esterases contribute to total enzymatic activity measured but differ in sensitivity towards ChE-inhibiting compounds, which may confound the study of ChE activity in plasma (Garcia et al., 2000). To assess if non-specific esterases were present in plasma of chicks and adults eserine hemisulphate, which is considered a specific inhibitor of ChEs within the concentration range of 10^{-6}–10^{-5}, was used as a selective inhibitor of all ChEs (Eto, 1974). To further disentangle which ChE forms were present in plasma of chicks and adults, Tetraisopropyl pyrophosphoramide (iso-OMPA) and 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) were used as selective inhibitors of BChE and AChE, respectively (Monteiro et al., 2005; Osten et al., 2005).

Stock solutions were prepared for each inhibitor in ultrapure water (Milli-Q® Academic Ultra-Pure Water System) or ethanol, as appropriate, with concentrations ranging from 6.25 to 200 μM (eserine and BW284C51, solutions prepared in ultrapure water) and from 0.25 to 8.0 mM (iso-OMPA, solutions prepared in ethanol). For each inhibitor concentration, 5 μL of inhibitor was then incubated with 495 μL of sample (30 min, 25 ± 1 °C) before substrate addition and determination of enzyme activity (for further details on determination of enzyme activity, see Section 2.6 Enzymatic assays). To distinguish between changes in enzymatic activity related to the inhibitors tested rather than the incubation procedure and/or solvent used, ultrapure water and/or ethanol (i.e. 5 μL solvent per 495 μL of sample) was also incubated before enzymatic determination. ChE activity was then assayed using both AcSCh and BuSCh as substrates. Ultrapure water was added to all inhibitor controls, and an additional control of ethanol was used in the experiments with iso-OMPA. Apparent K_m (substrate concentration needed to reach one-half of the maximum reaction velocity) and V_{max} (maximum rate of enzymatic activity when saturated with the substrate) of ChE(s) present in plasma was calculated for each substrate and age class, using the Lineweaver-Burk plot. Percentage of ChE inhibition (mean ± standard error) for each inhibitor concentration was calculated for each replicate by comparing the respective control activity (water control: eserine and BW284C5; ethanol control: iso-OMPA).

2.5. Protein determination

Protein concentrations in BC homogenates and their respective PMS were determined in quadruplicate at 595 nm by the Bradford method (Bradford, 1976) adapted to the microplate, using the Bio-Rad protein detection kit and bovine γ-globulin as standard. All photometric measurements were carried out using a Multiskan Spectrum (Thermo Scientific ®).

2.6. Lipid peroxidation

The LPO was assessed following an adaptation by Ferreira et al. (2010) of the Ohkawa (Ohkawa et al., 1979) and Bird and Draper methods (Bird and Draper, 1984). Briefly, 150 μL of homogenised sample was incubated (100 °C, 1 h) with 0.5 mL of sodium trichloroacetate (TCA, 12% w/v) and 0.4 mL of a Tris-HCl buffer (60 mM) containing diethylenetriaminepentaacetic acid (DTPA, 0.1 mM). Following this, samples were kept away from light, centrifuged (Heraeus Megafuge 8 centrifuge, rotor radius: 8.5 cm; 5 min, 11,500 rpm, 25 °C) and read at 535 nm, in quadruplicate. The LPO is expressed as nmol of TBARS hydrolysed per minute per mg of protein in BC homogenate, using a molar extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$.

2.7. Enzymatic assays

2.7.1. Cholinesterase activity

The ChE activity was determined at 412 nm, in quadruplicate, according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996), using 50 μL of sample and 250 μL of the reaction solution. The reaction solution was a mixture of 1 mL of 10 mM 5.50-dithiobis(2-nitrobenzoic acid) solution (DTNB), 0.2 mL of substrate solution (concentration range during characterization: 0.005–20.48 mM; concentration for determination of BChE activity: 5.12 mM) and 30 mL of phosphate buffer (0.1 M, pH = 7.2). Enzymatic activity is expressed in μmol of substrate hydrolysed per minute per mL of plasma (μmol/min/mL of plasma), using a molar extinction coefficient of $13.6 \times 10^{3}\ M^{-1}\ cm^{-1}$.

2.7.2. Lactace dehydrogenase activity

Activity of LDH was determined at 340 nm, in quadruplicate, by Vassault's method (Vassault, 1983) as adapted to microplate (Diamantino et al., 2001), using 40 μL of sample, 250 μL NADH (0.24 mM) and 40 μL of pyruvate (10 mM). Enzymatic activity is expressed in nmol of substrate (pyruvate) hydrolysed per minute per mL of plasma (μmol/min/mL of plasma), using a molar extinction coefficient of $6.3 \times 10^{-3}\ M^{-1}\ cm^{-1}$.

