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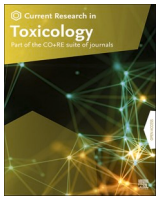

Research Paper

Molecular response of *Chironomus riparius* to antibiotics

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ABSTRACT

Antibiotics, like other pharmaceuticals, are continuously released into the environment as a result of human activities. Although designed to target harmful bacteria, they can also affect non-target organisms in aquatic ecosystems. Standard toxicological tests often fail to detect the subtle or long term antibiotic-induced effects, but newer methods are providing valuable insights into the molecular pathways and physiological responses they affect. *Chironomus riparius*, a dipteran with aquatic larvae, is widely used in toxicological testing due to its sensitivity to various toxicants. However, little is known about the molecular effects of antibiotics on this species.

This study investigated the gene expression profile of *C. riparius* in response to antibiotics from three classes – aminoglycosides, fluoroquinolones and penicillin. Fourth instar larvae were exposed to concentrations of 0.001, 0.1 and 10 mg/L for 24 and 72 h. The expression of genes involved in hormonal regulation, detoxification, stress response and DNA repair was analysed. The results showed that all antibiotics altered mRNA levels, with three of the four (amoxicillin, neomycin and levofloxacin) downregulating genes at 24 h and upregulating them at 72 h. Genes affected by gentamicin showed the opposite trend.

These transcriptional changes in response to different antibiotics highlight the complexity of the regulatory mechanisms involved in development, detoxification, stress response and DNA repair in aquatic insects. Further research is needed to better understand the molecular effects of antibiotics on this species.

1. Introduction

Antibiotics, like other pharmaceuticals, are continuously released into the environment as a result of human activities. While notable levels are expected in surface waters near densely populated areas, their presence has also been documented in protected areas (Boxall et al., 2024). Although designed to target harmful bacteria, antibiotics can also pose risks to non-target organisms in aquatic ecosystems (Almeida et al., 2021; Dionísio et al., 2020; Yang et al., 2020). The ecological consequences of such exposures are still not fully understood, particularly as traditional toxicological tests often focus on acute toxicity and may fail to detect more subtle or long-term effects (Hook et al., 2014).

In recent years, there has been increasing interest in integrating data from different toxicity endpoints to improve chemical safety assessments (Madia et al. 2021; Johnson et al., 2022; EFSA, 2024). This approach combines apical endpoints with molecular responses, allowing for the detection of changes in biological pathways before any alterations are observed in traditional toxicological endpoints. Recognizing the limitations of conventional tests, regulatory authorities are taking steps to implement new toxicological test methods to strengthen risk assessment strategies (Gant et al., 2023; Schmeisser et al., 2023).

In environmental toxicology, gene expression analysis has become a valuable tool for understanding how specific genes respond to chemical exposures (Schirmer et al., 2010). By focusing on a defined set of genes, this approach can reveal early signs of biological changes due to contaminants. While gene expression responses to antibiotics have been studied in some aquatic model organisms, such as Daphnia (Kim et al., 2017) and early developmental stages of zebrafish (Liu et al., 2020; Zhang et al., 2015), similar studies in aquatic insects are still limited. Given the ecological importance of aquatic insects in freshwater systems, studying their gene expression is crucial to understanding the wider impacts of environmental pollution on biodiversity and ecosystem health.

Chironomus riparius (Meigen, 1804) is a dipteran with aquatic larvae that is widely used in several standard toxicity tests (e.g. OECD, 2004a, b, 2010, 2011). This benthic species is abundant in freshwater ecosystems and plays a key role in many food chains. The life cycle consists of four instars/stages: embryo, larva, pupa and adult, with the larval stage

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– particularly the fourth instar – being the most metabolically active (Armitage et al., 1995). Due to the significant developmental events occuring during this stage, larvae are highly sensitive to toxicants, making them valuable indicators for environmental monitoring. Although the first instar is generally considered the most sensitive to contaminants, it is often associated with high mortality even at environmentally relevant concentrations, which can limit its use in molecular analyses. The fourth instar, by contrast, provides sufficient tissue for transcriptomic studies and allows for the assessment of sublethal effects. While much is known about the response of *C. riparius* to conventional pollutants, little is currently understood about how exposure to antibiotics affects this species at the molecular level.

Antibiotics receive considerable attention due to growing concerns about antibiotic resistance, yet their potential adverse effects on key species should not be overlooked. In this study, we selected four antibiotics from three different classes, each with a distinct mechanism of action, to investigate their effects on gene expression in *C. riparius* larvae. Amoxicillin, a penicillin antibiotic, inhibits bacterial cell wall synthesis, while levofloxacin, a fluoroquinolone, disrupts DNA synthesis. Gentamicin and neomycin, both aminoglycosides, inhibit bacterial protein synthesis by targeting the ribosome (Calvo and Martínez-Martínez, 2009). By selecting antibiotics with different mechanisms, we aimed to capture a range of gene expression changes in *C. riparius* larvae, which may respond differently to each compound. We selected fourteen genes covering essential metabolic pathways – hormonal regulation, detoxification mechanisms, stress response and DNA repair – to reveal molecular responses that have been largely unexplored in this model organism.

