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Environmental concentrations of sulfamethoxazole increase crayfish *Pacifastacus leniusculus* susceptibility to White Spot Syndrome Virus

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ABSTRACT

Antibiotics used for humans and livestock are emerging as pollutants in aquatic environments. However, little is known about their effect on aquatic organisms, especially in crustaceans. In the present study, the freshwater crayfish *Pacifastacus leniusculus* was exposed during 21 days to environmental concentrations of sulfamethoxazole (SMX) (100 ng/L and 1 μg/L). Subsequently, the crayfish susceptibility to infection was evaluated by using White Spot Syndrome Virus (WSSV) challenge, a well-known crustacean pathogen. The median survival time of the infected crayfish exposed to 100 ng/L SMX was one day, whereas the control and the group exposed to 1 μg/L SMX survived for two and three days, respectively. In order to elucidate the effect of SMX upon the crayfish immune response, new sets of crayfish were exposed to the same SMX treatments to evaluate mRNA levels of immune-related genes which are expressed and present in hemocytes and intestine, and to perform total and differential hemocyte counts. These results show a significant down-regulation of the antimicrobial peptide (AMP) Crustin 3 in hemocytes from the 100 ng/L SMX group, as well as a significant up-regulation of the AMP Crustin 1 in intestines from the 1 μg/L SMX group. Semigranular and total hemocyte cell number were observed to be significantly lower after exposure to 100 ng/L SMX in comparison with the control group. The present study demonstrates that environmentally relevant SMX concentrations in the water at 100 ng/L led to an increased WSSV susceptibility, that may have been caused by a reduction of circulating hemocytes. Nevertheless, SMX concentrations of 1 μg/L could marginally and for a few days have an immunostimulatory effect.

1. Introduction

Antibiotics represent around 40% of the most frequently encountered pharmaceuticals contaminating the freshwater systems all over the world [1]. Among them, sulfamethoxazole (SMX) is one of the most prominent, with concentrations ranging from ng/L to mg/L in different aquatic environments, including rivers and effluents of wastewater treatment plants [1–7]. In a previous study [1], it was estimated that, on a global scale, SMX has been detected in 67% of the studied freshwater ecosystems with a median concentration of 83 ng/L.

Sulfamethoxazole belongs to the sulfonamide group and it is a synthetic antibiotic that inhibits the bacterial synthesis of dihydrofolic acid, and thus preventing the survival of the bacteria. Sulfamethoxazole is particularly important due to its widespread use in human and veterinary medicine. It is usually administrated in combination with trimethoprim to treat diseases, and little information is available about pharmacodynamics, dosage, withdrawal period and turnover rate of SMX alone.

Sulfamethoxazole is considered a persistent pollutant in the aquatic environments since it is not completely eliminated after wastewater treatment [5,8]. Recent studies have shown that the major processes by which this antibiotic is degraded in the aquatic ecosystems are by photolysis [9] and to a lesser extent by hydrolysis [10]. Likewise, pH, salinity and dissolved organic matter in the water determine the photodegradation rate of SMX, and this rate is slower in environmental compartments, including estuarine and river water [11].

Notwithstanding the importance that SMX is a pollutant of the aquatic ecosystems, up to date, no information is available regarding the effect of environmental concentrations upon the wild fauna. However, over the past decades, many toxicological studies concerning the lethality of SMX have been carried out. For example, it has been demonstrated that this antibiotic has a potentially toxic to lethal effect at high concentrations to different species, including a marine bacterium (*Vibrio fischeri*) (> 70 mg/L), crustaceans (*Daphnia magna* and

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Ceriodaphnia dubia) (> 170 mg/L and > 15 mg/L, respectively), and a fish (*Oryzias latipes*) (> 750 mg/L) [12,13]. Nevertheless, in all these cases, the antibiotic concentrations were very high and not environmentally relevant.

In addition, studies regarding the monitoring of SMX bioaccumulation in the wild fauna are also scarce. Trace levels of sulphonamides, were detected in the muscle and gills of wild fish that inhabits highly-polluted rivers in China [7,14] and in a dam reservoir in Turkey [15]. Moreover, SMX was monitored in a study by Zhao et al., in 2015 [7], who reported a mean concentration of SMX of < 0.91 μg/kg wet weight in fish.

Due to the current lack of information, clarification of possible effects of SMX environmental concentrations upon the normal physiology of the wild fauna, represents a very important topic to study. Particularly on species considered as threatened, for example, freshwater crayfish which are native for Europe, and a majority of these populations have crashed in Europe over the last decades [16]. One of the reasons for this drastic reduction is infections by the crayfish plague fungus, *Aphanomyces astaci* and it is likely that environmental stressors, leads to a suppression of immune functions making them even more sensitive to pathogens in general, and to the crayfish plague fungus in particular, for example the introduced crayfish *Pacifastacus leniusculus* [17,18]. Therefore, the crayfish *P. leniusculus* is considered a convenient model for studies on crustacean immunity [19–21], and it can be used for this purpose.