2.7.3. Catalase acitivity

The CAT activity was determined at 240 nm, in quadruplicate, following the Claiborne method (Clairborne, 1985), using 15 μL of sample, 135 μL K-Phosphate buffer (0.05 M, pH = 7.0) and 150 μL of hydrogen peroxide (H_2O_2; 0.03 M). Enzymatic activity is expressed as μmol of substrate (H_2O_2) hydrolysed per minute per mg of protein in PMS of BC (μmol/min/mg of protein), using a molar extinction coefficient of $40\ M^{-1}\ cm^{-1}$.

2.7.4. Glutathione-S-transferase determination

The GST activity was determined at 340 nm, in quadruplicate, by the Habig's method (Habig et al., 1974), adapted to microplate (Frasco and Guilhermino, 2002), using 100 μL of sample and 200 μL of a reaction solution. The reaction solution was a mixture of 14.85 mL of phosphate buffer (0.1 M, pH = 6.5), 0.45 mL of 1-chloro-2,4-dinitrobenzene (CDNB) 10 mM solution and glutathione reduced (GSH) 10 mM. Enzymatic activity is expressed as nmol of substrate (GSH) hydrolysed per minute per mg of protein in PMS of BC (nmol/min/mg of protein), using a molar extinction coefficient of $9.6 \times 10^{-3}\ M\ cm^{-1}$.

2.8. Ethics statement

All experiments and manipulations carried out on chicks and adults were in accordance with the European/FELASA ethical guidelines for vertebrate animal experimentation and approved by the Ethical Committee of the University of Ghent. Additionally, significant effort was made to improve the general well-being of chicks raised in the aviary as well as during blood collection. Chicks that exhibited any sign of illness during rearing or blood sampling procedures were excluded from the experiment and given veterinary care (percentage of individuals excluded: 37%). To remove any effect of human imprinting during chick rearing, at the end of the experiment all chicks were kept together with wild gull chicks and juveniles that were under rehabilitation at the VOC care facilities. When deemed fully grown (about one month), fledglings were released into the wild.

2.9. Data analysis

An one-way analysis of variance (ANOVA) was used to test for the effects of the inhibitors assayed (i.e. eserine, BW284C51 and iso-OMPA)

on ChE activity, whereas pairwise differences between inhibitor treatments and assay controls assessed with a Dunnet's test. Similarly, a one-way ANOVA was used to test for variation in LDH, BChE, LPO, CAT and GST levels between chicks of varying age. Differences between levels of LPO, CAT and GST in adults between 2014 and 2015 were assessed using *t*-tests. When the criteria of normality and homogeneity of variance were not met, data were transformed using $\log_{10}$ or square root transformations when necessary. Whenever data transformation to meet both criteria of normality and homogeneity of variance was not successful, non-parametric tests were carried out on the original untransformed data. The Kruskal-Wallis ANOVA was then used to test for age-dependent differences in LPO, CAT and GST levels of chicks, followed by the multiple comparisons test. Similarly, a Kruskal-Wallis ANOVA followed by the Dunnett test was used to test for the effect of eserine inhibition on ChE activity of 12 and 30 day-old chicks, using AcSCh as a substrate.

Due to factors beyond our control, a significant number of samples collected during experimental procedures was lost during shipment, which led to a limitation on the number of samples available to carry out biochemical analyses. For the same reason, it was not possible to run any repeated measures ANOVA. Instead, a Mann-Whitney *U* Test was used to test for differences in CAT and LPO levels of adults between 2014 and 2015. All analyses were carried out using Statistica 8 (StatSoft Inc., Tulsa, OK, USA) and SigmaPlot® 12.5 software (Systat Software Inc.).

3. Results

3.1. Cholinesterase characterization

3.1.1. Substrate assays

Substrate preferences of the ChE form(s) in plasma of chicks and adults, using the substrates AcSCh, PrSCh and BuSCh at increasing concentrations, are shown in Fig. 1. Maximum enzyme activity in 12, 21 day-old chicks and adults was reached with PrSCh at 20.48 mM (12 days: 2.93 ± 0.10 μmol/min/mL; 21 days: 1.92 ± 0.14 μmol/min/mL; adults: 2.39 ± 0.16 μmol/min/mL), whereas in 30 day-old chicks maximum activity was reached with BuSCh at 5.12 mM (1.45 ± 0.08 μmol/min/mL). Apparent K_m and V_{max} for each substrate are shown in Table 1. Highest enzyme affinity, which is indicated by lower K_m, was reached in 12, 21 and 30 day-old chicks with PrSCh, whereas in adults it was reached with AcSCh.

