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Impact of emergent pollutants and multi-stress in *Chironomus riparius*: a molecular and cellular analysis

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PROGRAMA DE DOCTORADO EN CIENCIAS

José Luis Martínez Guitarte

“Un país sin investigación es un país sin desarrollo”

“Una investigación básica de calidad es fundamental para un posterior desarrollo, porque de ella saldrán resultados no previsibles a priori”

Margarita Salas

“El camino del progreso no es ni rápido ni fácil”

“No hay que temer a nada en la vida, sólo tratar de comprender”

Marie Curie

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ABBREVIATIONS

°C	Celsisus degrees
20-E	20-hydroxyecdysone
ABC transporters	ATP-binding cassette transporters
ABCB6	ABC transporter beta 6
AchE	Achetilcolinesterase
AMPs	Antimicrobial peptides
ATM	Ataxia telangiectasia mutated
BPA	Bisphenol A
C. riparius	*Chironomus riparius*
CAT	Catalase
CECs	Contaminant of emerging concern
CYPs	Cytochromes
Cyp 450	Cytochrome p450
Cyp12a2	Cyp 12 alfa 2
Cyp18a1	Cyp 18 alpha 1
Cyp4d2	Cyp 4 delta 2
Cyp6b7	Cyp 6 beta 7
Cyp9b2	Cyp 9 beta 2
DDR	DNA damage response
Decay	Death executioner caspase
Def	Defensin
DEPC	Diethylpyrocarbonate
Dis	Disembodied
DNA	Deoxyribonucleic acid
Dronc	Death regulator Neddd2 like caspase
DSB	Double Strand Break
E93	Ecdysone-induced protein 93
EcR	Ecdysone receptor
EDCs	Endocrine-disrupting compounds
ETS	Electron Transport System
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Gp93	Glycoprotein 93
GST	Glutathione-S-transferase
GSTd3	GST delta 3
GSTe1	GST epsilon 1
GSTo1	GST omega 1
GSTt1	GST theta 1
h	hours
hsc70	Heat-Shock-Constitutive 70
HSPs	Heat-Shock-Proteins
HYOU1	Hypoxia upregulated protein 1
ILPs	Insulin-like peptides
InR	Insulin-receptor
JH	Juvenile hormone
JHAMT	Juvenile- O-methyltransferase
Kr-h1	Krüppel homolog 1
LDPE	Low-density Polyethylene

ABBREVIATIONS

LPO	Lipid Peroxidation
MAPR	Membrane-associated Progesterone Receptor
Met	Methoprene-tolerant
min	minutes
MPs	Microplastics
mRNA	Messenger RNA
MRP-1	Multidrug resistance protein 1
NLK	Nemo Like Kinase
OC	Octocrylene
OD-PABA	2-Ethylhexyl 4-(dimethylamino) benzoate
OECD	Organisation for Economic Co-operation and Development
PARP	Poly(ADP-Ribose) Polymerase
PCPs	Personal Care Products
PG	Prothoracic gland
Phfk	Phosphofructokinase
PO	Phenoloxidase
POPs	Persistent Organic Pollutants
PPCPs	Pharmaceuticals and Personal Care Products
Proph	Prophenoloxidase
PTTH	Prothoracicotropic Hormone
RNA	Ribonucleotic Acid
RNApol	RNA polimerase
ROS	Reactive-Oxygen-Species
rpL11	Ribosomal protein 11
rpL13	Ribosomal protein 13
RT-PCR	Real-Time polymerase chain reaction
sHSPs	Small Heat-Shock-Proteins
SSB	Single Strand Break
T	Temperature
TBP	TATA-Binding-Box
TGSH	Total Glutathione reducted
UVFs	Ultraviolet Filters
WWTP	Waste Water Treatment Plants
XRCC1	X-ray repair cross-complementing protein 1

