Proteome changes in muscles, ganglia, and gills in Corbicula fluminea clams exposed to crude oil: Relationship with behavioural disturbances

A. Miserazzi, M. Perrigault, M. Sow, C. Gelber, P. Ciret, A.M. Lomenech, J.M. Dalens, C. Weber, S. Le Floch, C. Lacroix, et al.

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Proteome changes in muscles, ganglia, and gills in *Corbicula fluminea* clams exposed to crude oil: relationship with behavioural disturbances.

Miserazzi A.[1,2], Perrigault M.[1,2], Sow M.[1,2], Gelber C.[3], Ciret P.[1,2], Lomenech, A.M[4]., Dalens J.M.[3], Weber C.[3], Le Floch S.[5], Lacroix C.[5], Blanc P.[6], Massabuau J.C.[1,2,*]

[1] University of Bordeaux, EPOC, UMR 5805, Arcachon, France

[2] CNRS, EPOC, UMR 5805, Talence, France

[3] Pôles d'études et de Recherche de Lacq, TOTAL, Lacq, France

[4] Center of Functional Genomics, Bordeaux University, Bordeaux, France

[5] CEDRE, Brest, France

[6] CSTJF, TOTAL SA, Pau, France

[*] Corresponding author: jean-charles.massabuau@u-bordeaux.fr

Highlights

(1). Behavioural response was marked by the evolution of 3 parameters

(2). Valve closure-duration and valve agitation index increase, valve opening-amplitude decrease

(3). Gills were significantly contaminated by PAH but not muscles + ganglia pool

(4). Metabolic and cellular processes increased in gills and decreased in muscles + ganglia pool

(5). Toxicological impacts seem to be greater in gills than in muscles + ganglia pool

ABSTRACT

The use of online remote control for 24/7 behavioural monitoring can play a key role in estimating the environmental status of aquatic ecosystems. Recording the valve activity of bivalve molluscs is a relevant approach in this context. However, a clear understanding of the underlying disturbances associated with behaviour is a key step. In this work, we studied freshwater Asian clams after exposure to crude oil (measured concentration, 167 ± 28 $\mu g \cdot L^{-1}$) for three days in a semi-natural environment using outdoor artificial streams. Three complementary approaches to assess and explore disturbances were used: behaviour by high frequency non-invasive (HFNI) valvometry, tissue contamination with polycyclic aromatic hydrocarbons (PAH), and proteomic analysis. Two tissues were targeted: the pool adductor muscles – retractor pedal muscle – cerebral and visceral ganglia, which is the effector of any valve movement and the gills, which are on the frontline during contamination. The behavioural response was marked by an increase in valve closure-duration, a decrease in valve opening-amplitude and an increase in valve agitation index during opening periods. There was no significant PAH accumulation in the muscle plus nervous ganglia pool, contrary to the situation in the gills, although the latter remained in the low range of data available in literature. Major proteomic changes included (i) a slowdown in metabolic and/or cellular processes in muscles plus ganglia pool associated with minor toxicological effect and (ii) an increase of metabolic and/or cellular processes in gills associated with a greater toxicological effect. The nature of the proteomic changes is discussed in terms of unequal PAH distribution and allows to propose a set of explanatory mechanisms to associate behaviour to underlying physiological changes following oil exposure. First, the first tissues facing contaminated water are the inhalant siphon, the mantle edge and the gills. The routine nervous activity in the visceral ganglia should be modified by nervous information originating from these tissues. Second, the nervous activity in the visceral ganglia could be modified by its own specific contamination. Third, a decrease in nervous activity of the cerebral ganglia close to the mouth, including some kind of narcosis, could contribute to a decrease in visceral ganglia activity via a decrease or blockage of the downward neuromodulation by the cerebro-visceral connective. This whole set of events can explain the decrease of metabolic activity in the adductor muscles, contribute to initiate the catch mechanism and then deeply modify the valve behaviour.

KEYWORDS

Biomonitoring

Oil spill

Proteomics

Behavior

PAH

Asiatic clam

1. INTRODUCTION

Biomonitoring is key in estimating the environmental status of wildlife and their habitats (Goldberg et al., 1978). This is especially true in aquatic ecosystems. Within an environment subject to natural variations and/or deeply disturbed by human activity, recording the behaviour of organisms is a first response tool that requires consolidation to a mature stage. Disrupted behaviour can be used to establish a link between physiological and ecological alterations (Amiard-Triquet, 2009). Today, behavioural ecotoxicology appears reliable in hazard identification, release assessment, exposure assessment, consequence assessment and risk estimation (Hartmann et al., 2016; Pyle and Ford, 2017).

The analysis of valve activity of clams is of particular interest because it allows wireless, 24/7 insight into multiple aspects of the life history of groups of animals over long distances, for 1–3 years (Andrade et al., 2016; Danovaro et al., 2016). Clams are ecologically relevant because most of them are strictly sedentary, sessile, abundant and available year-round. They are filter feeders, which means there is no need to feed them, and using lightweight electrodes and soft cables, one can record artefact-free behaviour readings. Understanding the physiological changes associated with clam-based behavioural monitoring systems is a key step to improving their use for biomonitoring surveys. Hence, proteomic analysis was used in the current study. Protein identification and quantification of individuals subjected to different situations, completed by functional annotations and pathway analysis of proteins, are powerful tools providing access to a global or detailed view of altered biological processes (Campos et al., 2012; Lemos et al., 2009; Van Aggelen et al., 2010). This study was performed on the freshwater bivalve *Corbicula fluminea* in a semi-natural environment within outdoor artificial streams during the first 3 d of crude oil exposure (Miserazzi et al., 2020). Relatively few studies on the effects of crude oil have been carried out in freshwater environments (Bhattacharyya et al., 2003; Green and Trett, 1989; Monteiro et al., 2019). Today, such studies are still in the minority although multiple anthropogenic causes are responsible for oil pollution in freshwaters, including: oil spills, pipeline failure, urban runoff, waste oils or crude oil extraction, etc. (Green and Trett, 1989). Notably, the solubility of crude oil hydrocarbons is generally greater in freshwater than seawater (Eganhouse and Calder, 1975; Green and Trett, 1989; Rossi and Thomas, 1981) and contamination by polycyclic aromatic hydrocarbons (PAH) increases when salinity decreases (Ramachandran et al., 2006; Shukla et al., 2007).

In this study, we first examined key parameters describing the behavioural responses of clams exposed to crude oil by comparison to a reference situation (natural variations only). Thereafter, we focused on the impact of oil in their internal medium by examining tissue that are centrepieces to explain bivalve behaviour, including: (i) the pool adductor muscles-pedal retractor muscles-visceral ganglia-cerebral

ganglia and (ii) the gills. We first investigated their respective PAH contamination status and thereafter, explored associated disturbances of major underlying biological-processes by proteomic analysis. Finally, based on this set of indicators, behaviour, PAH contamination and proteomic analysis, we propose a couple of explanatory mechanisms linking behaviour and disturbance of the internal medium in crude oil exposed bivalve molluscs.

This report is the sister report of a recent work on distinguishable behavioural responses to crude oil in Asian clams *Corbicula fluminea* under semi-natural multiple stress conditions (Miserazzi et al., 2020). In brief, to mimic field conditions and for the purposes of the present experiment, clams were exposed in two outdoor artificial streams that were subject to natural variations and were continuously fed by freshwater from the Gave de Pau river (S.W. France). After a 26-day period, the clams (n = 16 per condition) were separately exposed for an additional period of 10 days to crude oil or natural changes alone. In each stream, a longitudinal survey was performed that involved repeated observations of the same behavioural parameters on the same individuals. The results presented here were obtained by sampling *C. fluminea* exposed for 3 days to crude oil alone or natural changes alone, once the behavioural response curve had flattened. In the presence of crude oil, the behaviour of clams was systematically characterised by an increase in valve-closure duration, a decrease in valve-opening amplitude and an increase in valve agitation index. We present here insights into the nature of the underlying associated proteomic changes.