3.1.2. Eserine assay

Enzymatic assays with specific inhibitors were performed with AcSCh and BuSCh as substrates (Fig. 2). Eserine significantly inhibited ChE activity in plasma of chicks and adults, when both AcSCh (Fig. 2A; $p < 0.05$) and BuSCh (Fig. 2B; $p < 0.05$) were used as substrates. When using AcSCh as a substrate, average plasma ChE inhibition rates at the highest eserine concentration were 96 ± 2% (12 days), 93 ± 2% (21 days), 89 ± 4 (30 days) in chicks and 97 ± 1% in adults. Similar average inhibition rates were observed when using BuSCh at the highest inhibitor concentration, i.e. 97 ± 1% (12 days), 92 ± 2% (21 days) and 91 ± 3% (30 days) in chicks and 88 ± 2% in adults.

3.1.3. BW284C51 assay

Effects of BW284C51 are presented in Fig. 2C and D. For up to 200 μM of BW284C51, no significant inhibition was observed in adults, using both AcSCh and BuSCh as substrates. However, a decrease of 48% and 35% in ChE activity relative to the control was observed using both AcSCh and BuSCh as substrates, respectively, at 200 μM. In chicks, the inhibition of plasma ChE by BW284C51, the specific inhibitor of acetylcholinesterase, varied with both substrates used. While using AcSCh as a substrate, BW284C51 inhibition was only observed at a concentration equal or higher than 50 μM (21 days) or 100 μM (30 days) (Fig. 2C; $p < 0.05$); ChE activity in 21 and 30 days-old chicks was inhibited at the lowest inhibitor concentration (Fig. 2D; $p < 0.05$) when using BuSCh. Significant inhibition of plasma ChE activity in 12 days-old chicks was observed at BW284C51 concentrations equal or higher than 25 μM, using AcSCh (Fig. 2C; $p < 0.05$), and 100 μM, using BuSCh (Fig. 2D; $p < 0.05$) as substrates. At maximum inhibitor concentration, ChE depression in chicks was 49 ± 5% (12 days), 88 ± 10% (21 days) and 46 ± 4% (30 days) with AcSCh as substrate, whereas with BuSCh inhibition was 52 ± 15% (12 days), 80 ± 3% (21 days) and 54 ± 15% (30 days).

3.1.4. Iso-OMPA assay

Effects of iso-OMPA are presented in Fig. 2E and F. Using AcSCh as a substrate, no significant differences in ChE activity of chicks and adults was apparent up to the highest inhibitor concentration (8 mM) tested (Fig. 2E; $p < 0.05$). In spite of this, inhibition rates at 8 mM of iso-OMPA ranged between 73 ± 16% in adults and chicks from 49 ± 14% (12 days), 57 ± 24% (21 days) to 64 ± 9% (30 days). Using BuSCh as a substrate, iso-OMPA (0.25–8 mM) significantly inhibited plasma ChE activity (Fig. 2F; $p < 0.05$) in 12 and 21 days-old chicks and adults. No significant inhibition of ChE present in plasma of 30 days-old chicks was reported up to 8 mM of iso-OMPA (Fig. 2F; $p > 0.05$). At the maximum inhibitor concentration used, inhibition rates of ChE were 84% in adults and chicks 94 ± 2% (12 days), 99 ± 0.3% (21 days) and 59 ± 54% (30 days).

3.2. Biomarker levels in plasma and BCs

Neither LDH activity nor BChE activity in plasma significantly varied with age (One-Way ANOVA, all $p > 0.11$) (Table 2). LPO, CAT and GST levels in BCs are shown in Table 3. Twelve days-old chicks showed average LPO levels of 1.83 ± 0.58 SE nmol TBARS/mg protein which decreased significantly with age to 0.24 ± 0.04 SE nmol TBARS/mg protein in 30 days-old chicks (Kruskal-Wallis ANOVA: $H_{2,29} = 16.15$, $p < 0.001$; Post-hoc: Multiple comparisons: $Z = 4.02$, $p < 0.001$). LPO levels in adults (see Table 3) increased significantly in 2015 (Mann-Whitney *U* Test: $Z = -2.83$, $p = 0.005$). No age-related differences in CAT activity levels of chicks (Kruskal-Wallis ANOVA, $p = 0.70$) as well as between years in adults (Mann-Whitney *U* Test, $p = 0.92$) were reported. Average GST activity decreased significantly with increasing chick age from 9.89 ± 3.49 SE nmol/min/mg protein (12 days-old) to 5.29 ± 0.29 SE nmol/min/mg protein (30 days-old) (Kruskal-Wallis ANOVA: $H_{2,30} = 12.71$, $p = 0.002$; Post-hoc: Multiple comparisons: $Z = 3.56$, $p = 0.001$). Moreover, average GST activity levels in adults decreased significantly in 2015 compared to values reported in 2014 (*t*-test: t-value = 3.47, $p = 0.002$).