ABSTRACT

ABSTRACT

In recent years there has been a strong over-the-food in the manufacture of new consumer products, increasing the number of waste and therefore the difficulty in managing them correctly. These new compounds include so-called contaminants of emerging pollutants (CECs), defined as synthetic or naturally occurring chemicals without legislation and whose effects on the environment and human health are unknown. Therefore, presenting a double risk. Due to the absence of specific legislation for use and disposal, they are returned to the environment in their initial state so that they are easily found in multiple environmental compartments such as surface water. On the other hand, it has been shown that it was due to its chemical properties being persistent and remaining for years in the environment and able to bind to the tissues of organisms and therefore be transmitted along the food chain. The danger of these CECs is that they can cause damage at low concentrations through chronic exposures.

In many cases, such effects are not detected until the final stages of development, so they can be misclassified as harmless if only parameters such as survival or reproduction are evaluated. In the current context, these compounds are not found individually in the environment, but in most cases, are presented in the form of mixtures, both compounds of the same nature and from different nature. This scenario increases the complexity of the analyses, as well as the possible effects on the environment and on the organisms. In addition to the mixtures themselves, physical factors such as pH temperature or humidity can alter the behavior of both organisms and xenobiotics themselves.

The goal of the present thesis was to evaluate the molecular effects of exposure to different xenobiotics in single or combined exposure in fourth-stage larvae of the aquatic organism *Chironomus riparius*. Several compounds of different chemical nature and belonging to CECs were studied as representatives of ultraviolet filters (OD-PABA, OC, BP-3), pharmaceuticals (ibuprofen), plasticizers (BPA), and pesticides (endosulfan). Besides, the transcriptional profile and biochemical biomarkers were analyzed in response to exposure to microplastics, as it is a class of emerging pollutant with a growing impact on the environment. For this purpose, the Real-time polymerase chain reaction has been used, designing a specific array for *C. riparius*. The array covers five essential routes for invertebrates, such as the endocrine system, detoxification response, stress response, immune system, and DNA repair mechanisms.

The results obtained have confirmed the usefulness of the array to evaluate compounds of different chemical nature since all pathways have been altered to a greater or lesser extent by each compound. Concerning the mixtures used, more significant toxicity has been observed compared to the single exposure of the compounds being higher, even using compounds of different origins (UV Filters + BPA). Also, a more significant effect has been observed in longer exposures 96h despite the low effect at 24h, which accentuates the need to evaluate several times. The pesticide endosulfan showed severe disruption of all the routes evaluated, as well as at the level of enzymatic activity. Confirming its danger and its possible effects on the population in the long term despite the low concentrations used. Ibuprofen modified survival in *C. riparius* in all concentrations used, actually substantial mortality was detected in the lowest concentrations. So *C. riparius* seems to be much more sensitive to this compound than other invertebrates. At the molecular level, ibuprofen appears to produce damage by oxidative stress by altering the endocrine system, stress response, and immune system, but with no effect on the response to detoxification. Although the effects vary according to the exposure time used. Finally, polyethylene microplastics altered both biochemical response and cell response. In general, particles of 32-45 m had increased toxicity. Similar to ibuprofen, these particles appear to produce toxicity in *C. riparius* by releasing reactive oxygen species and thus producing oxidative stress.

ABSTRACT

As observed by increased activity of catalase and Glutathione transferase, as well as the total amount of Glutathione reduced. At the transcriptional level, a substantial effect was observed in the endocrine system, immune, and in genes related to DNA repair, so it seems to develop some genotoxicity. However, it must be clarified whether the effects of these microplastics are due to mechanical alterations by their size and shape or the release of any of their chemical components.