Crustacean immune system is to a very large extent depending on their hemocytes. In the case of *P. leniusculus*, there are two subpopulations of hemocytes found as the most abundant circulating in the hemolymph, semigranular cells (SGCs) and granular cells (GCs) [22,23]. These cells are involved in the cellular response through processes such as phagocytosis, encapsulation, and melanization, as well as in the humoral response through the activation of molecules known as antimicrobial peptides (AMPs) and melanization [24]. For this reason, studies monitoring the crustacean immune system, including crayfish, relies on the evaluation of immune-related molecules, along with the total and differential count of hemocytes [20]. Some of the most well-known immune-related molecules in *P. leniusculus* include the AMPs Crustin 1, Crustin 2, Crustin 3 and Antilipopolysaccharide factor (ALF), as well as components of the prophenoloxidase (proPO) activating system, which is the effector of the melanization [25]. Moreover, the hemocyte homeostasis-associated-like protein (HHAP), and thioester-containing protein (TEP) are both related to intestinal immunity [26,27]. Therefore, the expression of these genes is considered suitable in order to monitor effects on the crayfish immune system. Moreover, previous studies performed in *P. leniusculus* have demonstrated that the suppression of *P. leniusculus* AMPs results in succumbing to bacterial and viral infections [28,29].

One of the most aggressive viruses affecting crustaceans is the White spot syndrome virus (WSSV). WSSV is the causal agent of a disease that provokes severe mortalities in cultured shrimps (up to 100% within 3–10 days [30]), causing important economic losses in the shrimp farming industry worldwide. This virus has a broad host and/or vector range (~100 species) [31], and has been detected in wild crustacean populations in different countries [32–37]. Although WSSV pathogenesis has been extensively studied, there is currently no effective treatment to cure the disease. Previous research conducted in *P. lenisuculus* has contributed to the understanding of the mechanisms this virus uses to infect its hosts [19,38]. For instance, it was demonstrated that this virus infects and replicates in the hematopoietic stem cells [39], and further can be detected in hemocytes [40] and that SGCs are more susceptible to this infection than GCs [41].

Due to the frequency of detection of SMX as a pollutant of the aquatic environments, as well as the imminent presence of WSSV in wild populations of crustaceans, it is important to address the potential risk that the virus could represent for wild crustacean populations during antibiotics pollution scenarios. Therefore, the objective of the present study is to reveal possible effects of concentrations of SMX that are found in aquatic environments on the susceptibility to WSSV of the crayfish, *P. leniusculus*. This study also represents the first evaluation regarding the effects of environmental concentrations of antibiotics upon crustacean's normal physiology.

2. Materials and methods

2.1. Chemicals

In order to expose the organisms, a fresh stock of SMX (Sigma-Aldrich, CAS: 723-46-6) was prepared by dissolving it in hot water [42]. Then, test solutions with final concentrations of 100 ng/L and 1 μg/L were prepared from the stock immediately. Selected SMX concentrations are environmentally relevant, and similar with those found in freshwater ecosystems [1].

2.2. SMX exposure

Freshwater crayfish, *P. leniusculus* (mean weight: 37 ± 2 g) were obtained from Lake Erken, Sweden. Only intermolt males and apparently healthy crayfish were used for the experiments. The animals were acclimatized in aerated aquaria (with a total capacity of eight L), at 18 °C (± 2 °C) during two weeks prior to starting the SMX exposure. During the acclimatization period, freshwater was completely renewed every two days, and the crayfish were fed with 3% of their live weight with potatoes and carrots once a week. After 14 days of acclimatization period, crayfish were exposed to one of the following treatments: Control (no antibiotic added in the water), 100 ng/L of SMX or 1 μg/L of SMX during 21 days (Fig. 1).

The water from all three treatments was renewed every two days. For the antibiotic-treated groups, freshly prepared SMX solution was added to the water every time the water was changed. The total volume of water in the aquaria during both acclimatization and SMX exposure period was four L. The crayfish were fed once a week as mentioned earlier. The photoperiod was maintained at 12:12 h light:dark during both acclimatization and experimental periods. This study was performed in accordance with international animal welfare guidelines.

2.3. WSSV challenge

In order to test the effect of SMX exposure on WSSV infection in crayfish, the 21-day SMX exposure was followed by WSSV injection in crayfish (Fig. 1a).

WSSV inoculum was obtained from crayfish gills. The purification of the virus was performed as described before [39], and the concentration of the inoculum was quantified by measuring the optical density (OD) [43] in a spectrophotometer (Eppendorf BioPhotometer) at an absorbance of 600 nm. Then, viral particles were resuspended in CPBS (10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 Mm NaCl, 10 mM $CaCl_2$, and 10 mM $MnCl_2$, pH 6.8) at a concentration of 2.6×10^6 copies/mL. Determination of the viral dose was established so that mortality should occur two days-post infection. This was done in order to avoid prolongation of the moribund stage of crayfish, which may give several irrelevant side-effects on crayfish immunity.