4. Discussion

4.1. Characterization of ChE form(s) present in plasma of chicks and adults

4.1.1. Presence of cholinesterases and/or other type of esterases

ChE activity is a known and widely used marker of effect to ChE-inhibiting contaminants, requiring prior identification of the ChE form (s) present in the tissue assayed. One of the aims of this work was to characterize the ChE form(s) present in plasma of chicks and adults. To achieve that, in a first step we aimed to distinguish ChE form(s) from non-specific esterases (e.g. CbE), whose presence may contribute to the total enzymatic activity measured, but differ in their sensitivities towards ChE-inhibiting compounds (Eto, 1974). Such distinction can be done using eserine, a specific inhibitor of ChEs within the concentration range of 10^{-6}–10^{-5} M (Eto, 1974). In both *L. fuscus* chicks and adults, enzymatic activity measured in plasma was almost completely inhibited by eserine within this range for both AcSCh and BuSCh. As such, we can conclude that enzymes assayed in the plasma of gulls are predominantly ChE(s), rather than other types of esterases such as CbEs. To further

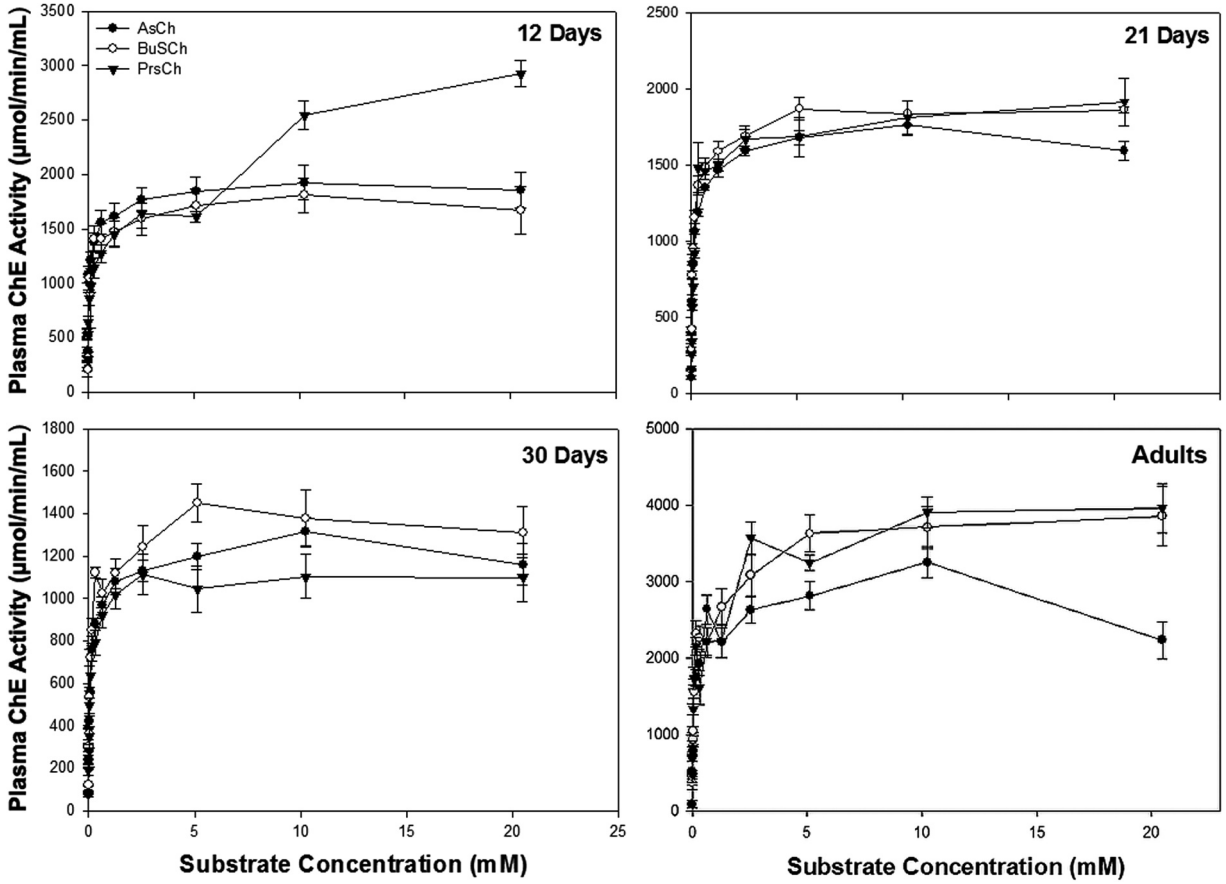

Fig. 1. ChE activity in plasma of *L. fuscus* chicks (12, 21 and 30 days old) and adults at increasing concentrations of acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh) and *S*-butyrylthiocholine iodide (BuSCh). Results are expressed as mean values ± standard error of four pooled samples (see methods for details).