2. MATERIALS & METHODS

2.1. Clam sampling and maintenance

Asian clams *C. fluminea* (height, 24–28 mm) were collected in the northern part of the Parentis - Biscarrosse lake, France. Clams were first maintained in 600 L tanks in an open circuit of freshwater for 10 d at the laboratory (Marine Biological Station, Arcachon, France). During this maintenance period, they were equipped with electromagnets to study behaviour by HFNI valvometry (Andrade et al., 2016). Thereafter, all clams (for behavioural, proteomic and PAH tissue contamination analyses) were introduced in artificial stream facilities of TOTAL (Lacq, southwest France; Bassères and Tramier, 2001).

2.2. Artificial stream facility

The artificial stream facility is an outdoor experimental site exposed to natural variations and supplied with freshwater from the Gave de Pau River. The water is derived upstream of a dam, flows by gravity in a flow control system, passes through a living organism "nursery" and is identically distributed in 16 experimental streams (length, 40 m; width, 0.5 m; depth, 0.5 m; water depth, 0.25 m) arranged in parallel (Cailleaud et al., 2019; Miserazzi et al., 2020). At the beginning of streams, a piston pump (Prominent) coupled to a high-pressure pump and a shearing valve (Netzsch; Nemo) allows to continuously inject and disperse crude oil in water as micro-droplets. For the requirements of the experiment, in each artificial stream used, quartz and silica sand zones (length, 1 m; depth, 0.1 m) were created to allow the natural burrowing behaviour of clams. The water velocity at clam's level was 6 $cm \cdot s^{-1}$ and the average residence time of water was ≈ 22 min in a stream.

2.3. Experimental protocol

The experiment was carried out from October to December 2016. The experiment was divided into three periods with t_0 being the beginning of the exposure period. Period 1 was a 16 d acclimation period (26th October –10th November) plus a 10 d reference period (11th November – 20th November; t_{-10}–t_{-1}). Period 2 (21st November–1st December; t_0–t_{10}) was the exposure period. The exposure period started on t_0 at 03:15 PM (GMT + 1; 21st November). It ended on t_{10} at 03:15 PM (GMT+1; 1st December). Period 3 (2nd–11th December; t_{11}–t_{20}) was a 10 d post-exposure period. During the experimental period, water temperature varied from 6.6–12.5 °C, O_2 content from 8–13 $mg \cdot L^{-1}$, pH from 6.5–9.5 and conductivity ≈ 200–300 $\mu S \cdot cm^{-1}$. For the requirements of the experiment, only two artificial streams were used. In one stream, clams were exposed to natural variations only. This is the control (CTRL) condition. In the other stream, clams were exposed to crude oil. This is the crude oil (OIL) condition. We have used non-replicated stream conditions due to logistical problems. For OIL condition, crude oil was continuously injected into the stream (6.5 $mL \cdot h^{-1}$) to obtain a nominal concentration of 400 $\mu g \cdot L^{-1}$. In each stream, the behaviour of clams was continuously recorded (n = 16 clams per stream, see section 2.6 for details). These clams were in a first sand zone located at 19–20 m from the crude oil injection. Clams used for tissue sampling were in a second sand zone, located at 29–30 m from the crude oil injection (n = 5 clams per stream for proteomic analysis, see section 2.7 for details; n = 5 clams per stream for contamination analysis, see section 2.8 for details). The spacing between the two zones made it possible to sample in the second zone without disturbing clams in the first zone (Miserazzi et al., 2020).

2.4. Crude oil exposure

2.4.1. Crude oil characteristics

Crude oil was a light oil from the North Sea. Its density was 0.77 (at 15 °C) and dynamic viscosity was 1.016 mPa·s (at 15 °C). Saturates, aromatics, resins and asphaltenes (SARA) analysis indicated that the residue fraction (representing 28.2 % of crude oil, compared to 71.8 % for the distillate fraction) contained 84 % saturated hydrocarbons, 15.5 % aromatic hydrocarbons and 0.5 % polar compounds.

2.4.2. Analysis of crude oil contamination

Crude oil injection rates were checked at t_1, t_3, t_4, t_7, t_8 and t_9. The measured average of crude oil injection rate was 6.6 ± 0.43 $mL \cdot h^{-1}$ (mean ± 1 SE). Water was sampled at t_1, t_3 and t_{10}, 35 m downstream of the crude oil injection in the centre of the stream and in the centre of water column in 1 L glass bottles (see Fig.1 in Miserazzi et al. (2020) for a detailed diagram with location of the sampling point). Water samples were stabilised with methanol and nitric acid. Total petroleum hydrocarbons (TPH) were extracted with 40 mL of hexane and concentrated under nitrogen flux for analysis by gas chromatography with flame-ionization detection (GC-FID), using an Agilent 7890 B GC system (equipped with a 15 m Agilent CP7491 column) and integration of total peak area between C10 and C40. Quality assurance and quality control (QA/QC) were ensured by the calibration curve method (Shrivastava and Gupta, 2011), the analysis of analytical blanks and the analysis of non-contaminated field samples. The mean measured concentration was 167 ± 28 $\mu g \cdot L^{-1}$.

2.6. Behaviour analysis

2.6.1. HFNI valvometry

In each stream, the behaviour of 16 clams was continuously assessed by High Frequency Non-Invasive (HFNI) valvometry (Andrade et al., 2016; Tran et al., 2003 for details). Briefly, a pair of lightweight electromagnets (< 1 g) were glued, face-to-face, on each valve. The voltage variation produced by the electromagnetic current generated between the two electromagnets was recorded every 1.6 s for each bivalve. Daily, data were automatically transmitted to a processing unit (Dell 8 core processor) based in the Marine Biological Station of Arcachon. Data were automatically handled and processed before daily publication on professional pages of the MolluSCAN *eye* website (https://molluscan-eye.epoc.u-bordeaux.fr/).

2.6.2. Behavioural parameters

Hourly valve closure-duration (VCD) and hourly valve opening-amplitude (VOA). For each hour and each bivalve, the VCD (below 5 % of valve opening amplitude) and the VOA were reported in percentage. For example, if the bivalve was opened for 1 h, the percentage of VCD was 0 % and if the bivalve was closed for 1 h, the percentage was 100 %. The hourly VOA values 100 % and 0 % were reported from the maximum and minimum values of valve opening, constantly slipping for each

bivalve over the previous 6 d. Thereafter, these percentages were determined for the group, averaging 16 bivalve values each hour.

Valve agitation index (VAI). An index of valve agitation weighted was calculated by dividing the average hourly distance travelled (mm) by electromagnets (i.e. by the valves) by the percentage of hourly VOA (%) for the group of 16 bivalves (Miserazzi et al., 2020).

2.6.3. Behavioural data treatment

Taking into account (i), the beginning of clam exposure to crude oil (21th November; t_0; 03:15 PM; GMT + 1), (ii) the time for 50 % of clams to react (465 min or 7 h and 45 min; Miserazzi et al., 2020) and (iii), the sampling time of clams exposed to crude oil for proteomic analyses (24th November; t_3; 03:00 PM GMT + 1), the bivalve behaviour was analysed from 21th November at 11:00 PM (GMT + 1; t_0) to 24th November at 03:00 PM (GMT + 1; t_3). It represents a 64 h exposure period (i.e. 2 days and 16 h) under oil-exposed conditions. Behavioural data were processed with R software (R Core Team, 2016). After verification of normality and homoscedasticity of error terms, comparisons between the two independent variables were investigated using the non-parametric Wilcoxon-Mann-Whitney test. Statistical differences were considered significant at $p < 0.05$.

