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RESEARCH ARTICLE

Impacts of chemical stress, season, and climate change on the flounder population of the highly anthropised Seine estuary (France)

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Abstract

The main objective of this study was to improve our knowledge on the responses of fish populations to multistress (diffuse pollution and warming waters) in estuaries. Adult flounders were caught in two estuaries in the Eastern English Channel: the heavily polluted Seine estuary vs the moderately contaminated Canche estuary. Fish samplings were conducted in January just before the reproduction period, and in July when gonads were at rest. The overall rise in coastal winter water temperatures detected over the Channel impairs the flounder's phenology of reproduction in the two estuaries, inducing a delay of maturation process and probably also spawning. The higher liver histopathology index in Seine vs Canche could be the consequence of the fish exposition to a complex cocktail of contaminants in a strongly industrialized estuary. Higher levels of neurotoxicity, gill lipid peroxidation, and liver EROD activity were observed in Seine vs Canche. Furthermore, a possible impairment in mitochondrial metabolism was suggested in the Seine flounder population. We confirmed in this study the potential role of two membrane lipids (sphingomyelin and phosphatidylserine) in the resistance towards oxidative stress in Seine and Canche. Finally, we suggest that the Seine flounder population (and possibly the connected Eastern English Channel flounder populations over the French Coast) could be seriously impacted in the future by multistress: higher winter temperatures and chemical contamination.

Keywords *Platichthys flesus* · Pollution · Biomarkers · Reproduction · Winter temperature

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Introduction

Large estuaries are impacted by interactive and cumulative effects among endogenic stressors (water pollution, coastal urbanization, flow changes, intertidal loss, eutrophication) and exogenic unmanaged stressor (e.g., warming waters linked to climate change). These stressors are seriously affecting the ecological quality of transitional waters and particularly their fish communities (Elliott et al. 2015; Teichert et al. 2016). Resident fish species that experience the estuarine environment throughout their life are more responsive to the local interaction of stressors, than non-resident fish using estuaries during a specific life stage (Teichert et al. 2017).

The flounder (*Platichthys flesus)* is an estuarine resident species excepted during its pelagic larval stage. This species lives principally in the oligo and mesohaline areas which are displaying the most stressful estuarine conditions (Dando 2011; Teichert et al. 2017). Thus, for the past ten years, numerous ecotoxicological studies were conducted in Europe using the flounder which was considered to be a very relevant species for the assessment of the water quality of transitional waters (Evrard et al. 2010; Williams et al. 2011; Henry et al. 2012; Kerambrun et al. 2013; Capela et al. 2016; Dabrowska et al. 2017; Borcier et al. 2020). The previous studies underlined alteration in gene expression patterns, biomarker signals, and fitness loss for the flounder populations living in chronically polluted estuaries, but few studies have explored the variation patterns in flounder response to chemical stress among seasons and genders (Laroche et al. 2013; Kopko and Dabrowska 2018).

Furthermore, *P. flesus* is a boreal species which usually displays a spawning peak in February over the English Channel; a swift ovary growth rate being mainly detected during the two previous months: December–January (Gallien-Landriau 2003). Climate change could induce winter spawner like *P. flesus* to have later spawning period (Sims et al. 2005; Elliott et al. 2015), particularly in the English Channel where a significant overall rise in winter sea surface temperature was detected between 1970 and 2010 (Fincham et al. 2013). Thus, during hot, positive phases of the North Atlantic Oscillation (NAO), flounders tend to spawn later (Sims et al. 2005). Warming waters could reduce the flounder recruitment in estuaries over the English Channel; a negative correlation being detected between the North Atlantic Oscillation Index (NAOI) and the juvenile *P. flesus* abundance in the Thames estuary, UK (Attrill and Power 2002).

In the present paper, the variability of fish chemical signatures, physiological characters, health status indicators, and biomarkers was analysed in two seasons (winter and summer), considering adults of *P. flesus* collected in contrasted systems located in the Eastern English Channel: the highly anthropised and polluted Seine estuary vs the northern moderately polluted Canche estuary (Fig. 1). Despite a significant improvement of the Seine water quality during the last decades (Fisson et al. 2014), the Seine estuary still shows very high levels of PCBs in flounder linked to intensive industrial activities, and to the high concentrations of persistent organic pollutants in the sediments (Tappin and Millward 2015). On the other hand, the Canche system is an agricultural catchment displaying reduced levels of pesticides, and limited sources of organic pollutants (Belles et al. 2019). Concerning the heavy metal pollution, the highest flux of metals over the Eastern Channel is related to the heritage of a strong historical pollution over the Seine basin (Fisson et al. 2017), and to the dumping of dredged spoil and domestic wastes or the discharge from the industry sector in the Seine estuary (Tappin and Millward 2015). Thus, important metal fluxes over the area from the Seine estuary (Le Havre) to the North of France (Dunkirk) could be related to the "coastal flow;" because of the tidal residual and the dominant south-west winds, particularly in winter, the residual circulation drifts northeastward and could play an important role in metal fluxes over the French coast (Brylinski et al. 1991; Tappin and Millward 2015).

The objectives of this study were (1) to improve our knowledge on the responses of fish populations to multistress (mainly diffuse pollution and warming waters), considering their reproductive stage, and (2) to explore the resilience of these populations facing a rapidly changing environment (Elliott et al. 2015).

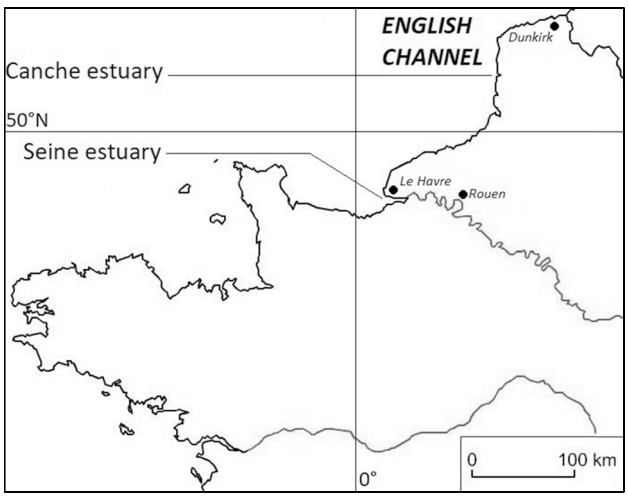

Fig. 1 Location of sampling sites (Seine estuary, Canche estuary) and industrial cities (Le Havre, Rouen)

Materials and methods

Study sites and fish sampling

The study area was located along the French coast of the Eastern English Channel. Two estuaries were sampled (Fig. 1). The Seine estuary (average annual flow 500 m^3/s), the largest one in the English Channel, shows a strongly urbanized and industrialized basin (water catchment 80,000 km^3) concentrating 40% of the French economic activity. Despite a significant improvement of the Seine water quality during the last two decades, the Seine is still considered one of the most chemically polluted estuaries in western northern Europe (Poisson et al. 2011; Burgeot et al. 2017). The Canche estuary (average annual flow 21 m^3/s) is a small system (water catchment 1,300 km^3) displaying a reduced anthropisation characterized by a low urban development without strong industrialisation; thus, this estuary is considered to be moderately polluted (Henry et al. 2012; Belles et al. 2019).

In mid-January 2018, a set of 30 adult flounders (total length > 25 cm) were collected by a beam trawl in the mouth of the two estuaries (Canche and Seine). The same procedure was repeated in early July 2018, but only 22 and 28 fish were, respectively, caught in both estuaries. Flounders were sampled during the reproduction period in winter and during the sexual rest period in summer (Kleinkauf et al. 2004b). Immediately after fishing, each fish was measured and weighted to determine the Fulton's condition factor (K), an indicator of well-being ($K = 100 \times (W/L^3)$, where W is the carcass weight in g, and L is the total length in cm). Different tissues were also collected in the field (muscle, liver, gills, brain, plasma) and immediately flash-frozen in liquid nitrogen. The otoliths were collected and air conserved, until their analysis by image processing to assess the growth rate.

Estuarine water temperature monitoring

For the two last decades, we explored the water temperatures measured in the Seine estuary in the harbour of Rouen (HAROPA-Port de Rouen / Seine-Aval) and in the mouth of the Canche estuary (Lefebvre 2015), during December and January, a critical period for the sexual maturation process of the flounder. Monthly means of water minimum day temperature were analysed for Seine and Canche estuaries, respectively, over the period 2000–2021 and 2004–2021.

Pollutant analysis

Liver PCBs

The concentration of 26 PCBs was assessed in fish tissues by stir bar sorptive extraction-thermal desorption-gas chromatography-tandem mass spectrometry (SBSE-GC–MS/MS) using a method adapted from Lacroix et al. (2014). Briefly, 100 mg wet weight (w.w.) of tissue was digested by saponification and analytes were extracted by stirring during 16 h at 700 rpm using polydimethylsiloxane stir-bars (Twister 20 mm × 0.5 mm, Gerstel). Bars were subsequently analysed using a gas chromatography system Agilent 7890A coupled to an Agilent 7000 triple quadripole mass spectrometer (Agilent Technologies) and equipped with a Thermal Desorption Unit (TDU) combined with a Cooled Injection System (Gerstel). Analytes were quantified relatively to deuterated compounds using a calibration curve ranging from 0.01 ng to 30 ng per bar. Method was validated by analysing mussel reference material NIST-SRM 1974c.