After the end of the 21-day treatments (described above), crayfish were alive and active and the water was renewed one time without addition of new SMX in all experimental groups. Then, crayfish from the three groups were injected in the base of the left fourth walking leg with 50 μL of the WSSV inoculum using a 1 mL syringe with a 0.4 mm needle. Survival was recorded during the next days. Neither water renewal nor feeding was performed during the infection/experimental period. This experiment was repeated with a total number of 6 animals for the control, 7 animals for the lower dose and 10 animals for the higher SMX dose.

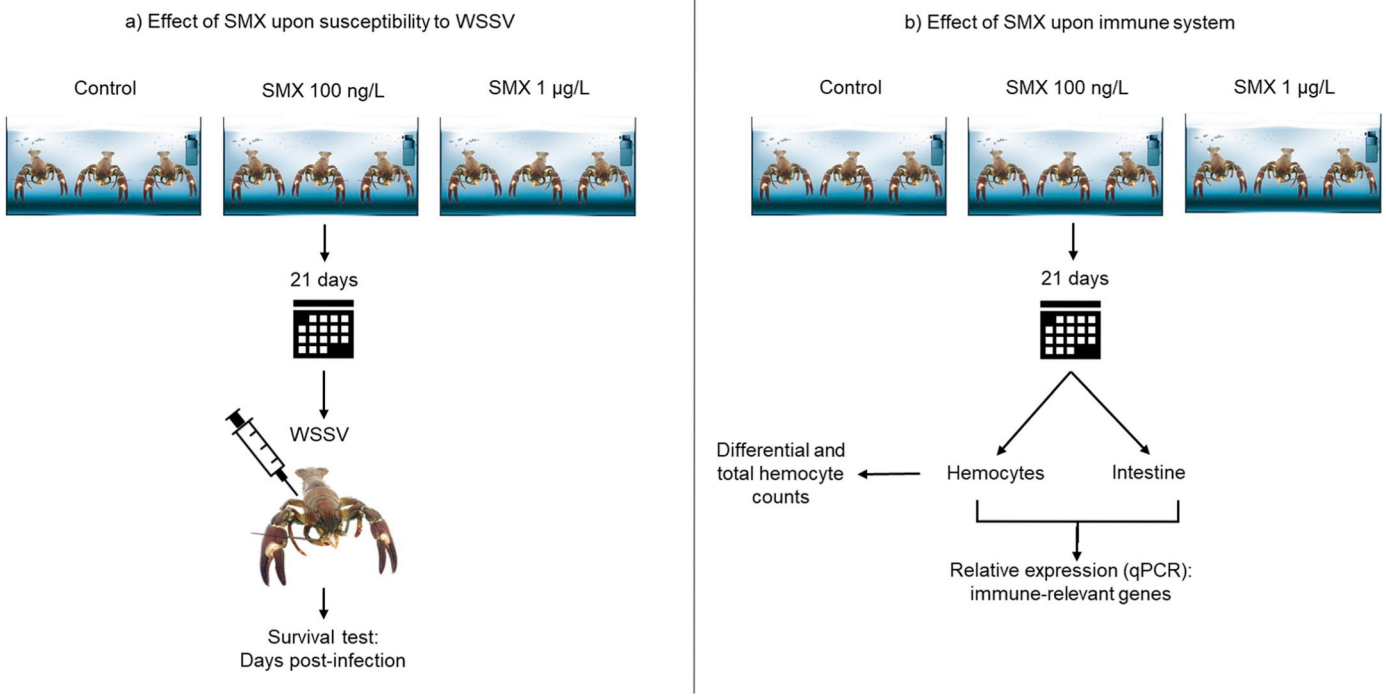

Fig. 1. Experimental setup of crayfish *P. leniusculus*. a) Exposure to environmental concentrations of SMX and then testing WSSV challenge survival. The time that the animals survived after the challenge was recorded and reported as days post-infection. b) Exposure to environmental concentrations of SMX, then isolation of hemocytes and intestine samples from individual animals were processed for determining differential and total hemocyte count and qPCR of relevant genes involved in immunity.

2.4. Biological sample collection

In order to explain the mortalities obtained after the WSSV challenge, new sets of crayfish were exposed to SMX for 21 days as mentioned above, and individual samples of hemocytes and intestine were taken from the crayfish in order to perform hemocyte count and to isolate RNA from hemocytes and intestine.

Hemolymph was collected from individual crayfish using a 1.2 mm needle into 2 mL tubes kept on ice. Immediately after, 100 µL of hemolymph were fixed with an equal volume of 4% paraformaldehyde and kept at 5 °C to perform total and differential hemocytes count. The rest of the hemolymph was then mixed with an equal volume of cold anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate 26 mM citric acid and 10 mM EDTA, pH 4.6) [23]. Samples with anticoagulant were centrifuged 800 × *g* for 5 min at 4 °C, the supernatant was discarded and the hemocyte pellet was kept at – 80 °C until RNA extraction.