Therefore, the present thesis has sought to address two of the most relevant current environmental problems. On the one hand, using a methodology that serves us to detect damage in short exposures at the molecular level and can serve as alarm signals and a source for putative future biomarkers. On the other hand, testing the effectiveness of the array against compounds of different chemical nature, as well as in multi-stress situations through various scenarios. The designed array has demonstrated the relevance of the simultaneous analysis of different pathways, which complements the growing interest of the scientific community in expanding the OECD database describing the adverse outcome pathways (AOP). The present thesis has highlighted the need to improve toxicity tests by including cellular and molecular variables and diversifying test conditions, such as multi-stress. Furthermore, mimicking environmental conditions and adding complexity to the analysis has also demonstrated the generation of random interactions and properties that are not observed in single exposures.

RESUMEN

RESUMEN

En los últimos años se ha observado un fuerte aumento en la fabricación de nuevos productos de consumo, incrementando el número de residuos y, por tanto, la dificultad para gestionarlos correctamente. Entre estos nuevos compuestos se encuentran los denominados contaminantes emergentes (CEs), definidos como productos químicos sintéticos o de origen natural, no legislados y cuyos efectos sobre el medio ambiente y la salud humana son desconocidos. Por tanto, presentan un doble riesgo. Debido a la ausencia de legislación específica para su uso y eliminación, son devueltos al medio ambiente en su estado inicial de modo que se encuentran fácilmente en múltiples compartimentos medioambientales como las aguas superficiales. Por otro lado, se ha demostrado que debido a sus propiedades químicas son persistentes, manteniéndose durante años en el medio ambiente y con capacidad de unirse a los tejidos de los organismos y, por tanto, ser transmitidos a lo largo de la cadena alimentaria. El peligro de estos CEs es que pueden generar daños a concentraciones bajas mediante exposiciones crónicas y, en muchos casos, dichos efectos no se detectan hasta estadios finales de desarrollo. Por ello, pueden ser erróneamente catalogados como inocuos si solo se evalúan parámetros como la supervivencia o la reproducción. En el contexto actual estos compuestos no se encuentran de forma individual en el medio, sino que en la mayoría de casos se presentan en forma de mezclas, tanto de compuestos de la misma naturaleza como de diferente origen. Este escenario aumenta la complejidad de los análisis, así como los posibles efectos en el medio y en los organimos. Además de las propias mezclas, los factores físicos como el pH, la temperatura o la humedad pueden alterar el comportamiento, tanto de los organismos como de los propios xenobióticos.

Esta tesis se centra en evaluar los efectos moleculares de la exposición a diferentes xenobióticos, de forma individual o combinada, en larvas acuáticas de cuarto estadío del insecto *Chironomus riparius*. Se estudiaron varios compuestos de diferente naturaleza química y catalogados como CEs como representantes de los filtros ultravioleta (OD-PABA, OC, BP-3), los fármacos (ibuprofeno), los plastificantes (BPA) y los pesticidas (endosulfan). Además, se analizaron el perfil transcripcional y los biomarcadores bioquímicos en respuesta a la exposición a microplásticos, ya que es una clase de contaminante emergente con un impacto creciente en el medio ambiente. Para ello se ha utilizado la reacción en cadena de la polimerasa en tiempo real, diseñando un panel de genes específico para *C. riparius*. Este panel incluye genes implicados en cinco rutas esenciales para los invertebrados como son el sistema endocrino, la respuesta de detoxificación, la respuesta al estrés, el sistema immune y los mecanismos de reparación del ADN.

