Science of the Total Environment 683 (2019) 681–689

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Fluoxetine suppresses the immune responses of blood clams by reducing haemocyte viability, disturbing signal transduction and imposing physiological stress

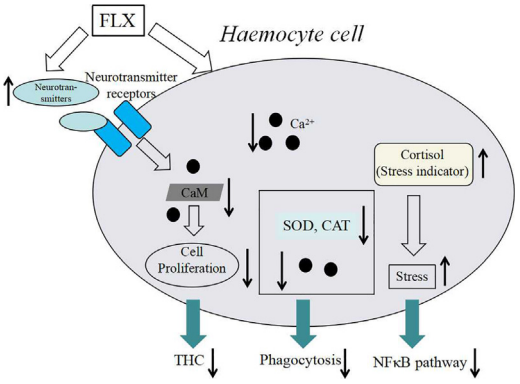
Wei Shi, Yu Han, Xiaofan Guan, Jiahuan Rong, Wenhao Su, Shanjie Zha, Yu Tang, Xueying Du, Guangxu Liu *

College of Animal Sciences, Zhejiang University, Hangzhou, PR China

HIGHLIGHTS

- FLX suppressed both cellular and humoural immune responses in *T. granosa*.
- FLX inhibited haemocyte viabilities and thus reduced the THC of *T. granosa*.
- FLX disturbed intracellular signal transduction and led to constrained phagocytosis.
- FLX aggravated physiological stress and thereafter hampers NFκB signalling pathways.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 January 2019
Received in revised form 16 May 2019
Accepted 20 May 2019
Available online 22 May 2019

Editor: Daniel Wunderlin

Keywords:
Fluoxetine
Immune responses
Ca^{2+} concentration
Neurotransmitters
Stress

ABSTRACT

The antidepressant fluoxetine (FLX), a selective serotonin reuptake inhibitor, is widely prescribed for the treatment of depression and anxiety disorders. Nowadays, measurable quantities of FLX have been frequently detected in the aquatic ecosystems worldwide, which may pose a potential threat to aquatic organisms. Although the impacts of FLX exposure on immune responses are increasingly well documented in mammals, they remain poorly understood in aquatic invertebrates. Therefore, to gain a better understanding of the ecotoxicological effects of FLX, the impacts of waterborne FLX exposure on the immune responses of blood clam, *Tegillarca granosa*, were investigated in this study. Results obtained showed that both cellular and humoural immune responses in *T. granosa* were suppressed by exposure to waterborne FLX, as indicated by total counts of haemocytes (THC), phagocytic rate, and activities of superoxide dismutases (SOD) and catalase (CAT), suggesting that waterborne FLX renders blood clams more vulnerable to pathogen challenges. To ascertain the mechanisms explaining how waterborne FLX affects immune responses, haemocyte viabilities, intracellular Ca^{2+} levels, *in vivo* concentrations of neurotransmitters, physiological stress conditions (as indicated by *in vivo* concentrations of cortisol), and expressions of key regulatory genes from Ca^{2+} and neurotransmitter signal transduction, as well as immune-related signalling pathways, were examined after 10 days of FLX exposure by blood clams *via* 1, 10 and 100 μg/L waterborne FLX. The results obtained indicated that immune response suppression caused by waterborne FLX could be due to (i) inhibited haemocyte viabilities, which subsequently reduce the THC; (ii) altered intracellular Ca^{2+} and neurotransmitter concentrations, which lead to constrained phagocytosis; and (iii) aggravated physiological stress, which thereafter hampers immune-related NFκB signalling pathways.

* Corresponding author at: Room 551, Agriculture-Environment-Biology Group, Zijingang Campus, Zhejiang University, 886# Yuhangtang Road, Hangzhou, Zhejiang 310058, PR China.
E-mail address: guangxu_liu@zju.edu.cn (G. Liu).

https://doi.org/10.1016/j.scitotenv.2019.05.308
0048-9697/

1. Introduction

Pharmaceutical compounds are a class of bioactive chemicals widely used in human and veterinary medicine (Kim et al., 2007). The increased production and usage of pharmaceuticals, as evidenced by the >3000 different substances used in human medicine in the European Union (Fent et al., 2006; Gardner et al., 2013), inevitably leads to their continuous release into the aquatic environment. For instance, according to the data from Organization for Economic Co-operation and Development (OECD; Organisation for Economic Co-operation and Development, 2017), the consumption of antidepressant drugs has doubled in OECD countries from 2000 to 2015. In this respect, these pharmaceuticals are often detected in different aquatic environments, especially in waters receiving wastewater effluents (Kolpin et al., 2002; Fent et al., 2006). Fluoxetine (FLX) is one of the most widely prescribed pharmaceuticals for the treatment of clinical depression and other compulsive disorders, with relatively few side effects (Milea et al., 2010). However, the high consumption, long half-life, and the relatively resistant to biodegradation of FLX often lead to it being found in high concentrations in aquatic environments (Kwon and Armbrust, 2006), which can range from 17 ng/L to 3.6 μg/L in waters polluted at different degrees (Kolpin et al., 2002; Fent et al., 2006; Salgado et al., 2011). Notably, FLX with an octanol/water partition coefficient (K_{ow}) log >4 can be accumulated in a much higher concentration in sediment (Johnson et al., 2005; Kwon and Armbrust, 2006). For instance, a fate study lasting for 44 days found that the percentage of detected fluoxetine was up to 10-fold higher in sediment than in overlaying water (Sánchez-Argüello et al., 2009). Similarly, FLX at 968-fold higher concentration was detected in sediment than in water due to the adsorption effect (Furlong et al., 2004; Kwon and Armbrust, 2006). In this respect, sessile benthic invertebrates such as bivalve mollusks live in proximity to sediment may experience FLX pollution at an even serious degree (Sánchez-Argüello et al., 2009). Furthermore, it has been suggested that waterborne FLX can easily accumulate in aquatic organisms through bioaccumulation, which subsequently exerts sublethal physiological effects on these organisms and poses a potential threat to the aquatic ecosystem (Brooks, 2014; Silva et al., 2015).

Though FLX as well as other pharmaceuticals are usually designed for specific metabolic and molecular pathways, there has been an increasing amount of evidence revealing that antidepressants including FLX can suppress the immune responses of mammalian species, such as rats and humans (Maes et al., 2012; Frick and Rapanelli, 2013). For example, a previous study conducted on rats found that acute FLX administration (10 mg/kg) can result in a dose- and time-dependent decrease in mitogen-induced lymphocyte proliferation and natural killer cell cytolytic activity (Pellegrino and Bayer, 1998). However, most of the previous research evaluating the ecotoxicity of FLX to aquatic organisms mainly focused on behavioural and physiological functions such as reproduction, foraging and stress responses (Bringolf et al., 2010; Ansai et al., 2016; Saaristo et al., 2017). Data regarding the immunotoxic effects of waterborne FLX on aquatic organisms are still relatively sparse (Munari et al., 2014). In addition, due to the differences in physiology and pharmacodynamics between terrestrial mammals and aquatic species, FLX may interfere with non-target pathways or systems in aquatic organisms, such as invertebrate bivalve mollusks, and therefore lead to serious physiological consequences on these organisms (Christen et al., 2010).

The mechanisms underlying the immunomodulatory functions of FLX still remain largely unknown especially in aquatic invertebrates; nevertheless, some of the intracellular pathways involved have been identified in mammals (Edgar et al., 1999; Maes et al., 2005). According to previous studies, FLX can trigger apoptosis and inhibit the proliferation of immune cells in humans (Edgar et al., 1999; Frick et al., 2008). Though the direct molecular targets of FLX remain unclear, these reported effects of FLX on immunity are thought to be mediated by neurotransmitter signalling pathways (Gordon and Barnes, 2003). It has been suggested that neurotransmitter signalling may also play an indispensable role in the immune systems of various invertebrates (Ottaviani et al., 2007; Liu et al., 2017). For example, it has been shown that neurotransmitters such as acetylcholine (ACh), norepinephrine (NE) and γ-aminobutyric acid (GABA) can induce apoptosis and phagocytosis of haemocytes and decrease inflammatory cytokine production in mollusks (Liu et al., 2015, 2016b; Li et al., 2016). Therefore, any changes in the *in vivo* contents of neurotransmitters' or the binding activities of neurotransmitters' receptors could result in disturbed immune responses (Li et al., 2016), which offers a potential explanation for FLX-induced immune response suppression in invertebrate species. However, empirical data regarding the effects of FLX on neurotransmitter signalling and subsequent neurotransmitter medicated immune responses are still unclear in aquatic invertebrates.

