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Subcellular effects and lipid metabolism alterations in the gilthead seabream *Sparus aurata* fed on ovatoxins-contaminated mussels.

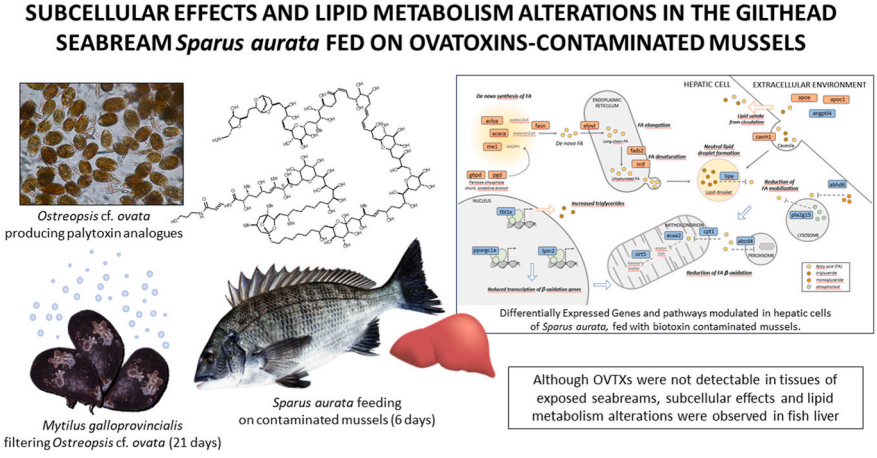
Maria Elisa Giuliani [a], Simone Bacchiocchi [a], Stefano Accoroni [b], Melania Siracusa [a], Debora Campacci [a], Valentina Notarstefano [b], Marica Mezzelani [b], Arianna Piersanti [a], Cecilia Totti [b], Maura Benedetti [b,c], Francesco Regoli [b,c], Stefania Gorbi [b,c,*]

[a] *Istituto Zooprofilattico Sperimentale Umbria e Marche "Togo Rosati", Via Cupa di Posatora 3, 60131 Ancona, AN, Italy*
[b] *Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy*
[c] *NBFC, National Biodiversity Future Center, Palermo 90133, Italy*

HIGHLIGHTS

- Direct filtering on *O.* cf. *ovata* causes the accumulation of OVTXs in mussel.
- Seabreams fed with OVTX-contaminated mussels started to reject the food after 6 days.
- No detectable levels of OVTXs were measured in seabream fed with contaminated mussels.
- OVTX-enriched diet induced alterations of lipid metabolism in seabreams liver.
- Although not bioaccumulated, OVTXs could be responsible of biological effects in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

The marine microalgae *Ostreopsis* cf. *ovata* are a well-known producer of palytoxin (PlTXs) analogues, *i.e.* ovatoxins (OVTXs) among others, which arouse concern for animal and human health. Both in field and laboratory studies, presence of OVTXs, detected in species directly feeding on *O.* cf. *ovata*, was frequently correlated with impairment on organisms' physiology, development and behaviour, while similar knowledge is still lacking for animals feeding on contaminated preys. In this study, transfer and toxicity of OVTXs were evaluated in an exposure experiment, in which gilthead seabream *Sparus aurata* was fed with bivalve mussel *Mytilus galloprovincialis,* contaminated by a toxic strain of *O.* cf. *ovata*. Mussels exposed to *O.* cf. *ovata* for 21 days accumulated meanly 188 ± 13 μg/kg OVTXs in the whole tissues. Seabreams fed with OVTX-contaminated mussels started to reject the food after 6 days of contaminated diet. Although no detectable levels of OVTXs were measured in

* Corresponding author. Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy.
E-mail address: s.gorbi@univpm.it (S. Gorbi).

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muscle, liver, gills and gastro-intestinal tracts, the OVTX-enriched diet induced alterations of lipid metabolism in seabreams livers, displaying a decreased content of total lipid and fatty acid, together with overexpression of fatty acid biosynthetic genes, downregulation of β-oxidation genes and modulation of several genes related to lipid transport and regulation. Results from this study would suggest the hypothesis that OVTXs produced by *O.* cf. *ovata* may not be subject to bioaccumulation in fish fed on contaminated preys, being however responsible of significant biological effects, with important implications for human consumption of seafood products.

1. Introduction

Marine biotoxins synthesized by microalgal and bacterial species represent a threat for both the environment and human health, since they can be accumulated by several organisms and transferred to humans through the food web. Due to their toxicity, some classes of biotoxins have long been regulated, and maximum permitted levels have been established in edible shellfish by the European Union (Reg. CE 853/2004). In the last years, increasing interest was paid to the so called Emerging Marine Biotoxins (EMBs), characterized by a limited knowledge of toxicological consequences and/or by an increasing occurrence in seafood that might pose a potential risk for human health. EMBs include, among the others, cyclic imines, tetrodotoxins, azaspiracids, brevetoxins, ciguatoxins and compounds belonging to the palytoxin (PlTX) group (Bacchiocchi et al., 2020, 2023; Giuliani et al., 2019; Otero and Silva, 2022). Dinoflagellates of the genus *Ostreopsis,* which are benthic epiphytes of macroalgae and other substrates, are known as producers of PlTX and PlTX-like compounds (Accoroni et al., 2016; Brissard et al., 2015; Ciminiello et al., 2006; Holmes et al., 1988; Lenoir et al., 2004; Meunier et al., 1997; Nakajima et al., 1981; Uchida et al., 2013; Yasumoto et al., 1987). The species of this dinoflagellate genus were initially reported in tropical areas, and only later in temperate latitudes (Parsons et al., 2012; Rhodes, 2011). Although in tropical areas they show the highest diversity, the maximum abundances (10^5 cells g^{-1} fw, Accoroni et al., 2020; Delgado et al., 2006; Díaz-Asencio et al., 2019; Faust, 2009; Okolodkov et al., 2007; Parsons and Preskitt, 2007; Tester et al., 2014) were lower than at temperate latitudes (up to 10^6 cells g^{-1} fw e.g., Vassalli et al., 2018; Mangialajo et al., 2011; Chomérat et al., 2022). The species *O.* cf. *ovata*, appearing during yearly seasonal blooms in the Mediterranean, synthesizes several ovatoxins (OVTXs: OVTX-a, -b, -c, -d, -e, -f, -g, and -h), classified as PlTX-like compounds, and low amounts of isobaric PlTX (Brissard et al., 2015; García-Altares et al., 2015; Tartaglione et al., 2017). Here, four toxin profiles were identified, with the most common one being dominated by OVTX-a, followed by OVTX-b, -d, -e, -c, and isobPlTX, in decreasing order of percentage, while very rarely non-producing strains were established (Tartaglione et al., 2017). In addition, other non-PlTX compounds (liguriatoxins, rivieratoxins) have been recently reported in specific Mediterranean *O.* cf. *ovata* strains (Ternon et al., 2022), highlighting a complex toxin profile for this species. The same species, when recorded outside the Mediterranean Sea, has been found to produce additional OVTXs (i.e. Ovatoxin k and j2 in South China Sea, Gu et al., 2022) and non-PlTX compounds (i.e. Ostreol A and B in South Korea, Hwang et al., 2013, 2018).

Previous studies reported the capability of several marine species to bioaccumulate OVTXs when exposed to algal bloom in field environment: e.g. crustaceans, molluscs (bivalves, gastropods, cephalopods), echinoderms and fishes, with highest levels ranging from 217 μg/kg in mussels whole flesh, 400 μg/kg in the sea urchin *Paracentrotus lividus*, up to 971 μg/kg in the octopus (Accoroni et al., 2022; Aligizaki et al., 2011; Amzil et al., 2012; Biré et al., 2013, 2015). Although less data are available for fish species, moderate levels of PlTX-like compounds (29.7 μg/kg) were detected in *Sarpa salpa* whole flesh during *O.* cf *ovata* blooms, while even higher concentrations were reported in the digestive tracts of *S. salpa* and other fish species (Biré et al., 2013, 2015). Biological effects were also documented on marine fauna exposed to algal bloom in field environment, with impairments evidenced by exoskeleton alterations, developmental effects or mass mortalities, in the worst cases (Accoroni et al., 2011; Aligizaki et al., 2011; Pavaux et al., 2020; Ramos and Vasconcelos, 2010; Sardo et al., 2021; Shears and Ross, 2009, 2010). OVTXs mechanism of action in marine organisms still remains scarcely characterized. Vertebrates and in particular fish species, have been so far poorly investigated (Giussani et al., 2016; Faimali et al., 2012; Pavaux et al., 2020; Pezzolesi et al., 2012; Ramos and Vasconcelos, 2010) with pivotal laboratory investigation highlighting a strong sensitivity of *Dicentrarchus labrax* fish juveniles (Faimali et al., 2012). On the other hand, invertebrates are more investigated and in mussels, particularly affected by *O.* cf *ovata* blooms due to their benthic, sessile and filter-feeding behaviour, accumulation of OVTXs in tissues was associated with the inhibition of the Na+/K+ pump, immunological and histological alterations, inflammation and neurotoxicity (Accoroni et al., 2022; Carella et al., 2015; Gorbi et al., 2012, 2013; Malagoli et al., 2008). Although accumulation of OVTXs has been mostly reported and experimentally confirmed in herbivores and omnivores organisms feeding directly on *Ostreopsis* cf. *ovata* or their substrates (e.g. macroalgae), their toxins have also been detected in few carnivorous species (fishes, crustaceans, gastropods, cephalopods), that had possibly fed on contaminated preys, like molluscs, crustaceans, worms and tunicates (Aligizaki et al., 2011; Biré et al., 2013, 2015). However, there is still no clear evidence of the transfer of OVTXs from lower to higher levels of the food chain, and no laboratory-based study has been performed so far.