2.7. Proteomic analysis

2.7.1. Sampling and dissections of clams

Five clams exposed to crude oil (OIL) and five clams exposed to natural variations only (CTRL) were dissected immediately after collection from artificial streams. Clams in OIL condition were dissected at 03:00 PM (GMT + 1) on t_3 (24th November). Clams in CTRL condition were dissected on the same day at 01:00 PM (GMT + 1). Muscles plus ganglia pool (n = 5) and gills (n = 5) were sampled and stored at -20 °C for 1 d and then at -80 °C until proteomic analysis was performed.

2.7.2. Sample preparation and protein digestion

Total protein extraction was performed by classical TCA-acetone method. Protein samples were solubilised in Laëmmli buffer and 10 µg per sample was deposited onto an SDS-PAGE gel for concentration and cleaning purposes. Separation was stopped once proteins entered the resolving gel. After colloidal blue staining, bands were cut out from the SDS-PAGE gel and subsequently cut in 1 mm × 1 mm gel pieces. Gel pieces were destained in 25 mM ammonium bicarbonate 50 % ACN, rinsed twice in ultrapure water and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature, covered with trypsin solution (10 ng·µl^{-1} in 50 mM NH_4HCO_3), rehydrated at 4 °C for 10 min, and finally incubated overnight at 37 °C. Spots were then incubated for 15 min in 50 mM NH_4HCO_3 at room temperature with rotary shaking. The supernatant was collected, and an H_2O/ACN/HCOOH (47.5:47.5:5) extraction solution was added onto gel slices for 15 min. The

extraction step was repeated twice. Supernatants were pooled and dried in a vacuum centrifuge. Digests were finally solubilised in 0.1 % HCOOH.

2.7.3. nLC-MS/MS analysis

Peptide mixture was analysed on an Ultimate 3000 nanoLC system (Dionex, Amsterdam, The Netherlands) coupled to a Electrospray Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA). Ten microliters of peptide digests were loaded onto a 300-µm-inner diameter × 5 mm C_{18} PepMap™ trap column (LC Packings) at a flow rate of 10 µL·min^{-1}. Peptides were eluted from the trap column onto analytical 75-mm id × 50-cm C18 Pep-Map column (LC Packings) with a 4–40 % linear gradient of solvent B in 108 min (solvent A was 0.1 % formic acid and solvent B was 0.1 % formic acid in 80 % ACN). The separation flow rate was set at 300 nL·min^{-1}. The mass spectrometer operated in positive ion mode at a 2 kV needle voltage. Data were acquired using Xcalibur 4.1 software in a data-dependent mode. MS scans (*m/z* 375-1500) were recorded at a resolution of R = 120 000 (@ m/z 200) and an automatic gain control (AGC) target of 4 × 10^5 ions collected within 50 ms. Dynamic exclusion was set to 60 s and top speed fragmentation in higher-energy collision dissociation cell (HCD) mode was performed over a 3 s cycle. MS/MS scans with a target value of 3 × 10^3 ions were collected in orbitrap (with a resolution of R = 30 000 (@ m/z 200)) with a maximum fill time of 54 ms. Additionally, only + 2 to + 7 charged ions were selected for fragmentation. Other settings were as follows: no sheath nor auxiliary gas flow, heated capillary temperature, 275 °C; normalised HCD collision energy of 30 % and an isolation width of 1.6 m/z. Monoisotopic precursor selection (MIPS) was set to Peptide and an intensity threshold was set to 2.5 × 10^4.

2.7.4. Database search and results processing

Data were searched by SEQUEST through Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc.) against a laboratory made protein database (69376 entries). Spectra from peptides higher than 5000 Da or lower than 350 Da were rejected. The search parameters were as follows: mass accuracy of the monoisotopic peptide precursor and peptide fragments set to 10 ppm and 0.6 Da respectively. Only b- and y-ions were considered for mass calculation. Oxidation of methionines (+ 16 Da) and protein N-terminal Acetylation (+ 42 Da) were considered as variable modifications and carbamidomethylation of cysteines (+ 57 Da) as fixed modification. Two missed trypsin cleavages were allowed. Peptide validation was performed using the Percolator algorithm (Käll et al., 2007) and only "high confidence" peptides were retained, corresponding to a 1 % False Positive Rate at the peptide level.

2.7.5. Label-Free Quantitative Data Analysis

Raw LC-MS/MS data from muscles plus ganglia pool and gills were imported in Progenesis QI for Proteomics 2.0 (Nonlinear Dynamics Ltd, Newcastle, U.K) in two separate datasets. Data processing

includes the following steps: (i) feature detection, (ii) feature alignment across 10 samples, (iii) volume integration for 2–6 charge-state ions, (iv) normalisation on feature ratio median, (v) import of sequence information, and (vi) calculation of protein abundance (sum of the volume of corresponding peptides). Then, data were processed with Perseus (version 1.6.2.3; Tyanova et al., 2016). Data analysis was partially based on the instructions published by Perseus developers (Tyanova and Cox, 2018). Normalised intensities were uploaded in the software, filtered to retain only quantified proteins in at least four out of five samples in at least one group (OIL or CTRL). Thereafter, the remaining missing values were replaced from normal distribution by maintaining the default settings. An additional filtration was performed to remove proteins labelled as "contaminant". A $\log_2$ transformation was subsequently performed for each sample. Data reliability was assessed by histogram visualisation and multi-scatter plot with Pearson correlation for each sample. For each protein, statistical analysis was performed between OIL (n = 5) and CTRL (n = 5) samples with a student t-test at FDR (False Discovery Rate; q-value) < 0.2 for muscles plus ganglia pool or < 0.25 for gills (Benjamini and Hochberg, 1995). For clustering only, protein abundances were normalised with Z-score. To calculate the protein abundance ratio between both conditions, normalised $\log_2$ intensities (before Z-scoring) was averaged for OIL (n = 5) and CRTL samples (n = 5). Fold change (FC) was obtained by the $2^{[\log2\ (\text{OIL mean}) - \log2\ (\text{CTRL mean})]}$ operation. To determine which pathways were involved by significant proteins, data were processed with Cytoscape software (version 3.7.1; Shannon et al., 2003) and ClueGO (version 2.5.4; Bindea et al., 2009) plus CluePedia (version 1.5.4; Bindea et al., 2013) plug-ins. Analyses were carried out independently for both tissues and protein clusters (i.e. proteins with highest or lowest abundance). Gene identifiers in the oyster *Crassostrea gigas*, corresponding to selected proteins, were uploaded in the software instead of the database on *Corbicula fluminea* (Chen et al., 2013). We used the *C. gigas* database because it is considered as the most complete reference transcriptome today in aquatic invertebrates. It was realized using embryo-larval development stages and adult organs as well and a large number of environmental stressors (Rivière et al., 2015; Zhang et al., 2012). Gene Ontology (GO) databases for Biological Processes (BP) were applied for gene annotation. All enriched terms were selected using the right-sided hypergeometric test with Benjamini-Hochberg (BH) adjustment at $p < 0.05$. Term fusion was activated for all protein clusters. The network was designed with a kappa score of at least 0.4. The ClueGO Layout was applied and manually adjusted in a minimal way.

2.8. Clams contamination

2.8.1. Sampling and dissections of clams

Sampling and dissections of clams for analysis of tissue contamination were performed on t_{10} (1st December; ≈ 02:00 PM; GMT + 1). Five clams exposed to crude oil (OIL) and five clams exposed to

natural variations only (CTRL) were dissected immediately after collection from artificial streams. Gills (n = 5) and muscles plus ganglia pool (n = 5) were stored at -20 °C until analysis was performed. Tissue contamination levels were analysed at t_{10} for analytical reasons (low tissue mass and low expected concentrations levels). However, based on literature data, it was assumed that contamination levels by PAH at t_{10} overestimate the contamination level at t_3 (Foster et al., 1987; Liu et al., 2014; Mason, 1988; Pruell et al., 1986; Stegeman and Teal, 1973; van Haren et al., 1994).