Los resultados obtenidos han confirmado la utilidad del panel diseñado para evaluar compuestos de diferente naturaleza química ya que todas las rutas se han alterado en mayor o menor medida por cada compuesto. Respecto a las mezclas empleadas, se ha observado una mayor toxicidad respecto a la exposición simple de los compuestos, siendo mayor incluso utilizando compuestos de diferente origen (Filtros ultravioleta + BPA). Además, se ha observado un mayor efecto en las exposiciones más largas a pesar del escaso efecto a tiempos cortos, lo que sugiere la necesidad de evaluar varios tiempos. El pesticida endosulfan mostró una fuerte disrupción de todas las rutas evaluadas, así como a nivel de actividad enzimática. Este resultado confirma su peligro y sus posibles efectos en la población a largo plazo, a pesar de las bajas concentraciones utilizadas. El ibuprofeno modificó la supervivencia en *C. riparius* en todas las concentraciones empleadas, detectándose una fuerte mortalidad en las concentraciones más bajas, lo que indica que *C. riparius* parece ser mucho más sensible a este compuesto que otros invertebrados. A nivel molecular, el ibuprofeno parece producir daño mediante estrés oxidativo alterando el sistema endocrino, la respuesta al estrés y el sistema immune, pero sin efecto en los mecanismos de detoxificación. Sin embargo, conviene tener presente que los efectos varían de acuerdo al tiempo de exposición empleado. Por último, los microplásticos de polietileno

alteraron tanto a la respuesta bioquímica como la respuesta celular. En general las partículas de 32-45 µm presentaron una mayor toxicidad. De forma similar al ibuprofeno, estas partículas parecen producir toxicidad en *C. riparius* mediante la liberación de especies reactivas de oxígeno y, por tanto, produciendo estrés oxidativo. El aumento de la actividad de la catalasa y la Glutation transferasa, así como la cantidad total de Glutation reducido, respaldan este mecanismo. A nivel transcripcional se produce un fuerte efecto en el sistema endocrino, el sistema inmune y en los genes relacionados con la reparación del ADN, por lo que es posible que se produzca cierta genotoxicidad. Sin embargo, debe dilucidarse si los efectos de estos microplásticos se deben a alteraciones mecánicas debidas a su forma y tamaño o por la liberación de alguno de los componentes que los forman.

La presente tesis ha tratado de abordar dos de los problemas medioambientales actuales más relevantes. Por un lado, utilizar una metodología que sirva para poder detectar daños en exposiciones cortas y a nivel molecular, pudiendo servir como señales de alarma y fuente de posibles futuros biomarcadores. Por otro lado, comprobar la efectividad del panel diseñado frente a compuestos de diversa naturaleza química, así como en situaciones de multi-estrés utilizando diferentes escenarios. El panel de genes ha demostrado la relevancia del análisis simultáneo de diferentes rutas, lo que complementa el interés creciente de la comunidad científica por expandir la base de la OECD que describe las denominadas rutas de resultado adversos (AOP). Esta tesis ha resaltado la necesidad de mejorar los test de toxicidad incluyendo las variables moleculares y celulares y diversificando las condiciones del test, como el multi-estrés. Además, mimetizando las condiciones ambientales y añadiendo complejidad al análisis se ha demostrado la generación de interaciones aleatorias y propiedades que no se observan en exposiciones simples.

INTRODUCTION

1. INTRODUCTION

Environmental pollution

Industrial development, together with the high population increase, has led to manufacturing many consumer products as well as chemical compounds, causing an ever-higher generation of waste. The treatment and handling of such waste have become an essential concern since, in many cases, their inefficiency leads to polluting the environmental compartments. Many of these compounds are xenobiotics capable of persistence for decades reaching to air, soil, or water and maintaining a continuous flow of pollutants between each other.

Water is one of the primary natural resources, an indispensable element for industrial, food, medical-sanitary processes, being vital for aquatic organisms and human beings. Therefore, we are currently facing a considerable challenge with the contaminating of our most important water sources, such as rivers, oceans, channels, lakes, and reservoirs. It is one of the significant concerns of our time because, without good quality water, it is impossible to guarantee the well-being of the environment, the human species, animals, and plants. Due to their long extension throughout the world, water resources have become one of the leading sinks of pollution. There are multiple sources of pollution, such as industrial or agricultural wastes, oil and petroleum leaks, sewage, and effluents from wastewater treatment plants (WWTP) or even the increased global temperature.