Ostreopsis cf. *ovata* blooms are associated with human toxicity due to inhalation of marine aerosol containing microalgal cell debris and/or toxins, with symptoms including skin/eyes irritation, respiratory disorders and fever (Brescianini et al., 2006; Casabianca et al., 2013; Chomérat et al., 2022; Ciminiello et al., 2014; Medina-Pérez et al., 2021; Poli et al., 2018). Food intoxications have not yet been reported for OVTXs worldwide (Accoroni and Totti, 2016; EFSA, 2009; Tubaro et al., 2011), while such events, including rare fatal/near-fatal cases, were ascribed to consumption of seafood contaminated by PlTX (Alcala et al., 1988; Kodama et al., 1989; Onuma et al., 1999; Taniyama et al., 2003). Since the toxicity of OVTXs through diet is unknown, no regulatory limits in seafood are available for this group of toxins. In fact, for OVTXs a lower toxicity has been hypothesised compared to PlTX (toxin maximum limit = 30 μg (PLTX + ostreocin-d)/kg shellfish), and a provisional limit of 250 μg/kg shellfish has been proposed by EFSA (EFSA, 2009), although ANSES reports the much lower reference concentration of 15 μg (PLTX + ostreocin-d)/kg (Lemee et al., 2023). A recent study, comparing the acute toxicity of PlTX and OVTXs on a cell culture model, showed that OVTX-a and -d have a lower toxic potential than PlTX (Gémin et al., 2022). However, an *in vivo* study on rats demonstrated a similar toxicity for PlTX and OVTX-a (Poli et al., 2018). A possible risk associated with human ingestion should not be underestimated, since i) the OVTX bioaccumulation is well documented in seafood during *O.* cf. *ovata* blooms; ii) OVTXs levels near and above the 250 μg/kg threshold have been reported in marine invertebrates (Aligizaki et al., 2011; Pavaux et al., 2020); iii) the hypothesis of a lower toxicity of OVTXs compared to PlTX is still controversial; iv) *O.* cf. *ovata* has been reported to produce also low amount of isobaric PlTX (García-Altares et al., 2015); v) the possible trophic-transfer and potential biomagnification of the OVTXs has not been investigated yet.

In this study, an integrated and multidisciplinary approach was applied to clarify the possible food transfer of OVTXs and to explore subcellular and molecular effects modulated by such exposure in fish.

The experiment was carried out using a strain of *O.* cf. *ovata* as OVTXs producer, the Mediterranean mussel *Mytilus galloprovincialis* both as primary consumer of microalgal cells and, subsequently as the prey for gilthead seabream *Sparus aurata*, a carnivorous fish naturally feeding on mussels. The Mediterranean mussel, *Mytilus galloprovincialis* was selected as test species due to its ecological, economic and ecotoxicological value (Musella et al., 2020), being recognized as a suitable bioindicator in the Mediterranean Sea, also toward the increasing occurrence of HABs. Similarly, seabream *Sparus aurata* has an important economic and commercial value considering its cardinal role in fulfilling the demand for safe and healthy food for a world population. Moreover, as omnivorous feeding species (Mhalhel et al., 2023), seabream could feed on mussels, thus making this species suitable for evaluating effects derived from OVTX-contaminated mussels. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis was used to determine the toxin profile and content of the *O.* cf. *ovata* strain and then to quantify the accumulation of OVTXs in mussels and seabreams. The fish liver was chosen as major metabolization/detoxification organ (Gorbi et al., 2014; Li et al., 2023) to highlight possible sublethal effects of OVTXs exposure. RNA sequencing technology (RNA-seq) was used to explore the hepatic seabream transcriptome and to determine molecular processes modulated by the OVTX exposure. Analyses on transcriptional profiles typically provide additional insights on activation of mechanisms of toxicity at molecular level, which might anticipate the progress of alterations to biochemical and cellular levels (Mezzelani et al., 2021). This information was integrated with functional effects both in terms of changes in macromolecular composition though Fourier-transformed infrared (FTIR) spectroscopy, directly related to the modulation of specific physiological processes (Carnevali et al., 2019) and further assessed by histological analysis. This study was expected to highlight possible implications also for human consumers, related both to food safety and to the nutritional value of edible Mediterranean species.

2. Materials and methods

2.1. Experimental design

2.1.1. Microalgal culture

Monoclonal cultures of the dinoflagellate *O.* cf. *ovata* and the diatom *Skeletonema marinoi* were obtained by isolating cells through the capillary pipette method from water samples collected along the Marche coast (Italy, NW Adriatic Sea) in August 2020 and November 2018, respectively. The diatom *S. marinoi*, a common phytoplanktonic species in the NW Adriatic Sea (Neri et al., 2022; Totti et al., 2019) was used as control microalgae (Gorbi et al., 2013). After an initial growth in microplates, cells of both species were cultured at 21 ± 0.1 °C under a 12:12 h L:D photoperiod and an irradiance of 90–100 μmol m^{-2} s^{-1}. *O.* cf. *ovata* was cultured in natural seawater with macronutrients added at a two-fold diluted f/2 concentration plus selenium, while for *S. marinoi* a replete f/2 growth medium was used. PlTX concentration and the toxin profile of the *O.* cf. *ovata* cultured strain were investigated by LC-MS/MS before its use for the exposure experiments. Regarding the cellular composition *O.* cf. *ovata* and *Skeletonema marinoi*, lipid content ranges from 24 to 29% (Pezzolesi et al., 2014) and from 24 to 27% (Olofsson et al., 2015), respectively.

2.1.2. Mussel exposure

Mussels *M. galloprovincialis* (480 specimens) were obtained from local farms (Marche coast, Italy, NW Adriatic Sea) and acclimated for 7 days to laboratory conditions in four recirculating tanks (40 L, 3 individuals L^{-1}) in artificial seawater (salinity 35, 20 ± 1 °C). After the acclimation period, a pool of three organisms' whole tissues, for each tank, were chemically tested for the absence of PlTXs bioaccumulation (t0 mussels). For the exposure experiment, carried out with the only purpose to obtain contaminated food for the subsequent fish exposure, 120 specimens (two tanks, 20 L, 3 individuals L^{-1}) were daily fed on *O.* cf. *ovata* (10^4 cells L^{-1}) and 120 specimens (two tanks, 20 L, 3 individuals L^{-1}) on *S. marinoi* (10^6 cells L^{-1}), in order to obtain an almost equivalent diet in term of biomass; water was changed every day and algae were re-dosed, after the water renewal. After 21 days, at the end of the exposure time, the organisms were dissected and the whole soft tissues were individually frozen to obtain contaminated and control food, respectively, for the subsequent fish exposure. Whole tissues (three pooled mussels) from control and exposed groups were sampled at day 5, 10, 14 and 21 and checked to monitor the PlTXs' bioaccumulation level (LC-MS/MS), over the time.

2.1.3. Fish exposure

Ten juvenile specimens of *S. aurata* (length = 20 ± 1 cm) were obtained from a local farm (Manfredonia, Italy) and acclimated to laboratory conditions (artificial seawater, salinity 34, 22 ± 1 °C) in six recirculating 200 L tanks (1/2 individual per tank) for 10 days, during which they were daily fed on t0 mussels (2 mussels/fish/day). After the acclimation period, three tanks were assigned to the control group and three to the exposed group (5 fish/group). Exposed fish were fed with mussels previously contaminated with *O.* cf. *ovata*, while control fish with mussels fed on *S. marinoi*. The whole soft tissues of two mussels of known weight were provided per fish daily and their ingestion by each fish was verified visually (specimens in shared tanks were identified through specific morphological traits): the number of items ingested by each fish was recorded (Supplementary Data) and non-ingested mussels were removed from the tank. At day 6 of exposure, most of the animals (4 of 5) exposed to contaminated mussels started to reject the food (see Video 1: feeding at day 2; Video 2: feeding at day 6) and the experiment was interrupted the following day (day 7). At the end of the experiment, fish were anesthetized on ice and dissected; livers, gills, muscles and gastrointestinal tracts were excised, frozen in liquid nitrogen and stored at – 80 °C.

Experimental procedures on vertebrate animals were approved by the Animal Care and Health Committee (OPBA, Organismo Preposto al Benessere Animale) of the Università Politecnica delle Marche, and by the General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health (Authorization No. 680/2018-PR), and the study was performed according to the EU Directive 2010/63/EU for animal experiments.

2.2. Chemical analysis of PlTX analogues

2.2.1. Chemicals and standards

Acetonitrile (MeCN, HPLC-MS grade) and methanol (MeOH, HPLC grade) were purchased from CARLO ERBA Reagents S.r.l. (Milano, Italy), acetic acid (CH_3COOH, HPLC-MS grade) from Sigma-Aldrich (Steinheim, Germany). Water was obtained through a MilliQ water purification system (DIW) (Millipore Ltd., Bedford, MA, USA). Analytical standard of PlTX was purchased from Wako Chemicals GmbH (Neuss, Germany) and dissolved in MeOH/H_2O (1:1, v/v) obtaining a stock solution of 20 μg mL^{-1}. Matrix matched PlTX standards at five levels of concentration (0.080, 0.125 0.250, 0.5, 1.5 μg mL^{-1}), using an extract of a blank mussel sample, were prepared to build calibration curves for quantitative analyses.