Table 1
Apparent K_m and V_{max} values for AcSCh, PrSCh and BuSCh in *L. fuscus* chicks and adults, as estimated by the Lineweaver-Burk (double-reciprocal) plot. K_m is expressed as µM and V_{max} is expressed as µmol/min/mL plasma.

Age		AcSCh		PrSCh		BuSCh	
		K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Chicks(days)	**12**	0.03	1.64	0.02	1.16	2.13	11.79
	21	0.07	1.76	0.04	1.28	0.05	1.62
	30	0.10	1.38	0.02	0.95	0.04	1.21
Adults		0.04	1.18	0.07	1.60	0.05	1.39

identify which ChE form(s) was (were) present, the substrate preference and sensitivity towards selective inhibitors of AChE and BChE activity were tested.

4.1.2. ChE form(s) present in plasma of chicks and adults

Substrate preferences of the ChE form(s) in plasma were qualitatively similar, increasing exponentially at low substrate concentrations, and stabilising and reaching maximum kinetic activity at higher substrate concentrations. In general, higher enzymatic activities were obtained with PrSCh, except for 30 days-old chicks in which the enzyme preferentially catalysed BuSCh. Moreover, ChE form(s) in plasma of chicks and adults showed similar affinities (K_m) towards the different substrates that were tested, except in 12 days-old chicks in which enzyme affinity was lower with BuSCh. AChE typically displays higher preference towards AcSCh, being unable to hydrolyse BuSCh, whereas BChE preferentially hydrolyses BuSCh but may display equally high rates of affinity towards other cholinesters such as PrSCh and AcSCh (Monteiro et al., 2005; Radic and Taylor, 2006). Given the similar preferences of the enzyme(s) assayed for the three substrates tested, results of our study seem to point towards the presence of either both AChE and BChE, or a BChE form with the ability to hydrolyse different cholinesters. Susceptibility of the ChE form(s) present towards BW284C51 and iso-OMPA inhibition was further investigated.

BW284C51 and iso-OMPA are two selective inhibitors of AChE and BChE activity, respectively (Monteiro et al., 2005; Osten et al., 2005). While in chicks BW284C51 significantly inhibited ChE activity in plasma using both AcSCh and BuSCh as substrates, little effect of BW284C51 was observed in adults. Furthermore, though highly variable, ChE activity in plasma was significantly inhibited by iso-OMPA (AcSCh: 6–64% inhibition; BuSCh: 44–71% inhibition) except in 12 and 21 days-old chicks (AcSCh as a susbtrate) or 30 days-old chicks (BuSCh as a substrate). In spite of an absence of statistical significance in 30 days-old chicks it should be noted, however, that data variability in this group was relatively high. Moreover, in spite of no statistical significance, at highest iso-OMPA concentration, the inhibition rate of ChE activity was about 59%. As such, it is likely that the elevated data variability was the main driving factor for the absence of statistical differences, as higher variability reduces the ability to detect statistical significance.

Sensitivity to BW284C51 and iso-OMPA of chicks ChE seem to suggest that both AChE and BChE occur in plasma of developing chicks. However, as higher rates of enzyme inhibition were observed with iso-OMPA across all age classes, BChE seems to be the predominant whereas AChE presence is likely to decline with individual age until adult stage, in which it seems to be absent. Similar co-existence of both ChE forms with higher prevalence of BChE was earlier shown for