Derived from the high manufacture production and life consumption, new chemicals that had not previously been detected were discovered at low levels in surface waters. These chemicals are known as contaminants of emerging concern (CECs) being defined as synthetic or naturally occurring chemicals without regulatory status and whose effects on the environment and human health are unknown (Deblonde et al., 2011). The CECs are a broad category, encompassing metals, pesticides, plastics, microplastics, phthalates, polycyclic aromatic hydrocarbons, pharmaceuticals, personal care products (PCPs) and endocrine-disrupting compounds (EDCs) (EU Water Framework Directive, 2000). These emerging contaminants have become a pressing issue given that the risk they pose to human health and the environment is not yet fully understood. Thus, the environmental agencies such as the United States Environmental Protection Agency (US EPA) have needed to renew their protocols; for example, developing a White Paper Aquatic Life Criteria for Contaminants of Emerging Concern (June 2008). The aims of these new guidelines and recommendations were focused on being able to evaluate the potential impact of CECs on aquatic life, determine protective levels for aquatic organisms, and the water quality determination.

Most CECs possess similar chemical characteristics as persistent organic pollutants (POPs), acting as pseudo-persistent compounds in the environment and with a tendency to bioaccumulate in the food chain (Bu et al., 2013; Zhang et al., 2017), developing potential damages on organisms and human health (Liu & Wong, 2013; Tan et al., 2018) Presence of CECs was observed in effluents of WWTPs containing PCPs, phthalates, and bisphenols (Kermia et al., 2016; Rodil et al., 2012), even in sediments (Lee et al., 2020). In this way, from all these sources, they end up in water reservoirs as indicated (Barrios-Estrada et al., 2018). These compounds are frequently present in surface waters like rivers and lakes (Čelić et al., 2020; Guruge et al., 2019; Thomas et al., 2017) or drinking water. Some of the classical CECs are Ultraviolet filters (UVFs), pharmaceutical compounds, pesticides, or plastics; hence this work will be focused on their effects on the invertebrate *Chironomus riparius*.

One crucial concern related to CECs has derived from its chronic toxicity; it causes significant effects at very low levels of exposure. Additionally, they could not cause detectable alterations during the early

stages of development but they can produce damage in the adult. Therefore, traditional toxicity test endpoints as survival or reproduction may not be sufficiently comprehensive for criteria derivation for these chemicals, opening the challenge to evaluate the effects at the sub-organismal level.

On the other hand, one of the present central problems related to the environment is climate change. It is an additional factor that should be considered in the assessment of the toxicity since the existence of multiple stressors in the environment, and their possible interactive nature is still neglected in the current toxicity test. Interactions between chemicals and natural stressors, such as e.g., low and high temperatures, drought, or oxygen depletion in aquatic environments, have been shown in some studies. As a consequence, it is a must to close the gap by studying the multi-stress scenarios, including factors such as temperature or pH. However, the mechanisms involved remain mostly unknown, although the evident importance on invertebrates because of their metabolic dependence of environmental conditions. The multi-stress scenario with mixtures of chemicals and diverse environmental conditions are typical for the wild populations, but standard (eco)toxicological tests are usually performed under conditions that do not resemble them. The present thesis tries to develop new approaches to analyze the damage produced by the pollutants and the response that the organisms activate to manage them at the cellular and molecular levels. Analysis of chemical mixtures and a multi-stress scenario is, thus, a must to establish reliable information to make decisions about the upcoming challenges involving the emerging contaminants and climate change.

Metabolic pathways in invertebrates

Classical toxicology, as well as the main guidelines for the evaluation of toxic compounds such as OECD tests, has focused on physiological parameters like survival or reproduction, ignoring the effects at the sub-organismal level. Therefore, some compounds can be classified as harmless by not affect these parameters, but still be modifying short, mid, and long-term metabolic pathways.

In the last years, due to the fast advance in genomics, the application of omics technologies for toxicology has increased dramatically. They have improved the way to evaluate the toxic effects on organisms, although they have not been included in the OECD test (Cefic, 2017). All these methodologies are englobed in the term “ecotoxicogenomics” (Kim et al., 2015). One of the main advantages is to reduce the use or even dispense with the animals themselves as well as the ability to predict effects in the short and medium-term (Verheijen et al., 2020).