In addition, FLX has been reported to modulate immune responses, such as T cell functioning by altering intracellular Ca^{2+} signalling in mammals (Maes et al., 2005; Frick and Rapanelli, 2013). To date, the crucial roles of intracellular Ca^{2+} homeostasis in immune responses have been well established (Vig and Kinet, 2009; Shi et al., 2018). For instance, phagocytosis, a critical mechanism of the innate immune system in aquatic invertebrates for eliminating various invading microorganisms or foreign particles, was shown to be regulated by intracellular Ca^{2+} signalling (Nunes and Demaurex, 2010). Therefore, it is possible that Ca^{2+} signalling could be another pathway through which FLX disrupts the immune responses of aquatic invertebrates. However, information about the effects of FLX on Ca^{2+} regulation is still unavailable in aquatic invertebrates at present.

The intrinsic relationship between stress and immune efficiency has long been well developed in aquatic organisms (Lacoste et al., 2002; Malham et al., 2003). For instance, stress conditions have been shown to suppress immune competence in both mammals and aquatic organisms, as indicated by elevated levels of cortisol, a key hormone regulating stress responses (Ader et al., 1991; Ottaviani et al., 1998; Hooper et al., 2007; Eames et al., 2010; Lagos et al., 2015). Recently, it has been reported that the presence of waterborne FLX can cause changes in responses to stress and may induce plasma cortisol in fish (Abreu et al., 2014), suggesting that FLX may hamper immune responses by causing aquatic organisms to become stressed as many other pollutants do (Meltzer et al., 1997). However, whether this presumption holds true or not in aquatic invertebrates still needs to be verified by empirical data.

Benthic filter feeders, such as bivalve mollusks, are some of the most effective sinks of pollutants in the aquatic food web (Fung et al., 2004; Shi et al., 2016; Su et al., 2019). Moreover, bivalve species are sensitive to waterborne pollutants and therefore excellent species in which to address the ecotoxicity of pharmaceuticals such as FLX (Gonzalez-Rey and Bebianno, 2013; Shi et al., 2018). The blood clam, *Tegillarca granosa*, is a traditional commercial bivalve species with a wide distribution throughout the Indo-Pacific region (Shao et al., 2016; Han et al., 2016). Due to their ecological importance in sediment nutrient cycling and ecosystem carbon flow, numerous studies have been performed on various aspects of *T. granosa* (Shi et al., 2017a; Zhao et al., 2017; Su et al., 2017). In addition, inhabiting the intertidal mudflat where surface runoff converges into the sea, the blood clam is often challenged by various pollutants and therefore deemed as a good model organism to investigate the toxicity of various pollutants on aquatic organisms (Shi et al., 2017b; Guan et al., 2018). However, little is currently known about the ecotoxicological impacts of pharmaceuticals on this species.

The present study was therefore conducted using the blood clam as a representative of aquatic bivalve mollusks to (i) determine the effects of waterborne FLX on the immune responses; (ii) examine how haemocyte viability will be affected by FLX exposure; (iii) investigate whether waterborne FLX weakens the immunity of bivalve species, if any, by affecting *in vivo* concentrations of neurotransmitters and the expression of neurotransmitter-modulatory genes; (iv) verify whether FLX hampers immune responses, if any, *via* disturbing intracellular Ca^{2}

$^{+}$ concentrations and Ca^{2+} signalling pathways; and (v) show whether waterborne FLX renders the organism into a more stressful condition.

2. Materials and methods

2.1. Collection and acclimation of bivalves

Adult blood clams, *T. granosa* (wet weight of 7.68 ± 0.61 g, mean ± SE), were obtained from Yueqing Bay (28° 280′ N and 121°110′ E), China in May 2018. To obtain the background concentration of FLX, seawater was sampled and analysed in triplicate using an HPLC system (1200 series, Agilent, Germany) equipped with a reversed C_{18} column at a wavelength of 226 nm following the method of previous studies (El-Dawy et al., 2002; Llerena et al., 2003). The environmental concentration of waterborne FLX was determined to be under the detection limits (<20 ng/mL). After scrubbing the shell surface to remove any debris and organisms attached, clam samples were allowed to acclimate in a 1000 L indoor tank with sand-filtered seawater for at least 7 days. During the process of acclimation, clams were fed twice daily with microalgae *Tetraselmis chuii* at a rate of 5% of the tissue dry weight and no mortality occurred during the experiment. Sand-filtered and UV-radiated seawater (temperature 21.5 ± 0.3 °C, pH 8.08 ± 0.07, salinity 20.9 ± 0.3‰) obtained from the clam-sampling site, Yueqing Bay, was used throughout the experiment. All experiments of this study were performed in accordance with the Animal Ethics Committee of Zhejiang University (ETHICS CODE Permit NO. ZJU2011-1-11-009Y).

2.2. Exposure experiment and sampling

The FLX hydrochloride (YZ-100513) used in the present study was purchased from the National Institutes for Food and Drug Control, China. On the basis of previous studies (Fent et al., 2006; Gros et al., 2010; Salgado et al., 2011) and taking the fact that blood clams live in proximity to sediment where FLX could occur in high concentrations into consideration, 1, 10 and 100 μg/L were chosen to simulate environmental concentrations of FLX at different pollution levels that the clams may experience. The stock solution of FLX at the dose of 1 g/L, a concentration high enough to avoid weighing errors, was prepared by dissolving FLX in 1 μm-sieve filtered seawater. In total, we utilized one control group, which had no added FLX and three experimental groups with the nominal 1, 10, and 100 μg/L concentrations of FLX, respectively, in the present study. After acclimatization, 360 clams were randomly divided into 12 plastic tanks (4 treatment groups × 3 replicates) with a total seawater volume of 40 L containing the corresponding FLX concentrations and with slight aeration. As described above, the working concentrations of FLX in each tank were measured every 2 days during the experiment using an HPLC system (1200 series, Agilent, Germany) at quantitation and detection limit of 50 ng/mL and 20 ng/mL, respectively (El-Dawy et al., 2002; Llerena et al., 2003) (Table 1). The clams were fed with *T. chuii* as descried previously and seawater was changed daily with newly added FLX. The exposure lasted for 10 days and no individual mortality was observed throughout the experimental period.

2.3. Haemocyte counts analysis

Haemocyte count analysis was performed after 10 days of experimental treatment following the methods described by Mackenzie et al. (2014) and Liu et al. (2016a). In brief, five individual clams from each replicate tank were randomly selected for analysis. After rinsing with 0.1 M phosphate buffer saline (PBS) solution to remove impurities, valves of the clam were opened with a scalpel and then 100 μL of haemolymph was extracted out of the cavity of each individual using a 1 mL syringe. The haemolymph was subsequently diluted with 800 μL of PBS and 100 μL of 2.5% glutaraldehyde in a 1.5 mL centrifuge tube and the remaining tissues of these clams were kept on ice for the measurements of contents of cortisol and neurotransmitters. Wet mount of fixed haemolymph was then prepared and estimated with a Neubauer haemocytometer (XB-K-25, Anxin Optical Instrument, China) under an Olympus BX53 microscope (Olympus, Japan) at a magnification of 400×. Similarly, blood smears were made and then stained with Wright's Gimesa stain (G1020, Solarbio, China) for haemocyte cell type analysis (Liu et al., 2016a). The counts of various cell types were determined using an Olympus BX53 microscope at 400× magnification and the percentage of each type of haemocyte was obtained by dividing the number of this type of cell identified by the total number of haemocytes examined. In all cases, >200 haemocyte cells for each sample were scored.

Table 1
Exposure concentrations of FLX (mean ± SE) measured in each treatment group every 2 days of the exposure experiment (limits of detection and quantitation were 20 ng/mL and 50 ng/mL, respectively).

Nominal concentration	Control	1 μg/L	10 μg/L	100 μg/L
FLX actual concentration (μg/L)	Not detected	1.02 ± 0.06	9.89 ± 0.09	101.12 ± 0.11

2.4. Phagocytosis assays

Following the methods described by Su et al. (2018) and Liu et al. (2016a), five individuals were randomly picked from each trial for phagocytosis assays after 10 days of experimental treatment. A yeast suspension (Instant dry yeast, AngelYeast, China) containing 1.35 ± 0.07 × 10^8 yeast cells per mL was prepared by dissolving 10 mg of yeast powder in 1 mL of Alsever's solution (R1016, Solarbio, China). After being extracted out of the cavity of each individual as described above, 100 μL of fresh haemolymph was mixed simultaneously (1:1) with pre-cooled Alsever's solution (R1016, Solarbio, China) in a 1.5 mL centrifuge tube. After adding yeast suspensions at a yeast-haemocyte ration of 10:1, the mixtures were kept at 25 °C for 30 min, incubated in a cool water bath at 4 °C for an hour and then stopped by the addition of 100 μL 2.5% glutaraldehyde. Blood smears were then made and stained with Wright's Gimesa stain, which colors the haemocyte cells and yeast particles into lavender and dark blue, respectively (Luengen et al., 2004). The phagocytic rate was measured microscopically at a magnification of 400 × with an Olympus BX53 microscope. Haemocyte cells that in the process of engulfment and had at least one yeast particle engulfed inside were deemed as phagocytic cells. The phagocytic rate was calculated by dividing the number of phagocytic cells by the total number of haemocyte cells counted. At least 200 haemocyte cells were scored for each sample to ensure accuracy.