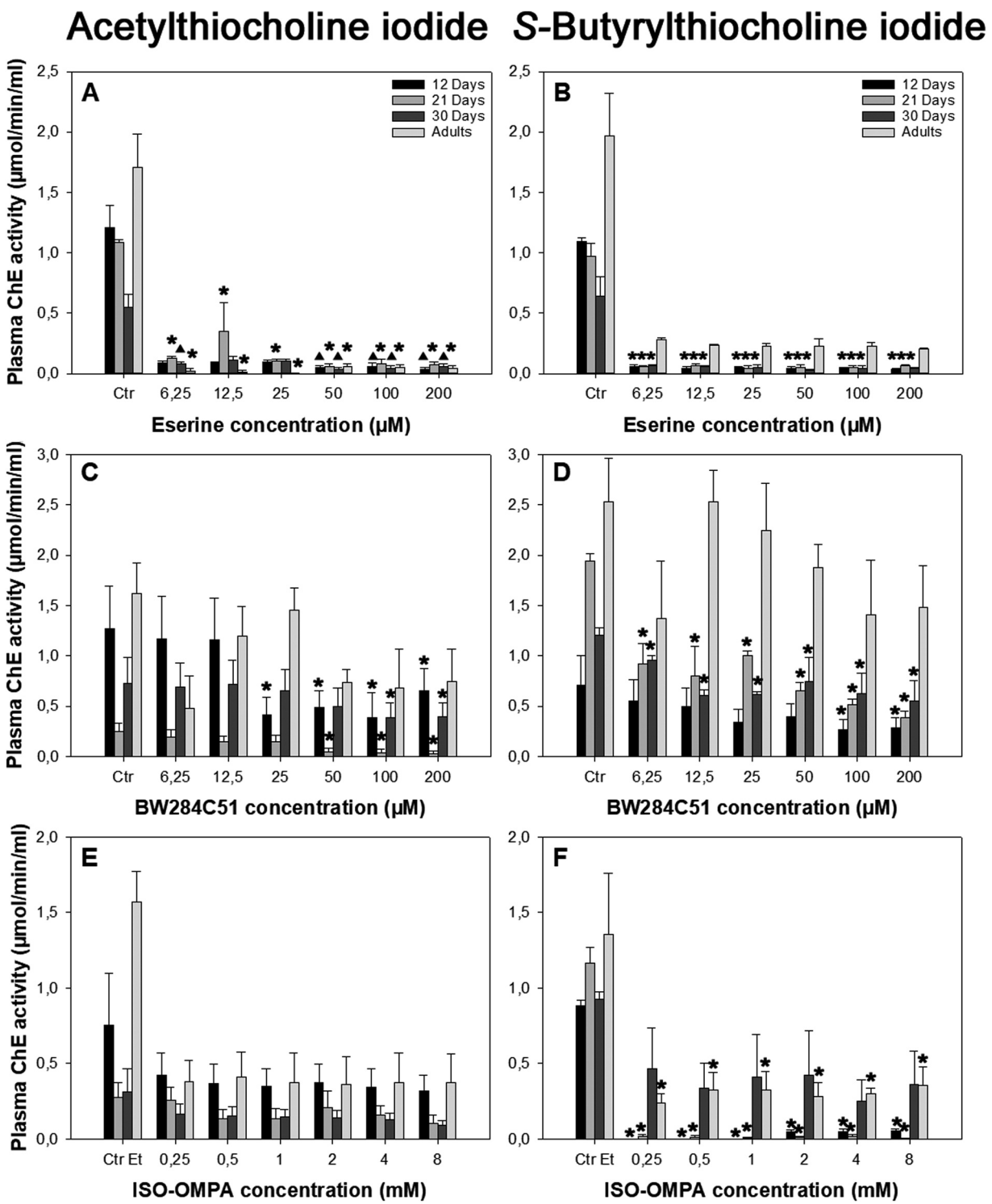

Fig. 2. Effects of the inhibitors eserine hemisulphate, BW284C51 and ISO-OMPA on the plasma cholinesterase (ChE) activity of chicks (12, 21 and 30 days) and adults of *Larus fuscus* using Acetylcholine iodide (2A, C and E) and *S*-Butyrylthiocholine iodide (2B, D and F) as substrates. Results are expressed as mean values ± standard error of four pooled samples (see methods for details); * = significantly different from control (One-Way ANOVA, Post hoc: Dunnett Test, $p < 0.05$); ▲ = significantly different from control (Kruskall-wallis ANOVA, Post hoc: Dunnett Test, $p < 0.05$); Ctr = water control; Ctr S = Ethanol control.

example in shorebirds (i.e., *Charadrius vociferus* and *Pluvialis dominica*) (Strum et al., 2008), owls (i.e. *Tyto alba* and *Strix aluco*) (Roy et al., 2005), and a number of Australian passerines (e.g. *Anthus novaeseelandiae* and *Taeniopygia bichenovii*) (Fildes et al., 2009). A similar decrease of plasma AChE with increasing age has also been found previously in Eastern bluebird (*Sialia sialis*) (Gard and Hooper, 1993), House wren (*Troglodytes aedon*) and European starling (*Sturnus vulgaris*) nestlings (Mayack and Martin, 2003).

A significant inhibition of ChE activity in the presence of BW284C51 was observed when using BuSCh as a substrate, suggesting that the BChE form present in plasma of gull chicks exhibits a certain degree of sensitivity towards BW284C51, the selective inhibitor of AChE, which is atypical. Though such type of atypical BChE form has been previously described in different fish species (Albendin et al., 2017; Monteiro

Table 2
Levels of lactate dehydrogenase (LDH) and butyrylcholinesterase (BChE) in plasma of *L. fuscus* of different ages. Data presented represent mean values ± standard error (SE), minimum (MIN) and maximum (MAX) values, and the number of replicates (N).