Liver metals

The lyophilized liver-tissue samples were precisely weighed (about 50–100 mg) into PTFE closed cups and predigested at room temperature for 24 h using 5 ml nitric acid (Merck, Suprapur) and 1 ml hydrogen peroxide (Merck, Suprapur) then digested on a heating block (HotBlock® SC100 Digestion System) at a temperature of 120 °C until total decomposition (~ 3 h). The solutions were then diluted to a final volume of 40 ml with ultrapure Milli-Q water. The metal concentrations were determined by ICP-AES (Agilent 5110, dual view) for the major elements and by ICP-MS (Varian, 820) for the trace elements. All samples were analysed in triplicates for reproducibility, accuracy, and precision. Procedural blanks and fish certified reference materials (CRMs): DORM-3 and IAEA-436 were analysed in the same conditions as the samples for quality assurance/quality control. The results were in good agreement with the certified values (> 90% confidence intervals).

Bile PAH metabolite: hydroxypyrene

The concentration of the bile PAH metabolite hydroxypyrene was determined by semi-quantitative analysis of the metabolites fluorescence (Aas et al. 2000). It consisted in measuring the fluorescence by a spectrophotometer with a 5-nm slit width on emission and excitation channels (Jasco FP-6200). Analyses were performed using excitation-emission wavelengths: 343 to 383 nm (4-ringed compounds including pyrene-type metabolites) (Aas et al. 2000).

Physiological and health status

Estimation of growth rate

The individual growth rate (GR) over one year was estimated using otoliths, between the annual marks of the first winter and second winter, a period displaying a very high and linear

growth for flounders. Otolith parameters were measured through image processing; fish lengths at the beginning of the first and second winter were computed by back-calculation (approximately between 8 and 20 months old fish) as described in Marchand et al. (2003).

Muscle lipid analysis

Lipid analyses were performed on flounder muscles by HPTLC (High-performance Thin Layer Chromatography) following a method adapted for *P. flesus* (Pédron et al. 2017b) using a CAMAG TLC Sampler 4 (CAMAG, Switzerland). Lipids were extracted, according to Mathieu-Resuge et al. (2019), from approximatively 150 mg of muscle crushed by a mixer mill (MM400, RETSCH, Germany). Six neutral lipid classes (sterol esters, glyceride ethers, triacylglycerol, free fatty acids, fatty alcohols, free sterols) and seven polar lipid classes (sphingomyelin, lysophospatidylcholine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylethanolamine) were identified by standard comparison and quantified relatively to standard calibration curves (Visioncats software, CAMAG). Neutral lipids, except free sterols, are storage lipids, whereas polar lipids and free sterols are membrane lipids.

The lipid storage index (triacylglycerol / free sterols: TG/FS) based on the ratio of the quantitative reserve lipid (TG) to the quantity of structural lipid (FS) was assessed for fish, because it was considered a relevant proxy of the fitness (Kerambrun et al. 2013). Furthermore, a focus on particular membrane phospholipids (sphingomyelin, phosphatidylserine, free sterols) was also conducted, because a previous study showed: (1) higher concentrations of these phospholipids in flounders from Seine vs a moderately contaminated bay, and (2) their potential role in the regulation of cellular functions of the plasma membrane as well as intracellular organelles (Borcier et al. 2020).

Gonadosomatic and sexual maturity indices

The gonadosomatic index (*GSI*) which reflects the maturation stage of each individual was assessed as follows: $GSI = (W_g/W_s) \times 100$, where W_g (g) and W_s (g) are, respectively, the gonad and the somatic weight.

The gonads were divided into 4 equal portions, in order to carry out the microscopic observations on three distinct cross sections (Minier et al. 2000) and fixed in 4% formalin solution. Tissues were transferred to 70% alcohol and dehydrated to 100% alcohol before being soaked in Histoclear clearing solution (Sigma, St Louis) for 12 h. Tissues were embedded in molten wax prior to sectioning at 3–5 µm thickness. All sections were stained with Mayers hematoxylin/eosin, mounted and carefully examined by light microscopy. Intersex for males was characterized by the presence of primary and secondary oocytes within the testes.

Oocyte maturation for females was classified from 1 to 4 as follows (Koç et al. 2008; Gallien-Landriau 2003): stage 1: previtellogenic primary oocytes. Size: 30 to 140 µm/stage 2: appearance of Cortical alveoli. Size: 100 to 200 µm/stage 3: active vitellogenesis showing an increasing number of yolk granule vesicles. Size: 200 to 300 µm/stage 4: hydrated oocytes. Size: 300 to 500 µm. A score of 1, 2, 3, and 4 was assigned to each oocyte according to its stage of maturation. On each section of a female ovary, the individual sexual maturity index (ISM = individual score) was assessed with the following formula: $IMS = [(N1 \times 1) + (N2 \times 2) + (N3 \times 3) + (N4 \times 4)] / Nt$ with $N1$ = number of oocytes at stage 1; $N2$ = number of oocytes at stage 2; $N3$ = number of oocytes at stage 3; $N4$ = number of oocytes at stage 4; Nt = total number of analysed oocytes. A sexual-maturity index for the different female populations (FSM) was estimated by averaging the ISM obtained for females at each estuary.

Plasma steroid concentrations and vitellogenin

Plasma was separated from blood by centrifugation (5 min, 10,000 rpm), and frozen at − 20 °C. Before the assay, the free steroids were extracted twice from plasma using a mixture made of ethyl acetate/cyclohexane (1:1, v:v). The samples were assayed by competitive enzyme immunoassay (ELISA) method, first developed for 11-ketotestosterone (11-KT) (Cuisset et al. 1994), then adapted for testosterone (T), 17β-estradiol (E2) and 17, 20 β-dihydroxy-4-pregnen-3-one (MIS) (Nash et al. 2000). The enzymatic competitors, made of acetylcholinesterase-coupled pure steroid, were purchased from Bertin Pharma (France).

The male flounder plasma VTG was quantified by ELISA method using anti-turbot VTG polyclonal antibody (Biosense laboratories) and flounder purified VTG as standard. Briefly, the 96-well microplates were coated overnight at 4 °C with male plasma or standard diluted in coating buffer (sodium carbonate buffer pH 9,6). After three washes with washing buffer (PBS-0.05% Tween 20), plates were blocked for one hour at 37 °C with a solution of PBS and 1% BSA. Then, plates were incubated with the anti-turbot VTG polyclonal antibody (1:500) for one hour and half at 37 °C. After three washes, plates were incubated with goat anti-rabbit IgG HRP conjugated antibody (1:50,000; Agrisera) for one hour at 37 °C. Colour development was performed with TMB reagent (Bio-Rad) in darkness at room temperature for 30 min. Then, reaction was stopped by addition of 0.1 mL per well of 1 N HCl. Absorbance was measured after 5 min at 450 nm, using a microplate reader (Synergy HT, Biotek Instruments).

Liver histopathology

A 5 mm × 5 mm portion of fish liver was stored in a histological cassette and fixed in 4% formalin solution. Tissues were transferred to 70% alcohol and dehydrated to 100% alcohol before being soaked in Histoclear clearing solution (Sigma, St Louis) for 12 h. Tissues were embedded in molten wax prior to sectioning at 3–5 µm thickness. All sections were stained with Mayers hematoxylin–eosin-safran (HES), mounted, and carefully examined by light microscopy. Liver damage was categorized according to the recommendations of Feist et al. (2004). A severity score ranging from 1 to 5 was arbitrarily assigned for each liver category of lesions (BEQUALM: Biological Effects Quality Assurances in Monitoring Programmes): (1) non-specific and inflammatory lesions considered to have an uncertain impact on the health of the individual, but must nevertheless be recorded: lipidosis, increased number and size of macrophage aggregates, lymphocytic infiltration; (2) non-neoplastic toxipathic lesions including non-nodular lesions such as hydropic vacuolation, fibrillar inclusions which characterize the body's exposure to contaminants (Myers et al. 1998); (3) foci of Cellular Alteration (FCA); (4) benign neoplasms: hepatocellular adenoma on different constituent cell types (cholangioma, hemangioma, and pancreatic acinar cell adenoma); (5) malignant neoplasms: hepatocellular carcinoma on different constituent cell types and hemangiosarcoma or angiosarcoma.

Each fish was scored with an individual histopathological index corresponding to the sum of the damage scores observed. Likewise, a global index was obtained for a sampling station by the average of the individual ratings.

Biomarkers

Acetylcholinesterase and EROD activities

The brain AChE and the liver EROD activities assays were already described by Borcier et al. (2020). Briefly, after protein extraction, AChE activity was determined within 24 h in quadruplicate, according to the colorimetric method of Ellman et al. (1961), and EROD activity was measured in quadruplicate with modification as described by Burke and Mayer (1974) and in accordance with the AFNOR standard XP T90-336–2. Brain AChE and liver EROD activities were, respectively, expressed as µmol of acetylthiocholine (AcSCh) hydrolysed per minute per milligram of protein and pmol of resorufin per minute per mg of protein.

Comet assays

Immediately after fishing, 10 µL of heparinized blood were added to 1 mL of cryopreservation medium (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6) in a cryogenic tube and immediately frozen in liquid nitrogen. Samples were stored at − 80 °C until analysis. The comet assay was performed on flounder erythrocytes according to the protocol described by Singh et al. (1988) with slight modifications. Ten microliters of cell suspension were mixed with 0.7% low melting point agarose in a 1:10 (v:v) ratio and layered on pre-coated slides. After lysis of cells with lysing solution (100 mM EDTA, 2.5 M NaCl, 1% *N*-Laurylsarcosine, 10 mM Tris, 10% DMSO, 1% Triton-100X), electrophoresis was performed using cold alkaline solution (300 mM NaOH, 1 mM ethylenediaminetetraacetic acid disodium salt (Na2 EDTA), pH > 13) at 300 mA and 23 V (ca. 0.8 $V.cm^{-1}$) for 20 min. Slides were stained with SYBR® Gold (Invitrogen) and comet cells were scored under a CellInsight CX5 HCS® System (Thermo) which automatically provides standardized percent of total cellular DNA in the tail (%DNA Tail).