After hemolymph was collected, crayfish were euthanized by decapitation and the whole intestine from midgut to rectum was collected from each individual animal, rinsed with cold CPBS and immediately stored separately at −80 °C until RNA extraction.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from hemocytes and intestine samples using TRIzol® LS reagent (Ambion) according to the manufacturer's protocol and treated with DNase I (RNase-free) (BioLab's®, New England) to eliminate contamination with DNA. Total RNA purity was established by calculating the ratio of the absorbance readings at 260 and 280 nm and quantified using the 260 nm absorbance reading [44]. Further, using equal amounts of RNA (500 ng), cDNA was synthesized using SuperScript IV Reverse Transcriptase (Thermo Fischer Scientific). Reactions without reverse transcriptase were run as controls. The cDNA was diluted 1:10 and stored at −20 °C until its use to detect gene transcription levels by qPCR.

2.6. Quantitative PCR (qPCR)

In order to evaluate the expression of the immune-relevant genes, primers for Crustin 1, Crustin 2, Crustin 3, ALF and TEP2 were designed using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Also, primers previously designed to evaluate HHAP [26] and proPO [45] were included in the analysis. The transcription of the 18S ribosomal gene was used as an internal control for each sample [46]. All primers used are listed in Table 1.

A standard curve for relative quantitation was prepared by mixing

Table 1
Primers used for qPCR analysis in the present study and their amplification conditions.

Gene	GenBank accession number	Primer sequences (5' – 3')	Tm (°C)	Product size
18S	GBYW01-019542	F: agtttcagcacatcctgcgt R: tcagtcatctctccagcacg	58	150 bp
Crustin1	EF523612	F: cctaggcctaaagtggacat R: cttgcagttgaggtggttag	56	192 bp
Crustin2	EF523613	F: ggtcctaacaagggctctaa R: gcaacacttctggttgtagg	56	171 bp
Crustin3	EF523614	F: gagtggacttcttccaggac R: ctacagttgccagtctgagg	56	162 bp
TEP	HQ596369	F: gtgaaagctgccatagattg R: tcatcgatgttatccagacc	58	233 bp
ALF	EF523760	F: cagcctcattatcagcaaac R: ttagttttggagttgccaga	58	171 bp
HHAP	HQ130432	F: ccataccagggcaagaggag R: ccacagaactgccctatttg	60	223 bp
proPO	X83494	F: acccatctcgaccatgcac R: agacgctgctccatgaagc	58	222 bp

equal amounts of total cDNA from all the samples per tissue. Serial dilutions of this stock were prepared (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) to be included in the curve. The reaction mixture of PCR was prepared using QuantiTect SYBR Green PCR kit (Qiagen) with a final volume of 25 μL, containing: 12.5 μL QuantiTect SYBR Green PCR Master Mix, 1.5 μL (0.5 mM) forward primer, 1.5 μL (0.5 mM) reverse primer, 4.5 μL RNase-Free Water and 5 μL of standard curve dilution or 5 μL 1:10 dilution of cDNA samples. Non-template controls were included as negative controls in all standard curves. Standard MIQE guidelines for RT-qPCR experiments were followed to conduct the analysis [44]. PCR amplification was performed as follows: 15 min at 95 °C, 45 cycles of 15 s at 95 °C, 20 s at 56–60 °C and 20 s at 72 °C. See Table 1 for specific Melting Temperature (Tm) and expected product sizes for each set of primers.

2.7. Hemocyte number count

Fixed hemocytes in 4% paraformaldehyde were used to perform GC, SGC and total hemocyte (THC) counts under microscope using a hemocytometer. The hemocyte counts are the result of the average of a blind test performed by two independent persons and expressed as the mean number of hemocytes/mL of hemolymph. Experimental replicates of three to five animals per treatment were conducted.

2.8. Statistical analysis

The comparison of survivals during the WSSV challenge was analyzed using the Log-rank (Mantel-Cox) Test. For statistical analysis of relative expression of immune-related genes, as well as the total and differential hemocytes counts, One-way Anova test was applied, followed by the Tukey post-test for normal distribution data. For non-normal distribution data, Kruskal-Wallis test was applied, followed by the Dunn's post-test. All results were expressed as mean ± standard deviation.

GraphPad Prism 8.1.2 software was used to analyze all data and differences were considered significant at $P < 0.05$, $P < 0.01$, $P < 0.001$.

3. Results

3.1. Survival of SMX exposed crayfish after challenge with WSSV

In order to investigate whether exposure to environmental-relevant concentrations of the antibiotic SMX had an effect on the crayfish survival to viral infection, crayfish were first exposed to SMX dissolved in water in aquaria for 21 days followed by an injection of WSSV. Animals maintained under the same condition, but no exposure to SMX were injected with WSSV as a control group.

The survival experiment showed that the 100 ng/L SMX exposed group had shorter survival period after WSSV challenge (all animals were dead one day post-infection), when compared with the control group (two days post-infection) and the 1 μg/L SMX exposed group (three days post-infection) (Fig. 2). Comparisons between all SMX groups and controls were significantly different ($P < 0.001$). It is important to note here, that after the 21-day period of SMX exposure, all crayfish survived and were as active as the control group (no SMX added).