Age		LDH (μmol/min/mL plasma)			BchE (μmol/min/mL plasma)		
		Mean ± SE (N)	MIN	MAX	Mean ± SE (N)	MIN	MAX
12		0.81 ± 0.05 (6)	0.68	1.01	2.38 ± 0.23 (6)	1.82	3.27
Chicks (days)	**21**	0.86 ± 0.14 (6)	0.42	1.33	2.20 ± 0.21 (6)	1.34	2.82
30		0.96 ± 0.09 (6)	0.72	1.24	1.78 ± 0.11 (6)	1.46	2.08
Adults (year)	**2015**	5.85 ± 0.84 (9)	2.08	10.01	1.54 ± 0.11 (12)	0.94	2.08

et al., 2005), to the best of our knowledge, this is the first time such a finding has been reported in birds.

Contrarily to chicks, BW284C51 did not significantly affect ChE activity in adults, a result that was more evident when using BuSCh as a substrate. Although not significant either, ChE inhibition at maximum BW284C51 concentration and with AcSCh as a substrate, approached 58% inhibition rate, which is moderately high. Moreover, plasma ChE activity in adults was strongly inhibited by iso-OMPA, the selective inhibitor of BChE, when using BuSCh as a substrate but not when using AcSCh. While not reaching statistical significance in relation to assay controls, inhibition rates with iso-OMPA ranged between 70% and 75%, which should not be considered as negligible. Hence, these results suggest that both ChE forms exist in the plasma of adult gulls, since a slight sensitivity towards BW284C51 was observed when using AcSCh as a substrate. However, the higher sensitivity of ChE to iso-OMPA, particularly when using BuSCh as a substrate, indicates that BChE is the main enzyme form present.

Based on these results, we recommend using BuSCh at ~10 mM when assessing ChE activity in plasma of Lesser Black-backed Gull chicks and adults, given that this substrate is specific to BChE and because maximum enzyme activity with this substrate was observed to be stable at this concentration.

4.2. Biomarker levels in plasma and BCs of Lesser Black-backed gulls

Plasma BChE (1.54–2.38 μmol/min/mL) and LDH activity (0.81–5.85 μmol/min/mL) in *L. fuscus* chicks and adults varied within the range of values reported for other free-ranging bird species. For example, mean BChE activities measured in the American golden plover (*Pluvialis dominica)* and the Least sandpiper (*Calidris minutilla)* ranged between 1.08 and 2.61 μmol/min/mL plasma, respectively (Strum et al., 2010). Barata et al. (2010) reported plasma LDH activities between 0.29 and 0.25 μmol/min/mL in nestlings of Purple heron (*Ardea purpurea)* and Little egret (*Egretta garzetta)*, whereas Champoux et al. (2006) found LDH activities ranging between 1.02 and 2.19 μmol/min/mL plasma in Great blue heron (*Ardea herodias)*. Likewise, the activity of CAT (4.69–10.84 μmol/min/mg) and GST (1.90–9.89 nmol/min/mg) assessed in this study were comparable with values reported by Espin et al. (2016), in Slender billed-gull (*Chroicocephalus genei*) and Audouin's gull (*Ichthyaetus audouinii*) (CAT: 10.09–14.36 μmol/min/mg; GST: 6.81–8.24 nmol/min/mg protein).

The LPO levels (0.13–0.83 nmol TBARS/mg) measured in blood cells of *L. fuscus* chicks and adults were, in contrast, considerably higher than those reported in the literature. For instance, average LPO levels in BCs were between 0.06 and 0.09 TBARS μmol/g in Audouin's gull and Slender billed-gull respectively (Espin et al., 2016), while those in Griffon vulture (*Gyps fulvus*) ranged between 0.03 and 0.05 TBARS μmol/g across different populations (Espín et al., 2014). The difference between our and previous studies could either reflect species-specific variation in general physiology or metabolic rates or be due to methodological differences as few studies so far used TBARS to assess LPO levels in avian BCs.

4.3. Age-related variation in biomarker levels

Variation in metabolic processes during chick development (such as growth and thermoregulation), and the energetic costs that these processes entail, can be expected to be reflected in age-related variation of biochemical blood parameters. For example, Mayack and Martin (2003) detected variation in ChE activity between fledglings and adult House wrens (*Troglodytes aedon*) and European starlings (*Sturnus vulgaris),* while no such age-related variation was observed for LDH or BChE activity in plasma in our study.