TBARS assays

Branchial arches were homogenized on ice in 500 µL of a chilled phosphate buffer (0.1 M; pH 7.5) using the MoBiTec G50 Tissue Grinder set at 3000 rpm and then centrifuged at 9,000 g for 25 min. The supernatant (S9 fraction) was isolated and stored at − 80 °C for protein and lipid peroxidation analyses. The total protein concentration was measured on the diluted S9 fraction (1:50 v/v in ultrapure water) following the method of Lowry et al. (1951) and using a BIO-TEK Synergy HT microplate reader. Lipid peroxidation (TBARS) was determined on S9 fraction using the method of Buege and Aust (1978) adapted to microplate readers (Weeks Santos et al. 2019). TBARS levels were measured using a UV-spectrophotometer (Biotek Synergy HT) at 530 nm. Results were expressed as nmoles of thiobarbituric acid reactive substance (TBARS) equivalents per mg of protein.

Metabolic activities: glucose-6-phosphate dehydrogenase and citrate synthase

Enzymatic activities were conducted from approximatively 250 mg of liver and muscle samples, according to Borcier et al. (2020). The G6PDH enzymatic activity was measured on liver tissues at 340 nm for 6 min, while the CS reaction rates were measured on liver and muscle tissues at 412 nm for 4 min, with a spectrophotometer OMEGA PolarStar (BMG Labtech). The protein concentration was determined according to the Coomassie blue method (Bradford 1976), with a bovine serum albumin (BIORAD Laboratories, USA) as standard. The absorbance was measured at 595 nm.

Statistical analysis

Since principal and interactive effects of three independent variables are often difficult to analyze and to interpret, the first step of the data analysis was to prioritize the relative contribution of each one of them (i.e., season, study site, and sex) conditionally to the two others on the global information contained in the samples × biological endpoint matrix. The variance explained by each factor is then tested to depict whether it can attain a significant level or not. Variance analysis was performed with a partial redundancy analysis (rda function, library vegan, Oksanen et al. 2020). Each computed RDA was then tested with an ANOVA. This preliminary analysis reveals a significant contribution of season (4.9% of variance explained, $p = 0.021$), of study site (6.9% of variance explained, $p = 0.002$), but not for sex (less than 1% of variance explained, $p = 0.634$). Thus, considering that sex effect is neglectable in this study, further analyses were performed considering only season and study site effects.

Statistical tests were performed in R (R Core Team (2021)). Normality and homoscedasticity of variances were controlled with a Shapiro–Wilk test and a Bartlett test, respectively. Since data were not normally distributed, a non-parametric Kruskal–Wallis test followed by a post hoc Dunn test (for multiple comparisons) was applied to compare means over all the conditions (season × study sites). A p value lower than 0.05 was considered a significant difference. The data integration was carried out by principal component analyses performed with the FactorMineR package with default settings (Lê et al. 2008).

Results

Water temperature monitoring

Globally, the water temperatures in the Seine and Canche estuaries (Fig. 2) showed convergent temporal trends over 2000–2020. The monthly mean of minimum day temperature in estuaries, during December–January, was rather variable from 2000–2001 to 2010–2011, then was stabilized to the highest values from 2017–2018 to nowadays.

Pollutants

Liver PCBs

The liver PCBs concentrations (Fig. 3a) showed considerably higher levels in Seine vs Canche with no significant difference between seasons within the two estuaries (Seine winter: 4771 ± 3221 µg.kg^{-1} dw – summer: 2216 ± 767 µg.kg^{-1} dw; Canche winter: 302 ± 288 µg.kg^{-1} dw – summer: 271 ± 261 µg.kg^{-1} dw).

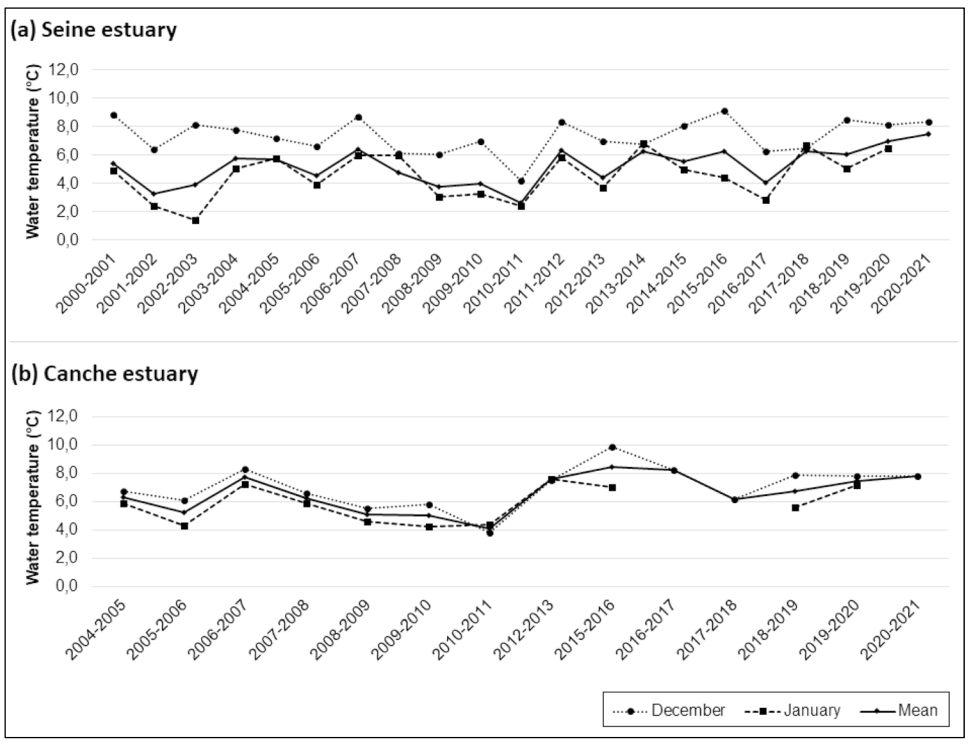

Fig. 2 Monthly mean of minimum day water temperature in the Seine and Canche, for December, January and December–January mean, over the last two decades

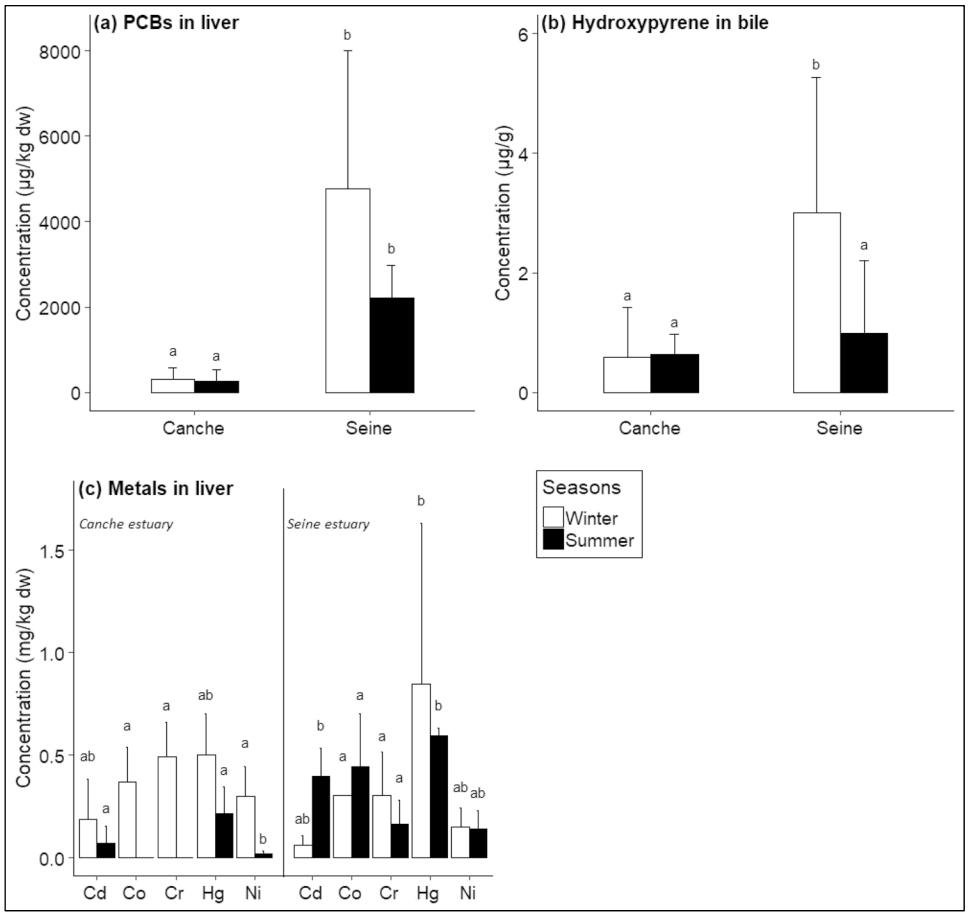

Fig. 3 Mean (and 95% confidence interval) of organic contaminants and heavy metals in *P. flesus* from Canche and Seine estuaries. **a** Polychlorobiphenyl (PCBs) concentrations in liver. **b** Hydroxypyrene in bile. **c** Cadmium (Cd), cobalt (Co), chromium (Co), mercury (Hg), and nickel (Ni) in the liver. (Statistics: Kruskal–Wallis test: p value < 0.05, letters correspond to significant differences among estuaries and seasons)

Liver metals

Globally, the metal concentrations are rather similar in Canche vs Seine, but a seasonal variation was observed for the Canche, particularly for Co, Cr, and Ni which showed decreasing concentrations in summer vs winter (Fig. 3c). The bioaccumulations of Hg were also the highest for Seine and Canche in winter (respectively 0.85 ± 0.79 mg.kg^{-1} dw, and 0.50 ± 0.20 mg.kg^{-1} dw). On the other hand, the highest Cd concentration was detected in Seine in summer (0.40 ± 0.14 mg.kg^{-1} dw).