The molecular approach involves the named biomarkers, defined as objective and quantifiable characteristics of biological processes employed as early warning signals for organisms and ecosystems (Booth et al., 2003; Silva et al., 2018). Two sources for molecular biomarkers are the analysis of the transcriptional profile of genes and the measurement of enzyme activities.

One methodology to study the transcriptional activity is the Real-Time polymerase chain reaction (RT-PCR). It is a specific PCR with a fluorescent dye that allows monitoring of the progression during the DNA amplification. In this thesis, retrotranscription and the RT-PCR have been used as the primary methodology to quantify messenger RNA levels. The analysis involves a specific array designed with genes obtained from a *C. riparius* transcriptome from the database (Marinkovic et al., 2012). Five different pathways focused the attention to get a picture of the molecular events caused by toxicants. It is worthy to note that cellular metabolism is an interconnection of pathways, so changes in one of them can trigger changes in others.

The processes analyzed are related to the endocrine system, the detoxification mechanisms, the stress response, the immunity, and the DNA repairing.

Endocrine system

The endocrine system is one of the best-studied systems in insects due to its essential role in the correct development of the organism. The development and metamorphosis are controlled by a wide range of hormones and neuropeptides. Two hormones, the 20-hydroxyecdysone (20-E) and the juvenile hormone (JH), work coordinately to regulate the process (Liu et al., 2018). Besides, the Insuline-like peptides are involved as mediators in metamorphosis regulation (Sheng et al., 2011). The 20-E and JH origin and action are resumed in figure 1.