2.2.2. PlTXs extraction from Ostreopsis cf. ovata

One litre of culture (10^6 cells L^{-1}) in the late exponential phase (i.e. after 7–9 days from the initial inoculum) was centrifuged for 20 min at 2500×*g* (4 °C) in 40 tubes (50 mL volume). Cell pellets of *O.* cf. *ovata* were combined and extracted with 5 mL of MeOH/H2O (80:20 v/v), vortex-mixed for 1 min, and sonicated for 10 min. The aliquot was then centrifuged for 10 min at 2500 *g* (4 °C), and the supernatant transferred to a 100 mL evaporation flask. Pellet extraction was repeated twice, and the supernatants combined and brought to near dryness under reduced pressure (T = 50–60 °C) (R-114 Büchi, Flawil, Switzerland). The residue was redissolved in 1 mL of MeOH/H_2O (80:20 v/v) and filtered through

a 0.2 μm–cellulose acetate syringe filter (Minisart, Sartorius, Germany) for LC-MS/MS analysis.

2.2.3. PlTXs extraction from mussels and seabream tissues

The extraction was implemented following the protocol already described by Ciminiello et al. (2011) and Accoroni et al. (2022). Briefly, 10.0g ± 0.5g of mussel homogenate was weighted in a 50 mL polypropylene tube and extracted with 30 mL of MeOH/H_2O (80:20 v/v). The sample was homogenized with an Ultra Turrax T25 mixer for 2 min at 9500 rpm and centrifuged for 15 min at 2000 *g* (20 °C). The supernatant was transferred in a 100 mL evaporation flask and the solid residue re-extracted twice. The supernatants were collected obtaining a final volume of 90 mL and evaporated to dryness. The residue was dissolved in 5 mL of MeOH/H_2O (80:20 v/v), filtered through a 0.2 μm syringe filter and analysed by LC-MS/MS.

Muscle, liver, gills and gastro-intestinal tracts of seabreams were extracted following the protocol already described for mussels, weighting 2.5g ± 0.5g of tissue homogenate. The matrix amount/extraction solvent ratio was kept constant lowering accordingly the solvent volume (7.5 x 3 mL of MeOH/H_2O 80:20 v/v, 1.25 mL of MeOH/H_2O 80:20 v/v for dry residue dissolving).

2.2.4. LC-MS/MS analysis

LC-MS/MS analyses of PlTX analogues (PlTX, isobaric PlTX, OVTX-a, -b, -c, -d, and -e) were performed using a hybrid triple-quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Turbo V source and an electrospray ionization (ESI) probe. The mass spectrometer was coupled to an Agilent 1200 HPLC (Palo Alto, CA, USA), including a solvent reservoir, inline degasser, quaternary pump, refrigerated autosampler, and column oven. Infusion experiments were performed using PlTX standard to set MS source parameters. The LC-MS/MS adopted conditions were reported in detail in Supplementary Data file.

2.2.5. Analytical performances

Method performances were studied through in-house validation experiments spiking mussels with PlTX reference material (the only reference material commercially available). Instrumental linearity was investigated injecting the matrix matched calibration curve three times in different days. The curves ($y = bx + a$) were obtained plotting the toxins chromatographic peak areas (y) against their concentrations (x), using the least squares regression model. Good correlation coefficients ($R^2 > 0.99$) and response factors variation (drift <10%) were obtained. The limit of quantification (LOQ) and the limit of detection (LOD) were calculated based on a signal/noise (S/N) ratio of 10 and 3 respectively. The estimated LOQ was experimentally confirmed by repeated analysis (n = 6) on a blank mussel sample spiked at 13 μg/kg (26 ng mL^{-1}). The LOD derived thereafter was 5 μg/kg (10 ng mL^{-1}), as already reported in Accoroni et al. (2022).

As regards the other matrices, seabream tissues and *O.* cf. *ovata* strain blank samples were spiked with the PlTX standard to check the sensitivity in terms of LOD and the LOQ. Both in case of algae and seabream tissues the LOD experimentally verified was of 13 μg/kg (26 ng mL^{-1}), the LOQ of 40 μg/kg (80 ng mL^{-1}). Accuracy in terms of recovery (R%) and precision in terms of intra-day repeatability (intra-day relative standard deviation - RSDr %) were calculated performing replicated analyses (N = 6 in the same day) on blank mussel samples spiked at 40 μg/kg (a low contamination level) and 250 μg/kg (EFSA guidance level). Good recoveries (R% = 69–81% at 40 μg/kg, R% = 78–97% at 250 μg/kg) and acceptable intra-day repeatability (RSDr% = 9% at 40 μg/kg and RSDr% = 7% at 250 μg/kg) were obtained. In seabream tissues and *O.* cf. *ovata* strain samples spiked at LOQ level the R% was also calculated (R% = 70 and 75 % respectively). Moreover, being the obtained recoveries comparable to those obtained for mussels, the matrix effect of all investigated matrices was considered comparable, then only matrix matching calibration curves in mussels was used. Assuming an equimolar response, PlTX standard was used to quantify all PlTXs included in the method, Relative Retetion Times (RRT) were used for identification.

2.2.6. Quality assurance

In each analytical batch for all investigated matrices (mussels, seabream tissues and algal pellets), a fortified sample at LOQ level was processed to guarantee method performances, in terms of matrix effect and losses due to the extraction.

2.3. Biological analyses

2.3.1. Transcriptomic analysis

The expression profiles in the livers of exposed and control fish were assessed by RNA sequencing. Total RNA was purified from *S. aurata* liver samples (~50 mg each) using the Hybrid-R™ purification kit (GeneAll®), according to the manufacturer's instructions. Total RNA concentrations were measured using Qubit RNA Assay Kit (Thermo Fisher). RNA integrity was checked using a 2100 Bioanalyzer (Agilent Technologies) with RNA 6000 NanoChip. Libraries were prepared from total RNA with Illumina Stranded mRNA Prep kit, according to manufacturer instructions. Libraries quality was verified on 2100 Bioanalyzer with a DNA HS Chip, and libraries concentrations were determined using Qubit DNA HS Assay Kit. Sequencing was performed on Illumina Novaseq 6000 in the 100 PE format.

2.3.2. FPA-FTIRI measurements and data analysis

Focal Plane Array (FPA)-Fourier Transform Infrared Spectroscopy Imaging (FTIRI) measurements were carried out in liver samples of control and exposed fish. For each liver sample, cryostat sections (6 μm) were deposited onto CaF_2 optical windows (1-mm thick, 13-mm diameter) and let air-dry for 30 min without any fixation process, for IR measurements. A Hyperion 3000 Vis–IR microscope coupled with a INVENIO interferometer and equipped with a FPA detector (164x164 μm; 4096 pixel/spectra; 2.56 μm spatial resolution) (Bruker Optics GmbH, Ettlingen, Germany) was used. On each section, 4 IR images were acquired in transmission mode (15X condenser/objective; 4000-900 cm^{-1}; 4 cm^{-1} spectral resolution, and 256 scans). Background spectra were obtained on clean regions of the CaF_2 optical windows with the same acquisition parameters. Raw IR images were corrected with Atmospheric Compensation and Vector Normalization routines (OPUS 7.5 software, Bruker Optics GmbH, Ettlingen, Germany). False colour images were generated by integrating IR images in specific spectral regions, to evaluate the spatial distribution and the relative amount of lipids (2995-2828 cm^{-1}, vibrational modes of lipid alkyl chains) and glycosylated compounds, mainly glycogen in liver (1065-1010 cm^{-1}, vibrational modes of C–O groups in carbohydrates and glycosylated compounds). All the spectra composing the chemical map were submitted to the integration procedure (integration Mode B, OPUS 7.5 software): 3031-3000 cm^{-1} (stretching vibration of = CH groups of unsaturated lipid alkyl chains, UNS), 2977-2841 cm^{-1} (symmetric and asymmetric stretching modes of CH3 and CH2 moieties of lipid alkyl chains, LIP), 1760-1727 cm^{-1} (stretching vibration of C═O ester moieties of fatty acids, FA), 1720-1480 cm^{-1} (vibrational modes of peptide linkage, PRT), and 1065-1010 cm^{-1} (stretching mode of C–O moieties in carbohydrates and glycosylated compounds, GLY). The integrated areas were used to calculate the band area ratios: LIP/PRT (total amount of lipids respect to total proteins); FA/PRT (total amount of fatty acids respect to total proteins); FA/LIP (relative amount of fatty acids respect to total lipids); UNS/FA (relative amount of unsaturated fatty acids among all fatty acids), and GLY/PRT (total amount of glycosylated compounds, mainly glycogen, respect to proteins). PRT was used for band area ratios since its area did not change significantly among the control and exposed samples.

2.3.3. Histological analysis

Neutral lipids content was determined in the liver on duplicate cryostat sections (8 μm thick), which were fixed in Beker's fixative and stained by Oil red O (ORO) before mounting in glycerol gelatin. Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of liver section. Five measurements were made on each section.

2.3.4. Statistical analyses

The total toxin intake for each *S. aurata* specimen was estimated by multiplying the total amount of ingested mussels (g) by the average toxin concentration detected in mussel tissues (188 μg/g); values were then expressed as μg of toxin per kg of fish weight.