Bile hydroxypyrene

In winter, the bile hydroxypyrene level was higher in Seine (3.00 ± 2.26 µg.g^{-1}) vs Canche (0.58 ± 0.83 µg.g^{-1}); the levels being not different in summer for Seine (0.99 ± 1.21 µg.g^{-1}) vs Canche (0.63 ± 0.33 µg.g^{-1}) (Fig. 3b).

Physiological and health status

Flounder total length, age, sex ratio

The collected adult-fish displayed a total length between 25 and 33 cm; the individual age was determined by reading otoliths (2 + ≤ cohorts detected in the fish samples ≤ 5 +). The sex ratios (males/females) per season were similar in the two estuaries (January: SR Canche = 0.87 / SR Seine = 0.58; July: SR Canche = 0.46 / SR Seine = 0.40).

Fulton's condition factor, growth rate, muscle lipids

The Fulton's condition factor (K) showed a significant difference between seasons but not between estuaries (Fig. 4a). K was significantly lower in winter (Canche: 0.88 ± 0.08, Seine: 0.95 ± 0.18) than in summer (Canche: 1.07 ± 0.13, Seine: 1.06 ± 0.14).

The average flounder growth rates (between 8- and 20-month-old-fish) estimated using otoliths were not significantly different between the Canche and Seine populations, with respectively 8.78 ± 2.37 cm/year and 9.78 ± 2.56 cm/year.

The amount of total lipids in flounder muscle was lower in winter than in summer for the two estuaries (Table 1). The triacylglycerols (TG) were the major reserves of lipids but the Seine population presented a higher winter concentration than the Canche population. Regarding the bioactive membrane lipids (free sterols (FS), phosphatidylserine (PS), sphingomyelin (SPG)), no significant difference was

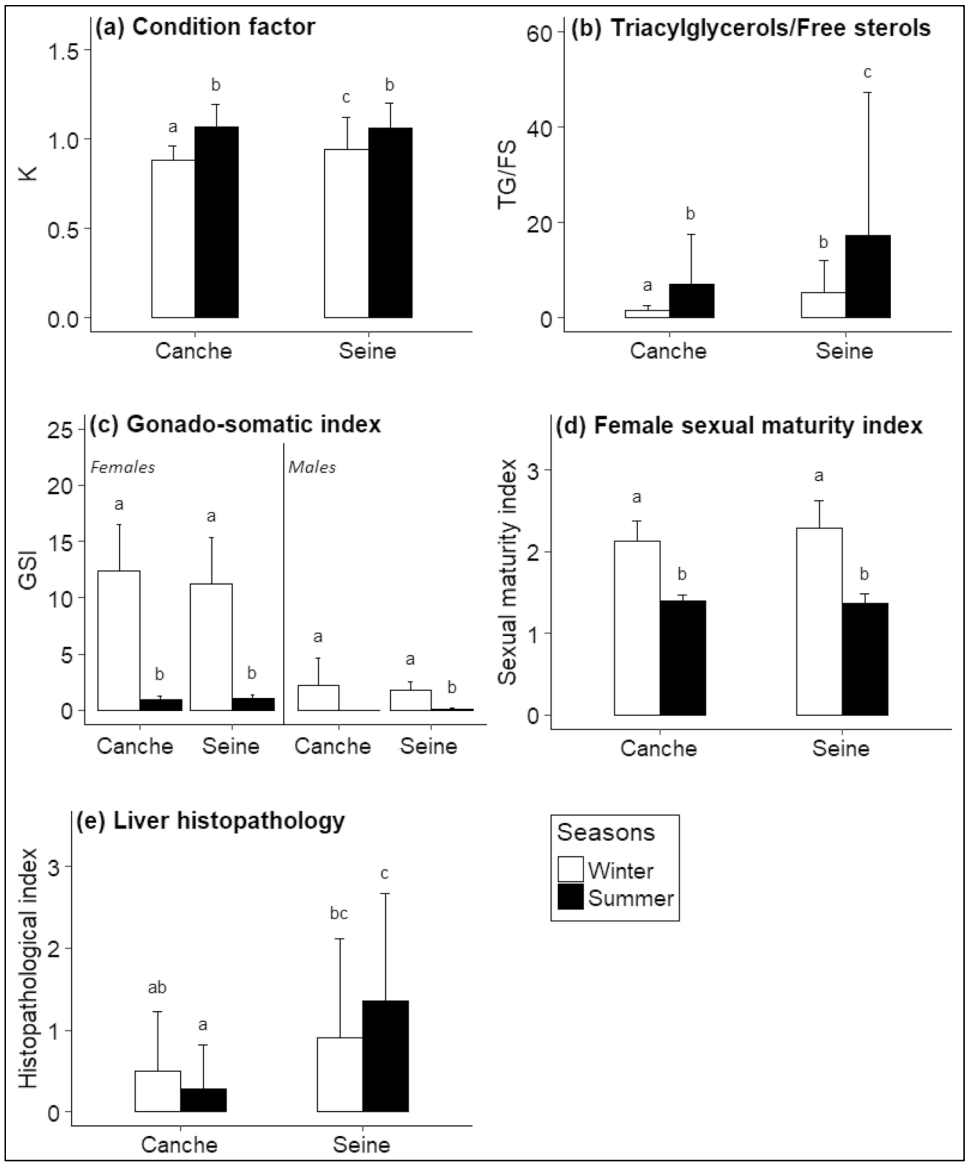

Fig. 4 Physiological and health status of *P. flesus* from Canche and Seine estuaries. Mean (and 95% confidence interval). **a** Fish condition factor. **b** Triacylglycerols/Free sterols: lipid storage index. **c** Gonadosomatic index. **d** Female sexual maturity index assessed by histology. **e** Liver histopathology. (Statistics: Kruskal–Wallis test: p value < 0.05, letters correspond to significant differences among estuaries and seasons)

detected for FS and PS levels between estuaries and between seasons; a higher winter SPG level being observed in Seine (0.38 ± 0.12 µg.mg^{-1} fw) vs Canche (0.30 ± 0.11 µg.mg^{-1} fw) (Table 1).

The lipid storage index: triacylglycerols/free sterols (TG/FS) was significantly higher in Seine vs Canche in winter and summer; the two estuaries displaying higher TG/FS levels in summer vs winter (Fig. 4b).

Gonadosomatic index, sexual maturity index, plasma steroids, and vitellogenin

The female *GSI* (Fig. 4c) showed a significant difference between the seasons, but not between the flounder populations. *GSI* was higher in the winter reproduction period (Canche: $12.42 \pm 4.11\%$; Seine: $11.26 \pm 4.09\%$) vs the summer rest period (Canche: $0.93 \pm 0.34\%$, Seine: $1.03 \pm 0.34\%$). The same pattern was observed for the male *GSI* in both estuaries (Fig. 4c).

The histology of male gonads showed no intersex fish, i.e. the absence of individuals characterized by the presence of primary and secondary oocytes within their testes.

The female sexual maturity index analysed by histology (Fig. 4d) displayed a higher level in winter, similar in Canche (2.12 ± 0.25) and Seine (2.28 ± 0.34). This index was lower in summer and again similar in Canche (1.39 ± 0.08) and Seine (1.37 ± 0.11).

The female E2 plasma level (Table 2) was significantly higher in winter (Canche: 3802.88 ± 3246.81 pg.mL^{-1}, Seine: 3958.11 ± 1960.74 pg.mL^{-1}) than in summer (Canche: 75.20 ± 72.23 pg.mL^{-1}, Seine: 81.3 ± 52.56 pg.

Table 1 Muscle lipid content measured in *P. flesus* from Canche and Seine estuaries. Muscle lipid content expressed in µg of lipid per milligram of fresh weight.

	Canche in winter	Canche in summer	Seine in winter	Seine in summer
Storage lipids (µg.mg^{-1})				
ALC	$0.03 \pm 0.02^{(a)}$	$0.03 \pm 0.05^{(ab)}$	$0.032 \pm 0.02^{(a)}$	$0.02 \pm 0.03^{(b)}$
FFA	0.05 ± 0.03	0.06 ± 0.07	0.054 ± 0.04	0.06 ± 0.05
GE	$0.003 \pm 0.01^{(a)}$	$0.06 \pm 0.08^{(b)}$	$0.006 \pm 0.01^{(a)}$	$0.05 \pm 0.07^{(b)}$
SE	0.077 ± 0.03	0.08 ± 0.07	0.07 ± 0.02	0.08 ± 0.07
TG	$0.62 \pm 0.38^{(a)}$	$4.05 \pm 5.18^{(bc)}$	$2.35 \pm 2.82^{(b)}$	$10.69 \pm 18.70^{(c)}$
$\sum$ storage lipids	$0.78 \pm 0.39^{(a)}$	$4.30 \pm 5.20^{(bc)}$	$2.51 \pm 2.82^{(b)}$	$10.90 \pm 18.71^{(c)}$
Membrane lipids (µg.mg^{-1})				
FS	$0.44 \pm 0.07^{(a)}$	$0.62 \pm 0.11^{(b)}$	$0.46 \pm 0.07^{(a)}$	$0.60 \pm 0.09^{(b)}$
CL	$0.13 \pm 0.11^{(ac)}$	$0.04 \pm 0.07^{(bc)}$	$0.08 \pm 0.05^{(c)}$	$0.12 \pm 0.05^{(a)}$
LPC	0.21 ± 0.08	0.21 ± 0.15	0.25 ± 0.10	0.23 ± 0.10
PC	5.02 ± 1.78	5.17 ± 2.65	4.97 ± 1.36	5.26 ± 2.52
PE	$1.87 \pm 0.68^{(ac)}$	$2.23 \pm 0.29^{(b)}$	$1.86 \pm 0.36^{(c)}$	$2.03 \pm 0.42^{(ab)}$
PI	0.71 ± 0.25	0.70 ± 0.13	0.66 ± 0.14	0.72 ± 0.16
PS	$0.24 \pm 0.08^{(a)}$	$0.94 \pm 0.82^{(b)}$	$0.28 \pm 0.08^{(a)}$	$0.70 \pm 0.71^{(b)}$
SPG	$0.30 \pm 0.11^{(a)}$	$0.33 \pm 0.17^{(ab)}$	$0.38 \pm 0.11^{(b)}$	$0.30 \pm 0.10^{(a)}$
$\sum$ Membrane lipids	$8.91 \pm 2.73^{(ac)}$	$10.25 \pm 2.22^{(b)}$	$8.95 \pm 1.78^{(c)}$	$9.98 \pm 2.44^{(bc)}$
Total lipids (µg.mg^{-1})	$9.69 \pm 2.71^{(a)}$	$14.55 \pm 5.48^{(b)}$	$11.46 \pm 3.51^{(a)}$	$20.87 \pm 19.14^{(b)}$

ALC fatty alcohols, *FFA* free fatty acid, *GE* glyceride ethers, *SE* sterol esters, *TG* triacylglycerols, *FS* free sterols, *CL* cardiolipins, *LPC* lysophosphatidylcholine, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SPG* sphingomyelin. (Statistics: Kruskal–Wallis test: p value < 0.05, letters correspond to significant differences among estuaries and seasons)

mL^{-1}). No significant E2 differences were detected between the two estuaries.