2.5. Activities of key immune-related enzymes

After 10 days of exposure, nine clams from each experimental group (3 individuals for each replicate) were selected to determine the activities of superoxide dismutase (SOD) and catalase (CAT) in the haemolymph. As described above, 100 μL haemolymph was extracted from the cavity of each individual. One part of the haemolymph (40 μL) was used to determine the activities of SOD and CAT (20 μL each), and the other part (60 μL) was used for Ca^{2+} concentration analysis (see details described in 2.8). In brief, the activities of SOD and CAT were measured using commercial kits (A001 and A007, Nanjing Jiancheng Bioengineering Institute, China) with a microplate reader (Multiskan GO, Thermo, USA) at the absorption wavelength of 450 and 405 nm, respectively. One unit of SOD activity (U_{SOD}) was defined as the enzyme amount inhibiting the rate of nitroblue tetrazolium reduction by 50% in 1 mL of reaction solution. One unit of CAT activity (U_{CAT}) was defined as the amount of 1 μmol of H_2O_2 decomposed per mL haemolymph per second.

2.6. Haemocyte viability analysis

Haemocyte viability was evaluated using a 3 - (4,5 - dimethylthiazol - 2 - yl) - 2,5 - diphenyl - tetrazolium bromide (MTT) reduction assay, a sensitive and quantitative colorimetric assay which measures the capacity of mitochondrial succinyl dehydrogenase in living cells by converting a yellow substrate (MTT) into a purple formazan product (Domart-Coulon et al., 2000). After 10 days of exposure, five individual clams from each replicate tank were randomly picked for haemocyte viability analysis. The assay was performed using commercial kits (C0009, Beyotime Biotechnology, China) according to the manufacturer's instructions. Briefly, 100 μL haemolymph obtained from each individual was seeded into a 96-well plate and mixed with 10 μL MTT solution. After an incubated at 37 °C for 4 h, 100 μL Formazan provided by the kit was added and incubated at 37 °C for another 4 h. The optical density was measured at 570 nm using a microplate reader (Multiskan GO, Thermo, USA). Following the method described by Ong et al. (2017) and Zha et al. (2019), relative percentages of cell viability were obtained by comparing against the control group.

2.7. Measurement of in vivo concentrations of cortisol and neurotransmitters

Following the methods of Guan et al. (2018) and Rong et al. (2018), the concentrations of ACh, GABA and cortisol in the gills of three individuals in each replicate tank were quantified by commercial ELISA kits (ML095412, ML086216 and ML003467, MLBIO biotechnology, China, respectively) according to the manufacturers' protocols. After dissection on ice, the gill of each clam was removed and homogenized in ice-cold PBS (0.01 M, pH 7.4, *w*/*v* (mg/mL) = 1/1) with an electric homogenizer (ART, MICCRA D-1, Germany) as described in previous studies (Guan et al., 2018). Homogenates were centrifuged at 2000 rpm for 20 min at 4 °C, and the supernatant was then used to determine the concentrations of neurotransmitters. Briefly, 10 μL of the testing sample was mixed with 40 μL of diluent in each well of a microplate and then incubated at 37 °C for 60 min. After five rounds of washing with wash buffer, the wells were then incubated at 37 °C for another 15 min in the dark followed by the addition of 50 μL of corresponding chromogenic reagent. The absorption values were measured at a wavelength of 450 nm using a microplate reader (Multiskan GO, Thermo, USA). The concentrations of ACh, GABA and cortisol in each sample were subsequently determined by comparing the obtained absorption values to corresponding standard curves.

2.8. Intracellular Ca^{2+} concentration analysis

Following the methods described in previous studies (Shi et al., 2017b), three individuals from each experimental tank were selected to determine the intracellular Ca^{2+} concentration of haemocytes. In brief, freshly extracted haemolymph from each individual was immediately transported into a glass beaker and incubated in calcium-free saline solution containing 50 mM fluo-4/AM (Dojindo Laboratories, Japan) in the dark for 20 mins. After incubation, the fluo-4/AM-loaded haemolymph was washed three times to remove extracellular dye and allotted an additional 20 min to allow for the de-esterification of the dye into its charged, Ca^{2+}-sensitive form in haemocytes. Then, the intracellular Ca^{2+} levels were determined using a fluorescence microscope (Nikon Eclipse 80i) at 1000 × magnification fitted with a Nikon DXM1200C digital video camera (Nikon, Tokyo, Japan). An excitation filter at a wavelength of 480 ± 40 nm and an emission filter at 535 ± 50 nm were used for the intracellular Ca^{2+} assays. The Ca^{2+} levels were then determined from the measured fluorescence intensities with the software Image-Pro Plus 6.0 (Rockville, MD, USA), using the following equation: F = IOD/area, where F is the fluorescence intensity of the haemolymph, integrated optical density (IOD) represents the total optical density value of the stained haemocyte area, and 'area' is the stained area of the haemocytes.

2.9. Gene expression analysis

Total RNA was isolated from haemocyte samples of 5 clams individually from each treatment replicate as described in our previous study (Shi et al., 2017b). RNA quality and concentration were verified with gel electrophoresis and a NanoDrop 1000 UV/visible spectrophotometer (Thermo Scientific, USA), respectively. First strand cDNA was synthesized from high-quality total RNA (>500 ng/μL) using the PrimeScript RT reagent Kit (TaKaRa, RR037A) according to the manufacturer's protocol. Real-time quantitative PCR was then conducted on the StepOnePlus Real-Time PCR System (Thermo Scientific, USA) in triplicate. Amplifications were carried out in a total volume of 10 μL consisting of 5 μL of SYBR Green Master Mix (Q111-2, Vazyme, China), 0.2 μL of each primer (10 μM), 0.2 μL of ROX Reference Dye (Q111-2, Vazyme, China), 1 μL of cDNA template, and 3.4 μL of double-distilled water. The following amplification protocol was used: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A melting curve analysis was used to confirm the specificity and reliability of the PCR products. The 18S rRNA gene was employed as an internal reference (Hüning et al., 2013; Wang et al., 2016) and the $2^{-\Delta\Delta CT}$ method was applied to analyse the relative gene expression of target genes (Livak and Schmittgen, 2001). In total, the expressions of five genes encoding calmodulin (CaM), muscarinic ACh receptor M3 (mAChR3), GABA transaminase (GABAT), nuclear factor kappa B subunit 1 (NFκB1), and TNF receptor associated factor 2 (TRAF2), respectively, were analysed in the present study. The primers used are listed in Table 2 and all primers were synthesized by TsingKe Biotech (Beijing, China).

2.10. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* comparison test were conducted to illustrate the effects of FLX exposure on haemocyte counts, phagocytosis, percentages of three major types of haemocytes, cell viability, Ca^{2+} concentrations and the activities of immune-related enzymes (SOD and CAT) among experimental groups. For all analyses, Levene's tests and Shapiro-Wilk's tests were used to verify the homogeneity of variances and normality, respectively. In cases where these assumptions were not satisfied by raw data, the data were arcsine square root transformed prior to analysis (McDonald, 2014). Gene expression levels were analysed using the Duncan multiple range test. All of the analyses were conducted using the Origin-Pro 8 software package. All of the data are presented as the mean ± SE and a p value < 0.05 was accepted as significant difference.

Table 2
Primer sequences for the genes investigated.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession no.
CaM	AGCTGAACTTCGCCATGTTATGAC	ACTTGTCCGTCACCATCTATATCAG	JZ898325
mAChR3	GCCCGTGAGTAACTTCCCATAAACA	CCAGACAACATCGTTCTTCGCAAAT	MH156847
GABAT	GGCACCTGACAACAGAGGCTAT	GGGAGCTTCGGGATAACCTGTT	MH156847
NFκB1	AATCAAGCAGGTGTAGTAGAC	CAGACAGGACAGCCAGAT	MH507319
TRAF2	CGTAATAGAAGAGCCGATCA	GCGAATAGATACTGGTCACT	JZ898323
18S	CTTTCAAATGTCTGCCCTATCAACT	TCCCGTATTGTTATTTTTCGTCACT	JN974506.1

Table 3
Total counts (THC), percentages of three major cell types and phagocytosis of haemocytes of *T. granosa* after 10 days exposure to various doses of waterborne FLX. Different uppercase letters indicate statistically significant differences among concentrations.