2. Materials and methods

2.1. Materials

Amoxicillin (CAS: 26787–78-0), gentamicin sulphate (CAS: 1405–41-0), neomycin trisulfate salt hydrate (CAS: 1405–10-3) and levofloxacin (CAS: 100986–85-4) were purchased from Merck (Spain). TRIzol™ and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) were obtained from Invitrogen (Germany). Oligonucleotide dT18 primer and gene-specific primers were supplied by Macrogen (Korea). RNase-free DNase was purchased from Sigma (Germany). DNA polymerase and dNTPs were obtained from Biotools (Spain), and EvaGreen was purchased from Biotium (USA). The rest of reagents used were from Merck (Spain).

2.2. Test organisms

Cultures of *C. riparius* have been maintained in the laboratory for several generations. The original population was obtained from Massamagrell (Valencia, Spain). The animals are reared in glass vessels in reconstituted water (0.5 mM CaCl2, 1 mM NaCl, 1 mM MgSO4, 0.1 mM NaHCO3, 0.025 mM KH2PO4) supplemented with commercial fish food (TetraMin, Germany) and cellulose tissue, which provided suitable conditions for larval burrowing in the absence of sediment. The culture medium is renewed twice a week, and the animals are fed ad libitum at the same time. Cultures are maintained at 18.5 °C with constant aeration and a standard 16:8 light:dark cycle.

2.3. Exposure to antibiotics

Exposure concentrations were selected to encompass a wide range of environmentally relevant and high-exposure scenarios, from levels typically observed in treated municipal wastewater to those reported in untreated effluents and industrially impacted surface waters (Larsson and Flach, 2022). Stock solutions of amoxicillin, gentamicin and neomycin (10 mg/mL) were prepared in ultrapure water, while levofloxacin was dissolved in chloroform at the same concentration. As amoxicillin did not dissolve completely in ultrapure water, HCl was added to the stock solution (0.4 % of the final volume) to facilitate dissolution. Working solutions were then prepared by diluting the stock solutions 1:100 in ultrapure water. The dilutions of antibiotics were freshly prepared for each experiment. For exposure, 50 µL of each working solution was added to 50 mL of culture medium to give final concentrations of 0.001, 0.1 and 10 mg/L. In all cases where additives (e.g. acid or solvent) were used, the same volume was added to the control samples (0.0004 % of the final volume) to account for their potential effects.

Prior to the main exposure experiments, preliminary assays were conducted to confirm that the test conditions did not induce mortality or visible signs of toxicity (e.g., immobility, abnormal behaviour) in the larvae. For the main exposures, larvae were transferred to test vessels containing the respective treatments. In each treatment, six fourth-instar larvae were exposed in a test vessel for either 24 or 72 h under the same conditions as during maintenance. Larvae were fed at 48 h. Three larvae were collected at 24 h and the remaining three at 72 h. Larvae were individually frozen in microcentrifuge tubes on dry ice and prepared for subsequent RNA extraction. Samples were stored in TRIzol™ reagent at −80 °C until RNA extraction, which was performed within 72 h of collection. Each treatment (i.e., each antibiotic concentration and the control) was independently replicated three times using different sets of larvae, resulting in three biological replicates per time point. Thus, for each treatment and time point, a total of n = 9 individual larvae were analyzed for gene expression.

2.4. RNA extraction and retrotranscription

RNA extraction was performed using TRIzol™ reagent according to the manufacturer's instructions. Frozen larvae were homogenized and incubated with 0.2 volumes of chloroform for 3 min at room temperature. The sample was then centrifuged for 10 min, and the upper aqueous phase was collected. The RNA was precipitated with 0.7 vol of isopropanol and washed with 75 % ethanol. The RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water and incubated with RNase-free DNase for 45 min. DNAse was removed by a phenol:chloroform:isoamyl alcohol extraction and the RNA was re-precipitated with isopropanol and resuspended in 28 µL DEPC-treated water.

Retrotranscription was performed using MMLV reverse transcriptase in a final volume of 40 µL with 24 µL RNA, according to the manufacturer's instructions. The primer used was poly dT_{18}. Retrotranscribed samples were stored at −20 °C until further use.