3.2. Expression of immune-relevant genes after SMX exposure

In order to explain the effect of environmental relevant SMX concentrations (100 ng/L and 1 μg/L) upon the survival rates during WSSV infection, we decided to evaluate the immune system of *P. leniusculus* after 21 days of SMX exposure. Due to the role that hemocytes and intestine play in the immune response of the crayfish *P. leniusculus*, both tissues were chosen to quantify the relative expression of AMPs, proPO, HHAP, and TEP, using RT-qPCR. The principal functions and the tissue distribution of the immune-related genes analyzed in the present study are listed in Table 2.

Fig. 2. Survival curve of SMX-exposed crayfish challenged with WSSV. Median survivals: 2 days post-infection (dpi) for control group; 1 dpi for 100 ng/L of SMX exposed group; 3 dpi for 1 μg/L of SMX exposed group. Significant differences ($P < 0.001$) were found between both SMX exposed groups in comparison with the control. The data were analyzed by Log-rank (Mantel-Cox) test. In total for the three replicate experiments 6 crayfish for control, 7 for the lower dose of SMX and 10 for the higher dose of SMX were used.

Fig. 3 shows the relative expression of AMPs, proPO and HHAP quantified in hemocytes from the three treatments. Regarding the group of crayfish exposed to 100 ng/L of SMX, the expression of Crustin 3 gene was significantly down-regulated when compared with the control group ($P < 0.05$) (Fig. 3A). However, the expression of Crustin 1, Crustin 2, HHAP, ALF and proPO was not significantly different ($P > 0.05$) from that of the control. No significant differences were found for the results obtained from hemocytes collected from the 1 μg/L SMX for any of the genes ($P > 0.05$).

Likewise, as shown in Fig. 4, the relative gene expression of AMPs, proPO, HHAP and TEP2 in intestine from the three treatments, none of the seven genes analyzed showed any significant difference in expression between the group exposed to 100 ng/L of SMX and the control group ($P > 0.05$). However, samples from the 1 μg/L of SMX exposed group showed up-regulation of the AMP Crustin 1 ($P < 0.05$) (Fig. 4A), while the rest of the genes from this group did not show any significant changes ($P > 0.05$).

3.3. Hemocyte count

In order to explain the regulation of AMPs and proPO observed in hemocytes and intestine from both SMX exposed groups, differential and total counts of hemocytes were performed. The results showed that crayfish exposed to 100 ng/L of SMX had a significantly lower number of SGCs and THC compared with the control group ($P < 0.05$), as well as a significantly lower number of GCs ($P < 0.01$), SGCs ($P < 0.05$) and THC ($P < 0.01$) in comparison with the group exposed to 1 μg/L of SMX (Fig. 5). No significant differences were obtained between the group exposed to 1 μg/L of SMX and the control ($P > 0.05$).

4. Discussion

In this study, we report the effect of two different environmental concentrations of the antibiotic SMX upon the susceptibility to WSSV and upon the immune system of the model crustacean *P. leniusculus*. Herein the results of the expression of AMP Crustins were the most affected by the environmental concentrations of SMX in both hemocytes and intestine. Crustins are proteins that are found in the hemocytes of many species of crustaceans, including crayfish, crabs, shrimps and lobsters [28,47,48]. Crustins have an important function in the innate immune system, since they have a Gram-positive and Gram-negative antimicrobial activity [28,47,49–52]. In the case of *P. leniusculus*, three Crustins have been previously characterized, *Pl*Crustin1 – 3 [28].

Table 2
Principal functions and tissue distribution of antimicrobial peptides (AMP), HHAP, ProPO and TEP mRNA from *Pacifastacus leniusculus* evaluated in the present study.

Gene	Function	mRNA expression	Reference
18S	Ribosomal gene	Constitutively in all tissues	[46]
Crustin1	Anti-bacterial defense	Hemocytes	[22,26]
Crustin2	Anti-bacterial defense	Hemocytes	[22,26]
Crustin3	Anti-bacterial defense	Granular hemocytes	[22,26]
TEP	Gram-negative bacteria defense	Stomach, foregut and hindgut	[27]
ALF	Anti-WSSV defense	Hematopoietic tissue, semigranular and granular hemocytes	[29]
HHAP	Intestinal microbiota regulation	Hemocytes, gills, foregut and midgut	[26]
proPO	Melanization, antibacterial defense	Semigranular and granular hemocytes	[69]