The same pattern was observed for the male 11-KT (Table 2) (Canche winter: 4170.54 ± 3246.81 pg.mL^{-1}, Seine winter: 2405.45 ± 1444.91 pg.mL^{-1}, Canche summer: 325.28 ± 59.55 pg.mL^{-1}, Seine summer: 436.13 ± 180.51 pg.mL^{-1}).

The VTG measured in males (Table 2) was not different in Canche winter (24.94 ± 1.13 ng.mL^{-1}), Canche summer (23.53 ± 1.47 ng.mL^{-1}), and Seine Summer (25.15 ± 4.32 ng.mL^{-1}). The lowest level of male VTG was detected in Seine winter (19.97 ± 1.62 ng.mL^{-1}).

Liver histopathology

The main alterations detected by histology on flounder livers were displayed in Fig. 5. Overall, the most observed liver lesions were assigned to grade 1. Thus, an excessive accumulation of lipids (lipidosis or steatosis) was observed in winter and summer in the Seine and Canche estuaries; this accumulation being not considered a pre-neoplastic lesion in fish.

During both seasons, livers showed a higher percentage of melanomacrophagic aggregates in Seine vs Canche (winter: 23% vs 7%; summer: 17% vs 0%, respectively).

In summer, the hydropic vacuolation considered a non-nodular lesion was detected in the Seine fish (28%) but was not observed in the Canche.

A FCA-type nodular lesion, a pre-tumour liver damage, was only detected in the Seine (one in summer and one in winter).

Finally, the global histopathological index was significantly higher in Seine vs Canche, in summer (Fig. 4e).

Table 2 Hormones and vitellogenin in plasma of *P. flesus* from Canche and Seine estuaries.

	Canche in winter	Canche in summer	Seine in winter	Seine in summer
FemaleE2 pg.mL^{-1}	$3802.87 \pm 3246.81^{(a)}$	$75.20 \pm 72.23^{(b)}$	$3958.11 \pm 1960.74^{(a)}$	$81.30 \pm 52.56^{(b)}$
Male 11KT pg.mL^{-1}	$4170.54 \pm 3246.81^{(a)}$	$325.28 \pm 59.55^{(b)}$	$2405.45 \pm 1444.91^{(a)}$	$436.12 \pm 180.51^{(b)}$
Male [VTG] ng.mL^{-1}	$24.94 \pm 1.13^{(a)}$	$23.53 \pm 1.46^{(a)}$	$19.97 \pm 1.62^{(b)}$	$25.15 \pm 4.32^{(a)}$

Mean (and 95% confidence interval). Female E2: 17ß-estradiol; Male 11-KT: 11-ketotestosterone; Male VTG: Vitellogenin. (Statistics: Kruskal–Wallis test: p value < 0.05, letters correspond to significant differences among estuaries and seasons)

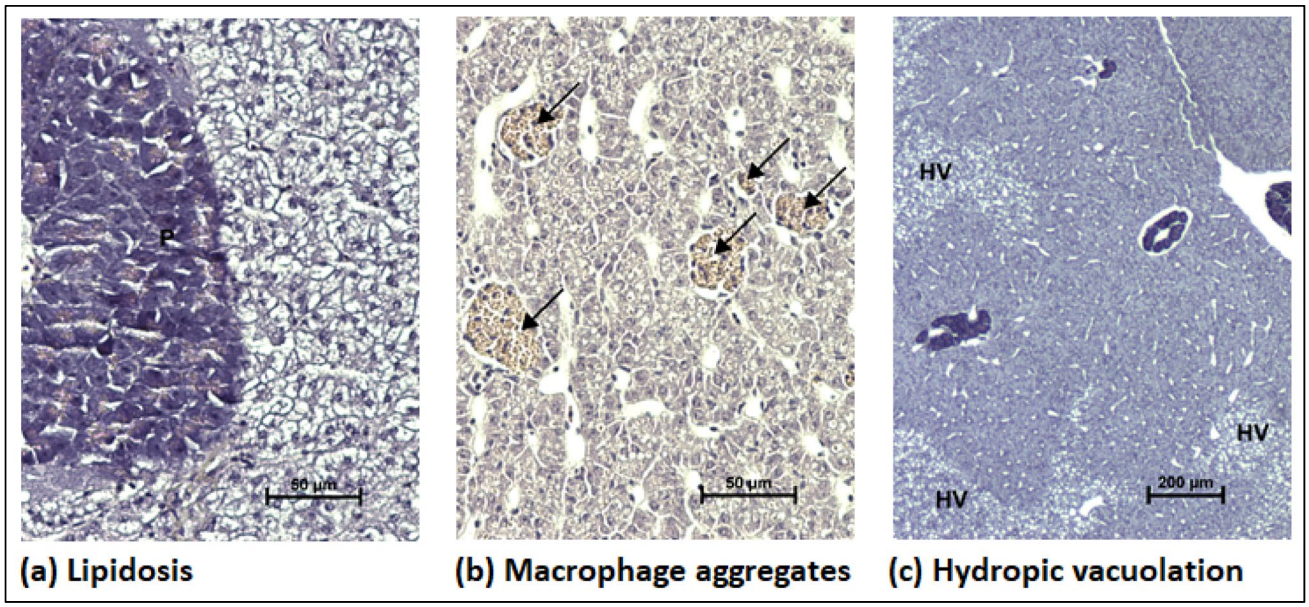

Fig. 5 Histological presentation of the main European flounder liver alterations. **a** Lipidosis: parenchymal cells (hepatocyes) containing few large lipid vacuoles (P = exocrine pancreatic component). **b** Increase in number and size of macrophage aggregates (arrows). **c** Focal region of hydropic vacuolation (HV) of hepatocytes

Biomarkers

Acetylcholinesterase and EROD activities

The brain AChE activity (Fig. 6a) was the highest in winter in both estuaries; this winter activity being higher in Canche (0.31 ± 0.11 µmol.min.mg prot^{-1}) than in Seine (0.19 ± 0.05 µmol.min.mg prot^{-1}). No significant difference was detected in the brain AChE activities in summer between both estuaries.

The liver female EROD activity (Fig. 6b) was the highest in summer, with the level in Seine (7.88 ± 5.21 pmol.min.mg prot^{-1}) being twice as strong as the level in Canche (3.55 ± 2.37 pmol.min.mg prot^{-1}). In winter, the female EROD activity was three times higher in Seine (3.56 ± 2.45 pmol.min.mg prot^{-}1) than in Canche (1.18 ± 0.45 pmol.min.mg prot^{-1}). An inverse seasonal trend was detected for the male EROD activities (Fig. 6b) which were higher in winter vs summer; the winter EROD activity being higher in Seine (12.27 ± 8.54 pmol.min.mg prot^{-1}) than in Canche ($8.50 \pm 13{,}16$ pmol.min.mg prot^{-1}).

Genotoxicity and lipid peroxidation

In winter, a higher blood cell DNA damage expressed as % of DNA tail (Fig. 6c) was detected in Canche (49.99 ± 7.87%) vs Seine (29.895 ± 4.56%), The levels of DNA damage were not different in summer for Canche and Seine (from 44 to 50%).

The gill lipid-peroxidation (Fig. 6d) was higher in winter for both estuaries; a significant higher peroxidation level being only observed in winter in Seine (3.55 ± 0.39 nmol eq. MDA.mg prot^{-1}) vs Canche (2.58 ± 0.55 nmol eq.MDA.mg prot^{-1}).

Metabolic activities

The liver G6PDH activity (Fig. 7a) was the highest in summer in Seine (0.20 ± 0.15 IU.mg^{-1}), with no significant difference detected between the three other conditions ($0.087 <$ G6PDH activity < 0.1 IU.mg^{-1}).

Globally, the liver CS activity (Fig. 7b) was lower in Seine vs Canche, whatever the season. A significant increase of this CS activity was detected from winter to summer in the Canche (0.0014 ± 0.0006 to 0.0028 ± 0.0017 IU.mg^{-1}), while an inverse trend was observed in the Seine (0.0010 ± 0.0003 to 0.0006 ± 0.0006 IU.mg^{-1}). The muscle CS activity (Fig. 7c) was the highest in Canche in summer (1.122 ± 0.081 IU.mg^{-1}); the other conditions showing a similar activity (0.080 IU.mg^{-1}).