2.5. Real-Time PCR

In this study, Real-Time PCR analyses targeted 14 genes involved in hormonal regulation (*EcR, Cyp18a1, Met, JHAMT, MAPR*), detoxification mechanisms (*Cyp4d2, Cyp6b7, Cyp12a2, GSTd3, GSTe2, MRP-1*), stress response (*Hsp70, Hsp90*) and DNA repair (*XRCC-1*), along with one reference gene (*L13*). Analysis was performed in a 96-well plate and each gene was analyzed in duplicate. Gene specific primer sequences and their efficiencies can be found in the supplementary material (Table S1). First, the primers (250 nM) were added (1 µL) to each well. A master mix containing cDNA, DNA polymerase (0.2 units/µL final concentration), Evagreen (0.5x), dNTPs (200 µM), 1x buffer, and 2.5 mM $MgCl_2$ was prepared and 9 µL were added to each well. Two technical replicates were run for each sample.

The program used was an initial denaturation at 95 °C for 2 min and then 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 15 s, repeated 39 times. This was followed by a melting curve from 60 to 85 °C in 0.5 °C increments to confirm a single product. The Maestro software (Bio-Rad, USA) used the regression option to determine the threshold cycle (Ct). The Ct value was used for subsequent data analysis by the 2 ΔΔCt method (Pfaffl, 2001).

2.6. Statistics

Statistical analysis was performed using SPSS 27 (IBM, USA). Normal distribution was tested using the Shapiro-Wilk test ($n < 50$). As the data did not follow a normal distribution, the non-parametric Kruskal-Wallis test with the Bonferroni correction was used for data analysis. Significance was set at $p \leq 0.05$.

3. Results and discussion

In general, all antibiotics altered the transcriptional activity of genes involved in hormonal, detoxification, stress, and DNA repair related responses in *C. riparius* (Table 1 and Figs. 1-4). Some differences were observed among the antibiotics and exposure times. While amoxicillin (penicillin), levofloxacin (quinolone) and neomycin (aminoglycoside) downregulated the expression of the affected genes at 24 h and upregulated them at 72 h, gentamicin (aminoglycoside) had the opposite effect. These contrasting patterns may reflect differences in cellular uptake (Martin and Beveridge, 1986), intracellular stability of the antibiotics, or their interaction with molecular targets (Sandoval et al. 2000), with gentamicin possibly triggering a faster or more robust response that activates gene expression earlier.

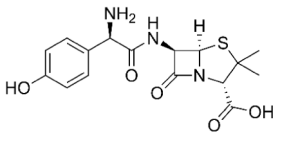
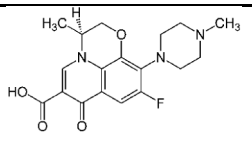
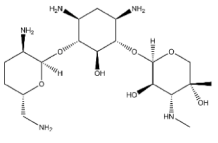
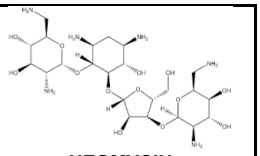
3.1. Endocrine system

In the present study, the endocrine system was affected in different ways by amoxicillin and gentamicin, as evidenced by changes in the expressions of two of the genes studied, *EcR* and *Met* (Table 1, Figs. 1 and 3). While the effects of amoxicillin were only observed at a concentration of 10 mg/L for both genes, gentamicin affected *EcR* at a lower concentration (0.1 mg/L). The downregulation of the *EcR* and *Met* genes at 24 h by amoxicillin suggests that receptors of ecdysone and juvenile hormone could be altered in their levels. The levels are recovered at 72 h, so the effect is transitory. In the case of gentamicin, an initial upregulation of *EcR* is followed by downregulation at 72 h, which may reflect a more prolonged or toxic effect of this antibiotic. The change could suggest an accelerated development, but the unchanged levels of the other endocrine genes tested make it difficult to determine the underlying mechanisms. Activation of *EcR* transcriptional activity has also been observed with the antibiotics sulfadiazine (a sulfonamide) as well as tetracycline after 48 h of exposure at concentrations ranging from 2 to 200 μg/L (Xie et al., 2019a,b), and with the antibacterial agent triclosan after 24 h at 1 mg/L (Martínez-Paz et al., 2017). This suggests a common pattern of endocrine disruption for different compounds in the same organism. Whether *EcR* is up- or downregulated by antibiotics in *C. riparius*, disruption of normal endocrine function can significantly affect developmental processes. For example, Galarza et al. (2021) observed a growth-promoting effect in wood tiger moth (*Arctia plantains*) larvae exposed to a mixture of tetracycline and ciprofloxacin during development. Although our experiments showed no changes in the expression of the genes studied in organisms exposed to levofloxacin (a quinolone antibiotic, like ciprofloxacin), we observed a notable increase in pupation rate after 72 h (data not shown). In contrast, gentamicin did not have a similar effect, nor did it affect another dipteran species, *Chrysomya putoria*, which was exposed to antibiotic-containing agar at concentrations up to 66 mg/mL (Ferraz et al., 2014). No significant changes in gene expression were observed for neomycin under the tested conditions for any of the endocrine-related genes, suggesting that this antibiotic may not interfere with endocrine signalling pathways in *C. riparius*, or that the selected markers were not sufficiently responsive to detect subtle effects.