Treatments		Control	1 μg/L FLX	10 μg/L FLX	100 μg/L FLX
THC ($\times 10^8$/mL)		3.88 ± 0.07^a	3.79 ± 0.04^a	3.27 ± 0.03^b	2.67 ± 0.04^c
Percentage (%) of the	Red granulocyte	88.31 ± 0.31^a	87.03 ± 0.56^a	83.79 ± 0.41^b	82.51 ± 0.22^b
	Basophil granulocyte	10.02 ± 0.33^a	11.96 ± 0.88^b	14.63 ± 0.44^c	16.23 ± 0.32^c
	Hyalinocyte	1.67 ± 0.22^a	1.01 ± 0.13^a	1.58 ± 0.27^a	1.26 ± 0.16^a
Phagocytosis (%)		29.49 ± 0.81^a	28.78 ± 0.30^a	17.93 ± 0.36^b	13.43 ± 0.71^c

3. Results

3.1. Impact of waterborne FLX exposure on the total counts and cell type composition of haemocytes

A significant reduction in the total counts of haemocytes (THC) was detected after 10 days of exposure to 10 and 100 μg/L FLX (Table 3, ANOVA, $F_{3,59} = 133.7$, $p < 0.05$). The THCs of the clams exposed to 10 days of 10 and 100 μg/L FLX significantly decreased to approximately 83.1% and 69.8% of that of the control group, respectively. In addition, compared to the control, a significant impact on the cell type composition exerted by FLX exposure was also detected (Table 3, ANOVAs, $F_{3,59} = 44.8$ and $F_{3,59} = 40.2$ for red granulocyte and basophil granulocyte, respectively, $p < 0.05$). After 10 days of exposure to 10 and 100 μg/L FLX, the percentages of red granulocyte significantly dropped to approximately 94.9% and 93.4% of the control, respectively. In contrast, the percentages of basophil granulocyte significantly increased to approximately 1.2, 1.5, and 1.6 times of those seen in the control for clams exposed to 1, 10 and 100 μg/L FLX, respectively. Compared to the control, exposure to waterborne FLX at 10 and 100 μg/L led to a significant decrease in phagocytosis, which declined to approximately 60.8% and 45.5% of the level seen in the control, respectively (Table 3, ANOVA, $F_{3,59} = 185.7$, $p < 0.05$).

3.2. Impact of FLX exposure on the activity of key immune-related enzymes

Remarkable decreases in the activities of SOD and CAT in *T. granosa* were observed after 10 days of exposure to waterborne FLX at 10 and 100 μg/L (Fig. 1, ANOVAs, $F_{3,35} = 64.8$ and $F_{3,35} = 21.3$ for SOD and CAT, respectively, $p < 0.05$). Clams exposed to 10 and 100 μg/L FLX for 10 days had significantly lower SOD activities, which were approximately 66.8% and 63.4% of the control, respectively (Fig. 1A). Similarly, CAT activities also sharply decreased to approximately 80.5% and 72.8% of the control after 10 days exposure of blood clams to FLX at 10 and 100 μg/L, respectively (Fig. 1B).

3.3. Impact of waterborne FLX exposure on the haemocyte viability

Exposure to waterborne FLX for 10 days was shown to exert significant impacts on the haemocyte viability of *T. granosa* (Fig. 2, ANOVA, $F_{3,59} = 24.5$, $p < 0.05$). Though there was no significant difference in haemocyte viability found between the control and 1 μg/L treatment, the haemocyte viabilities of *T. granosa* were significantly declined to 85.2% and 78.9% of that of the control after 10 days of exposure to FLX at 10 and 100 μg/L, respectively.

3.4. Impact of waterborne FLX exposure on the in vivo concentrations of cortisol and neurotransmitters

No significant changes in the *in vivo* concentrations of cortisol were detected between the control and those exposed to 1 or 10 μg/L FLX for

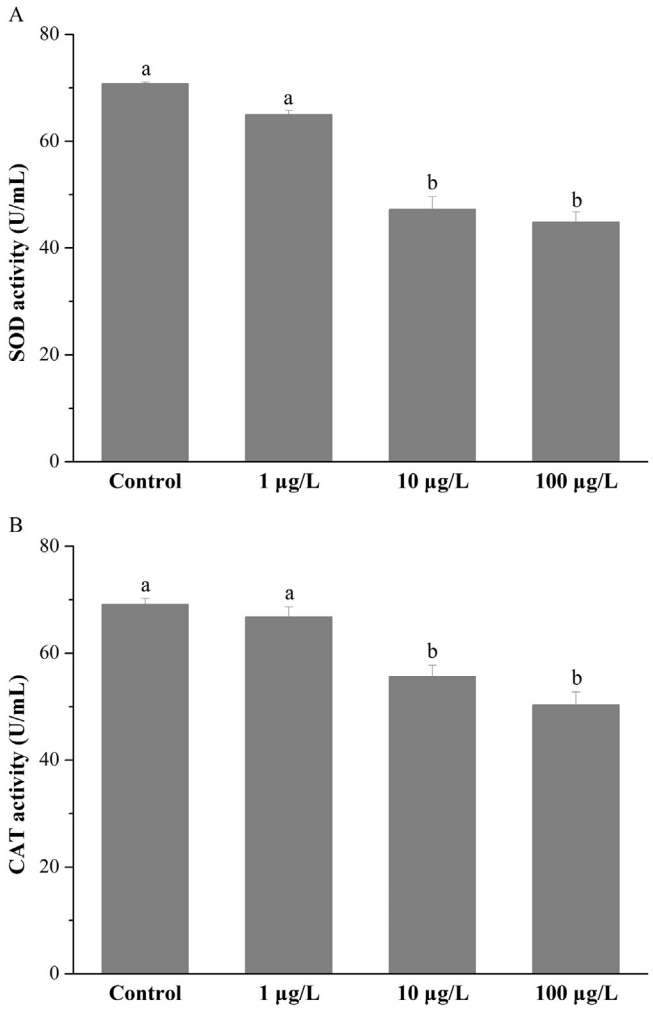


Fig. 1. The activities of SOD (A) and CAT (B) in *T. granosa* after 10 days of exposure to various doses of waterborne FLX. Mean values that do not share the same superscript were significantly different ($p < 0.05$).

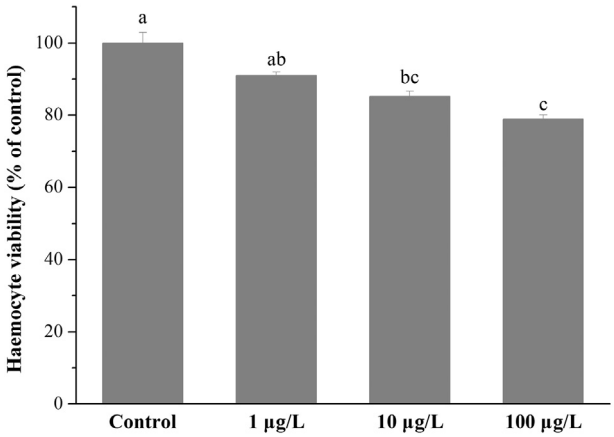


Fig. 2. Haemocyte viabilities of *T. granosa* after 10 days of exposure to various doses of waterborne FLX. Mean values that do not share the same superscript were significantly different ($p < 0.05$).

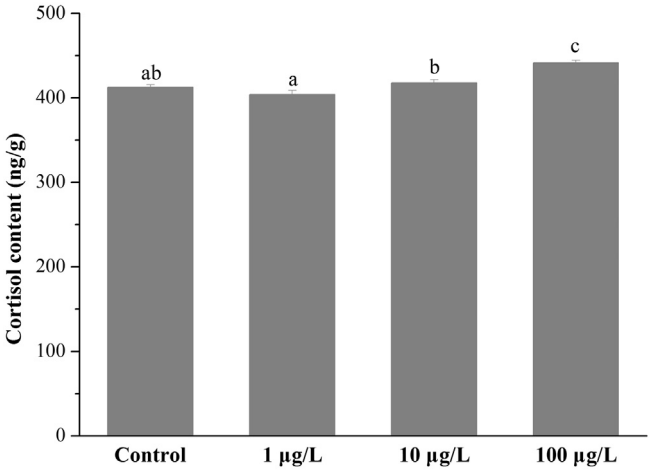


Fig. 3. The *in vivo* concentrations of cortisol in *T. granosa* after 10 days of exposure to various doses of waterborne FLX. Mean values that do not share the same superscript were significantly different ($p < 0.05$).

10 days. However, when the exposure concentration of FLX increased to 100 μg/L, the *in vivo* concentration of cortisol significantly increased to approximately 1.07 times that of the control (Fig. 3, ANOVA, $F_{3,35} = 46.6$, $p < 0.05$). In addition, exposure to waterborne FLX at 10 and 100 μg/L led to significant increases in the *in vivo* concentrations of ACh, which were approximately 1.12 and 1.18 times of that of the control group (Fig. 4A, ANOVA, $F_{3,35} = 56.6$, $p < 0.05$). Similarly, the *in vivo* GABA concentrations were significantly increased by the exposure of clams to waterborne FLX for 10 days (Fig. 4B, ANOVA, $F_{3,35} = 188.1$, $p < 0.05$), by approximately 1.09, 1.17 and 1.30 times of that in the control for exposure groups exposed to 1, 10 and 100 μg/L FLX, respectively.