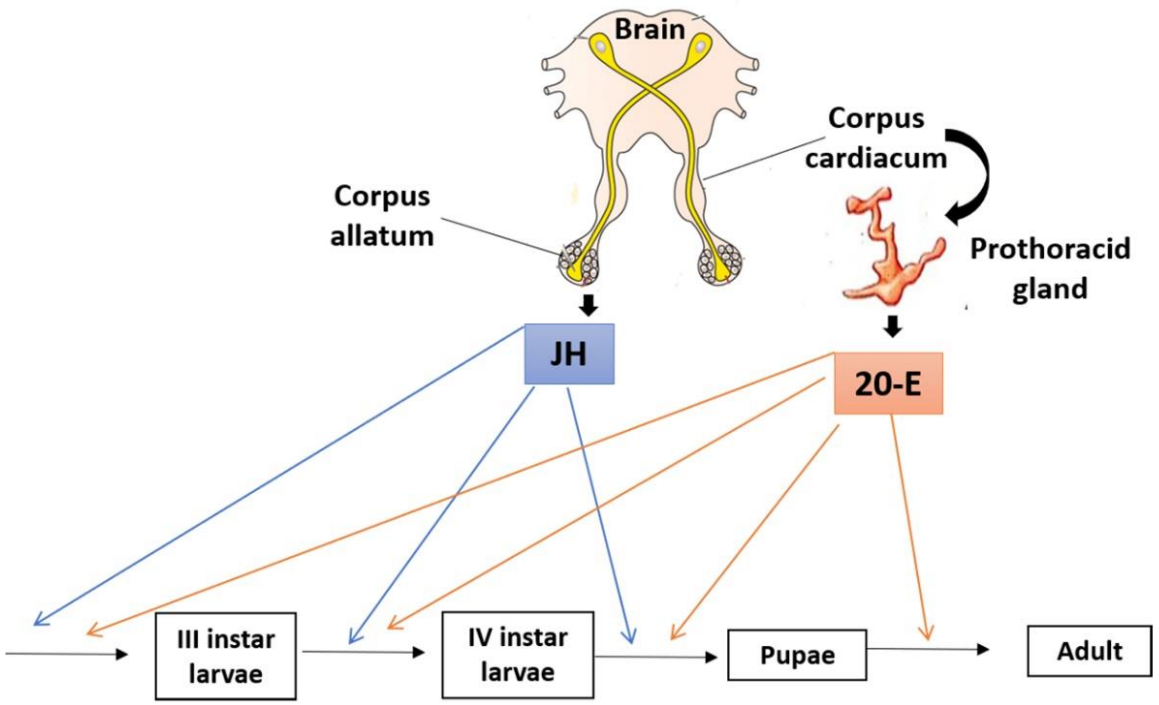

Figure 1. Ecdysone and JH synthesis and their action in insect metamorphosis/growth.

The process for the ecdysteroid hormone synthesis begins in the brain. Firstly, the *corpus cardiacum* synthesizes the prothoracicotropic hormone (PTTH), which stimulates the prothoracic gland (PG), producing the ecdysone as an inactive form of 20-E (Gilbert et al., 2002). It is released to the hemolymph, and the activation and transformation to functional 20-E happen in the target tissues like fat body or midgut. The 20-E synthesis requires the cholesterol molecule as an initial substrate. In some arthropods as insects, the absence of specific enzymes essential for cholesterol synthesis is solved using plant sterols or cholesterol from the diet (Niwa & Niwa, 2011). The first step generates 7 dehydrocholesterol (7dC) as a product through a conversion process. Secondly, a variety of reactions are developed to obtain 5-β cholesterol. These steps are not well defined; therefore, they are called "black box." From this product and through four consecutive hydroxylations, 20-E is synthesized. The 25, 22, 2, and 20 carbon are hydroxylated, and the reactions depend on Phantom (Phm), Disembodied (Dis), Shadow (Sad), and Shade (Shd) enzymes, respectively. These four enzymes belong to the Halloween group, and all of them are Cyp 450 enzymes discovered firstly in *Drosophila* (Gilbert, 2004) and, later, described in other insects (Rewitz et al., 2006) as critical enzymes in 20-E synthesis.

The hormone must join its receptor to fulfill its role in the cell. The receptor is a heterodimer of two proteins, the ecdysone receptor (EcR) and the ultraspiracle (Usp) (Hill et al., 2013). Once bound to the receptor, it promotes and regulates the molting process by activating the ecdysone response pathway (Spindler et al., 2009). It is a signaling cascade triggered by the 20-E - EcR/Usp junction, generally

divided into three sets of genes. Two of them, the early response genes (*E75*, *E74*, *E93*, or *Broad*) and the late response genes (*HR3*, *HR4*, *H38*, or *Kr-h1*), are transcription factors. The last one, the effector genes (*Dronc* or *vitellogenin*), groups the genes involved in the effective molting and metamorphosis processes (Yamanaka et al., 2013).

The synthesis and releasing pattern of ecdysone is done in pulses as a response to the environment conditions (Tennessen & Thummel, 2011). The entire process undergoes continuous regulation. On the one hand, positive stimulation through increased EcR and Usp transcription by 20-E. On the other hand, negative feedback is controlled by *Cyp18a1*, a cyp450 able to metabolize 20-E to acid 20-E, decreasing the hormone activity in the tissues (Guittard et al., 2011). Furthermore, 20-E is also involved in the regulation of other processes, including reproduction, behavior, and stress resistance (Schwedes & Carney, 2012).