In insects, the hormones ecdysone and juvenile hormone play a crucial role in regulating moulting and metamorphosis (Nation, 2008). EcR (Ecdysone receptor) is involved in the ecdysone signalling pathway, while Cyp18a1 (Cytochrome P450 enzyme) regulates ecdysone metabolism (Aquilino et al., 2016; Guittard et al., 2011). On the other hand, Met (Methoprene-tolerant) and JHAMT (Juvenile Hormone Acid Methyltransferase) are involved in the juvenile hormone pathway, mediating its signalling and synthesis, respectively (Jindra et al., 2013; Shinoda and Itoyama, 2003). MAPR (Membrane-Associated Progesterone Receptor) is an orphan receptor whose function in insects is still under investigation, but it may bind steroids (Kimura et al., 2012).

Additionally, we observed differences in gene expression levels between time points (e.g. between 24 and 72 h), even within the same treatment conditions (Figs. 1–4 and Fig. S1-4). These variations likely reflect the dynamic nature of gene regulation in *C. riparius* larvae, as this metabolically active stage involves significant developmental changes in preparation for pupation. Such shifts in gene expression over time

Table 1
Changes in the transcriptional activity of genes involved in the hormonal, detoxification, stress and DNA repair related responses in *C. riparius* exposed to four different antibiotics for 24 and 72 h. ↑ Upregulation. ↓ Downregulation respect to controls. $p < 0.05$.

		 AMOXICILLIN						 LEVOFLOXACIN						 GENTAMICIN						 NEOMYCIN					
	Time	**24H**			**72H**			**24H**			**72H**			**24H**			**72H**			**24H**			**72H**		
	[mg/L]	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**	**0.001**	**0.1**	**10**
Endocrine system	***EcR***	-	-	↓	-	-	-	-	-	-	-	-	-	-	↑	↑	-	↓	↓	-	-	-	-	-	-
	Cyp18a1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Met	-	-	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	JHAMT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MAPR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Detoxification mechanisms	***Cyp4d2***	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	↓	-	-	-	-
	Cyp6b7	-	-	↓	-	-	-	-	-	-	-	-	-	-	-	↑	-	-	-	-	-	-	-	-	-
	Cyp12a2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GSTd3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GSTe2	-	-	-	-	-	-	-	-	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	MRP-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↓	-	-	-	-
Stress response	***Hsp70***	-	-	↓	-	-	-	-	-	-	-	-	-	-	↑	↑	-	↓	-	-	↓	-	-	-	-
	Hsp90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	↑	↓	↓	-	↓	↓	-	-	-	-
DNA repair	***XRCC-1***	-	-	↓	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