2.8.2. PAH analysis

In brief, 21 PAH (acenaphthylene, acenaphthene, anthracene, benzo(a)anthracene, benzothiophene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene, biphenyl, chrysene, dibenzo(a,h)anthracene, dibenzothiophene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, perylene, phenanthrene, and pyrene), among which, 16 listed as priority pollutants by the US EPA (United States Environmental Protection Agency; underlined in the list) were analysed in muscles plus ganglia pool (n = 5) and gills (n = 5) of five clams per condition (Miserazzi et al., 2020). The analyses were performed by stir bar sorptive extraction-thermal desorption-gas chromatography-tandem mass spectrometry (SBSE-GC-MS/MS) as described in Lacroix et al. (2014). Analytes were quantified relative to deuterated compounds using a calibration curve ranging from 0.01 ng to 10 ng per bar. Two compounds, benzo(b)fluoranthene and benzo(k)fluoranthene, were quantified as a sum named benzo(b+k)fluoranthene due to poor resolution. The limits of quantification (LOQ) were calculated by the calibration curve method (Shrivastava and Gupta, 2011), and the limit of detection (LOD) was estimated by dividing the LOQ by 3.

2.8.3. Statistics

Tissue contamination data were processed with R software (R Core Team, 2016). After verification of normality and homoscedasticity of error terms, comparisons between the two independent variables were investigated using the non-parametric Wilcoxon-Mann-Whitney test. Statistical differences were considered significant at $p < 0.05$.

3. RESULTS

No mortality occurred during the entire experiment (acclimation period, reference period, exposure period and post-exposure period).

3.1. Behavioural disturbances and tissue contamination

The behaviour of clams was deeply modified by the presence of crude oil. It was characterised by three major changes: an increase of valve closure duration, VCD (Fig. 1A), a decrease of valve opening amplitude, VOA (Fig. 1B) and an increase of valve agitation index, VAI (Fig. 1C). The hourly VCD was 9.5 times greater in the presence of crude oil (Fig. 1A). The hourly VOA was 5.3 times lesser (Fig. 1B) and the hourly VAI was 2.8 times greater (Fig. 1C). The level of contamination in muscles plus ganglia pool and in gills after a 10 d exposure period is presented in Fig. 1D. All values are in the low range. The median contamination level in muscles plus ganglia pool was not different between exposed and control clams (6.1 and 7.4 $ng \cdot g^{-1}$ ww). In contrast, it was different in gills. It was 70.2 $ng \cdot g^{-1}$ ww in gills of OIL clams and 18.9 $ng \cdot g^{-1}$ in CTRL clams.

3.2. Analysis of proteomic changes

A total of 1860 proteins were quantified in muscles plus ganglia pool and 5469 in gills. Among them, in the muscles plus ganglia pool, 139 showed a significant difference between the both conditions (7.5 %; FDR < 0.2) and 144 in gills (2.6 %; FDR < 0.25). As an exploratory analysis, Principal Component Analyses (PCA) was applied to significantly changing proteins. PCA showed a clear individualisation according to the experimental conditions, which explained 81.3 % (Component 1, 74.2 % + Component 2, 7.1 %) variability in the muscles plus ganglia pool (Fig. 2A) and 80 % (Component 1, 74 % + Component 2, 6 %) in the gills (Fig. 2B). Thereafter, we turned to functional analyses (hierarchical clustering and corresponding pathways analysis) to explore observed differences (Figs. 3A, 3B, 3C and 4A, 4B, 4C). Firstly, in agreement with the PCA, upper vertical clustering shows a clear segregation between crude oil and control conditions (OIL, five left-side columns; CTRL, five right-side columns) in muscles plus ganglia pool (Fig. 3A) and in gills (Fig. 4A). In hierarchical clustering, each horizontal line represents one differentially expressed protein. In both tissues, horizontal hierarchical clustering revealed two major row clusters: in the upper ones, proteins were less abundant in the presence of crude oil and in the lower one, proteins were more abundant in the presence of crude oil. Fig. 3A shows that in muscles plus ganglia pool, 90 proteins (65 % of the total) were less abundant and 49 proteins (35 % of the total) were more abundant in crude-oil exposed clams. In contrast, in gills (Fig. 4A), only 59 proteins (i.e. 41 % of the total) were less abundant in crude-oil exposed clams while 85 proteins (59 % of the total) were more abundant. Thereafter, a functional analysis was performed for each row cluster within the two tissues (Figs. 3B, 3C and 4B, 4C). In muscles plus ganglia pool, biological processes associated to less abundant proteins (Fig. 3B) were relatively numerous. There were essentially related to metabolic and/or cellular activities namely generation of precursor metabolites and energy, small molecule metabolic process, organonitrogen compound metabolic process, cellular ketone metabolic process, alpha-ketoacid dehydrogenase activity, proteolysis and glutamate biosynthetic process. GO enrichment analysis also indicated that

this set of proteins was associated with the biological regulation of neurotransmitter levels. The most abundant proteins were statistically represented through the categorical term system process, which makes it possible to establish a bridge between regulation of muscle contraction and nervous system through the term synapse assembly (Fig. 3C). The less abundant proteins in oil-contaminated gills were statistically enriched only through two biological processes, negative regulation of endopeptidase activity and regulation of hydrolase activity (Fig. 4B). The functional network associated to proteins more abundant in contaminated gills was relatively richer and the GO terms were also mainly related to metabolic and/or cellular processes (Fig. 4C). More precisely, GO terms were related to cell signalling pathway with activation of MAPKK activity, to energy or nucleotide metabolism with tricarboxylic acid cycle, GTP metabolic process or nucleoside diphosphate phosphorylation. Finally, GO terms were also related to cell signal transduction with adenylate cyclase-modulating G-protein coupled receptor signalling pathway and to protein processing with the term protein polymerization.

4. DISCUSSION

In the current study, we used three complementary approaches to further increase analytical and empirical understanding of crude oil-induced behaviour in the Asian clam *C. fluminea*: behaviour, PAH contamination and proteomics. The behavioural response of clams exposed to crude oil was unequivocal. It was characterised by a significant increase of VCD, a significant decrease of VOA and a significant increase of VAI (Figs. 1A, 1B, 1C). We sought, through a proteomic analysis focused on two target tissues (the adductor muscles – pedal retractor muscles – visceral ganglia – cerebral ganglia pool and the gills), to explore the underlying disturbances that occur in the internal medium of exposed clams. To complete the analysis, we used data on PAH accumulation that showed significant contamination in gills but no contamination in muscles plus ganglia pool. Importantly, these contamination levels were in the low range of literature data (Webster et al., 2006). The major proteomic changes included a decrease of metabolic and/or cellular processes and nervous functions in muscles plus ganglia pool with a minor toxicological impact, and an increase of metabolic and/or cellular processes in gills with a higher toxicological impact.

4.1 Non-replicated stream conditions

In this work, we have used non-replicated stream conditions due to logistical problems but we individually analysed five Asian clams exposed to crude oil in one stream and five other individuals exposed to control conditions in another stream. A PCA analysis showed first a clear individualisation

between streams (Figs. 2A and 2B). Then we turned to functional analysis and upper vertical clustering that also showed a clear segregation between crude oil and control streams (Figs. 3A and 4A). Thus, despite the use of only one stream per treatment, that is a standard situation in field sampling, our data set shows the amplitude of individual variations and strongly supports significant differences between oil and control conditions.

4.2. The target tissues

We focused our interest on the muscles plus ganglia pool and the gills for evaluating their functional role in setting out Asian clam's behaviour.