Data integration

Globally, the present results (3.1 to 3.5) highlight in winter vs summer (1) higher levels of chemical contamination in the flounder populations of Seine and Canche and (2) more contrasted phenotypic responses between the two fish populations. Thus, two principal component analyses (PCA)

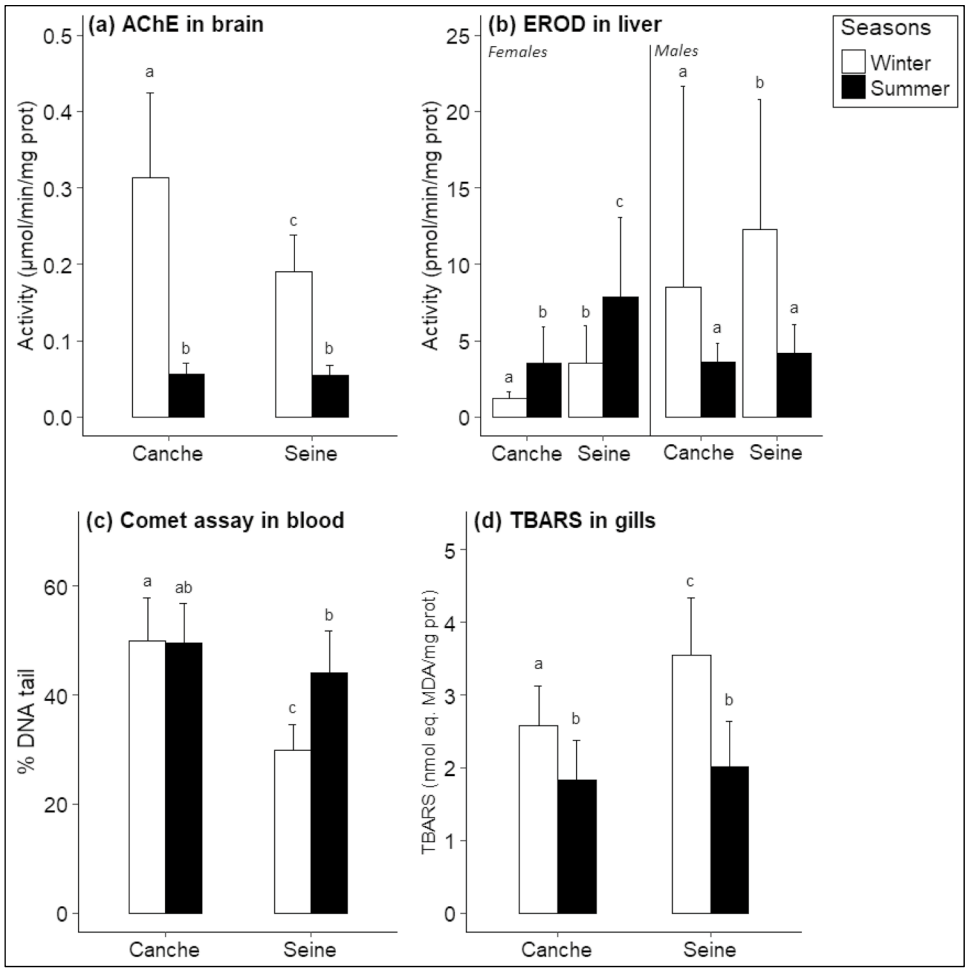

Fig. 6 Biomarkers measured in *P. flesus* from Canche and Seine estuaries. **a** AChE: acetylcholinesterase enzymatic activity in brain. **b** Male and female EROD activities measured in liver. **c** Comet assay measured in blood. **d** Lipid peroxidation (TBARS) measured in gills. (Statistics: Kruskal–Wallis test: p value < 0.05, letters correspond to significant differences among estuaries and seasons)

were performed for the flounder populations in winter, to integrate fish markers not directly linked to reproductive status and sex. The relationships between the following markers were analysed by PCA: bile hydroxypyrene, physiological and health status (fish total length TL, condition factor K, lipid storage index TG/FS, muscle bioactive membrane lipids (free sterols FS, phosphatidylserine PS, sphingomyelin SPG), liver histopathology index, biomarkers (brain acetylcholinesterase: AChE, blood DNA damage: GENOTOXICITY, gill lipid peroxidation: TBARS), metabolic activities (G6PDH and CS enzymatic activities).

The two first axes of the PCA explained 40.5% and 34.3% of the total variance of the data set, respectively, for Canche and Seine populations (Fig. 8). In the Canche estuary (Fig. 8a), two variables (TBARS and K) were negatively correlated with another group of variables (Genotoxicity, AChE, PS, and SPG). In the Seine estuary (Fig. 8b), a group of variables (TBARS, Histopathology, CS_liver, G6PDH_liver) was negatively correlated with another group of variables (PS and SPG).

Discussion

Temporal trends in winter water temperature in estuaries

The annual winter flounder migration from colder estuarine habitats to warmer coastal and offshore spawning grounds is considered to be a fish response to maintain higher gonadal growth prior to spawning (Graham and Harrod 2009); the Seine flounder spawning peak being classically observed in February, in 2000–2001 (Gallien-Landriau 2003).

The temporal variability in Seine and Canche minimum water temperatures in December–January (two months before the average spawning peak) showed high stabilized values, from the winter 2017–2018 to nowadays. This temporal trend of the winter climate over the Seine and Canche estuaries confirms that the overall rise in coastal winter sea surface temperatures between 1970 and 2010 over the Eastern English Channel (Fincham et al. 2013) was maintained over the last decade 2010–2020. Over the English Channel

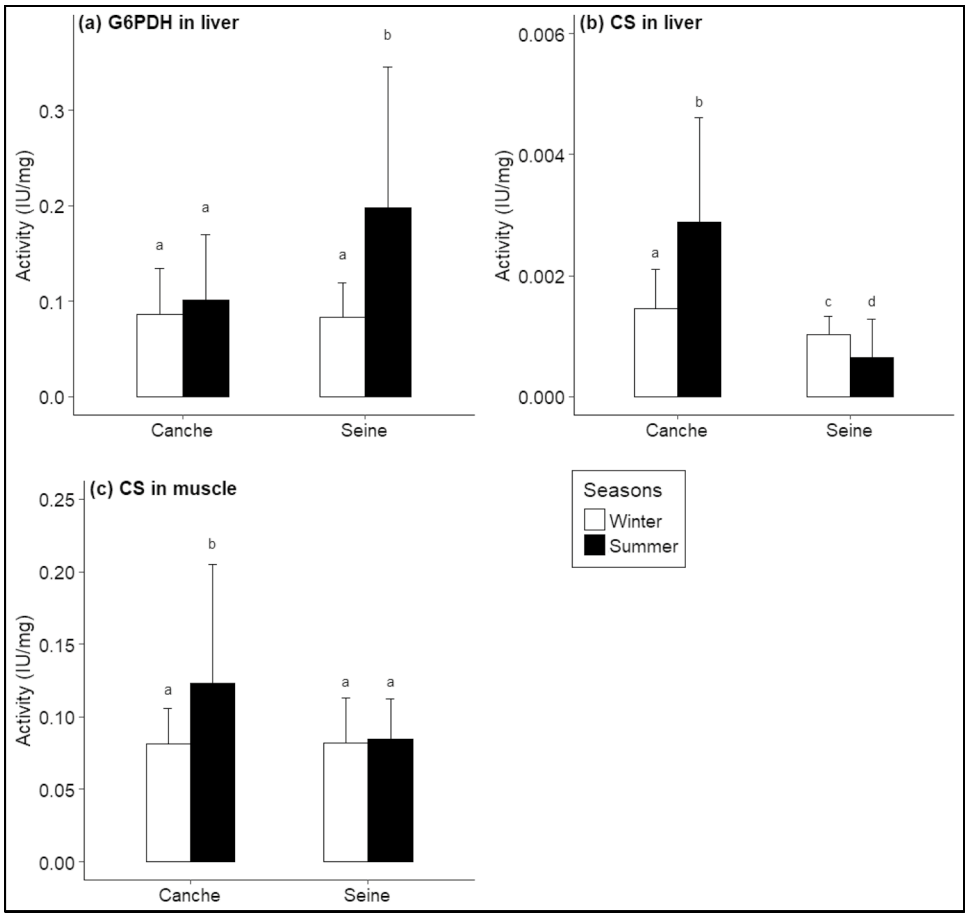

Fig. 7 Metabolic activities measured in *P. flesus* from Canche and Seine estuaries. **a** G6PDH enzymatic activity measured in liver. **b** CS enzymatic activity measured in liver. **c** CS enzymatique activity measured in muscle. (Statistics: Kruskal–Wallis test: p value < 0.05, letters correspond to significant differences among estuaries and seasons)

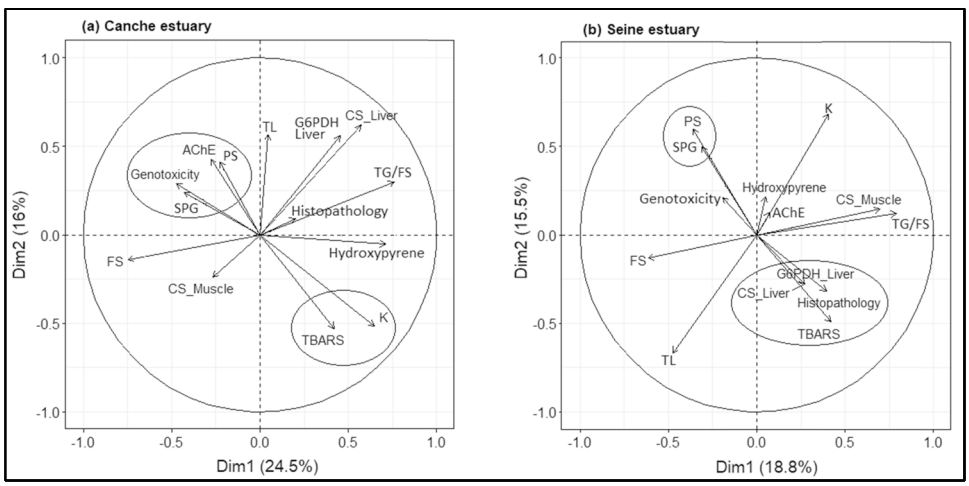

Fig. 8 Principal component analysis (axe 1 and 2). Distribution of 15 markers on the correlation circle in the Canche and Seine flounder populations. G6PDH_liver: glucose-6-phosphate deshydrogenase; CS_liver, CS_muscle: citrate synthase; AChE: brain acetylcholinesterase; Genotoxicity: DNA damage on blood cells; liver histopathology index; bile hydroxypyrene; TBARS: lipid peroxydation in gills; K: condition index; TL: fish total length; SPG: sphingomyelin; FS: free sterols; PS: phosphatidylserine; TG: triacylglycerols; TG/FS: lipid storage index

and the North-Sea, the shift in the timing of spawning in sole (classically timed in late winter or spring) was linked to warming sea temperatures (Fincham et al. 2013). We suggest that the phenology of the flounder spawning could also be modified by such climate change in winter.