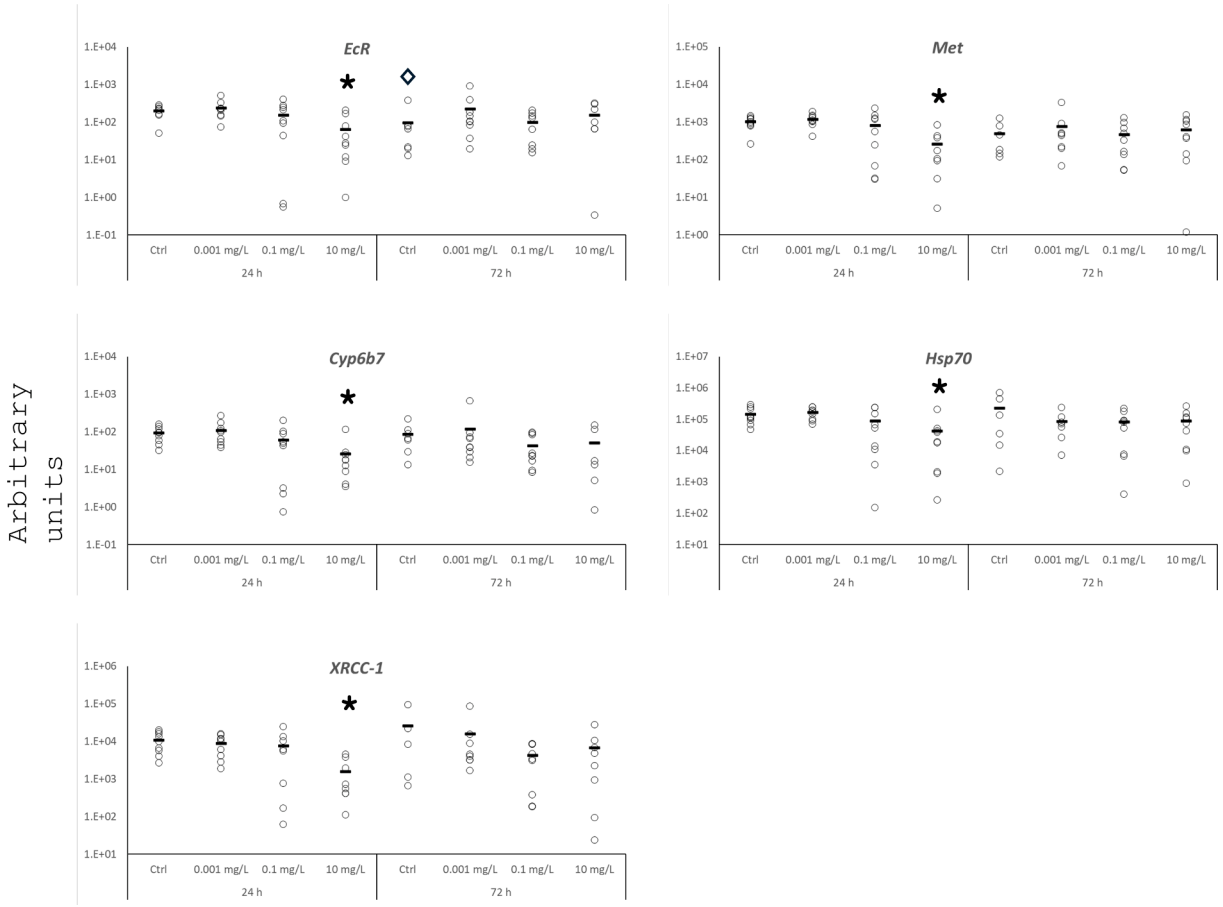


Fig. 1. Expression of genes involved in hormonal regulation, detoxification, stress response and DNA repair in *C. riparius* exposed to amoxicillin for 24 and 72 h. Horizontal line corresponds to the mean. Significant differences ($p < 0.05$): * vs. control; ◇ between controls; △, ▲, □ within 0.001, 0.1 and 10 mg/L treatments, respectively, between time points.

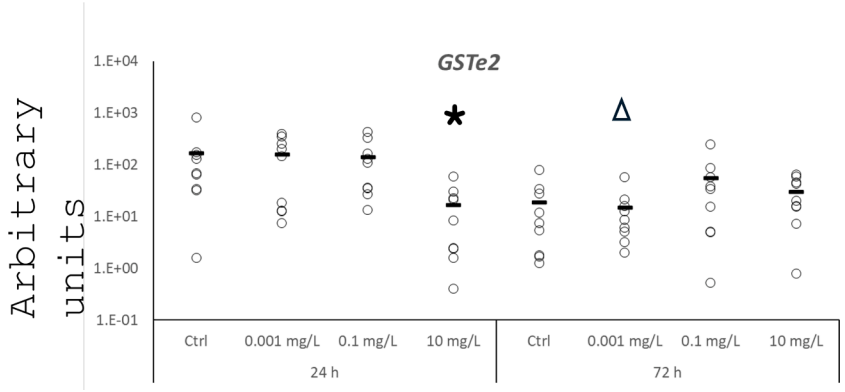


Fig. 2. Expression of genes involved in hormonal regulation, detoxification, stress response and DNA repair in *C. riparius* exposed to levofloxacin for 24 and 72 h. Horizontal line corresponds to the mean. Significant differences ($p < 0.05$): * vs. control; ◇ between controls; △, ▲, □ within 0.001, 0.1 and 10 mg/L treatments, respectively, between time points.

highlight the influence of both exposure duration and larval growth stage on molecular responses to antibiotics.

These alterations in endocrine function could have significant ecological implications, as disruptions in larval development and growth may affect the survival and reproductive success of *C. riparius*, potentially impacting freshwater food webs and ecosystem health.

3.2. Detoxification mechanisms

All antibiotics affected the detoxification system at the highest concentrations, with the exception of neomycin, which had an effect at lower concentrations (Table 1, Figs. 1-4). The detoxification processes in organisms, including insects, typically occurs in three phases, each aimed at neutralizing and eliminating harmful compounds such as xenobiotics (Yu, 2008). Phase I involves mainly enzymes of the cytochrome P450 (CYP) family, which modify xenobiotics to increase their