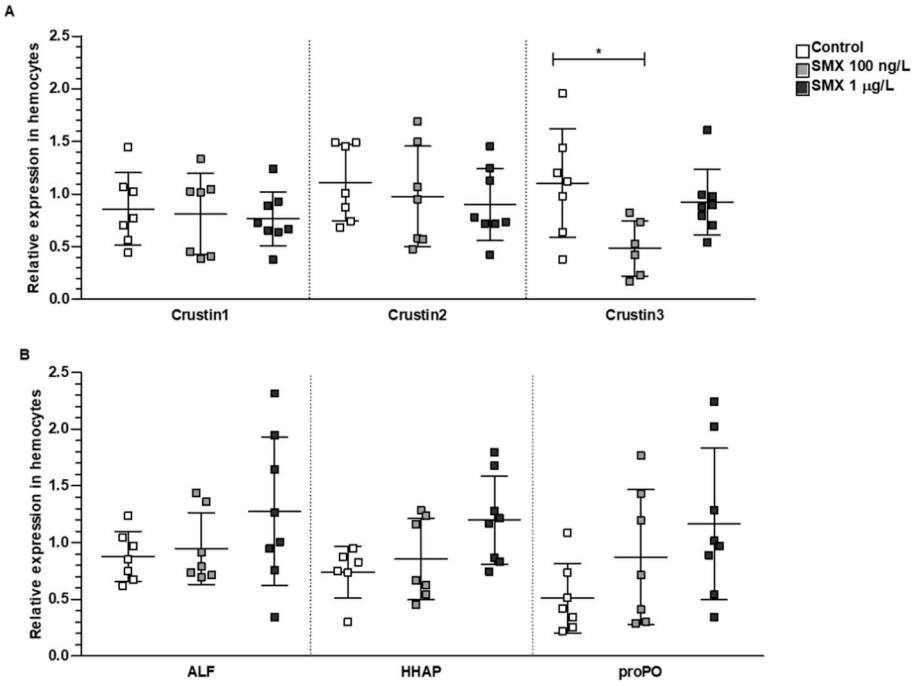

Fig. 3. Relative expression of immune-relevant genes in hemocytes of SMX-exposed crayfish determined by real-time qPCR. (A) Crustin 1, Crustin 2, Crustin 3 and (B) Antilipopolysaccharide factor (ALF), Hemocyte homeostasis-associated-like protein (HHAP) and Prophenoloxidase (proPO). Crustin3 from 100 ng/L of SMX group showed significant down-regulation (*P < 0.05) when compared with control. Data were analyzed by One-way ANOVA analysis followed by Tukey-test for normal distribution data, and Kruskal-Wallis test followed by the Dunn's test for non-normal distribution data. At least 6 individual samples were included per treatment, and samples were taken individually from each animal. Each dot represents value from one individual animal. Error bars represent standard deviation.

They are highly expressed in hemocytes, but only *Pl*Crustin 2 is expressed in the hematopoietic tissue [28]. *Pl*Crustins 1–3 are also expressed at the egg stage during embryo development in *P. leniusculus* [48]. More recently, through a proteomic approach, it was found that *Pl*Crustin1 and *Pl*Crustin3 are the most abundant proteins in granular hemocytes and that the former is exclusively produced in this type of hemocyte cell [22].

Our results show that when crayfish exposed to SMX at low but environmentally relevant concentration (100 ng/L) were challenged with WSSV, the mortality rate was faster (1 dpi) in comparison with the control group (2 dpi) (Fig. 3). When mRNA expression of the immune-related genes from these SMX exposed animals were evaluated in the hemocytes, *Pl*Crustin3 was down-regulated, whereas, *Pl*Crustin1 and 2 were maintained at similar level of expression when compared with control (Fig. 3A). Moreover, the result obtained from the number of circulating hemocytes count from this crayfish group exposed to SMX at 100 ng/L, showed lower number of SGCs, and THC (Fig. 5). Since the hemocytes play an important role for the well-being of crustaceans, differential and total hemocyte count are considered important parameters to evaluate the immunological system [20]. Consequently, the results obtained in our study indicate that the low number of SGCs and THC negatively affects the host antiviral response, giving an advantage to WSSV to establish an infection. Why low concentration of the antibiotic SMX affects the total hemocyte number or the expression of Crustin mRNAs is not resolved in this study, but since AMPs are known to be induced in expression by the presence of different bacteria [28], changes in expression may be caused by a change in the internal composition of microbes in the intestine and/or other internal surfaces exposed to the surrounding water.

There is no information about a role for *Pl*Crustins during antiviral response since previous studies focused only on their anti-bacterial function [28,52]. However, studies conducted in *Penaeus monodon* showed that *Pm*Crustin3 was gradually up-regulated during a 10-day WSSV infection [53]. The same effect was observed for crustins from *Macrobrachium rosenbergii* [54], *Marsopenaeus japonicus* [55,56], and *Sp*Crustin from mud crab, *Scylla paramamosain* [57]. In this last study, the authors also found that *Sp*Crustin suppressed replication of WSSV to some extent. All these evidences, therefore, indicates that crustins may affect WSSV replication most likely together with other unknown factors. Taken together, our results suggest two possibilities: 1) concentration of 100 ng/L of SMX depletes *P. leniusculus* hemocytes from circulation, or 2) concentration of 100 ng/L of SMX inhibits cell differentiation. In either case, the same outcome is obtained: the decreasing of the number of total circulating hemocytes and the diminishing of the anti-WSSV response making the crayfish more prone to death. Still, a SMX effect upon cell differentiation remains to be elucidated.