3.5. Impact of waterborne FLX exposure on the intracellular Ca^{2+} concentration

Results obtained showed that intracellular Ca^{2+} concentration of haemocyte was significantly affected by the exposure of clams to waterborne FLX for 10 days (Fig. 5, ANOVA, $F_{3,35} = 267.6$, $p < 0.05$). After 10 days of exposure, Ca^{2+} fluorescence intensities significantly declined to approximately 45.9% and 11.3% of that in the control for clams exposed to FLX at 10 and 100 μg/L, respectively.

3.6. Impact of waterborne FLX exposure on the expressions of tested genes

Exposure to 10 days of waterborne FLX at 1, 10 and 100 μg/L led to a significant suppression of the expressions of genes encoding for the intracellular receptor for Ca^{2+} (CaM) (Fig. 6, $p < 0.05$). The expressions of genes encoding for the neurotransmitter modulatory enzyme and receptor were significantly altered by exposure of clams to FLX as well. The expression of the GABA modulatory enzyme, GABAT, was significantly induced whereas that of ACh receptor, mAChR3, was significantly downregulated upon exposure to waterborne FLX (Fig. 6, $p < 0.05$). In addition, the expressions of genes encoding NFκB1 and TRAF2 from the immune related NFκB signalling pathway were markedly suppressed by the exposure of clam individuals to 1, 10 and 100 μg/L of waterborne FLX.

4. Discussion

In recent years, the direct effects of FLX on the immunity of mammals have been demonstrated (Maes et al., 2005; Frick and Rapanelli, 2013). At the same time, a widespread occurrence of FLX in aquatic environments, as well as its ability to elicit adverse impacts on aquatic

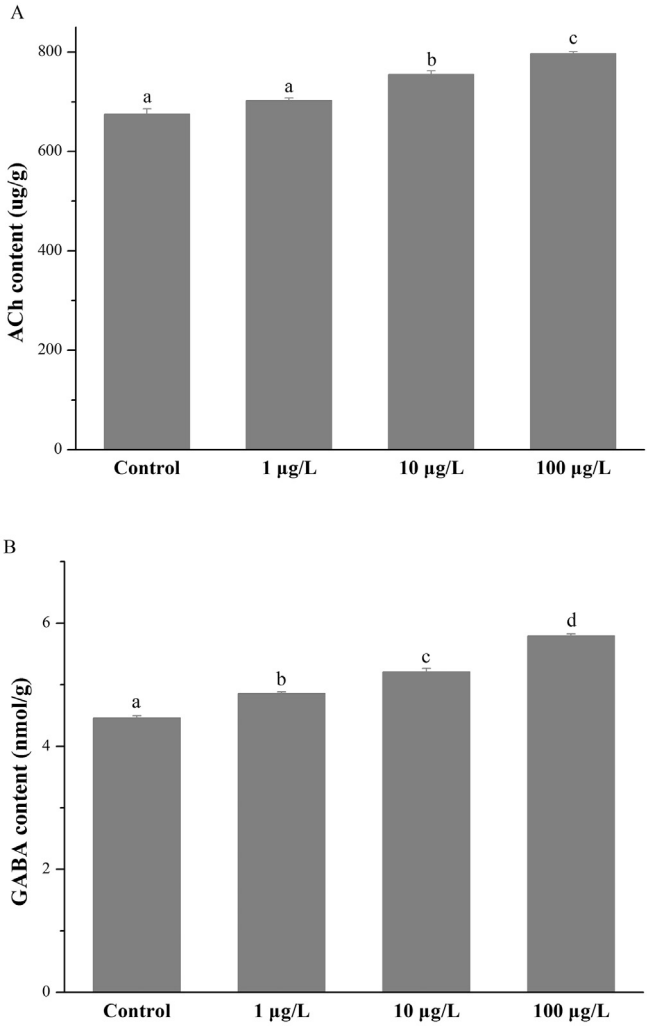


Fig. 4. The *in vivo* concentrations of ACh (A) and GABA (B) in *T. granosa* after 10 days of exposure to various doses of waterborne FLX. Mean values that do not share the same superscript were significantly different ($p < 0.05$).

organisms were also increasingly revealed (Brooks, 2014; Silva et al., 2015). However, whether waterborne FLX will affect the immune responses of aquatic organisms and, if any, the affecting mechanism underpins its immunotoxicity are still largely unknown. The results

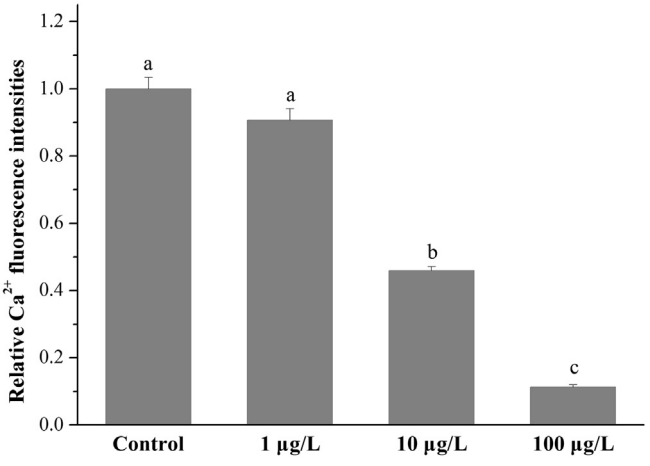


Fig. 5. Haemolymph Ca^{2+} concentration of *T. granosa* after 10 days of exposure to various doses of waterborne FLX. Mean values that do not share the same superscript were significantly different ($p < 0.05$).

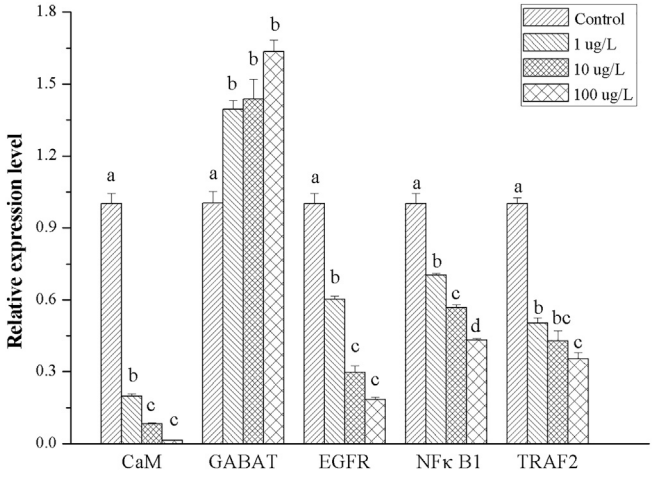


Fig. 6. Expression of genes encoding for CaM, GABAT, mAChR3, NFκB1, and TRAF2 after 10 days of exposure to various doses of waterborne FLX. Mean values that do not share the same superscript were significantly different.

obtained in this study showed that the immune responses of *T. granosa*, as indicated by THC, cell type composition, and phagocytosis, were significantly hampered by exposure to waterborne FLX, which may be due to the integrated effects of FLX on haemocyte viabilities, neurotransmitter signalling, intracellular Ca^{2+} levels, and physiological stress conditions.

Haemocytes are regarded as the main immune cells responsible for carrying out the host defence response in many aquatic organisms including marine bivalve mollusks (Loker et al., 2004). In the present study, reduced THCs were observed after *T. granosa* was exposed to waterborne FLX (at the concentrations of 10 and 100 μg/L), which implied an evident suppression of immune response. This reduction in THC might be a result of an FLX-induced cell apoptosis and suppression in proliferation (Edgar et al., 1999; Frick et al., 2008), which can be supported by the significantly decreased percentages of viable haemocytes after FLX exposure observed in the present study. Similar inhibitory effects of FLX on immune cells have also been revealed by previous studies in mammal species (Xia et al., 1999; Frick and Rapanelli, 2013). For example, it has been shown that treatments with FLX (10–100 μM) induced cell death, loss of mitochondrial membrane potential and formation of reactive oxygen species (ROS) in Hep3B human hepatocellular carcinoma cell line (Mun et al., 2013).

Phagocytosis of haemocytes is considered as the primary line of cellular defence against invaded pathogens in marine invertebrates (Canesi et al., 2012; Su et al., 2018). In the present study, the phagocytosis rate of *T. granosa* decreased markedly after exposure to waterborne FLX at 10 and 100 μg/L. Since the red granulocyte was predicted to have the highest ability of phagocytosis among all major cell types of haemocytes in blood clams (Zhu et al., 2011), the FLX induced declines in both the proportion and number of red granulocytes may partially explain the constrained phagocytosis detected.