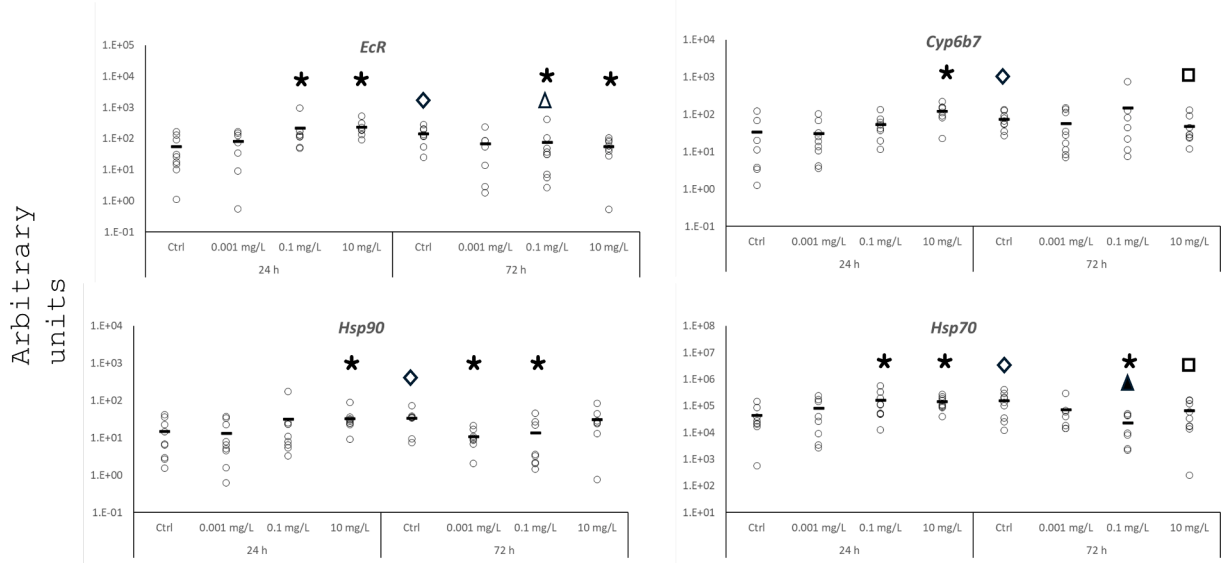


Fig. 3. Expression of genes involved in hormonal regulation, detoxification, stress response and DNA repair in *C. riparius* exposed to gentamicin for 24 and 72 h. Horizontal line corresponds to the mean. Significant differences ($p < 0.05$): * vs. control; ◇ between controls; △, ▲, □ within 0.001, 0.1 and 10 mg/L treatments, respectively, between time points.

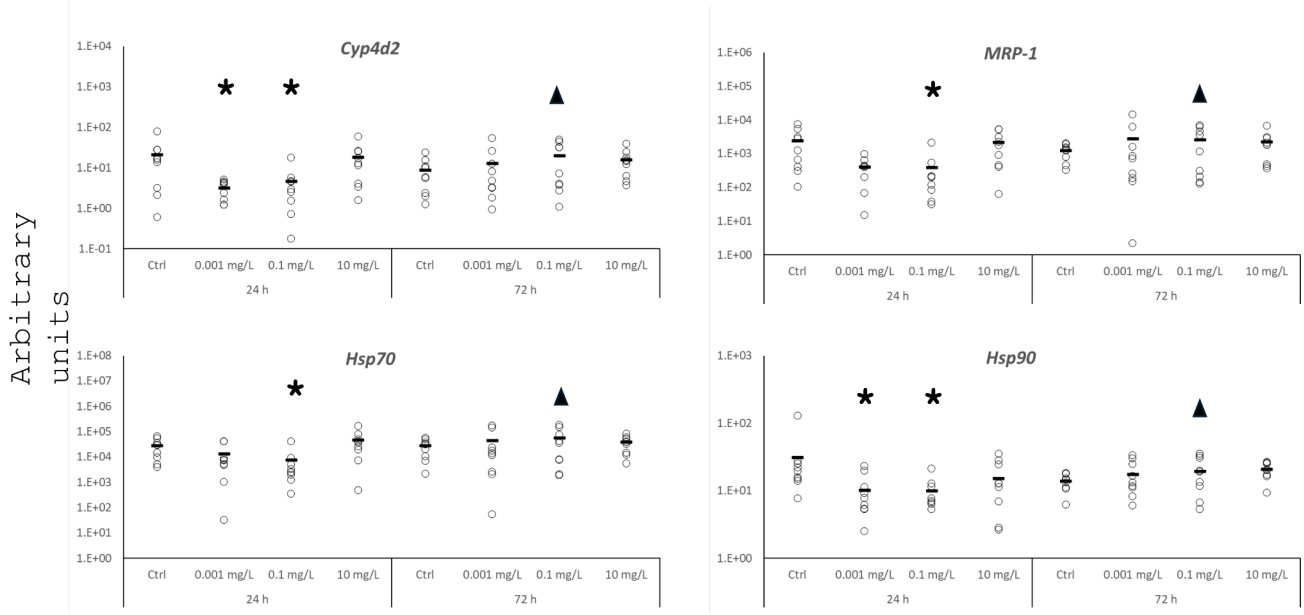


Fig. 4. Expression of genes involved in hormonal regulation, detoxification, stress response and DNA repair in *C. riparius* exposed to neomycin for 24 and 72 h. Horizontal line corresponds to the mean. Significant differences ($p < 0.05$): * vs. control; ◇ between controls; △, ▲, □ within 0.001, 0.1 and 10 mg/L treatments, respectively, between time points.

reactivity or hydrophilicity, making them available for further detoxification. In Phase II, these modified compounds undergo conjugation with hydrophilic molecules to increase their solubility. Finally, in phase III, the conjugated compounds are transported out of the cells and eliminated from the body, with transport proteins such as ATP-binding cassette (ABC) transporters playing a key role (Martínez-Guitarte, 2018).