The pool was composed of the anterior and posterior adductor muscles (which is a mix of striated and smooth muscles), anterior and posterior pedal retractor muscles, paired cerebral ganglia, paired visceral ganglia and its innervations to muscles (Kraemer and Lott, 1977; Britton and Morton 1982). In *C. fluminea*, cerebral ganglia and visceral ganglia are two of the three major ganglia in the nervous system. Cerebral ganglia lie flat against the anterior pedal muscles while visceral ganglia nestle in posterior pedal muscles. Visceral ganglia innervate gills through a gill nerve while cerebral ganglia are connected to the digestive system (oesophagus, stomach, intestine, etc.) and pedal ganglia. The visceral and cerebral ganglia are also interconnected with each other. In bivalves, adductor muscles are mechanical effectors of valve movements with the elastic ligament situated along the edge of the hinge between the valves. When these muscles are impacted, the valve activity is expected to be modified and *vice versa*. The fast muscle (or striated muscle) is responsible for rapid contractions, while the slow muscle (or smooth muscle) is responsible for the state of catch, which is the maintenance of valve closure for long periods with minimal energy demand (Sun et al., 2018).

Gills were sampled because they are, together with the inhalant siphon and the mantle edge, on the frontline during any contamination. Gill tissue is particularly complex, and composed of respiratory exchange tissue, ion exchange tissue, immune tissue, ciliary and muscles for water movements and feeding purposes (Britton and Morton, 1982; Gainey et al., 2003; Medler and Silverman, 2001; Mommsen, 1984). It is richly innervated via a branchial nerve originating from the visceral ganglia (Britton and Morton, 1982; Kraemer and Lott, 1977).

4.3. Disturbances in muscles + ganglia pool

4.3.1. Proteins with a lower abundance

Metabolic activity. Within the muscles plus ganglia pool (Fig. 3), proteins with a lower abundance were a majority. These proteins were mainly involved in metabolic processes. This strongly suggests a decrease in metabolic activity, which is consistent with the change in behavioural activity. Indeed, in exposed clams, there was an increase in valve closing duration and thus an increase in the catch

mechanism. In bivalves, catch is a passive state of the smooth muscle that allow valves to remain closed for long periods with minimal energy consumption (Galler, 2008; Yamada et al., 2013). The valve opening amplitude was also lower, which is consistent with the decline in valve activity. Altogether, this behaviour represents probably a defence mechanism to limit exposure of the inner soft tissues. With regard to the VAI, exposed clams were more agitated when opened. However, valve closing duration was significantly greater in exposed clams, suggesting that transient valve agitation behaviour has a limited weight in the overall energetic balance of valve activity.

Neurotransmitters. Taking a close look at the lower abundant proteins impacted in the muscles plus ganglia pool, it can be seen that some elements were linked to the metabolism of glutamate. Glutamate is a major metabolic hub involved in diverse processes (Walker and van der Donk, 2016) and, is an excitatory neurotransmitter, whose deregulation can lead to some toxicity in the nervous system (Choi, 1988; Wu and Wang, 2010). The network analysis of lower abundant proteins highlights the disruption of the "glutamate biosynthetic process" and, interestingly, glutamate synthase (FC, 0.018; FDR, 0.082) was found among the top five of less abundant proteins in this tissue pool. Its decrease decreases glutamate synthesis (from 2-oxoglutarate, an intermediate of the tricarboxylic acid cycle), which is in agreement with results from Müller et al. (2018) on oysters. These authors observed a decrease in glutamate 4 receptor abundance in gill microsomes of oysters *Crassostrea brasiliana* exposed during 24 h to water contaminated by diesel fuel.

With regard to the regulation of neurotransmitter levels, we identified two other proteins through network analysis of lower abundant proteins: synaptotagmin-1 (FC, 0.12; FDR, 0.187) and 4-aminobutyrate aminotransferase or gamma-aminobutyric acid (GABA) transaminase (FC, 0.08; FDR, 0.161). Synaptotagmin-1 plays an important role in neurotransmission by being a major sensor of Ca^{2+} within excitatory synapses (Brose et al., 1992; Chapman, 2002). A decrease of synaptotagmin-1 suggests a decrease of excitatory synapse activity. This protein is also implicated, but to a lesser extent, in the transmission of the nervous message within GABAergic neurons (Kerr et al., 2008). GABA transaminase allows the synthesis of glutamate from 2-oxoglutarate - an intermediate of the tricarboxylic acid cycle - and from GABA. Its lower abundance is consistent with the lower abundance of glutamate synthase, thus supporting the hypothesis of a decrease in glutamate synthesis. Finally, this protein is a key enzyme in GABA catabolism (Walls et al., 2015), a neurotransmitter that inhibits the nervous system of vertebrates and invertebrates (Lunt, 1991; Miller, 2019). We suggest that inhibition of this enzyme, which leads to increased levels of GABA (inhibitor neurotransmitter; Abel and Kohli, 1999), and inhibition of glutamate synthase, responsible for glutamate synthesis (excitatory neurotransmitter), should contribute to the decrease in clam's activity in the presence of crude oil. Indeed, whether in vertebrates or invertebrates, GABA is able to regulate locomotor patterns by modulating underlying neural circuits (Shen et al., 2016). In addition, the change in abundance of the enzyme GABA transaminase in the brain could cause mitochondrial muscle disruption, independent of the GABAergic system. Besse et al. (2015) showed that a deficiency of this enzyme in the brain can

cause mitochondrial DNA (mtDNA) depletion, a syndrome associated with various symptoms including muscle weakness and hypotonia. Note, all this forms a coherent whole with the tissue pool analysed and the decrease in behavioural activity.

Mitochondrial process. The most impacted protein, in the muscles plus ganglia pool, is the ClpX protein (FC, 0.001; FDR, 0.024). This protein belongs to the AAA+ superfamily (ATPases Associated with various cellular Activities), a family of proteins that is found in all kingdoms of life and participates in a wide range of biological processes (Ammelburg et al., 2006; Ogura and Wilkinson, 2001; Sysoeva, 2016). More specifically, ClpX protein is involved in dependent ATP proteolysis by being a component of Clp proteases (Baker and Sauer, 2012). It is involved in mitochondrial processes by affecting the distribution and morphology of mtDNA nucleoids (Bogenhagen et al., 2008; Kasashima et al., 2012; Ogura and Wilkinson, 2001). Its lower abundance highlights an effect on proteolysis, in a coherent way with the network analysis of the lower abundant proteins in the muscles plus ganglia pool as well as a potential mitochondrial dysfunction due to the stress generated on the mtADN. In turn, this mitochondrial dysfunction can be apprehended by network analysis, which shows a decrease in the "generation of precursor metabolites and energy". The three proteins associated with this process within the network were electron transfer flavoprotein-ubiquinone oxidoreductase (FC, 0.689; FDR, 0.147), an enzyme from the respiratory electron transport chain, succinyl-CoA ligase[GDP-forming] subunit beta (FC, 0.06; FDR, 0.104), a tricarboxylic acid cycle enzyme, and xanthine dehydrogenase (FC, 0.107; FDR, 0.168), an enzyme leading to the generation of reactive oxygen species (ROS). A lower abundance of xanthine dehydrogenase has already been shown in gills of oysters *Crassostrea gigas* exposed to various stresses: thermal stress, hypo-salinity and aerial exposure (Zhang et al., 2015). The lower expression of these three proteins suggests a decrease in mitochondrial activity, a decrease in number of mitochondria, or both simultaneously. Recently, several studies highlighted the effect of crude oil on mitochondria, particularly concerning their role in aerobic metabolism (Johansen and Esbaugh, 2019; Kirby et al., 2019; Salazar-Coria et al., 2019). Following a 24 h exposure period to crude oil, Kirby et al. (2019) proposed that crude oil may alter the mitochondrial respiratory chain, thus the ATP supply of myocardial cells in mahi-mahi (*Coryphaena hippurus*). This dysfunction was also implied in the impairment of cardiac muscle function observed in the presence of oil in fish (Cox et al., 2017; Johansen and Esbaugh, 2019; Kirby et al., 2019; Nelson et al., 2016; Nelson et al., 2017;). We suggest that the dysfunction of mitochondrial processes within the muscles plus ganglia pool could be at the origin of the decrease, or at least, the alteration in muscular and nervous function of clams, and therefore, by extension, in their behavioural activity.