Fish contamination by metals and organic pollutants

The flounder liver contamination by metals was rather similar in Seine and Canche estuaries, particularly in winter; the summer contamination levels being however depleted in particular metals (Co, Cr, Ni) in Canche vs Seine. Convergent levels of metal contamination in flounder were also detected in Seine and Canche in the past, considering adults (Henry et al. 2004) and juveniles (Henry et al. 2012). In the present study, we suggest that the lower levels of metals in the Canche flounder in summer could be related to a strong reduction of metal fluxes between the Seine and the Southern Bight of the North Sea, linked to weak river flow.

In this study, the levels of toxic metals such as Cd in Seine and Canche (0.2–0.4 $\mu g.g^{-1}$ dw) were higher than those observed in adult flounders from moderately anthropised coastal systems in the Ushant Sea, Bay of Douarnenez (Cd = 0.09 $\mu g.g^{-1}$ dw in Borcier et al. 2020) or in the Bay of Biscay, Ster estuary (Cd = 0.01 $\mu g.g^{-1}$ dw in Evrard et al. 2010). However, the liver flounder levels of Cr, Cd, Ni, Hg in Seine and Canche presented concentrations around five times lower than those detected in flounder from the highly polluted UK estuaries: Tyne and Mersey (Williams et al. 2011).

The flounder liver concentrations in PCBs were 10–15 times higher in Seine vs Canche; a PCBs decrease being observed in summer vs winter, particularly in the Seine estuary. The PCBs level in the Canche estuary ($\approx$ 300 $\mu g.kg^{-1}$ dw) was similar to the status of moderately polluted systems as the Ster estuary in the Bay of Biscay ($\approx$ 200 $\mu g.kg^{-1}$ dw in Evrard et al. 2010). On the other hand, the Seine PCBs level observed in winter (4700 $\mu g.kg^{-1}$ dw) was considerably higher than those observed in large polluted French estuaries with $500 < PCBs < 800$ $\mu g.kg^{-1}$ dw in Loire and Gironde (Evrard et al. 2010), or in heavily polluted systems in the UK with $400 < PCBs < 1600$ $\mu g.kg^{-1}$ dw in Tyne and Mersey (Williams et al. 2011).

In the Seine system, the lower Flounder PCBs level in summer (rest period) vs winter (reproduction period) could be related to a probable decontamination of adult fish during the reproduction. One part of the lipophilic pollutants was transferred to oocytes then eliminated by spawning (Ostrach et al. 2008), or directly metabolized during the reserve mobilization for the reproduction.

The higher value of bile hydroxypyrene detected in Seine vs Canche, particularly in winter, highlights a strong flounder metabolization of the pyrene, a widely distributed PAH in the Seine. The levels of flounder bile hydroxypyrene showed that concentrations were 4 to 17 times higher, respectively, in Seine vs North Sea—Holland and in Seine vs Iceland (Kamman et al. 2017). We suggest that the intensive dredging activities conducted in the Seine to maintain the navigation channel depth could be a major factor increasing the flounder contamination by PAHs in this estuary, as described in other industrial estuaries in Europe (Vethaak et al. 2016).

Fish health indicators: condition factor, storage and membrane lipids, growth rate, liver histopathology

The flounder condition factor was similar in Seine vs Canche, with a lower value in winter vs summer. This decrease in January flounder condition was due to fasting during mobilization of body energy stores required for the final gonad maturation; a restauration of the condition factor being observed in post-spawning fish in July. The temporal variability of muscle lipids showed globally the same trend with lower values in January vs July, but the lipid storage index (TG/FS: triacylglycerols/free sterols) was significantly higher in Seine vs Canche, whatever the season. In previous studies conducted on the adult flounders in November–December, in the UK and the Baltic Sea, heavily polluted sites displayed the lowest fish conditions (Kleinkauf et al. 2004b; Dabrowska et al. 2017). In the present study, we suggest that the higher muscle lipid storage in Seine vs Canche could be related to the higher benthic prey production in the Seine estuarine system, where larger intertidal mudflats could produce the major part of the flounder diet.

The concentrations of bioactive muscle membrane lipids (free sterols (FS), phosphatidylserine (PS), sphingomyelin (SPG)) were also analysed in this study and showed a significant increase of winter SPG in Seine vs Canche. SPG is a type of sphingolipids which are located in plasma membrane and endoplasmic reticulum membrane; they are associated with sterols to form heterogeneities in the membrane called lipid rafts which show an important proportion of Cytochrome P450s (Brignac-Huber et al. 2016). This sphingolipids-sterols association is essential for maintaining endoplasmic reticulum homeostasis (Kajiwara et al. 2012). Thus, in the present study, we suggest that the increase of SPG in winter, in Seine vs Canche could be related to an increase of the lipid rafts density in the endoplasmic reticulum to improve the cell capacity for xenobiotic metabolism. This result confirms a previous study on the flounder responses in Seine vs Douarnenez Bay, a moderately polluted system (Borcier et al. 2020) where the authors suggest that the variation in the content of muscle SPG could become a relevant marker in ecotoxicology.

An increase of FS and PS levels in flounder lipids was also reported in Seine vs Douarnenez Bay (Borcier et al. 2020); this trend was not confirmed in the present study and could be related to different sampling seasons between the two studies. PS is an anionic phospholipid that should facilitate protein insertion in membrane and thus should also play

a key role in the regulation of cellular functions in plasma membranes and organelles (Brignac-Huber et al. 2016). In the present study, the general increase of the PS and FS detected in summer vs winter for Seine and Canche could be related to their role in the maintenance of the membrane fluidity (Leventis and Grinstein 2010).

The similar 8- to 20-month-old flounder growth rates (≈ 9 cm/year) estimated in both studied estuaries was previously observed in polluted large French estuaries such as Seine, Loire, and Gironde. However, less polluted systems showed higher flounder growth rates (14–16 cm/year) (Marchand et al. 2004; Evrard et al. 2010).

The higher liver histopathological-index measured in fish caught in Seine vs Canche in summer was mainly explained by the high prevalence of melanographic aggregates (17%) and hydropic vacuolations (28%) in Seine; these non-nodular lesions were not detected in Canche and could reflect the exposure of Seine fish to toxic substances and particularly hydrocarbons (Feist et al. 2004). An FCA-type nodular lesion was detected once in winter and in summer, only in the Seine estuary; this lesion being generally considered a precursor to benign or malignant liver tumours in polluted environments (Myers et al. 1998). The frequency of the pre-tumour FCA lesion is correlated with the size-age of the fish sampled, insofar a neoplastic progression can take several years (Rhodes et al. 1987). Furthermore, in the present study, the prevalence of histopathological lesions was shown to vary according to sampling season; this result being confirmed in French Atlantic estuaries where liver necrosis occurs more frequently in flounders during summer (Cachot et al. 2013). A significant association between ulcer occurrence and measured body burdens, PAH metabolites and toxicopathic liver lesions was observed in a flounder population in the Dutch Wadden Sea (Martínez-Gómez and Vethaak 2019). The flounder displayed higher prevalence (particularly of melanomacrophage aggregates, inflammation, and necrotic foci) in contaminated vs reference sites in the UK (Lyons et al. 2004); thus, we suggest that the higher histopathological index in Seine vs Canche was probably related to a higher chemical contamination.

Phenology of the reproduction

The flounder gonad maturation cycle appeared very similar in both estuarine systems, Seine and Canche. For males and females, high *GSI* in January followed by low *GSI* in July were observed in this study. They confirmed the general trend detected for the flounder in the Eastern Channel where growing and maturing gonads were observed in November–January, normally preceding a reproduction peak in February; gonads at rest being observed in summer (Gallien-Landriau 2003). However, the Seine female *GSI* obtained in the present study (January 2018, $GSI = 11.26\%$) was lower than the *GSI* assessed in our previous study in Seine (January 2003, $GSI = 18.32\%$) (Marchand et al. 2004). Furthermore, this Seine January female flounder *GSI* appeared relatively weak compared to the range of female *GSI* observed in January 2003 for four French Atlantic estuaries ($14.1\% < GSI < 26.9\%$) (Marchand et al. 2004).

In the present study, a similar trend was observed for the female sexual maturity index (FSM) assessed by ovary histology, with higher value in January vs July. Furthermore, the low value of the FSM detected for both estuaries (FSM ≈ 2.2) in January 2018 showed that, this year, the majority of the oocytes were not at the stage 3 (active vitellogenesis) or at the stage 4 (hydrated oocytes). This low index is the mark of a weak vitellogenesis in January 2018 compared to previous studies conducted in January (over the period 1975–2002) where 70% of Seine flounders showed advanced vitellogenesis (Gallien-Landriau 2003) and all the Douarnenez Bay flounders displayed final oocyte maturation (Déniel 1981).