Notably, in this study all observed gene expression changes occurred at 24 h, with no significant effects at 72 h, suggesting a possible early response to the antibiotics. Phase I detoxification genes were influenced by amoxicillin, gentamicin and neomycin, while levofloxacin and neomycin affected phase II and III detoxification genes, respectively. Interestingly, with the exception of *cyp6b7*, which was upregulated in response to gentamicin exposure, all other affected genes were downregulated. While upregulation of the cytochrome P450 genes is a common response to xenobiotics (Huang et al., 2013; Liang et al., 2015; Poupardin et al., 2010), downregulation could indicate that the insect either cannot or does not need to activate these pathways to detoxify the specific compound, or that the compound is interfering with the normal detoxification process. A similar downregulation of several genes in the Cyp450 family was reported by Martínez-Guitarte (2018) in *C. riparius* larvae exposed to the UV filter component 4-methylbenzylidene camphor (1 mg/L, 24 h). The downregulation of the phase II *GSTe2* gene following levofloxacin exposure may indicate a compromised detoxification response in larvae, potentially reducing their ability to cope with prolonged or combined environmental stressors. Such

detoxification impairments could ultimately affect larval fitness and, consequently, population dynamics and ecological functions of *C. riparius* in freshwater ecosystems. Similar effects have been reported in *C. riparius* larvae after 24-h exposure to polystyrene microplastics (Kalman et al., 2023) or the pesticide fipronil for 24 and 96 h (Pinto et al., 2024). Likewise, sublethal exposure of adult *Anopheles coluzzii* mosquitoes to pyrethroid insecticides for 4 to 48 h induced similar detoxification impairments (Ingham et al., 2021). Neomycin appears to specifically target the initial and final stages of detoxification processes. The downregulation of phase I and III genes may result in the intracellular accumulation of unmetabolized toxic compounds. Overall, each antibiotic triggered distinct responses in the detoxification pathways. However, it is important to note that these findings are based on a limited set of detoxification genes, raising the question of the involvement of other enzymes in the detoxification of antibiotics in this species.

3.3. Stress response

In addition to hormonal and detoxification responses, the stress response of *C. riparius* larvae was further investigated by examining the expression of heat shock proteins (HSP). In particular, we focused on the *hsp70* and *hsp90* genes, which are known indicators of stress and are frequently upregulated in response to various abiotic and biotic stressors (Jeyachandran et al., 2023; Zhao and Jones, 2012).

Levofloxacin had no significant effect on gene expression at the concentrations and time points tested, which indicates that it may not activate this stress response pathway in *C. riparius*. In contrast, amoxicillin and neomycin caused a downregulation of *hsp70* after 24 h, potentially compromising the ability of larvae to respond to stress (Figs. 1 and 4). A similar effect was observed following exposure to endosulfan (up to 10 μg/L, 24 h) by Muñiz-González et al. (2021), further suggesting that certain compounds may weaken the stress response and increase the susceptibility of organisms to environmental challenges. Moreover, neomycin also caused downregulation of *hsp90* after 24 h (Fig. 4). This reduced ability to respond to stress could ultimately affect the overall health and survival of organisms. In the case of gentamicin, an initial upregulation of both *hsp70* and *hsp90* was observed at 24 h, indicating an acute stress response consistent with the protective role of heat shock proteins against cellular stress (Fig. 3). However, this was followed by a downregulation of both genes at lower concentrations after 72 h, possibly indicating that the larval stress response was overwhelmed or that the organisms were beginning to acclimate to prolonged exposure. This shift may reflect a transition from acute stress to a compromised ability to maintain the defence response, potentially making the larvae more vulnerable to continued stress. Similarly, in the studies by Xie et al. (2019a,b), exposure to sulfonamide and tetracycline antibiotics resulted in upregulation of *hsp70* after 48 h at concentrations up to 200 μg/L. Interestingly, although gentamicin and neomycin belong to the same group of antibiotics, they have different effects on the stress response. This variability in response is similar to observations made with polystyrene microplastics (Kalman et al., 2023), which showed no significant effect on *hsp70* and *hsp90*, compared to polystyrene nanoplastics (Martin-Folgar et al., 2004), which increased *hsp70* and decreased *hsp90* levels after 24 h. Such differences highlight the complexity of stress responses in *C. riparius* and suggest that even compounds of the same class can have different physiological effects, emphasising the need for further investigation of their effects on aquatic organisms. These highly conserved proteins play a crucial role in assisting proper protein folding and repair. Beyond their involvement in protein metabolism, HSPs participate in fundamental biological processes such as cell cycle, apoptosis, diapause, immunity and development (Denlinger et al., 2001; Jeyachandran et al., 2023). Given the ecological role of *C. riparius* as a key species in freshwater ecosystems, disturbances in HSP expression may have cascading effects on ecosystem functioning, Due to their sensitivity to a wide range of chemical substances, HSPs have been recommended as effective biomarkers for monitoring environmental pollution (Gupta et al., 2010; Moreira-de-Sousa et al., 2018).