Activity levels of oxidative stress markers GST and LPO were significantly lower in older chicks, while a similar trend was observed in CAT, although not statistically significant. These results are in accordance with a previous study in kestrel (*Falco tinnunculus*) nestlings by Costantini et al. (2006). Higher levels of the enzymatic oxidative stress defence in younger chicks could be due to their higher metabolic rates and resulting higher production of ROS, in response to rapid growth (Costantini et al., 2006; Monaghan et al., 2009). In *L. fuscus*, chick growth has been shown to follow a sigmoidal function with an exponential phase between 5 and 20 days of age (Sotillo et al., unpubl.; see also: Harris, 1964). This period coincides with the high oxidative

Table 3
Oxidative stress biomarkers in blood cells of *L. fuscus* of different ages. Levels of lipid peroxidation (LPO) and enzymatic activities of catalase (CAT) and glutathione-*S*-transferase (GST) are presented as mean values ± standard error (SE), minimum (MIN) and maximum (MAX) values, and the number of replicates (N). Chicks were reared in captivity while adults were free-living at the Outer Port of Zeebrugge, Belgium (51°21′N, 03°11′E).

Age		LPO (nmol TBARS/mg protein)			CAT (μmol/min/mg protein)			GST (nmol/min/mg protein)		
		Mean ± SE (N)	MIN	MAX	Mean ± SE (N)	MIN	MAX	Mean ± SE (N)	MIN	MAX
12		1.83 ± 0.58 (9)	0.48	5.94	7.39 ± 1.88 (10)	2.35	18.58	9.89 ± 3.49 (10)	5.76	15.36
Chicks (days)	**21**	0.60 ± 0.18 (10)[a]	0.16	1.98	6.00 ± 1.05 (10)	2.50	14.19	7.53 ± 0.99 (10)	3.90	15.17
30		0.24 ± 0.04 (10)[a]	0.10	0.63	4.69 ± 0.66 (10)	1.73	7.64	5.29 ± 0.29 (10)[b]	4.01	6.74
Adults (year)	**2014**	0.13 ± 0.01 (11)	0.06	0.22	10.84 ± 1.97 (10)	3.44	24.31	5.46 ± 0.97 (11)	2.60	14.30
	2015	0.19 ± 0.01 (12)[c]	0.15	0.23	10.04 ± 0.88 (10)	5.78	14.78	1.90 ± 0.32 (11)[d]	0.51	3.64

[a] = Significantly different from 12 days-old chicks (Dunn's Method, $p < 0.05$).
[b] = Significantly different from 12 days-old chicks (Tuckey Test, $p < 0.05$).
[c] = Significantly different from 2014 (Mann-Whitney Test, $p < 0.05$).
[d] = Significantly different from 2014 (*t*-Test, $p < 0.05$).

stress levels reported in 12 days-old chicks compared to lower levels at days 21 and 30, hence likely reflecting variable ROS production during chick development.

4.4. Between-year variation in biomarker levels

LPO levels in adults were significantly higher in 2015 compared to 2014, whereas GST activities were significantly lower. In both years, adults were sampled early in the breeding season, periods of high metabolic activity (e.g. notably incubation) in which levels of ROS are expected to be elevated (Selman et al., 2012). As excessive production of reactive ROS, and the inability of antioxidant defences to remove these radicals, are known to cause oxidative stress (Yoshikawa and Naito, 2000), these differences could suggest that individuals sampled during the breeding season of 2015 had higher rates of metabolic activity than those sampled in 2014. Alternatively, differences in environmental conditions (e.g. in contaminant exposure, nutritional stress, abiotic stressors, etc.) between both breeding seasons could also have prompted differences in activation of antioxidant defences mechanisms by adults, such as reduced GST mediated detoxification. This, in turn, may have resulted in a differential accumulation of ROS and oxidative damage via lipid peroxidation.

5. Conclusions

The high level of variability of ChE activity and oxidative stress markers reported in the literature in different species and in the present study between different developmental stages of *L. fuscus* individuals supports the need to characterize ChE form(s) present in plasma before its application in environmental studies. The present study shows that BChE is the predominant ChE form present in plasma of *L. fuscus* chicks as well as in adults. Hence, at least for this species, it is recommended to use BuSCh to assess BChE activity in plasma when quantifying exposure to anti-ChE contaminants. Furthermore, this study also supports the need to assess baseline variation in both sets of markers and standardise assay conditions at different specimen ages. BChE and LDH activity in plasma, and oxidative stress markers (LPO, CAT and GST) in blood cells can likely be used as a set of non-destructive tools to monitor effects of environmental contamination across the lifecycle of *L. fuscus*.

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Author contributions

CSAS and AS designed and performed the experiments; CSAS, LL, MSM and SL analysed the data; CSAS wrote a first draft of the paper, and all authors contributed to discussing and writing subsequent drafts. MSM and LL contributed equally to the paper.

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