For the analysis of transcriptomic data, the reads pre-processing was performed by using fastp v0.20.0 (Chen et al., 2018), applying specific parameters to remove residual adapter sequences and to keep only high quality data (qualified_quality_phred = 20, unqualified_percent_limit = 30, average_qual = 25, low_complexity_filter = True, complexity_threshold = 30). The percentage of uniquely mapped reads resulted high with the mean value of 85% (mean value for sample: unmapped reads 9%, quality base > q30 94%). Passing filter reads were mapped to the genome reference (*Spaurus aurata*) using STAR v2.7.0 (Dobin et al., 2013) with standard parameters, except for sjdbOverhang option set on read length. Genome and transcripts annotation provided as input were downloaded from v103 of Ensembl repository. Alignments were then elaborated by RSEM v1.3.3 (Li and Dewey, 2011), to estimate transcript

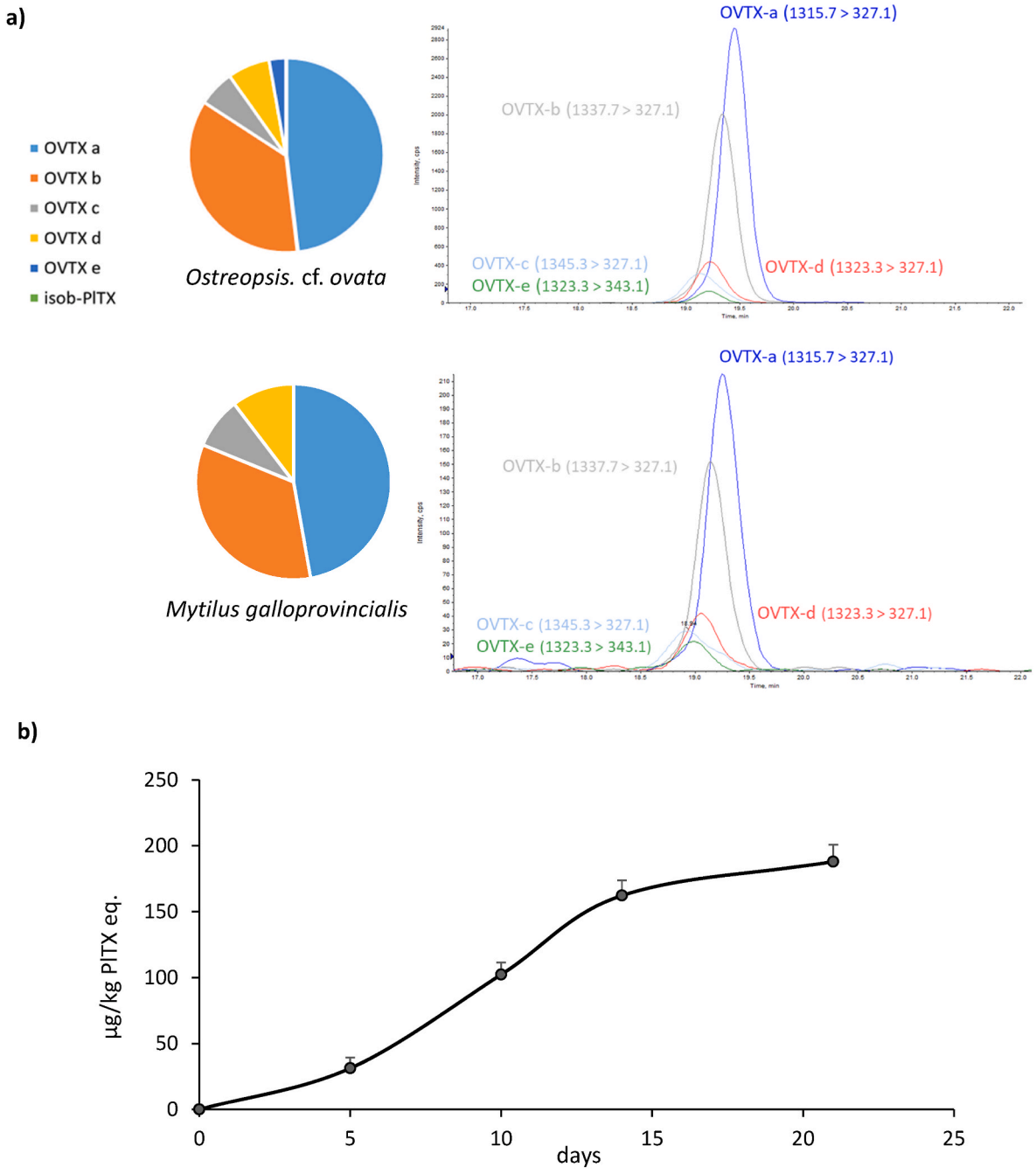

Fig. 1. a) Toxic profile and LC-MS/MS chromatograms of *O.* cf. *ovata* strain used for the exposure (above), and of *M. galloprovincialis* exposed to the toxic algae (below): the plots show the percentage of the different analogues detected by LC-MS/MS; **b)** Temporal trend of toxin accumulation in *M. galloprovincialis* whole tissues during exposure; values are expressed as μg of toxins per kg of whole tissue (mean ± s.d.).

and gene abundances. Subsequently, the sample-specific gene-level abundances were merged into a single raw expression matrix applying a dedicated RSEM command (rsem-generate-data-matrix). Genes with at least 10 counts in 50% of samples were then selected. Differential expression (pairwise comparisons) was computed by edgeR (McCarthy et al., 2012) from raw counts in each comparison. Multiple testing controlling procedure was applied and genes with a p-value $\leq$0.05 and log Fold-Change (FC) $>$ |0.5| were considered differentially expressed. Annotation of differential expressed genes (DEGs) was performed using the bioMart package (Durinck et al., 2009) into R 4.1, querying available Ensembl Gene IDs and retrieving Gene Names and Entrez gene IDs. Uncharacterized sequences were represented with the LOC# identifier. The functional enrichment analysis of DEGs was performed by the DAVID tool (Database for Annotation, Visualization, and Integrated Discovery; Huang et al., 2009) to identify the most enriched Gene Ontology (GO) terms. In the absence of a well-annotated genome for *S. aurata*, the DEGs were matched to *Danio rerio* genome, and only results with corrected p-value (ease) $<$ 0.05 were considered. Protein-protein interaction (PPI) analyses were performed by bash scripts on public database STRING, which consist of PPI information including most of the annotated genes for eukaryotes. To conclude, graphical diagrams of PPI including interactions involving annotated differential expressed genes were generated using Cytoscape (Shannon et al., 2003).

For FTIR data and ORO staining data, statistical significances between control and exposed group were calculated by Student's t-test and set at $p < 0.05$, after checking the assumptions of homoscedasticity and normality.

3. Results

3.1. Chemical characterization of O. cf. ovata strain and exposed mussels

Chemical characterization of *O.* cf. *ovata* strain demonstrated a total content of 5.35 pg/cell of toxins, among which OVTX-a (48%) and –b (36%) were predominant, with lower amount of OVTX-c (6%), -d (7%) and –e (3%); PlTX and isobaric PlTX were undetectable (Fig. 1a). At the end of the exposure period no mussels' death was evidenced for any of the tested conditions. LC-MS/MS analysis of *M. galloprovincialis* daily fed on *O.* cf. *ovata* confirmed that the organisms accumulated OVTXs with major contribution of OVTX-a (47%) and –b (34%), followed by OVTX-c (9%), OVTX-d (10%) and with a toxic profile similar to that of *O.* cf. *ovata* (Fig. 1a). OVTXs levels, monitored throughout the exposure, were 31 $\pm$ 2 μg/kg at day 5 and reached 188 $\pm$ 13 μg/kg at the end of the exposure (21 days) (Fig. 1b). No toxins accumulation was observed in control mussels.

3.2. Toxin levels in Sparus aurata fed with OVTX-contaminated mussels

Levels of OVTXs measured in liver, muscle, gills and gastro-intestinal tracts of both control and exposed seabreams were always below the Level of Detection (LOD = 13.3 μg/kg).

The estimated OVTXs fish intake, calculated considering the total amount of mussels ingested by each fish and the mean OVTXs levels in mussels, was between 48.5 and 78.4 μg/kg b.w. (Supplementary Data). Experimental observation indicated that *S. aurata* fed with OVTX-contaminated mussels suddenly changed their feeding behaviour after 6 days of exposure, when they started to reject the food items, which were repeatedly taken and then expelled from the mouth (see Video 1: feeding at day 2; Video 2: feeding at day 6).

3.3. Transcriptomic responses in livers of fish fed with OVTX-contaminated diet

To understand if the exposure to OVTXs caused subcellular alterations, even in the absence of a clear bioaccumulation of the toxins, RNA sequencing technology (RNAseq) was used to explore the seabream transcriptome in liver, chosen as the main metabolization/detoxification organ.

The analysis of differential expression between exposed and control livers resulted in 268 DEGs ($p < 0.05$), among which 115 were upregulated and 153 downregulated in the exposed group (Supplementary Data). The hierarchical cluster heatmap of the top 50 DEGs (35 upregulated, 15 downregulated, $\log_2FC \geq |1.73|$) revealed a good clustering of control samples; four of five exposed samples clustered together, while one was separated from the others (#6, Fig. 2). Ptprq, me1, cusr, fasn, aclya and acaca were among the most over-expressed genes in the exposed group ($\log_2FC > 3$; $FC > 8$); among the most under-expressed genes we found wdr49 ($\log_2FC > -3$; $FC < 0.125$) and angptl4 ($\log_2FC > -2$; $FC < 0.25$) (Fig. 2). Four non-coding RNA (three upregulated, 1 downregulated) were among the most variable transcripts (Fig. 2). The analysis of protein-protein interaction (PPI), performed on the whole DEGs set (268 DEGs) and represented through the PPI network, revealed 72 interactions involving 67 proteins (Fig. 3). The proteins with the highest number of interactions were tblx1, fads2 (8 interactions), ppargc1a, smarcd3 (6 interactions), ctnnb1, me1 and stat 1 (5 interactions).