3.4. DNA repair

Among the antibiotics tested, amoxicillin was the only one to affect DNA repair mechanisms (Fig. 1, Table 1). Maintaining genome stability is essential for all cells, as it ensures proper functioning and survival of the organism. DNA is constantly exposed to endogenous and exogenous agents, which can result in various types of damage, including single-strand breaks (SSB), double-strand breaks (DSB), base modifications, and cross-links, among others (Huang and Zhou, 2021). To prevent long-term genomic instability, cells rely on efficient DNA repair mechanisms to correct these damages. Interestingly, previous studies have shown that amoxicillin can induce DNA damage in other organisms (Chowdhury et al., 2020; Orozco-Hernández et al., 2019). Building on this, we focused on the X-ray repair cross-complementing protein 1 (*XRCC-1*) gene to investigate the potential DNA damage caused by antibiotic exposure in *C. riparius*. This gene plays a key role in the repair of SSB (Caldecott, 2003), one of the most common forms of DNA damage induced by oxidative stress and xenobiotics.

Amoxicillin significantly affected the expression of the *XRCC-1* gene, showing a downregulation at the highest concentration tested after 24 h of exposure (Fig. 1). This observation suggests that the activation is concentration dependent, as the cells could have been overwhelmed. Supporting this idea, another study found that vinclozolin caused a general inhibition of DNA damage-related genes in *C. riparius* at its highest concentration compared to lower concentrations (Aquilino et al., 2018). The impact of amoxicillin on DNA integrity may vary across species, as exposure to this antibiotic did not result in genotoxic effects in *Drosophila melanogaster* (de Sousa et al., 2019). In contrast, levofloxacin, gentamicin and neomycin had no significant effect on *XRCC-1* expression under the conditions tested, possibly due to a lack of DNA damage, insufficient damage, or their limited impact on repair pathways. Although not analysed in the present study, previous research suggests that antibiotics can activate oxidative stress, potentially leading to indirect DNA damage (Xie et al., 2019b). Such interference with DNA repair mechanisms could compromise organismal fitness and, over time, affect population dynamics in aquatic ecosystems exposed to antibiotic contamination.

4. Conclusions

In this study, we investigated the molecular responses of *C. riparius* larvae to four commonly used antibiotics by assessing the expression of genes related to endocrine function, detoxification, stress response, and DNA repair. This study demonstrates that antibiotic exposure, particularly to amoxicillin and gentamicin, can disrupt developmental pathways in *C. riparius*, potentially affecting growth, metamorphosis and survival under environmental stress. All antibiotics affected detoxification pathways, predominantly downregulating genes within 24 h of exposure, suggesting either a disruption of normal detoxification processes or a limited capacity to process toxic compounds. Heat shock protein responses were variable, indicating both acute and adaptive stress responses. Elevated levels of amoxicillin may overwhelm cellular DNA repair mechanisms, thereby compromising genomic integrity. The contrasting patterns of gene expression in response to different antibiotics, as observed under the experimental conditions, highlight the complexity of the mechanisms regulating developmental processes, detoxification, stress management and DNA repair in aquatic insects and underline the need for further investigation. Due to the ecological importance of *C. riparius* as a key species in freshwater ecosystems, such molecular perturbations may have wider implications for aquatic food webs and ecosystem functioning. Future research should prioritise the investigation of the long-term and cumulative effects of antibiotic pollution, with particular attention to its impact on critical species such

as *C. riparius*. In addition, it is essential to incorporate molecular-level biomarkers into ecological risk assessments to better understand the broader consequences of antibiotic contamination in aquatic environments. Given the increasing presence of antibiotics in freshwater systems, addressing these research gaps is critical to safeguarding aquatic biodiversity and ensuring the resilience of ecosystems under increasing environmental pressures.

CRediT authorship contribution statement

Judit Kalman: Conceptualization, Investigation, Resources, Writing – review & editing, Methodology, Supervision, Formal analysis, Writing – original draft, Project administration, Visualization. **Yolanda Valcárcel-Rivera:** Conceptualization, Investigation, Resources, Writing – review & editing, Funding acquisition. **José Luis Martínez-Guitarte:** Conceptualization, Investigation, Resources, Writing – review & editing, Methodology, Supervision, Formal analysis, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2025.100239.

Data availability

Data will be made available on request.

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