4.3.2. Proteins with a higher abundance

Neuromuscular synapses. The detail of higher abundant proteins impacted in the muscles plus ganglia pool shows some disturbances in the nervous system through the "synapse assembly" process as well

as a disturbance of the muscular system with the "regulation of muscle contraction" processes. The alteration of the "synapse assembly" process indicates a disruption in the formation of synapses, thus in the potential number of mature and functional synapses. Disruption of muscle contraction involved the disruption of any process able to modulate frequency and/or the extent of muscle activity. The protein linking the synapse assembly process and the system process was neurexin 1α. Neurexins are transmembrane proteins located mainly at synaptic extremities of neurons and are involved in neurotransmission and formation of synapses (Reissner et al., 2013). Kang et al. (2007) found that α-neurexins promote recruitment and stabilisation of GABAergic synapses. This again supports the role of GABA activity in the global increase of valve closing duration in oil-contaminated clams. Concerning the regulation of muscle contraction, the protein involved was troponin T of skeletal muscle. In the adductor muscle of bivalve molluscs, striated muscle is responsible for rapid valve contractions (Chantler, 2006; Millman, 1967; Sun et al., 2018). Within this tissue, troponin T will bind troponin I and troponin C to tropomyosin (Farah and Reinach, 1995; Tanaka et al., 2008; Zot and Potter, 1987). To conclude, the whole data set above showed that both nervous and muscle tissues were impacted by the inspired crude oil.

4.3.3. Stress markers and oxidative stress

In their meta-analysis of the literature, Anderson et al. (2015) identified the top five genes most commonly affected by environmental stressors. In the category "intracellular stress and detoxification", we find genes encoding superoxide dismutase, metallothioneins, glutathione-S-transferases, heat shock protein (HSP) 70's and glutathione peroxidases. Among these key stress markers, in the muscles plus ganglia pool, three were present: a chaperone protein, the heat shock cognate 71 kDa protein (FC, 1.58; FDR, 0.053) and two enzymes involved in defence against oxidative stress, superoxide dismutase Cu/Zn (FC, 16.5; FDR, 0.151) and glutathione peroxidase (FC, 0.119; FDR, 0.196). Cu/Zn superoxide dismutase catalyses the transformation of the superoxide anion into hydrogen peroxide, outside of the mitochondrial matrix. Its higher abundance would indicate an excess of the superoxide anion (despite global decrease in metabolic activity). Glutathione peroxidase catalyses the transformation of hydrogen peroxide into water within the mitochondrial matrix. Its lower abundance consequently indicates either a deficit in hydrogen peroxide within the mitochondrial matrix or a failure in reducing hydrogen peroxide toxicity. Regardless, the respective abundance of these two enzymes suggests an excess of hydrogen peroxide, a key metabolite of oxidative stress. Hydrogen peroxide is a messenger of the redox signal at the enzymatic and transcriptional levels (Sies, 2017). It is able to pass through biological membranes, affect other molecules (lipids, proteins, DNA), whether near or distant from its original site, and interfere with other biochemical reactions (Tomanek, 2015). In a manner coherent with the literature (Baussant et al., 2009; Boutet et al., 2004; Jiang et al., 2017; López-Landavery et al., 2019 for examples in bivalves), there was a disruption of enzymes involved in defence against oxidative stress in response to crude oil exposure.

4.3.4. A specific presence of PAH in the nervous system?

Multiple enzymatic responses to oxidative stress were shown in the muscle plus ganglia pool despite an absence of local PAH accumulation in this specific tissue set. However, the non-significant accumulation in the muscles plus ganglia pool (Fig. 1D) should not be directly interpreted as an absence in ganglia. Indeed, the fraction of nervous tissue was minor in our samples. An unequal PAH distribution between muscle and ganglia could occur. Lipid content is not the only biological determinant governing PAH accumulation in tissue (Frapiccini et al., 2018), but the nervous tissue is made of ≈ 80 % of lipids in invertebrates and vertebrates (McColl and Rossiter, 1951), while there are only ≈ 4 % of lipids in the adductor muscles in bivalve molluscs (Dongre and Sonwane, 2014). We suggest in bivalves, that the nervous tissue could be a PAH sink as in fishes. In the latter, the brain accumulates largest quantities of PAH by comparison to muscles (Wu et al., 2012; Xu et al., 2011). However, considering the low amount of tissue available for PAH analysis, the latter remains an open challenge in small bivalve molluscs such as *Corbicula*.

4.4. Disturbances in gills

In gills, where accumulation of PAH was highly significant, there was a deregulation of two HSPs 70's only: 12A (FC, 1.23; FDR, 0.114) and 12B (FC, 0.079; FDR, 0.153), that are among the top five most commonly affected genes in the category "intracellular stress and detoxification" listed by Anderson et al. (2015). HSP plays a key role in protein regulation and were considered proteins produced or deregulated by all cellular organisms in a stressful situation (Roberts et al., 2010). However, network analysis of higher abundant proteins also shows the activation of MAPKK activity (mitogen-activated protein kinase kinase; Fig. 4). MAPKK is part of the MAPK (mitogen-activated protein kinase) phosphorelay system, a group of serine/threonine kinases that participate in gene expression regulation, mitosis, movement, metabolism and apoptosis (Johnson and Lapadat, 2002). Multiple studies have highlighted the disruption of this pathway in the presence of hydrocarbons or other pollutants in aquatic organisms (Burlando et al., 2006; Châtel et al., 2011; Won et al., 2016). In addition, we were able to notice the presence of other proteins reflecting (i) the disruption of the cell cycle, (ii) the disruption of the apoptotic mechanism or (iii) the accumulation of potential DNA damage. RecQ1, a protein that plays an important role in cellular DNA metabolism (Wu and Brosh, 2010) by being involved in maintaining genome stability, was lower abundant in exposed clams (FC, 0.064; FDR, 0.248). There was also the lower abundance (FC, 0.041; FDR, 0.193) of Cdc5-like protein, a DNA-binding protein involved in the regulation of the mitotic cell cycle and in DNA damage response (Bernstein and Coughlin, 1998; Mu et al., 2014). Lastly, caspase 7, a protease involved in programmed cell death and the inflammatory process (Lamkanfi and Kanneganti, 2010),

was lower abundant (FC, 0.427; FDR, 0.223). Finally, there was the higher abundance of the tumour protein D54 (FC, 1.90; FDR, 0.218) which belongs to the protein family D52 (Nourse et al., 1998). Proteins in this family, in addition to being involved or suspected of being involved in many cellular processes, are frequently overexpressed in multiple cancers where they are thought to play a role in cell proliferation and thus promote tumour progression (Boutros et al., 2004). In addition, network analysis of higher abundant proteins in gills show a general increase in energy and nucleotide metabolism, an increase in transduction of cellular signals and an increase in protein processing. The analysis thus shows an increase in cellular activity in gills including a substantial mobilisation of energy resources. This increase may be necessary to promote cell maintenance in response to crude oil exposure and PAH accumulation in gills of clams after a 3 d exposure period. This general metabolic change could also promote or support the potential carcinogenesis process initiated in this tissue (Lunt and Fendt, 2018).