Among the biological processes affected in fish fed on *O.* cf. *ovata*-contaminated mussels (Supplementary Data), a clear alteration was observed in the expression of genes involved in lipid metabolism, in both biosynthetic and catabolic process. In particular, several genes involved in fatty acid biosynthesis were upregulated and those involved in fatty acid degradation were mainly downregulated in the exposed group. Other processes related to lipid metabolism were enriched, including glycerolipid metabolic process (e.g. neutral lipids such as tryglicerides, see Supplementary Data), carboxylic acid transport, plasma lipoprotein clearance, and regulation of ketone metabolism. Other biological processes related to metabolic activities were significantly enriched, namely tricarboxylic acid, sulfur compound and organophosphate (including nucleotide) metabolisms, and the pentose-phosphate shunt. Additional DEGs were involved in regulation of transcription and in protein phosphorylation. "Response to stimuli" processes were also observed, such as response to lipid, oxygen-containing compounds, hormone, iron ion, hypoxia, and biotic stimulus (Table 1).

3.4. Lipid content in liver of fish fed with OVTX-contaminated diet

To further elucidate the effects of the OVTX-diet on metabolic processes including lipid metabolism, the content of lipids and other macromolecules was investigated in fish livers by comparing FTIR images of control and exposed livers. False colour images, representing the distribution of lipids (LIP), glycogen (GLY) and proteins (PRT), showed a lower content of LIP and GLY in liver of exposed fish, while PRT amounts were nearly unchanged (Fig. 4a). The spectra composing the IR maps confirmed a variation of LIP and GLY in exposed samples, and highlighted differences in total and unsaturated FA (Fig. 4b). Given the unvaried profile of PRT between exposed and control samples, the band area ratios showed a significantly lower amount of total lipids (LIP/PRT), a lower amount of fatty acids, both compared to proteins (FA/PRT) and to the overall lipids (FA/LIP), and higher levels of unsaturated fatty acids (UNS/FA). The relative amount of glycogen (GLY/PRT) showed a slight but not significant decrease in exposed group (Fig. 4c). The decrease of lipid content in the liver of fish fed with contaminated mussels was further confirmed by histochemical analysis (Oil Red O staining), which revealed a significant lower amount of neutral lipid droplets in hepatic sections of exposed organisms (Fig. 5).

4. Discussion

The genus *Ostreopsis* includes species largely distributed worldwide, from tropical to temperate marine areas (Delgado et al., 2006; Faust, 2009; Mafra Jr. et al., 2023; Mangialajo et al., 2011; Okolodkov et al.,

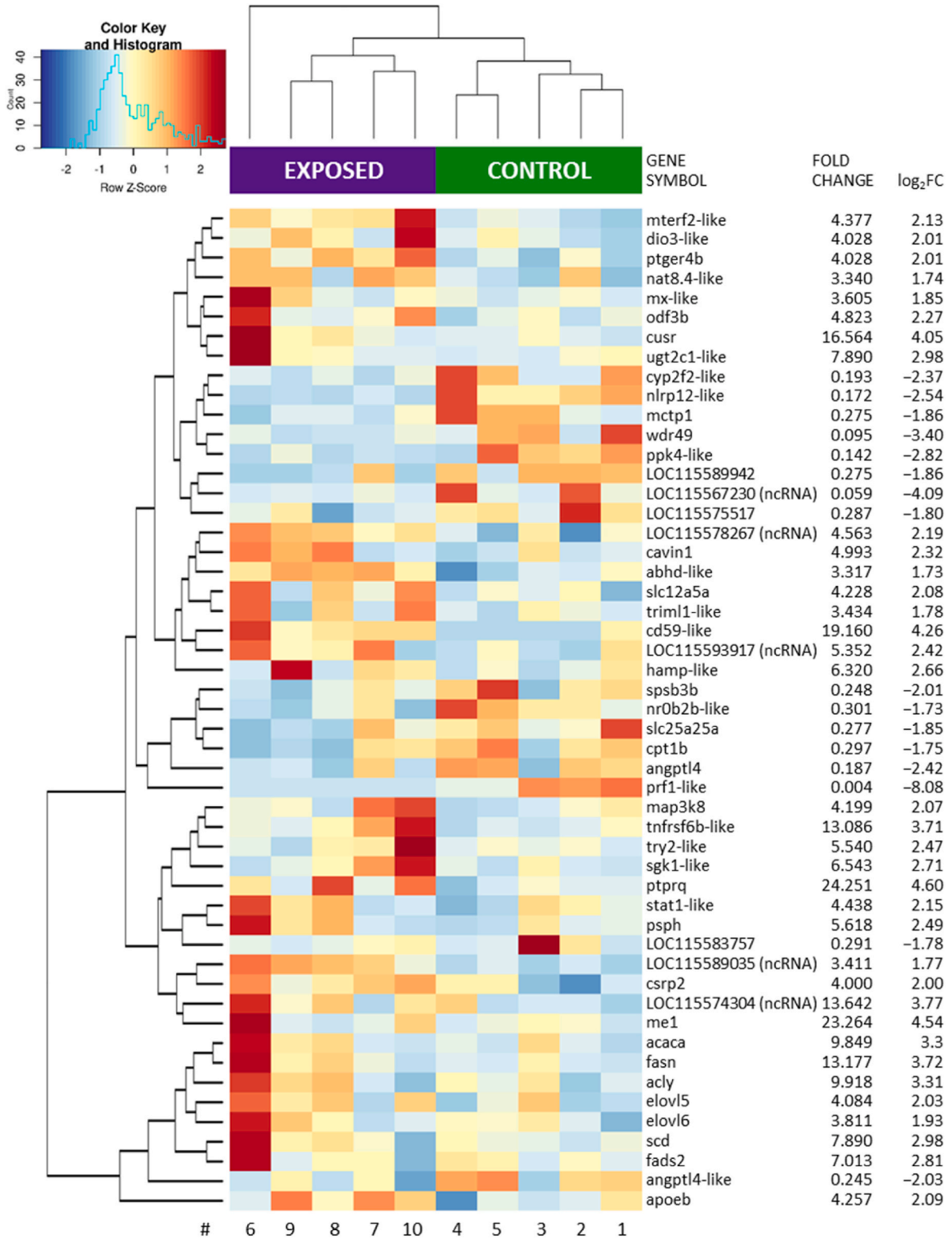

Fig. 2. Hierarchical cluster heatmap of the top 50 most variable DEGs ($log_2FC \geq |1.73|$) in the hepatic transcriptome of control and exposed *S. aurata*. Colors from blue to red represent increasing expression: orange/red colour (Z-score >1) represents gene abundance higher than the mean, blue colour (Z-score <1) represents gene abundance lower than the mean. # represents specimen numbers (1–5: controls; 6–10: exposed). Gene symbols of annotated genes are defined as in GenBank database (www.ncbi.nlm.nih.gov); sequences with putative annotation are indicated with "-like"; uncharacterized sequences are represented with the LOC# identifier; ncRNA = non-coding RNA. FC = fold change of exposed over control (mean value, n = 5). FC > 1 and positive log_2FC represent upregulated genes; FC < 1 and negative log_2FC represent downregulated genes. The complete DEGs list is given in Supplementary File S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