The convergent decreasing trend detected in Seine and Canche, for the *GSI* and the vitellogenesis in January 2018 vs 2003 and anterior years, confirms that the higher water temperatures during the vitellogenesis period in November–December 2018 could cause a delay in oocyte maturation and spawning of the flounder in the Eastern Channel. Thus, warming sea temperatures over the English Channel during winter significantly affect the date of peak spawning, leading to earlier spawning for the Lusitanian species like the sole *Solea solea* (Fincham et al. 2013), and to later spawning for boreal species like the flounder and halibut (Sims et al. 2005; Brown et al. 2006).

Undetected reproductive endocrine disruption

In this study, no male flounders were intersex, i.e. had gonads with both male and female tissues in 2018, whereas they were 8% in the Seine in 1998 (Minier et al. 2000); this decrease could be related to the general improvement of the Seine water quality during the last decades (Fisson et al. 2014). This lessening in the degree of estrogenic endocrine disruption phenomenon was also suggested in polluted estuaries in the UK, where only 0.5% of the male flounders contained ovotestes (Kleinkauf et al. 2004a).

The presence of vitellogenin (VTG; egg yolk protein) in the male plasma is considered to be a very sensitive biomarker of exposure to exogenous estrogen in fish (Kar et al. 2021). In the present study, the male VTG levels were very low (20 to 25 $ng.mL^{-1}$ in plasma) in Seine and Canche compared to the average concentrations of 125 $\mu g.mL^{-1}$ and 5 $mg.mL^{-1}$ detected in the past, respectively, in the Seine (Minier and Amara 2008) and in heavily polluted UK estuaries (Kleinkauf et al. 2004a); they confirmed the actual low

impact of endocrine disruptors on male gonads of flounder in Seine and Canche.

The flounder steroid plasma levels in Seine and Canche decreased considerably in summer vs winter, with spermatogenesis, vitellogenesis, and oocyte maturation, as classically described in the literature (Blazer et al. 2012). The January female E2 assessed in this study for both estuaries (≈ 3900 pg.mL^{-1}) appeared higher than the one observed in Seine 2001 (1500 pg.mL^{-1}) by Gallien-Landriau (2003); however, we suggest that this limited difference is probably not related to the impact of estrogenic endocrine disruptors on ovaries. The January male 11KT of this study was very close to the Seine 2001 study (Gallien-Landriau 2003) (respectively ≈ 3300 vs 3000 pg.mL^{-1}).

Finally, no major impact of endocrine disruptors on gonads was detected in the flounder populations of Seine and Canche, in the present study.

Biomarkers and metabolic activities

Three biomarkers of damage were explored in the present study (AChE in brain, comet assay in blood, TBARS in gills). The winter lower brain AChE activity detected in Seine vs Canche underlined a higher flounder neurotoxicity induced by the cocktail of pollutants in the Seine system characterized by a very complex mixture of pesticides, metals, detergents and hydrocarbons (Poisson et al. 2011). The AChE level in Seine was particularly stable in autumn and winter, from 2008 to nowadays (AChE activity ≈ 0.2 µmole/min/mg prot) (Burgeot et al. 2017; Borcier et al. 2020; this study).

On the other hand, the blood cell DNA damage assessed by the comet assay in winter showed reduced DNA damages in Seine vs Canche. As numerous chemicals in the field cause DNA damage, the reduced genotoxicity in Seine in winter confirms that in this heavily and chronically contaminated estuary, a selection is acting on the organisms' ability to protect and/or repair their DNA (Marchand et al. 2004; 2013). In the moderately polluted Canche, this selection is probably not active; thus, the flounder displays higher DNA damage.

Pollution induces oxidative stress in aquatic organisms since many pollutants are redox cycling compounds and can induce lipid peroxidation (Lushchak and Bagnyukova 2006). Thus, we suggest that the major fluxes of pollutants in estuaries in winter could explain the higher gill lipid peroxidation in Seine vs Canche.

The EROD activity, a biomarker of defence, showed low values in the Seine and Canche estuaries (EROD activity < 12 pmol/min/mg prot), compared to other polluted but open coastal zones in the Baltic Sea (flounder EROD ≈ 250 pmol/min/mg prot) (Dabrowska et al. 2017). We suggest that the low EROD activity particularly in the Seine estuary could be related to a possible adaptation of the flounder population inhabiting chronically contaminated waters with organic pollutants (Brammell et al. 2013). However, in the present study, the EROD activity remained globally higher in Seine vs Canche, whatever the sex. Furthermore, in Seine and Canche, the reduced female EROD activity in winter vs summer was probably linked to the suppressive effect of the hormone 17 β-estradiol on this activity during the flounder ovary maturation (Kirby et al. 2007).

The glucose-6-phosphate dehydrogenase (G6PDH: a key regulatory enzyme of the pentose-phosphate shunt, essential for the regeneration of NADPH for biotransformation and detoxification reactions) and the Citrate Synthase (CS: a key enzyme in the Krebs cycle) could be considered pertinent proxies of defence against oxidative damage and aerobiosis in flounder populations (Pédron et al. 2017a, 2017b). Furthermore, the CS activity could be considered a proxy of mitochondrial amount in tissues and thus was related to aerobic metabolic rate of fish (Norin and Malte 2012). In the present study, we suggest that the higher liver G6PDH activity in July in Seine vs Canche could be related to the fish response to a marked oxidative stress in the Seine system in summer. A convergent trend was observed for the CS activities in liver and muscle within each estuary, with an increase vs a decrease or maintenance of the activities, respectively, in Canche and Seine, from winter to summer. We hypothesize that a possible impairment in mitochondrial metabolism of the Seine flounders prevents them to increase their metabolic rate in response to the increase of water temperature in July.

Potential relationships between sphingomyelin, phosphatidylserine, and lipid peroxidation

The fish markers integration was carried out within the Canche and Seine estuaries in winter, *i.e.* in the season displaying the highest levels of contaminants in fish tissues. A negative correlation between the group SPG-PS and the lipid peroxidation TBARS was clearly detected in the two estuaries. We suggest that the increase of the particular bioactive lipids sphingomyelin (SPG) and phosphatidylserine (PS) in plasma membrane and organelles could conduct to a better resistance towards the oxidative stress induced in heavily polluted or moderately contaminated sites, respectively, the Seine and the Canche estuary. Thus, the present study confirms the potential role of SPG and PS in the flounder antioxidant defence against pollutants detected in a previous study (Borcier et al. 2020). Furthermore, within the Seine estuary, higher levels of lipid peroxidation were positively and strongly correlated with the histopathology index and the liver G6PDH activity, confirming the possible links between oxidative stress and liver pathology in flounders living in chronically polluted sites (Koehler 2004).

Conclusions

During the flounder reproduction period in winter, a major coastal flow of the English Channel drifts north-eastward in the English Channel, from Le Havre to Dunkirk, carrying pollutants and probably also many flounder eggs and larvae produced by a large spawning site in the Seine mouth estuary. The connectivity between flounder populations over the Eastern-Channel could be enhanced by maximum Seine river discharge, favourable wind conditions, and long flounder larval duration (70 days) (Barbut et al. 2019). Thus, the fluxes of pollutants over the Channel could explain similar metal levels in flounders from the Seine and Canche particularly in winter; high organic pollutants levels in flounder being only detected in the industrial Seine estuary. The overall rise in coastal winter water temperatures detected over the Channel impairs the flounder reproduction phenology in both estuaries, inducing a delay of maturation process and probably also of reproduction. In cold winters (Gallien-Landriau 2003), the flounder reproduction peak was usually detected in February and we suggest now that the spawning period in the Channel could be delayed and extended by several weeks or even months (March–April or possibly later May–June). In the past, the major recruitment event of young of the year juvenile flounders (2–3 cm long) along the French Atlantic estuaries was classically detected in May (Masson 1987). However, this last decade, we observed a second recruitment peak of 2–3 cm juveniles in September. We suggest that flounder embryos produced at the end of the spawning season might have a lower survival over those produced earlier, as observed in the winter flounder in the USA (Buckley et al. 1991). This hypothesis could explain the lowest juvenile flounder abundances associated to hot winters in the UK estuaries (Attrill and Power 2002).

Finally, the present study shows that the warming waters of coastal systems could impair the flounder phenology of reproduction, and possibly its future recruitment in estuaries over the Eastern English Channel. The impact of chemical stress was also clearly detected in the Seine flounder population and was probably not negligible in the Canche population. Furthermore, the Seine juvenile flounders could display a lower tolerance to thermal stress compared to moderately contaminated fish from the Bay of Biscay (Lavergne et al. 2015). Thus, we suggest that the Seine flounder population (and possibly the connected Eastern Channel flounder populations over the French Coast) could be seriously impacted in the future by multistress: higher winter temperatures and chemical contamination.

Author contribution Formal analysis was conducted by J. Laurent, E. Lavergne, J. Couteau, S. Devin, and C. Fisson. Investigations were performed by J. Laurent, J. Couteau, S. Le Floch, B. Ouddane, J. Cachot, B. Davail, C. Clérandeau, A. Devaux, R. Amara, M. Diop, V. Pichereau, and J. Laroche. Ressources were provided by J. Couteau, S. Le Floch, B. Ouddane, J. Cachot, A. Devaux, and R. Amara. The original draft was written by J. Laurent and J. Laroche, then reviewed and edited by E. Lavergne, J. Couteau, J. Cachot, B. Davail, and V. Pichereau. Funding was acquired thanks to R. Amara and J. Laroche. Finally, J. Laroche was in charge of the conceptualisation and the supervision of the project.

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Data availability Not applicable.

Declarations

Ethics approval and consent to participate All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication All authors have read and agreed to the published version of the manuscript.

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