4.5. Mechanisms that may explain the behavioural response to crude oil

To conclude and propose a link between behavioural observations, proteome modifications and PAH contamination reported in this study, we suggest two mechanisms or scenarios to relate behavioural disturbances to those of the internal medium (Figs. 5A, 5B, 5C). Before that, one must recall that in clams, the first tissue facing contaminated water are the inhalant siphon and to a lesser extent the rest of the mantle edge (Fig. 5A). These areas have many sensory papillae or tentacles capable of tasting the ambient water and thus leading to the valve closure (Britton and Morton, 1982). In the first scenario (Fig. 5B), the routine activity of visceral ganglia would be disrupted first by nervous information generated by the contact of the contaminants with sensory papillae of the inhaling siphon and/or mantle edges and then the contaminated gills. In a second step, the nervous activity of visceral ganglia could be disrupted by its own specific contamination. According to proteomic analyses within the muscles plus ganglia pool, the list of disturbances includes a decrease of nervous and metabolic activity. We propose that the decrease of nervous activity in visceral ganglia participated to decrease metabolic activity in muscles, to decrease valve activity and contributed to the catch mechanism. In the second scenario (Fig. 5C), we include a neuromodulatory role for cerebral ganglia. The nervous activity of the cerebral ganglia could also be disrupted by its own specific contamination. Moreover, cerebral ganglia have a special location near the mouth, which exposes them more to hydrocarbon compounds. Indeed, all contaminated mucus collected on gills converges to the mouth and then to the cerebral ganglia area, which could be affected by a passive diffusion of hydrocarbons. The decrease in nervous activity of the cerebral ganglia, including some kind of narcosis, would cause an additional decrease in nervous activity of visceral ganglia via a decrease or blockage of neuromodulation (mechanism reviewed by Hamood and Marder, 2014). This would

630 strengthen the effects already presented in the first scenario on visceral ganglia, muscles and thus
631 valve activity.

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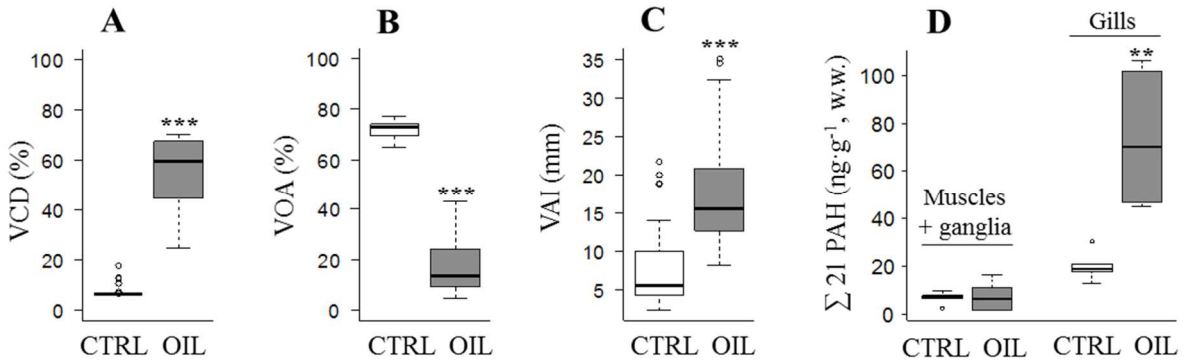
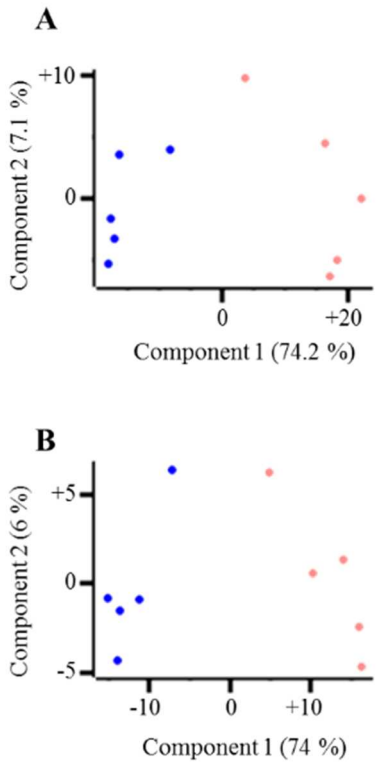


Fig. 1. Behavioural analysis and tissue contamination. (A) Mean hourly valve closure-duration VCD (%; n = 64 for 64 h), (B) mean hourly valve opening-amplitude VOA (%; n = 64 for 64 h), (C) mean hourly valve agitation index VAI (mm; n = 64 for 64 h) of 16 clams by condition, (D) sum of individual concentrations of 21 PAH analysed in muscles plus ganglia pool and gills after 10 d of exposure (ng·g^{-1}, wet weight; n = 5 clams per condition. Redrawn from Fig. 7 in Miserazzi et al., 2020). Data are given as a boxplot. The top line, middle line and lower line of the box indicate upper quartile, median and lower quartile respectively. The ends of the whiskers indicate the lowest and highest values. The dots are outliers. **, $p < 0.01$; ***, $p < 0.001$; Wilcoxon-Mann-Whitney. CTRL, clams under control condition; OIL, clams exposed to crude oil.



666 **Fig. 2. Principal Component Analysis.** PCA performed with significant proteins in (A), muscles plus
667 ganglia pool and (B) gills. It clearly separates the five OIL clams (exposed to crude oil; pink circles,
668 on the right) from the five CTRL clams (control conditions; blue circles, on the left).