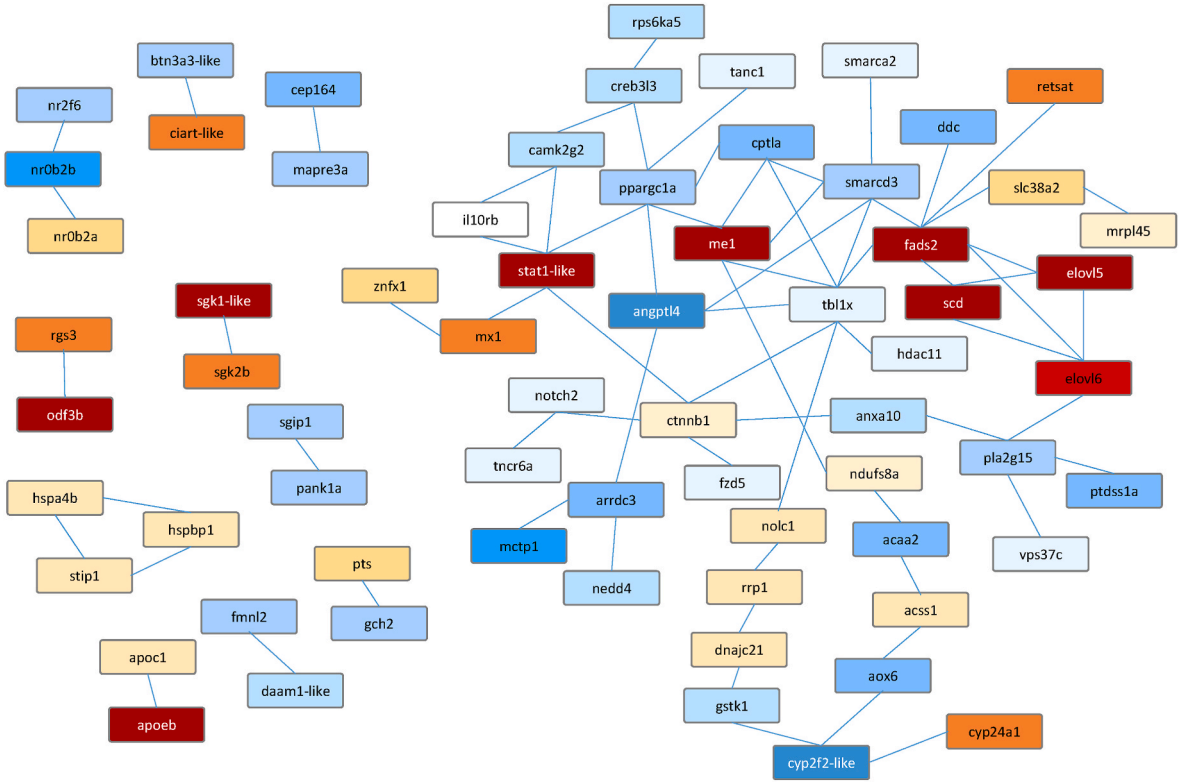

Fig. 3. Protein-protein interaction network of DEGs. Nodes represent proteins; connecting edges represent protein-protein associations. Blue scale colors: down-regulated DEGs. Red scale colors: upregulated DEGs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2007; Parsons and Preskitt, 2007; Rhodes, 2011; Parsons et al., 2012; Tester et al., 2014; Vassalli et al., 2018; Díaz-Asencio et al., 2019; Accoroni et al., 2020; Chomérat et al., 2022). In the Mediterranean Sea, blooms of toxic *Ostreopsis* spp. (mostly caused by *O.* cf. *ovata*, although *O.* cf. *siamensis* and *O. fattorussoi* have also been identified; Accoroni et al., 2016; Penna et al., 2012) are a recurrent seasonal event during summer-autumn (Accoroni and Totti, 2016). Although both marine organisms and humans are threatened by the produced toxins (palytoxin-like compounds, among which the ovatoxin family is the most relevant), little is known on their trophic transfer and effects, which may proceed from the subcellular level toward individual physiology, up to population and ecosystem changes. Here, we selected *O.* cf *ovata*, mussels and seabream as a model of Mediterranean trophic chain, to explore the possible transfer of OVTXs and effects at the lower levels of biological organization, i.e. molecular, biochemical and histological.

To simulate an environmental scenario, our feeding experiments were based on *O.* cf. *ovata* exposure concentrations comparable to those found during bloom peaks, *i.e* 10^4 cells L^{-1} (Accoroni et al., 2022; Vassalli et al., 2018). During the 21 days of exposure of *M. galloprovincialis* to microalgae, OVTXs bioaccumulation in mussels' tissues increased up to 188 μg/kg (on average), similar to the maximum levels detected in wild mussels (217 g/kg, Amzil et al., 2012). Such values are close or higher respect to the provisional values hypothesised for PlTX-group toxins. The OVTXs accumulation was not linear with time but rather showed a plateau, possibly because of a reduced filtration rate or an equilibrium between uptake and excretion, in agreement with previous insights from both laboratory and field investigations (Gorbi et al., 2013; Accoroni et al., 2022; Tibiriçá et al., 2019). The analysis of the toxic profile demonstrated a non-preferential accumulation of specific OVTXs in *M. galloprovincialis* since the main OVTXs analogues were found in similar proportions in *O.* cf. *ovata* cells and mussels' tissues; this behaviour is different from that already evidenced for other marine biotoxins, such as Azaspiracids produced by the Mediterranean dinoflagellate *Azadinium dexteroporum,* for which a preferential bioaccumulation of specific analogues has been demonstrated in exposed mussels (Giuliani et al., 2019).

Despite OVTX analogues were previously found in carnivorous fishes (Aligizaki et al., 2011; Biré et al., 2013, 2015), in our experiment *S. aurata* fed on OVTX-contaminated mussels seems not to accumulate detectable levels of the toxins in the main target tissues (liver, gills, muscle and gastrointestinal tract), despite the estimated total seabream toxin intake (48–78 μg/kg) was above the detection limit of the LC-MS/MS analytical method (LOD = 13.3 μg/kg). The fact that OVTXs was not detectable in *S. aurata* may depend on the sensitivity of the analytical method applied in this study; however, the observed biological effects, suggest the activation of biotransformation and detoxification processes, particularly effective in vertebrate compared to invertebrate marine organisms (Mezzelani et al., 2021; Regoli, 2012; Regoli and Giuliani, 2014), with a subsequent production of different metabolites which could have been more easily excreted.

Even in the absence of toxins detection in fish tissues, the biological reactivity of OVTXs was highlighted after a few days of OVTX-enriched diet, when seabreams stopped feeding on contaminated mussels, which they tested and then refuse it; unfortunately it was impossible to administer to the fish higher doses of toxins because of the described rejection mechanism. Food rejection is an important defence against harmful compounds (Kasumyan, 2019) which can influence food selection and intake, *e.g.* by decreasing food palatability and interacting with the fish taste system (Azizishirazi et al., 2013; Lürling and Scheffer, 2007; Kasumyan, 2019). The rejection of contaminated food was already reported in fish feeding on polychaetes hyperaccumulating methylated arsenic compounds (Fattorini and Regoli, 2004). Behavioural studies demonstrated that exposure to metals or insecticides suppresses the taste response in fish, inducing food rejection without loss of feed motivation, being the food ingested and then refused (Kasumyan, 2019; Abtahi et al., 2018; Bryan et al., 1995), similarly to

Table 1
Main enriched Biological Processes (BP) and corresponding DEGs in livers of *S. aurata* exposed to *O.* cf *ovata*-contaminated diet. Red: upregulated genes; blue: downregulated genes. FE: fold-enrichment. Colour backgrounds indicate related BP (yellow: BP related to "lipid metabolic process−GO:0006629"; orange: BP belonging to other "metabolic process−GO:0008152"; green: BP related to "response to stimulus−GO:0050896"; blue: BP belonging to "biological regulation−GO:0065007); white: other specific biological processes). The complete list is given in Supplementary File S1.

Biological Process (GO term)	Genes	p-value	FE
fatty acid metabolic process (GO:0006631)	aclya, elovl5, elovl6, acaca, cyp24a1, fads2, fasn, gcdh-like, scd abcd4, acaa2, cpt1a, cpt1b, lpin2	7.3E-8	7.2
lipid metabolic process (GO:0006629)	aclya, elovl5, elovl6, acaca, apoc1, apoeb, cyp24a1, fads2, fasn, gcdh-like, gpat3-like, scd, retsat abcd4, abhd6, acaa2, angptl4, cpt1a, cpt1b, dolk, hsd17b7, lipe, lpin2, ptdss1a, pla2g15	7.1E-6	2.9
lipid catabolic process (GO:0016042)	apoc1, apoeb, cyp24a1, gcdh-like abcd4, abhd6, acaa2, cpt1a, cpt1b, lipe, lpin2	1.4E-5	6.1
fatty acid catabolic process (GO:0009062)	gcdh-like abcd4, acaa2, cpt1a, cpt1b, lpin2	1.5E-4	11.9
fatty acid biosynthetic process (GO:0006633)	aclya, elovl5, elovl6, acaca, fads2, fasn, scd	2.6E-4	7.9
fatty acid beta-oxidation (GO:0006635)	gcdh-like abcd4, acaa2, cpt1a, cpt1b	4.0E-4	14.2
lipid biosynthetic process (GO:0008610)	aclya, elovl5, elovl6, acaca, fads2, fasn, gpat3-like, scd dolk, hsd17b7, lpin2, ptdss1a	7.0E-4	3.5
glycerolipid metabolic process (GO:0046486)	apoc1, gpat3-like abhd6, lipe, lpin2, ptdss1a, pla2g15	3.4E-2	2.9
sulfur compound metabolic process (GO:0006790)	aclya, elovl5, elovl6, acaca, acss1, gcdh-like, mat2ab gstk1, sult-like	2,6E-3	3.8
organophosphate metabolic process (GO:0019637)	dnph1, cap1, ndufs8a, cmpk, g6pd, gpat3-like, pgd, pgam1b pfkfb4-like, gch2, dolk, gpd1-like, pank1a, ptdss1a, pla2g15	1.1E-2	2.1
peptidyl-serine phosphorylation (GO:0018105)	rps6ka5 mknk2b, sgk2b, sgk1-like, ulk2	3.3E-2	4.1
pentose-phosphate shunt, oxidative branch (GO:0009051)	g6pd, pgd	3.5E-2	55.4
nucleotide metabolic process (GO:0009117)	dnph1, cap1, ndufs8a, cmpk, g6pd, pgd, pgam1b gpd1-like, pank1a	3.7E-2	2.4
tricarboxylic acid metabolic process (GO:0072350)	aclya, aco1 ogdh	4.1E-2	9,2
response to lipid (GO:0033993)	cyp24a1, lgals17, hamp-like, ptger4b, scd esr1, nfe2l2a	5.8E-3	4.3
response to oxygen-containing compound (GO:1,901,700)	cyp24a1, lgals17, hamp-like, ptger4b, stat 1-like, scd esr1, lpin2, nfe2l2a	1.3E-2	2.9
response to hormone (GO:0009725)	hamp-like, ptger4b, stat 1-like esr1, lpin2, nfe2l2a, nr1d-like	1.8E-2	3.4
response to iron ion (GO:0010039)	aco1 slc11a2-like	3.5E-2	55.4
response to hypoxia (GO:0001666)	ddit4, ldha camk2g2, ppk4-like	3.6E-2	5.5
response to biotic stimulus (GO:0009607)	cd59-like, lgals17, hamp-like, map3k5-like, mx-like agbl5, angptl4, cpt1b, pank1a	4.6E-2	2.3
negative regulation of transcription, DNA-templated (GO:0045892)	cipcb, ciart-like, nr0b2a, znfx1 cbx4, id3, nr0b2b-like, nr1d-like, nr2f2, nr2f6	2.0E-2	2.5
positive regulation of transcription, DNA-templated (GO:0045893)	ctnnb1 smarca2, smarcd3, creb3l3, hnf4b, lpin2, nfe2l2a, nr1d-like, ppargc1a, ocln-like, mafgb	3,4E-2	2.1
regulation of cellular ketone metabolic process (GO:0010565)	acaa2, cpt1a, sirt5	2.8E-2	11.5
plasma lipoprotein particle clearance (GO:0034381)	apoc1, apoeb	4.4E-2	44.3
carboxylic acid transport (GO:0046942)	fabp4a, slc38a2 abcd4, cpt1b, slc16a6a-like, slc16a13	3.1E-2	3.4

what happened in the present study. The hypothesis of an alteration of the chemosensory/taste function by OVTXs may be supported by the "bitter metallic taste" reported by several victims of PlTX intoxication (Alcala et al., 1988; Aligizaki et al., 2011).