It is also important to highlight the relevance of this result at an ecological level. Due to the fact that the most common concentrations of SMX found in the aquatic ecosystems are ranging in the scale of nanograms per liter, along with the presence of viral diseases including WSSV and Infectious hypodermal and hematopoietic necrosis virus (IHHNV) on wild crustaceans [33,34,36,37,58,59], SMX pollution can represent a threat for the natural population of these organisms.

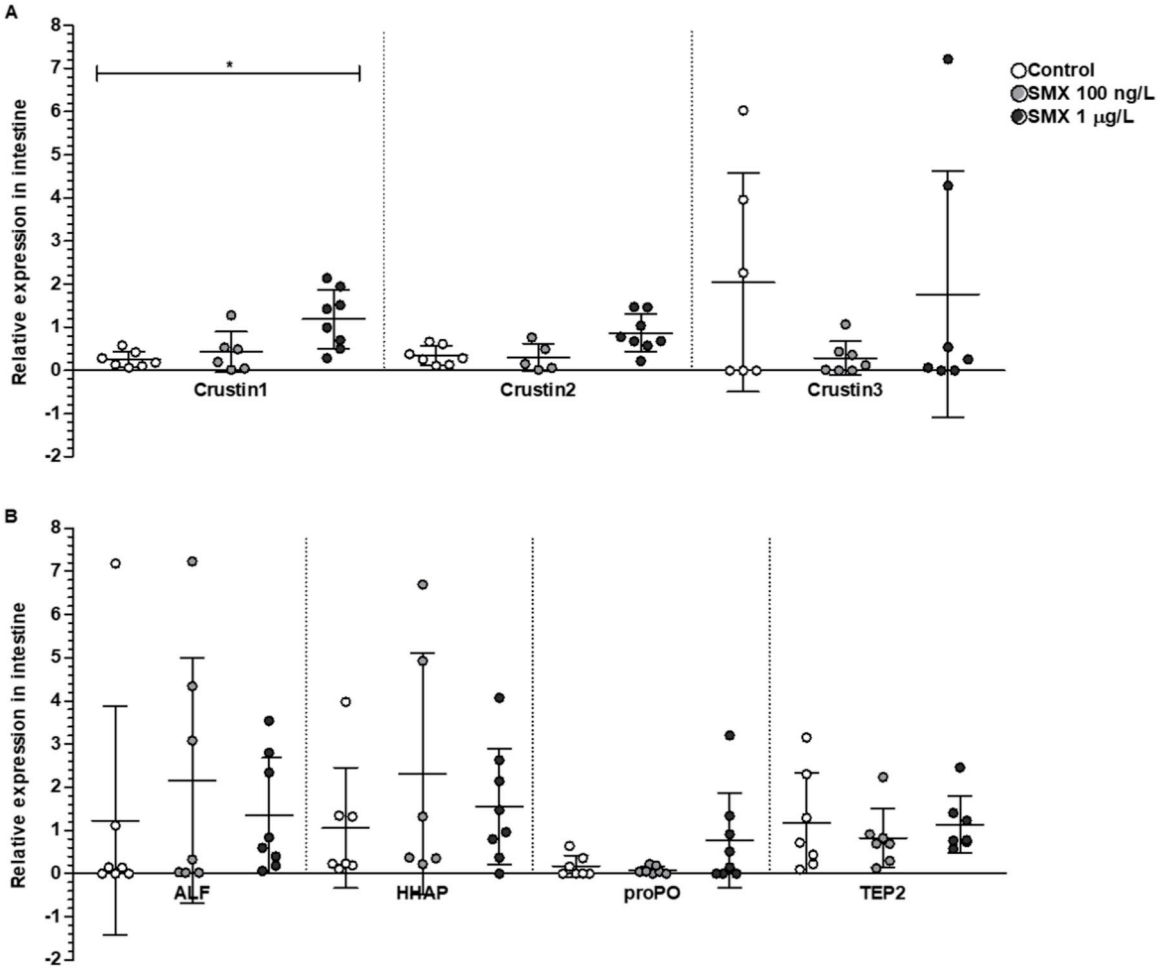

Fig. 4. Relative expression of immune-relevant genes in intestine of SMX-exposed crayfish determined by real-time qPCR. (A) Crustin 1, Crustin 2, Crustin 3 and (B) Antilipopolysaccharide factor (ALF), Hemocyte homeostasis-associated-like protein (HHAP), Prophenoloxidase (proPO) and Thioester-containing protein 2 (TEP2). Crustin 1 from 1 µg/L of SMX group showed significant up-regulation (*$P < 0.05$) when compared with control. Data were analyzed by One-way ANOVA analysis followed by Tukey-test for normal distribution data, and Kruskal-Wallis test followed by the Dunn's test for non-normal distribution data. At least 5 individual samples were included per treatment, and samples were taken individually from each animal. Each dot represents a value from one individual animal. Error bars represent standard deviation.