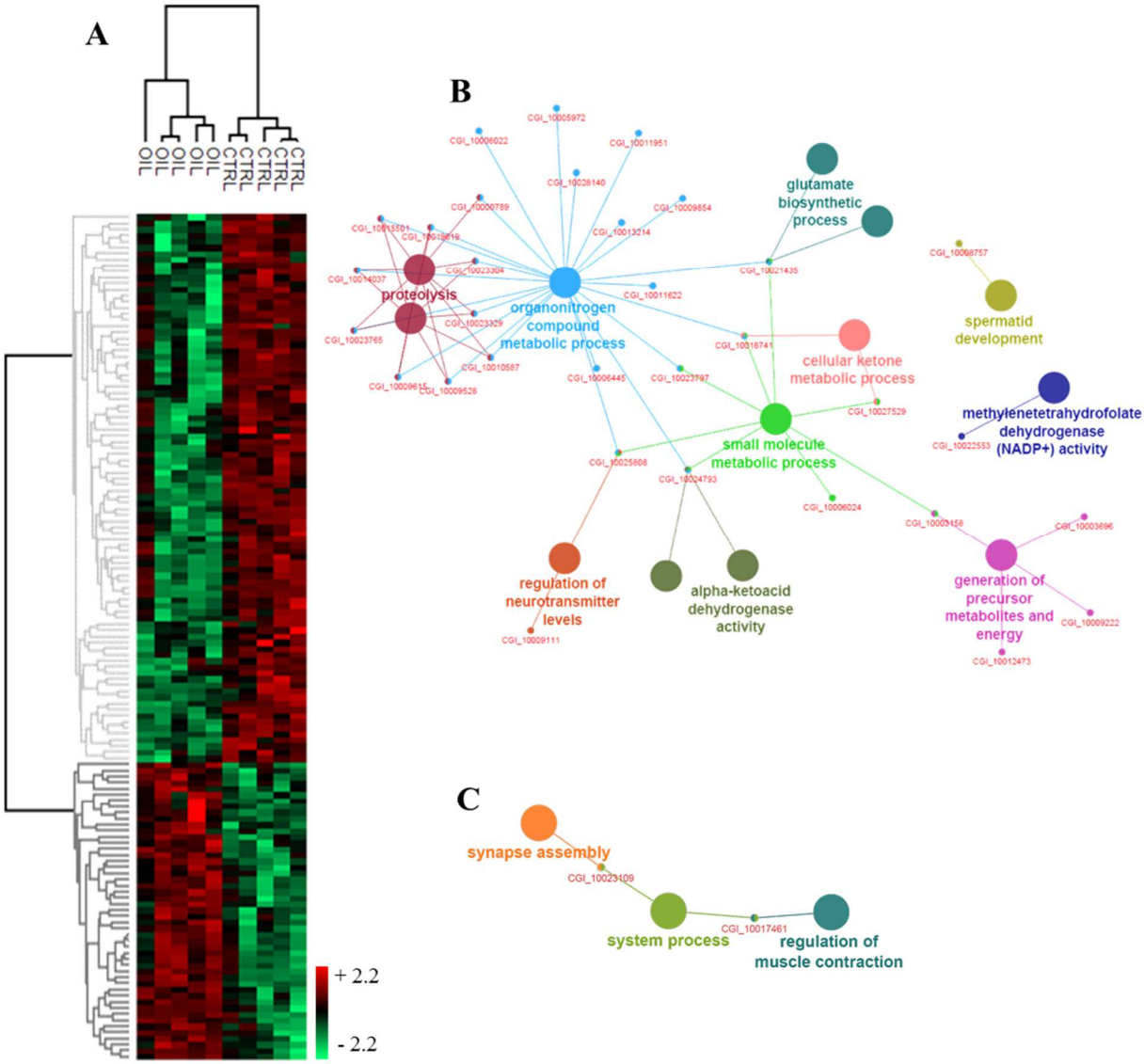


Fig. 3. Differential proteomic analysis performed in muscles plus ganglia pool of crude oil exposed clams vs control. (A) Hierarchical clustering with significant proteins (FDR < 0.2). Normalized z-scored protein abundance are depicted in red (relatively higher abundance) and green (relatively lower abundance). Two row clusters of protein profiles are highlighted in light and dark grey. (B) Functional network of the proteins with relatively lower abundance in OIL clams ($p < 0.05$). (C) Functional network of proteins with relatively higher abundance in OIL clams ($p < 0.05$). The dashed line separates the analyses between lower and higher abundances.

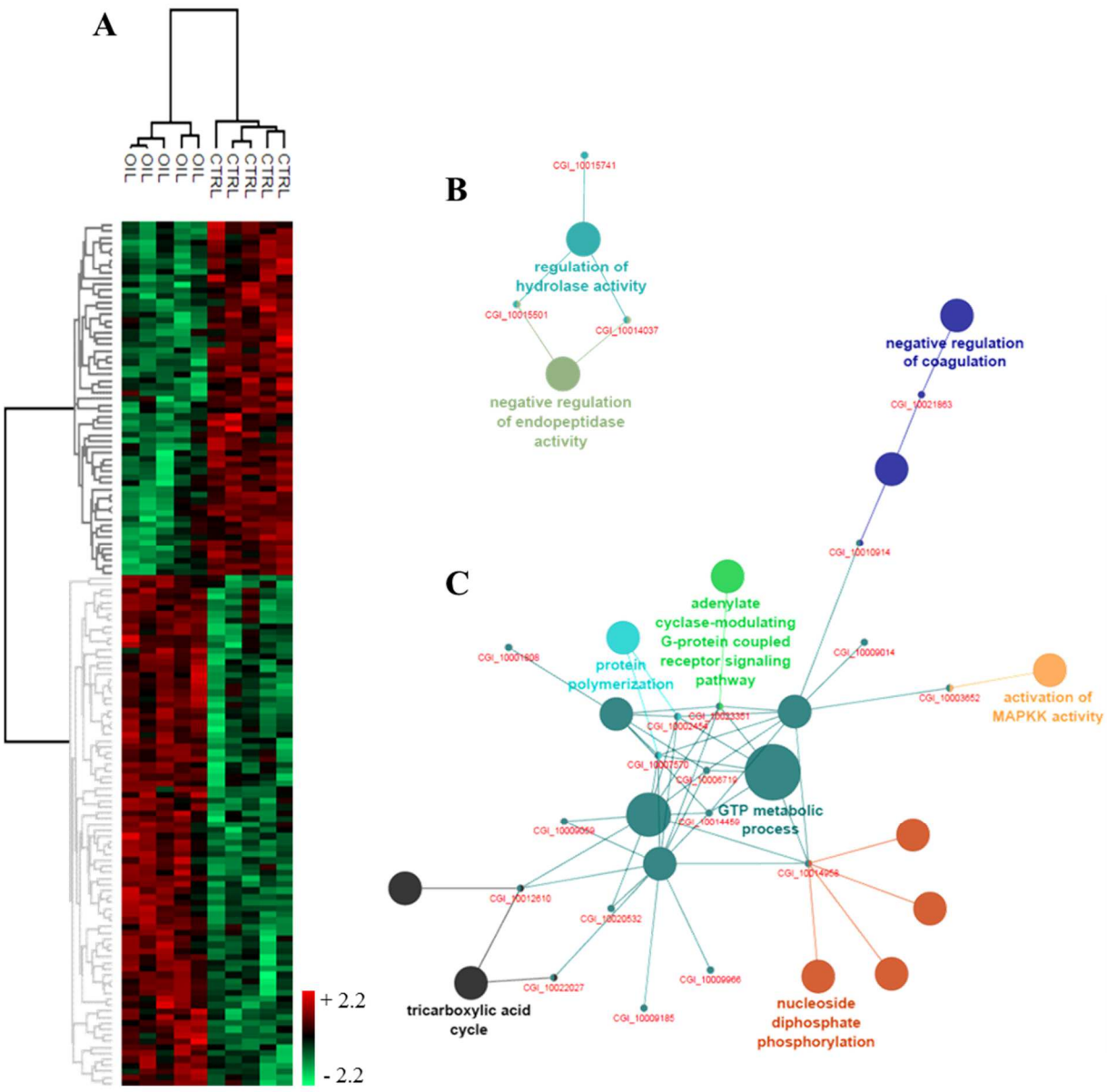


Fig. 4. Differential proteomic analysis in gills of crude oil exposed clams vs control. (A) Hierarchical clustering with significant proteins (FDR < 0.25). Normalized z-scored protein abundance are depicted in red (relatively higher abundance) and green (relatively lower abundance). Two row clusters of protein profiles are highlighted in light and dark grey. (B) Functional network of proteins with a relatively lower abundance in OIL clams ($p < 0.05$). (C) Functional network of proteins with a relatively higher abundance in OIL clams ($p < 0.05$). The dashed line separates analyses between lower and higher abundances.

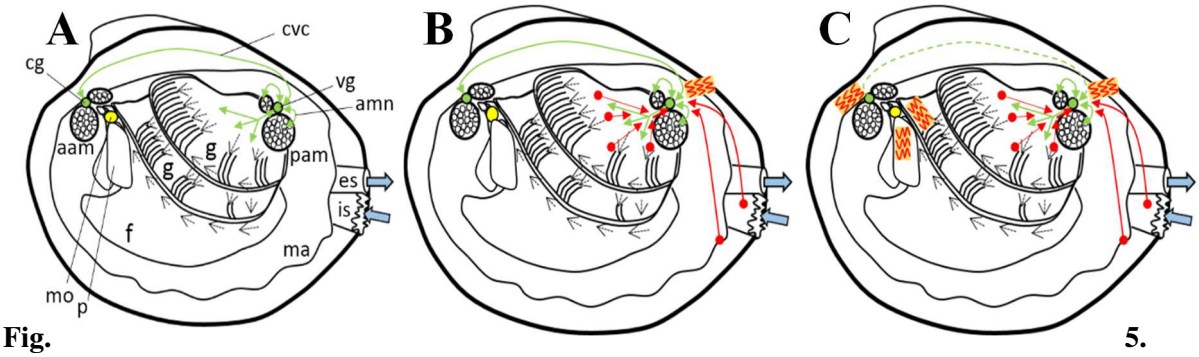


Fig. 5. **Schematic diagrams that potentially explain behavioural changes in crude oil exposed Asian clams.** (A) Background information on the anatomy of C. *fluminea* and two nonexclusive disturbance scenarios (B and C) explaining behavioural changes reported in this work, see text. aam, anterior adductor muscle; cg, cerebral ganglia; cvc, cerebro-visceral commissure; es, exhalant siphon; f, foot; g, gills; is, inhalant siphon; ma, mantle; mo, mouth; p, labial palp; pam, posterior adductor muscle; vg, visceral ganglia. Green arrows, efferent nerves from visceral ganglion to adductor muscles, gills and between visceral and cerebral ganglia. Red arrows, afferent nerves from inhalant siphon, mantle edge and gills to visceral ganglia. Wavy red lines represent the presence of hydrocarbon compounds directly acting on ganglia.

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