The innate immune responses of invertebrates including bivalve mollusks are largely under the regulation of neuroendocrine-immune (NEI) network (Ottaviani et al., 2007; Liu et al., 2017). Upon the activation of immune system in response to pathogen, neuroendocrine system will excrete neurotransmitters to modulate the behavior of immunocytes (Sternberg, 1997). Therefore, any changes in the concentration of neurotransmitters would exert significant impact on the immune responses of the organisms (Shi et al., 2014; Liu et al., 2017). For instance, it has been demonstrated that ACh treatment can significantly decrease the haemocyte phagocytic activity of oyster, *Crassostrea gigas* (Shi et al., 2014). Therefore, the reduction in haemocyte phagocytosis detected in this study may also result from the increased concentrations of neurotransmitter (GABA and ACh) and the altered expressions of neurotransmitter related genes (GABAT, and mAChR3). Previous studies carried out in mammals also showed that FLX can affect intracellular Ca^{2+} signalling and neurotransmitter transporters on the surface of lymphoid cells (Gordon and Barnes, 2003; Frick and Rapanelli, 2013). Similarly, disturbed intracellular concentration of Ca^{2+} as well as down-regulated expression of CaM was observed in the present study upon FLX exposure. Since intracellular Ca^{2+} signals play crucial roles in almost every step of the phagocytosis process including pathogen recognition and adhesion, phagosomal maturation, and phagocytic ingestion in both vertebrates and invertebrates (Nunes and Demaurex, 2010), the FLX induced aberrant Ca^{2+} homeostasis and disrupted CaM expression may account for the constrained haemocyte phagocytosis detected as well. Therefore, other than directly affecting THC and cell type composition, exposure to FLX may also hamper the phagocytosis of haemocytes through disrupting intracellular neurotransmitter and Ca^{2+} signals.

Upon pathogen challenge, relevant immunogenic pathways of bivalves such as the NFκB signalling pathway would be activated and subsequently lead to non-specific and specific immune responses such as oxidative and inflammation reaction (Medzhitov, 2007). It has been suggested by a series of studies that the downregulation of genes in NFκB signalling pathway would suppress the innate immune responses and result in increased susceptibility to pathogens (Li and Verma, 2002; Dev et al., 2011). Therefore, the hampered immunity upon FLX exposure detected in this study may be due to the inhibited expression of NFκB1 and TRAF2 of NFκB signalling pathway as well.

In addition, consistent with previous studies conducted in other species (Meltzer et al., 1997), the results obtained in this study showed that *T. granosa* had significantly higher levels of cortisol after 10 days of exposure to waterborne FLX. Since cortisol is a key hormone regulating stress responses and widely used as a typical stress indicator in both vertebrates and invertebrates (Ader et al., 1991; Eames et al., 2010), the induction of cortisol detected suggested that FLX exposure rendered clams into a stressful condition. It has been suggested that the NFκB signalling pathway is involved in stress-related disorders and any changes in the stress condition of an organism would affect its immune responses by influencing the NFκB signalling pathway (Pace et al., 2012; Zhang et al., 2014). Therefore, exposure to FLX may also hamper the immune response of *T. granosa* by imposing physiological stress and thereafter disrupting the NFκB signalling pathway.

In addition to cellular immunity, humoural pathways also play important roles in the immune responses of many invertebrate species to pathogen or foreign particle challenges (Galloway and Depledge, 2001; Song et al., 2010). For instance, various types of non-specific immune-related humoural defence molecules (Song et al., 2010; Liu et al., 2016b), including enzymes such as SOD and CAT, have been reported in bivalve mollusks. Containing high proportion of polyunsaturated fatty acids in plasma membranes, immune cells are particularly sensitive to oxidative stress such as reactive oxygen species (ROS) (Meydani et al., 1995; Knight, 2000). ROS can alter the membrane fluidity and physical state through oxidation of the polyunsaturated fatty acids components of membrane and therefore disrupt membrane-dependent functions of the immune cells such as membrane-related signalling and membrane-mediated defence against exogenous substance (Knight, 2000). Under normal physiological conditions, the harmful effects of ROS are neutralized by the antioxidant defence system comprising SOD and CAT, which act to remove oxygen radicals effectively (Knight, 2000; Cheung et al., 2001). Therefore, the significant decline in SOD and CAT activities upon FLX exposure detected in the present study indicated a hampered antioxidant ability to eliminate reactive free radicals, which may disrupt the normal structure and function of the membranes of immune cells and therefore lead to a depressed immunocompetence of haemocytes (Wootton et al., 2003).

In the present study, some immuno-markers were not significantly affected upon exposure to the lowest dose of FLX (1 μg/L) tested, which indicated that the immunotoxicity of FLX to blood clam could

be dose-dependent, a phenomenon that are widely reported for many biomarkers in response to all kinds of toxic pollutants (Meng et al., 2011; Hu et al., 2014). It has been suggested that species may manage to maintain hemeostasis under moderate stress through physiological compensation such as adjusting the energy allocated to different physiological processes (Pook et al., 2009; Roberts et al., 2013). Therefore, the lowest dose of FLX tested in this study did not exert significant impacts on some immuno-markers examined simply indicated that FLX at this dose is less toxic to the blood clam.

In conclusion, the results obtained in the present study suggest that exposure to waterborne FLX (10 and 100 μg/L) could hamper both cellular and humoural immune responses of *T. granosa*, which may render this species more susceptible to pathogen infections. In addition, this study indicates that FLX may exert immunotoxic impacts on *T. granosa* through a comprehensive mechanism *via* reducing cell viability, altering Ca^{2+} homeostasis, disturbing neurotransmitter signal transduction, and generating a stressful condition.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

This work was financial supported by National Key R&D Program of China (NO. 2018YFD0900603) and National Natural Science Foundation of China (No. 31672634).

References

Abreu, M.S., Koakoski, G., Ferreira, D., Oliveira, T.A., Rosa, J.G., Gusso, D., Giacomini, A.C., Piato, A.L., Barcellos, L.J., 2014. Diazepam and fluoxetine decrease the stress response in zebrafish. PLoS One 9, e103232.

Ader, D.N., Johnson, S.B., Huang, S.W., Riley, W.J., 1991. Group size, cage shelf level, and emotionality in non-obese diabetic mice: impact on onset and incidence of IDDM. Psychosom. Med. 53 (3), 313–321.

Ansai, S., Hosokawa, H., Maegawa, S., Kinoshita, M., 2016. Chronic fluoxetine treatment induces anxiolytic responses and altered social behaviors in medaka, *Oryzias latipes*. Behav. Brain Res. 303, 126–136.

Bringolf, R.B., Heltsley, R.M., Newton, T.J., Eads, C.B., Fraley, S.J., Shea, D., Cope, W.G., 2010. Environmental occurrence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. Environ. Toxicol. Chem. 29, 1311–1318.

Brooks, B.W., 2014. Fish on Prozac (and Zoloft): ten years later. Aquat. Toxicol. 151, 61–67.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., Gallo, G., 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. Mar. Environ. Res. 76, 16–21.

Cheung, C.C.C., Zheng, G.J., Li, A.M.Y., Richardson, B.J., Lam, P.K.S., 2001. Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. Aquat. Toxicol. 52, 189–203.

Christen, V., Hickmann, S., Rechenberg, B., Fent, K., 2010. Highly active human pharmaceuticals in aquatic systems: a concept for their identification based on their mode of action. Aquat. Toxicol. 96, 167–181.

Dev, A., Iyer, S., Razani, B., Cheng, G., 2011. NF-kB and innate immunity. In: Karin, M. (Ed.), NF-kB in Health and Disease. Springer Berlin Heidelberg, Berlin, Heidelberg.

Domart-Coulon, I., Auzoux-Bordenave, S., Doumenc, D., Khalanski, M., 2000. Cytotoxicity assessment of antibiofouling compounds and by-products in marine bivalve cell cultures. Toxicol. in Vitro 14, 245–251.

Eames, S.C., Philipson, L.H., Prince, V.E., Kinkel, M.D., 2010. Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. Zebrafish 7, 205–213.

Edgar, V.A., Sterin-Borda, L., Cremaschi, G.A., Genaro, A.M., 1999. Role of protein kinase C and cAMP in fluoxetine effects on human T-cell proliferation. Eur. J. Pharmacol. 372, 65–73.

El-Dawy, M.A., Mabrouk, M.M., El-Barbary, F.A., 2002. Liquid chromatographic determination of fluoxetine. J. Pharmaceut. Biomed. 30, 561–571.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159.

Frick, L.R., Rapanelli, M., 2013. Antidepressants: influence on cancer and immunity? Life Sci. 92, 525–532.

Frick, L.R., Palumbo, M.L., Zappia, M.P., Brocco, M.A., Cremaschi, G.A., Genaro, A.M., 2008. Inhibitory effect of fluoxetine on lymphoma growth through the modulation of antitumor T-cell response by serotonin-dependent and independent mechanisms. Biochem. Pharmacol. 75, 1817–1826.

Fung, C.N., Lam, J.C.W., Zheng, G.J., Connell, D.W., Monirith, I., Tanabe, S., Richardson, B.J., Lam, P.K.S., 2004. Mussel-based monitoring of trace metal and organic contaminants along the east coast of China using *Perna viridis* and *Mytilus edulis*. Environ. Pollut. 127, 203–216.