The observed alterations in the feeding behaviour were reflected at molecular and cellular levels by significant changes of lipid metabolism in liver, where metabolization/detoxification activities may be responsible for observed effects, even without an evident toxin accumulation. After 6 days of exposure to contaminated mussels, the amounts of lipids (FA and neutral lipids) in fish liver decreased significantly respect to the control ones. Since the algal species used in this exposure experiment are rich in neutral lipid droplets, mainly composed of polyunsaturated lipids (Honsell et al., 2013; Olofsson et al., 2015), the decrease of those substances in seabream tissues seems a direct effect of the OVTXs, rather than a reduced lipid content in the diet. Indeed, in this study the dinoflagellate *O.* cf. *ovata* and the diatom *S. marinoi* were used for exposed and control mussel food, and previous studies identified a lipid content ranging from 24 to 29% (Pezzolesi et al., 2014) and from 24 to 27% (Olofsson et al., 2015) for these two species, respectively. The nutritional values of filter-feeding organisms (and indirectly of fish feeding on them) could be affected by the quality of phytoplankton. In this regard, the proportional fatty acid profiles of marine and brackish diatoms and dinoflagellates are rather similar to each other in comparison with their freshwater counterparts. Nevertheless, regardless of the habitat, diatoms generally show higher EPA than DHA, while dinoflagellates exhibit higher concentrations of DHA than EPA (Peltomaa et al., 2019). In complicating matters, many phytoplankton species are known to produce toxins, and little is known about the effects on nutritional values of edible marine organism in their presence (Svensson and Förlin, 2004).

The erosion of lipid content after exposure to marine biotoxins was previously observed for azaspiracid-1, which caused a 50% decrease of cholesterol content in human lymphocytes together with the upregulation of genes for lipid biosynthesis (Twiner et al., 2008), similarly to our study. In this respect, the modulation of genes involved in lipid metabolism in OVTX-treated seabreams may represent a response to the decrease of lipids and a possible mechanism to restore their physiological levels: in particular, our results highlighted an upregulation of genes related to lipid biosynthesis and a downregulation of genes related to lipid degradation (Fig. 6). Some of the most strongly upregulated genes encode enzymes for the *de novo* synthesis of FA (me1, aclya, acaca, fasn), the elongation of long-chain FA (elovl5, elovl6), and the biosynthesis of unsaturated FA (scd, fads2). Among the others, the retsat gene, overexpressed in OVTX-treated fish, positively regulates *de novo* lipogenesis and FA desaturation (Heidenreich et al., 2017), while the pentose phosphate shunt (g6pd, pgd) is also involved in lipid biosynthesis by generating NADPH. The centrality of FA biosynthetic pathways in exposed seabreams was demonstrated also by the high number of protein interactions for fads2 and me1, which are connected to enzymes (elovl5, elovl6, scd, retsat), transcriptional regulators (ppargc1a, tbl1x, smarcd3), and transporters (cpt1a) involved in lipid metabolism.

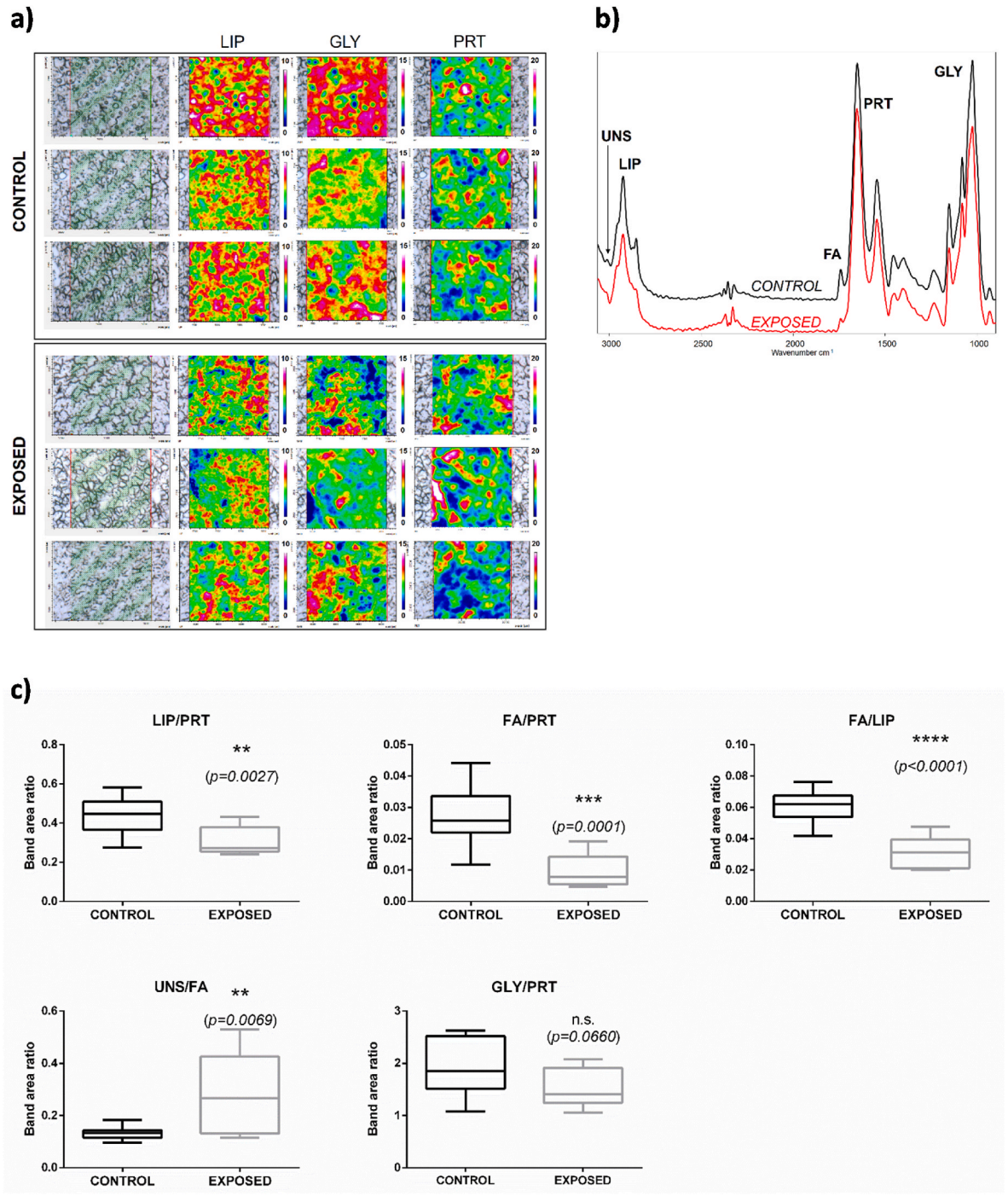

Fig. 4. **a)** Representative microphotograph and IR image displaying the distribution of lipids (LIP), glycogen (GLY) and proteins (PRT) of three liver samples from control and exposed groups. Colour scales range from blue (low values) to magenta (high values). **b)** Representative IR spectra of control and exposed groups. The bands of interest are highlighted (UNS = unsaturated fatty acids; FA = fatty acids). **c)** Box plots show the values of the LIP/PRT, FA/PRT, FA/LIP, UNS/FA, and GLY/PRT band area ratios calculated in liver samples of control and exposed liver samples: centre line marks the median, edges indicate the 25th and 75th percentile, whiskers indicate the min and max values. Asterisks indicate a statistically significant difference between CONTROL and EXPOSED groups; statistical significance was set at 0.05 and calculated by Student's *t*-test (n.s. = not significant difference); exact p values are reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

On the other hand, several genes with lipid catabolic function were downregulated, further confirming the need for reconstituting the intracellular lipid amount. The main downregulated processes were related to the FA β-oxidation, which was affected at different levels: FA degradation reactions (acaa2), FA transport into mitochondria and peroxisomes (cpt1, abdc4), FA mobilization from phospholipids, mono- and triglycerides (pla2g15, abhd6, lipe), and transcription of β-oxidation genes (ppargc1a, lpin2) (Grabner et al., 2021). The transcriptional coactivators ppargc1a and lpin2 are key genes for lipid metabolism, and their downregulation reduces the amount of FA oxidation enzymes (Knutti and Kralli, 2001; Chen et al., 2015). In particular, ppargc1a is a key "energetic regulator" highly expressed in tissues with elevated energy demand like the liver (Knutti and Kralli, 2001), and its downregulation suggests a reduced use of lipids for energy production, to