In the case of crayfish exposed to 1 µg/L of SMX, the results from the WSSV challenge showed that this group of crayfish survived for a longer period (3 dpi) (Fig. 2). Also, we found that *Pl*Crustin1 was significantly up-regulated in intestine samples in these animals (Fig. 4A). In addition, GCs, SGCs and THC were significantly increased in this group in comparison with 100 ng/L group (Fig. 5), suggesting that this concentration could have an immunostimulatory effect on the crayfish *P. leniusculus* making the crayfish less susceptible to the viral infection to some extent, and for a relatively short time.

*Pl*Crustin1 is mainly expressed in the granular hemocytes, and detection of mRNA levels in other tissues, including intestine is most likely attributed to hemocyte infiltration [22,60]. It is also well known that *Pl*Crustin1 has an important protective role during bacterial infections [28,52]. However, as mentioned before, it is currently not known the role of *Pl*Crustins during viral infections.

It is likely that the crayfish survived longer since the concentration

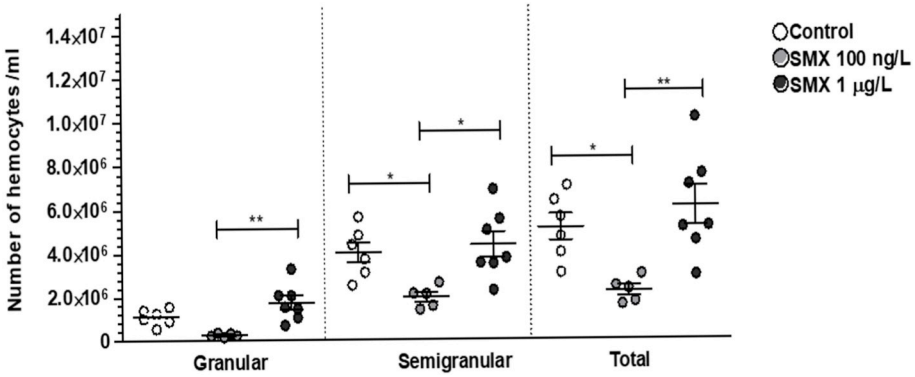

Fig. 5. Differential and total hemocyte count (THC) of SMX-exposed crayfish. Semigranular (SGCs) and THC were significantly lower in comparison with control group ($P < 0.05$) and significantly lower number of granular cells (GCs) ($P < 0.01$), SGCs ($P < 0.05$) and THC ($P < 0.01$) in comparison with the group exposed to 1 µg/L of SMX. No significant differences were obtained between the group exposed to 1 µg/L of SMX and the control ($P > 0.05$). At least 5 individual samples were included per treatment, and samples were taken individually from each animal. Each dot represents value from one individual animal. Error bars represent standard deviation.

of 1 μg/L of SMX stimulated the up-regulation of *Pl*Crustin1, perhaps by changing the internal microflora, since similar results were previously obtained during the evaluation of immunostimulant substances. For example, after the administration of marine yeast *Candida haemulonii*, the expression of crustin-like transcripts in *P. monodon* hemocytes was enhanced, and the shrimp showed a longer survival during WSSV infection [61].

Our result indicates that the concentration of 1 μg/L of SMX acts as external immunostimulant, maybe only for a short time. However, the efficiency of immunostimulants, including antibiotics, in crustaceans is reported as controversial [62], since a protective effect during the administration of these substances has not been completely demonstrated. Similarly, repeated application of antibiotics in the long term promotes antibiotic resistance [63,64].

It is estimated that more than 80% of the world's wastewater is released to the environment without any treatment [65]. Therefore, the harm that pharmaceutical substances may represent to the aquatic environments and towards organisms is an increasingly important topic to study. Our investigation, along with recent works, including those conducted in European eel (*Anguilla anguilla*) evaluating illicit drug cocaine [66], and in rainbow trout (*Oncorhynchus mykiss*), evaluating antibiotic erythromycin [67] and cardiovascular drug diltiazem [68], demonstrates the damage that environmental concentrations of pharmaceuticals have upon fresh-water species, and that the chronic effect rather than the acute toxic effects of these substances should be evaluated, especially in the normal functioning of aquatic organisms.

5. Conclusions

The present study indicates that low concentration of SMX interfere with the immune system of *P. leniusculus*. Thus, the presence of such low concentration in aquatic environments may increase the susceptibility of the organisms to WSSV infection by diminishing its cellular response, specifically by the down-regulation of *Pl*Crustin3 and by a low number of circulating hemocytes. On the contrary, higher concentration of SMX provided longer survival during WSSV infection, probably due to a higher number of circulating hemocytes (especially granular cells), and expression of an AMP, which leads to an enhanced crayfish humoral response for a short time.

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CRediT authorship contribution statement

Ariadne Hernández-Pérez: Methodology, Formal analysis, Investigation, Conceptualization, Writing - original draft, Writing - review & editing. **Chadanat Noonin:** Methodology, Writing - original draft. **Kenneth Söderhäll:** Conceptualization, Writing - review & editing. **Irene Söderhäll:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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