Furlong, E.T., Kinney, C.A., Ferrer, I., 2004. Pharmaceuticals and personal-care products in solids: Analysis and field results for sediment, soil, and biosolid samples. Proceedings, 228th American Chemical Society National Meeting, Philadelphia, PA, USA, August 22–26.

Galloway, T.S., Depledge, M.H., 2001. Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. Ecotoxicology 10, 5–23.

Gardner, M., Jones, V., Comber, S., Scrimshaw, M.D., Coello-Garcia, T., Cartmell, E., Lester, J., Ellor, B., 2013. Performance of UK wastewater treatment works with respect to trace contaminants. Sci. Total Environ. 456, 359–369.

Gonzalez-Rey, M., Bebianno, M.J., 2013. Does selective serotonin reuptake inhibitor (SSRI) fluoxetine affects mussel *Mytilus galloprovincialis*? Environ. Pollut. 173, 200–209.

Gordon, J., Barnes, N.M., 2003. Lymphocytes transport serotonin and dopamine: agony or ecstasy? Trends Immunol. 24, 438–443.

Gros, M., Petrović, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environ. Int. 36, 15–26.

Guan, X., Shi, W., Zha, S., Rong, J., Su, W., Liu, G., 2018. Neurotoxic impact of acute TiO_2 nanoparticle exposure on a benthic marine bivalve mollusk, *Tegillarca granosa*. Aquat. Toxicol. 200, 241–246.

Han, Y., Shi, W., Guo, C., Zhao, X., Liu, S., Wang, Y., Su, W., Zha, S., Wu, H., Chai, X., Liu, G., 2016. Characteristics of chitin synthase (CHS) gene and its function in polyspermy blocking in the blood clam *Tegillarca granosa*. J. Mollus. Stud. 82, 550–557.

Hooper, C., Day, R., Slocombe, R., Handlinger, J., Benkendorff, K., 2007. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. Fish Shellfish Immunol 22, 363–379.

Hu, W., Culloty, S., Darmody, G., Lynch, S., Davenport, J., Ramirez-Garcia, S., Dawson, K., Lynch, I., Blasco, J., Sheehan, D., 2014. Toxicity of copper oxide nanoparticles in the blue mussel, *Mytilus edulis*: a redox proteomic investigation. Chemosphere 108, 289–299.

Hüning, A.K., Melzner, F., Thomsen, J., Gutowska, M.A., Krämer, L., Frickenhaus, S., Rosenstiel, P., Pörtner, H.O., Philipp, E.R., Lucassen, M., 2013. Impacts of seawater acidification on mantle gene expression patterns of the Baltic Sea blue mussel: implications for shell formation and energy metabolism. Mar. Biol. 160, 1845–1861.

Johnson, D.J., Sanderson, H., Brain, R.A., Wilson, C.J., Bestari, K.T., Solomon, K.R., 2005. Exposure assessment and microcosm fate of selected selective serotonin reuptake inhibitors. Regul. Toxicol. Pharma. 42, 313–323.

Kim, Y., Choi, K., Jung, J.Y., Park, S., Kim, P.G., Park, J., 2007. Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. Environ. Int. 33, 370–375.

Knight, J.A., 2000. Review: free radicals, antioxidants, and the immune system. Ann. Clin. Lab. Sci. 30, 145–158.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Kwon, J.W., Armbrust, K.L., 2006. Laboratory persistence and fate of fluoxetine in aquatic environments. Environ. Toxicol. Chem. 25, 2561–2568.

Lacoste, A., Malham, S.K., Gélébart, F., Cueff, A., Poulet, S.A., 2002. Stress-induced immune changes in the oyster *Crassostrea gigas*. Dev. Comp. Immunol. 26, 1–9.

Lagos, L., Herrera, M., Sánchez-Lazo, C., Martínez-Pita, I., 2015. Effect of larval stocking density on growth, survival and whole body cortisol of the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819) larvae reared under laboratory conditions. Aquac. Res. 46, 1648–1656.

Li, Q., Verma, I.M., 2002. NF-κB regulation in the immune system. Nat. Rev. Immunol. 2, 725–734.

Li, M., Qiu, L., Wang, L., Wang, W., Xin, L., Li, Y., Liu, Z., Song, L., 2016. The inhibitory role of γ-aminobutyric acid (GABA) on immunomodulation of pacific oyster *Crassostrea gigas*. Fish Shellfish Immunol 52, 16–22.

Liu, Z., Zhou, Z., Wang, L., Jiang, S., Wang, W., Zhang, R., Song, L., 2015. The immunomodulation mediated by a delta-opioid receptor for [Met 5]-enkephalin in oyster *Crassostrea gigas*. Dev. Comp. Immunol. 49, 217–224.

Liu, S., Shi, W., Guo, C., Zhao, X., Han, Y., Peng, C., Chai, X., Liu, G., 2016a. Ocean acidification weakens the immune response of blood clam through hampering the NF-kappa β and toll-like receptor pathways. Fish Shellfish Immunol 54, 322–327.

Liu, Z., Zhou, Z., Wang, L., Dong, W., Qiu, L., Song, L., 2016b. The cholinergic immune regulation mediated by a novel muscarinic acetylcholine receptor through TNF pathway in oyster *Crassostrea gigas*. Dev. Comp. Immunol. 65, 139–148.

Liu, Z., Zhou, Z., Jiang, Q., Wang, L., Yi, Q., Qiu, L., Song, L., 2017. The neuroendocrine immunomodulatory axis-like pathway mediated by circulating haemocytes in pacific oyster *Crassostrea gigas*. Open Biol. 7, 160289.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Llerena, A., Dorado, P., Berecz, R., González, A., Jesús Norberto, M., de la Rubia, A., Cáceres, M., 2003. Determination of fluoxetine and norfluoxetine in human plasma by high-performance liquid chromatography with ultraviolet detection in psychiatric patients. J. Chromatogr. B 783, 25–31.

Loker, E.S., Adema, C.M., Zhang, S.M., Kepler, T.B., 2004. Invertebrate immune systems-not homogeneous, not simple, not well understood. Immunol. Rev. 198, 10–24.

Luengen, A.C., Friedman, C.S., Raimondi, P.T., Flegal, A.R., 2004. Evaluation of mussel immune responses as indicators of contamination in San Francisco Bay. Mar. Environ. Res. 57, 197–212.

Mackenzie, C.L., Lynch, S.A., Culloty, S.C., Malham, S.K., 2014. Future oceanic warming and acidification alter immune response and disease status in a commercial shellfish species, *Mytilus edulis* L. PLoS One 9, e99712.