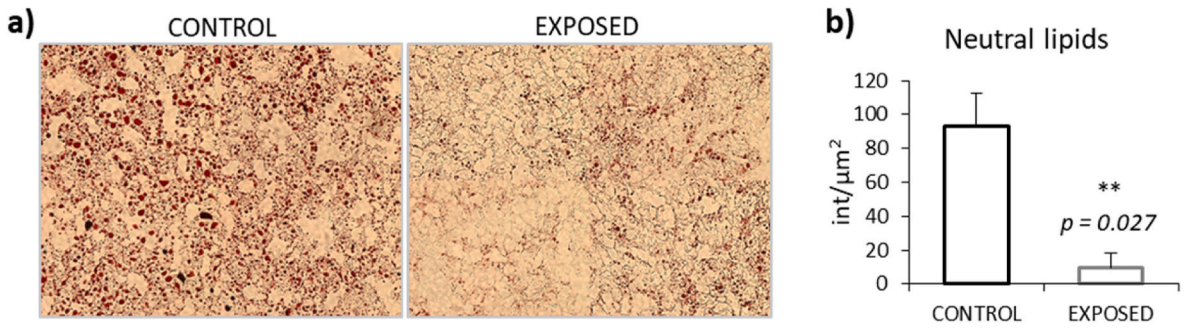

Fig. 5. Neutral lipid content in the liver of *S. aurata* exposed to OVTX-contaminated mussels. **a)** Representative pictures of histological sections stained with Oil Red O from control and exposed livers; **b)** Neutral lipid content in control and exposed livers determined by image analysis and expressed as colour intensity per µm^2 of tissue; statistical significance, calculated by Student's *t*-test, was set at 0.05; the exact p value is reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

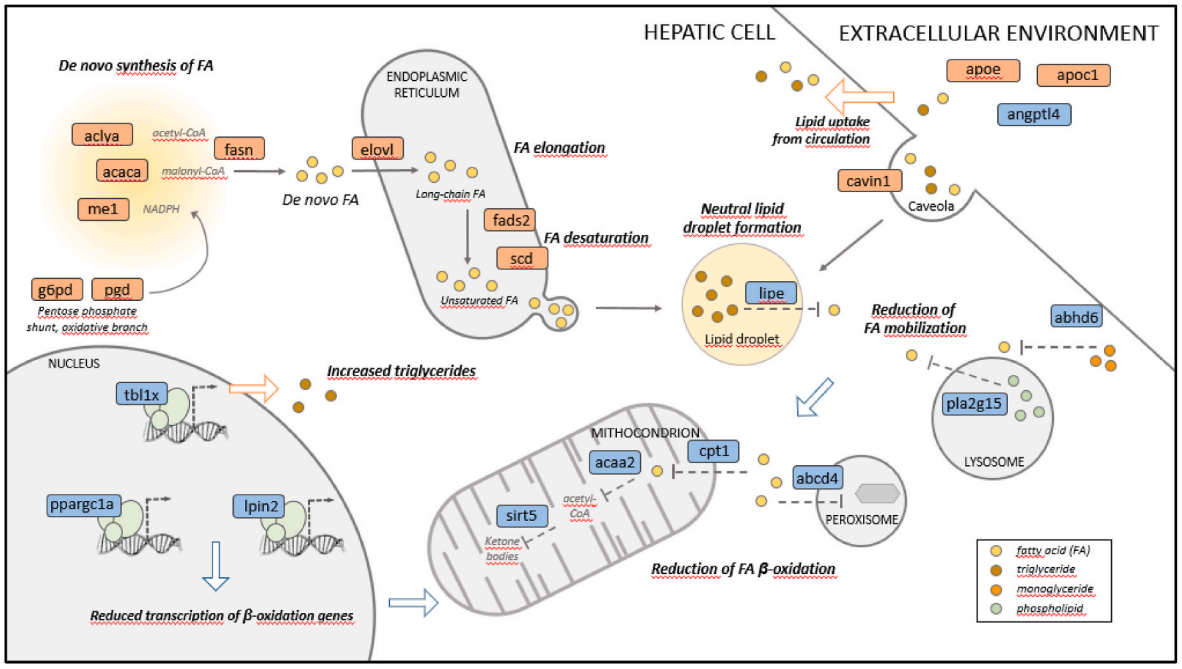

Fig. 6. Scheme of the main lipid-related DEGs and pathways modulated by the OVTX exposure in hepatic cells of *S. aurata* in response to FA and neutral lipids reduction. DEGs are represented by the gene symbol in orange or blue rectangles (for up-regulated and down-regulated genes, respectively). Aclya, acaca and me1 contribute to the *de novo* synthesis of FA through the production of acetyl-CoA, malonyl-CoA and NADPH, respectively, which are then used by fasn to synthesize new FA molecules. NADPH required for lipid synthesis is also generated by the pentose-phosphate pathway, oxidative branch (g6pd, pgd). In the endoplasmic reticulum, FA chains can then be elongated (by the elongases elovl) to produce long-chain FA, and in turn desaturated (by the desaturases fads2 and scd). Apolipoproteins (apoe, apoc1) and angptl4, secreted in the extracellular environment, modulate the lipid uptake from circulation. Extracellular lipid may also be internalized through the formation of caveolae, mediated by cavin1. Neutral lipids from the endoplasmic reticulum and the caveolae are then stored in the lipid droplets. The supply of triglycerides may also derive from downregulation of the tbl1x transcriptional cofactor. In the lipid droplets, downregulation of the lipase lipe decreases the degradation of triglycerides into FA; similarly, low levels of the lysosomal phospholipase pla2g15 and the hydrolase abhd6 reduce FA mobilization from phospholipids and monoglycerides. Reduced FA mobilization, together with the negative regulation of key effectors of mitochondrial (cpt1, acaa2) and peroxisomal (abcd4) β-oxidation, slows down the FA β-oxidation processes. β-oxidation is also inhibited at the transcriptional level, due to the downregulation of key transcriptional coactivators (ppargc1a, lpin2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

preserve and restore their storages. Among other transcriptional regulators modulated in treated seabream, tbl1x is involved in the hepatic metabolism of lipids, and reduced tbl1x mRNA levels were previously associated to increased levels of triglycerides in mouse liver (Kulozik et al., 2011). Being among the protein with the highest number of protein-protein interactions, we may hypothesise that tbl1x represents an essential protein in the seabream response to lipid depletion.

The upregulation of the apolipoprotein genes in the liver of exposed fish (apoeb, apoc1) suggests that the OVTX-diet stimulated the lipid uptake from circulation. Specifically, apoe is involved in lipoprotein uptake from circulation to hepatocytes (Subramanian et al., 2017), and downregulation of angptl4, one of the most under expressed genes in OVTX-exposed fish, would increase the clearance of plasma triglycerides and the cellular uptake of triglycerides-derived FA (Kersten, 2021).

Other DEGs and enriched processes confirmed the cellular demand for reconstituting the lipid pool in the hepatocytes of OVTX-treated seabreams. On one side, the downregulation of genes involved in ketone bodies formation (acaa2, cpt1ab, sirt5), which derive from FA breakdown, is in accordance with the reduced β-oxidation profile (Grabacka et al., 2016). On the other side, the increase of cavin1, involved in the formation of neutral lipid droplets, through triglycerides synthesis from FA (Yokomori et al., 2020), and the simultaneous

downregulation of lipase lipe (responsible for triglycerides degradation) reflects a cellular pathway for restoring the neutral lipid reserves.

Even if predominant, the alteration of gene expression in exposed seabream was not limited to lipid metabolism. The upregulation of genes involved in the *de novo* nucleotide biosynthesis (*e.g.* genes of pentose phosphate shunt and organophosphate metabolism) may support the hypothesis of an overall modulation of fish transcriptional activity in response to OVTXs contaminated diet. Indeed, the numerous processes related to "responses to stimuli" could be a further indication that, despite the absence of toxin accumulation, the exposure to contaminated food represented a challenge which stimulated several mechanisms of biological response.

5. Conclusion

This study demonstrates that, while the direct feeding on *O.* cf. *ovata* causes the accumulation of OVTXs in mussel tissues at environmentally realistic concentrations, seabream fed on such contaminated bivalves did not show detectable levels of toxins in tissues. However, adverse effects were highlighted in fish, by alteration of their feeding behaviour after short-term exposure (6 days) and the significant reduction of lipid content and metabolism.

The absence of a detectable levels of OVTXs in exposed seabream suggests limited effects as regards the risk associated to human consumption, at least for this species. Nonetheless, the altered lipid profile could represent an issue for the organoleptic properties and the nutritional value of the fish flesh; the food avoidance and the induction of a transcriptional profile for lipid synthesis could represent a mechanisms to restore the physiological status. The role of OVTXs in modulating lipid metabolism, if further confirmed in future studies, might open to a possible application of such biotoxins for pharmaceutical and nutraceutical purposes, similarly to other marine biotoxins.

CRediT authorship contribution statement

Maria Elisa Giuliani: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Simone Bacchiocchi:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Stefano Accoroni:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization. **Melania Siracusa:** Writing – original draft, Validation, Investigation. **Debora Campacci:** Investigation. **Valentina Notarstefano:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Marica Mezzelani:** Writing – review & editing, Investigation. **Arianna Piersanti:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Cecilia Totti:** Writing – review & editing, Supervision, Resources. **Maura Benedetti:** Writing – review & editing. **Francesco Regoli:** Writing – review & editing, Supervision, Resources. **Stefania Gorbi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefania Gorbi reports financial support was provided by Polytechnic University of Marche.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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