Maes, M., Kenis, G., Kubera, M., De Baets, M., Steinbusch, H., Bosmans, E., 2005. The negative immunoregulatory effects of fluoxetine in relation to the cAMP-dependent PKA pathway. Int. Immunopharmacol. 5, 609–618.
Maes, M., Fišar, Z., Medina, M., Scapagnini, G., Nowak, G., Berk, M., 2012. New drug targets in depression: inflammatory, cell-mediated immune, oxidative and nitrosative stress, mitochondrial, antioxidant, and neuroprogressive pathways. And new drug candidates-Nrf2 activators and GSK-3 inhibitors. Inflammopharmacology 20, 127–150.
Malham, S.K., Lacoste, A., Gélébart, F., Cueff, A., Poulet, S.A., 2003. Evidence for a direct link between stress and immunity in the mollusc *Haliotis tuberculata*. J. Exp. Zool. Part A 295, 136–144.
McDonald, J.H., 2014. Handbook of Biological Statistics. 3rd ed. Sparky House Publishing, Baltimore, Maryland.
Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449, 819–826.
Meltzer, H., Bastani, B., Jayathilake, K., Maes, M., 1997. Fluoxetine, but not tricyclic antidepressants, potentiates the 5-hydroxytryptophan-mediated increase in plasma cortisol and prolactin secretion in subjects with major depression or with obsessive compulsive disorder. Neuropsychopharmacology 17, 1–11.
Meng, X., Dai, X., Liao, T., D'Ambrosio, M., Wang, F., Yang, J.J., Yang, X., 2011. Dose-dependent toxic effects of high-dose estrogen on renal and cardiac injury in surgically postmenopausal mice. Life Sci. 88, 178–186.
Meydani, S.N., Wu, D., Santos, M.S., Hayek, M.G., 1995. Antioxidants and immune response in aged persons: overview of present evidence. Am. J. Clin. Nutr. 62, 1462S.
Milea, D., Verpillat, P., Guelfucci, F., Toumi, M., Lamure, M., 2010. Prescription patterns of antidepressants: findings from a US claims database. Curr. Med. Res. Opin. 26, 1343–1353.
Mun, A.R., Lee, S.J., Kim, G.B., Kang, H.S., Kim, J.S., Kim, S.J., 2013. Fluoxetine-induced apoptosis in hepatocellular carcinoma cells. Anticancer Res. 33, 3691–3697.
Munari, M., Marin, M.G., Matozzo, V., 2014. Effects of the antidepressant fluoxetine on the immune parameters and acetylcholinesterase activity of the clam *Venerupis philippinarum*. Mar. Environ. Res. 94, 32–37.
Nunes, P., Demaurex, N., 2010. The role of calcium signaling in phagocytosis. J. Leukoc. Biol. 88, 57–68.
Ong, T.H.D., Yu, N., Meenashisundaram, G.K., Schaller, B., Gupta, M., 2017. Insight into cytotoxicity of Mg nanocomposites using MTT assay technique. Mat. Sci. Eng. C Mater. 78, 647–652.
Organisation for Economic Co-operation and Development, 2017. Health at a Glance 2017: OECD Indicators. OECD Publishing, Paris.
Ottaviani, E., Franchini, A., Franceschi, C., 1998. Presence of immunoreactive corticotropin-releasing hormone and cortisol molecules in invertebrate haemocytes and lower and higher vertebrate thymus. Histochem. J. 30, 61–67.
Ottaviani, E., Malagoli, D., Franceschi, C., 2007. Common evolutionary origin of the immune and neuroendocrine systems: from morphological and functional evidence to in silico approaches. Trends Immunol. 28, 497–502.
Pace, T.W., Wingenfeld, K., Schmidt, I., Meinlschmidt, G., Hellhammer, D.H., Heim, C.M., 2012. Increased peripheral NF-κB pathway activity in women with childhood abuse-related posttraumatic stress disorder. Brain Behav. Immun. 26, 13–17.
Pellegrino, T.C., Bayer, B.M., 1998. Modulation of immune cell function following fluoxetine administration in rats. Pharmacol. Biochem. Be. 59, 151–157.
Pook, C., Lewis, C., Galloway, T., 2009. The metabolic and fitness costs associated with metal resistance in Nereis diversicolor. Mar. Pollut. Bull. 58, 1063–1071.
Roberts, D.A., Birchenough, S.N., Lewis, C., Sanders, M.B., Bolam, T., Sheahan, D., 2013. Ocean acidification increases the toxicity of contaminated sediments. Glob. Chang. Biol. 19, 340–351.
Rong, J., Su, W., Guan, X., Shi, W., Zha, S., He, M., Wang, H., Liu, G., 2018. Ocean acidification impairs foraging behavior by interfering with olfactory neural signal transduction in black sea bream, *Acanthopagrus schlegelii*. Front. Physiol. 9, 1592.
Saaristo, M., McLennan, A., Johnstone, C.P., Clarke, B.O., Wong, B.B., 2017. Impacts of the antidepressant fluoxetine on the anti-predator behaviours of wild guppies (*Poecilia reticulata*). Aquat. Toxicol. 183, 38–45.
Salgado, R., Marques, R., Noronha, J., Mexia, J., Carvalho, G., Oehmen, A., Reis, M., 2011. Assessing the diurnal variability of pharmaceutical and personal care products in a full-scale activated sludge plant. Environ. Pollut. 159, 2359–2367.
Sánchez-Argüello, P., Fernández, C., Tarazona, J.V., 2009. Assessing the effects of fluoxetine on *Physa acuta* (Gastropoda, Pulmonata) and *Chironomus riparius* (Insecta, Diptera) using a two-species water–sediment test. Sci. Total Environ. 407, 1937–1946.
Shao, Y., Chai, X., Xiao, G., Zhang, J., Lin, Z., Liu, G., 2016. Population genetic structure of the blood clam, *Tegillarca granosa*, along the pacific coast of Asia: isolation by distance in the sea. Malacologia 59, 303–312.
Shi, X., Wang, L., Zhou, Z., Liu, R., Li, Y., Song, L., 2014. Acetylcholine modulates the immune response in Zhikong scallop *Chlamys farreri*. Fish Shellfish Immunol 38, 204–210.
Shi, W., Zhao, X., Han, Y., Che, Z., Chai, X., Liu, G., 2016. Ocean acidification increases cadmium accumulation in marine bivalves: a potential threat to food safety. Sci. Rep. 6, 20197.
Shi, W., Han, Y., Guo, C., Zhao, X., Liu, S., Su, W., Wan, Y., Zha, S., Chai, X., Liu, G., 2017a. Ocean acidification hampers sperm-egg collisions, gamete fusion, and generation of Ca^{2+} oscillations of a broadcast spawning bivalve, *Tegillarca granosa*. Mar. Environ. Res. 130, 106–112.
Shi, W., Han, Y., Guo, C., Zhao, X., Liu, S., Su, W., Zha, S., Wang, Y., Liu, G., 2017b. Immunotoxicity of nanoparticle $nTiO_2$ to a commercial marine bivalve species, Tegillarca granosa. Fish Shellfish Immunol 66, 300–306.
Shi, W., Guan, X., Han, Y., Guo, C., Rong, J., Su, W., Zha, S., Wang, Y., Liu, G., 2018. Waterborne Cd^{2+} weakens the immune responses of blood clam through impacting Ca^{2+} signaling and Ca^{2+} related apoptosis pathways. Fish Shellfish Immunol 77, 208–213.
Silva, L.J., Pereira, A.M., Meisel, L.M., Lino, C.M., Pena, A., 2015. Reviewing the serotonin reuptake inhibitors (SSRIs) footprint in the aquatic biota: uptake, bioaccumulation and ecotoxicology. Environ. Pollut. 197, 127–143.
Song, L., Wang, L., Qiu, L., Zhang, H., 2010. Bivalve immunity. In: Söderhäll, K. (Ed.), Invertebrate Immunity. Springer, pp. 44–65.
Sternberg, E.M., 1997. Neural-immune interactions in health and disease. J. Clin. Invest. 100, 2641–2647.
Su, W., Zha, S., Wang, Y., Shi, W., Xiao, G., Chai, X., Wu, H., Liu, G., 2017. Benzo[a]pyrene exposure under future ocean acidification scenarios weakens the immune responses of blood clam, *Tegillarca granosa*. Fish Shellfish Immunol 63, 465–470.
Su, W., Rong, J., Zha, S., Yan, M., Fang, J., Liu, G., 2018. Ocean acidification affects the cytoskeleton, lysozymes, and nitric oxide of hemocytes: a possible explanation for the hampered phagocytosis in blood clams, *Tegillarca granosa*. Front. Physiol. 9, 619.
Su, W., Shi, W., Han, Y., Hu, Y., Ke, A., Wu, H., Liu, G., 2019. The health risk for seafood consumers under future ocean acidification (OA) scenarios: OA alters bioaccumulation of three pollutants in an edible bivalve species through affecting the in vivo metabolism. Sci. Total Environ. 650, 2987–2995.
Vig, M., Kinet, J.P., 2009. Calcium signaling in immune cells. Nat. Immunol. 10, 21–27.
Wang, Q., Cao, R., Ning, X., You, L., Mu, C., Wang, C., Wei, L., Cong, M., Wu, H., Zhao, J., 2016. Effects of ocean acidification on immune responses of the Pacific oyster *Crassostrea gigas*. Fish Shellfish Immunol 49, 24–33.
Wootton, E.C., Dyrynda, E.A., Pipe, R.K., Ratcliffe, N.A., 2003. Comparisons of PAH-induced immunomodulation in three bivalve molluscs. Aquat. Toxicol. 65, 13–25.
Xia, Z., Lundgren, B., Bergstrand, A., DePierre, J.W., Nässberger, L., 1999. Changes in the generation of reactive oxygen species and in mitochondrial membrane potential during apoptosis induced by the antidepressants imipramine, clomipramine, and citalopram and the effects on these changes by Bcl-2 and Bcl-XL. Biochem. Pharmacol. 57, 1199–1208.
Zha, S., Shi, W., Su, W., Guan, X., Liu, G., 2019. Exposure to 2, 3, 7, 8-tetrachlorodibenzo-paradioxin (TCDD) hampers the host defense capability of a bivalve species, *Tegillarca granosa*. Fish Shellfish Immunol 86, 368–373.
Zhang, X., Huang, Y., Cai, X., Zou, Z., Wang, G., Wang, S., Wang, Y., Zhang, Z., 2014. Identification and expression analysis of immune-related genes linked to Rel/NF-κB signaling pathway under stresses and bacterial challenge from the small abalone Haliotis diversicolor. Fish Shellfish Immunol 41, 200–208.
Zhao, X., Shi, W., Han, Y., Liu, S., Guo, C., Fu, W., Chai, X., Liu, G., 2017. Ocean acidification adversely influences metabolism, extracellular pH and calcification of an economically important marine bivalve, *Tegillarca granosa*. Mar. Environ. Res. 125, 82–89.
Zhu, Z., Xu, L., Wu, X., Zhang, Z., Wu, L., Lou, H., 2011. Morphological, structural characteristics and phagocytic and enzymatic activities of haemocytes in blood clam *Tegillarca granosa*. J. Fish. China 35, 1494–1504.