The JH is a sesquiterpenoid hormone, secreted by the *corpora allatum* before each molt. The JH is produced from acetate, following the mevalonate pathway through oxidation reactions to produce farnesoic acid (FA) (Nouzova et al., 2019). This product is transformed by epoxidase (CYP15C1) or juvenile- O-methyltransferase (JHAMT), according to the insect group, to juvenile hormone (Qu et al., 2018). The enzyme JHAMT is limiting as previously checked; its decreased expression is related to precocious metamorphosis events (Minakuchi et al., 2008). The JH is released in the hemolymph, next its signaling pathway becomes in the cytoplasm by interaction with its intracellular receptor Methoprene-tolerant (*Met*) as a critical factor controlling its functions. The JH's primary function is to postpone the metamorphosis until larvae reach the appropriate stage and size (Smykal et al., 2014). JH/Met complex is transported to the nucleus by the heat shock protein 83 (Hsp83) to stimulate the gene effectors expression being the main the Krüppel homolog 1 (*Kr-h1*) acting as a regulator to the larvae stages transformation (Qu et al., 2018). At the end of the last instar larva, the *corpora allatum* starts to atrophy, decreasing the JH releasing, repressing the *Kr-h1* expression, and therefore stimulating the metamorphosis (Jindra & Bittova, 2020). Similarly to 20-E, JHs also influence behavior, reproduction, diapause, stress resistance, and aging (Flatt et al., 2005; Qu et al., 2018).

Both hormones act coordinated, showing an interdependent control by some genes such as Ecdysone-induced protein E93 (*E93*) and *Kr-h1*. The later possess the role of anti-metamorphic component, being expressed by the stimulation of JH/Met. *Kr-h1* represses the metamorphosis by inhibiting the transcription of E93, which belongs to the 20-E pathway (Kayukawa et al., 2014). *E93* is an early response gene in the ecdysone pathway, activating the synthesis of genes like *Dronc*, an effector gene. In holometabolous insects, *E93* stimulates the molting process acting on the Broad complex (BR-C) on the prepupal stage (Belles, 2020). Once it reaches the pupa stage, *Dronc* gene, among others, regulates the metamorphosis process to be transformed into an adult.

On the other hand, the membrane-associated progesterone receptor (MAPR) is an orphan receptor. It is a member of a family with a common heme/steroid binding domain, present in multiple Eukaryotes. This domain gives them various functions in lipid metabolism and cancer processes (Kimura et al., 2012). *MAPR* is capable of binding to steroid hormones, for example, in invertebrates (Fujii-Taira et al., 2009). However, its specific role in the endocrine system in insects has not been defined yet (Mifsud & Bateman, 2002) but seems to act in the 20-E pathway. Previous studies in *C. riparius* showed altered expression in embryos by UVFs (Ozáez et al., 2016) and no effects after vinclozolin (Vz) and UVFs in larvae (Ozáez et al. 2016c; Aquilino et al., 2016).

Finally, the Insulin-like peptides (ILPs) are essential hormones in invertebrates, present in all of them. Equally to JHs, they are secreted in *corpora allatum*, concretely in the median neurosecretory cells. The ILPs have different functions on the cells being the main to regulate sugar metabolism (Johnson et al., 2014). Moreover, its role in the molting and growth in insects has been demonstrated, increasing its importance in insect development (Okamoto et al., 2009). ILPs can interact to 20-E for regulating carbohydrates reserves and participate in its synthesis, besides, alter JH for the reproduction system (Andreenkova et al., 2016).

The contaminants can impact the hormonal system, either altering the hormone metabolism or affecting the signal response pathway. By studying the transcriptional activity of genes involved in those processes, it can be elucidated the putative mechanisms altered. Any array focused on the analysis of endocrine disruption should be include genes that participate in these processes, so ten genes were selected. The genes covering the response pathways are *EcR* and Death regulator Neddd2 like caspase (*Dronc*) for ecdysone, *Met* for Juvenile hormone, and *Kr-h1* and *E93* for both. The hormone metabolism genes are Disembodied (*Dis*) and cytochrome p450 18 alpha 1 (*Cyp18a1)* for ecdysone and *JHAMT* for JH. The Insulin receptor (*InR*) provides information about ILPs response. The last gene is *MAPR*, an orphan receptor that has been observed to be responsive to some pollutants. Figure 2 shows the functional process of both enzymes and the moment of activity of each gene evaluated.

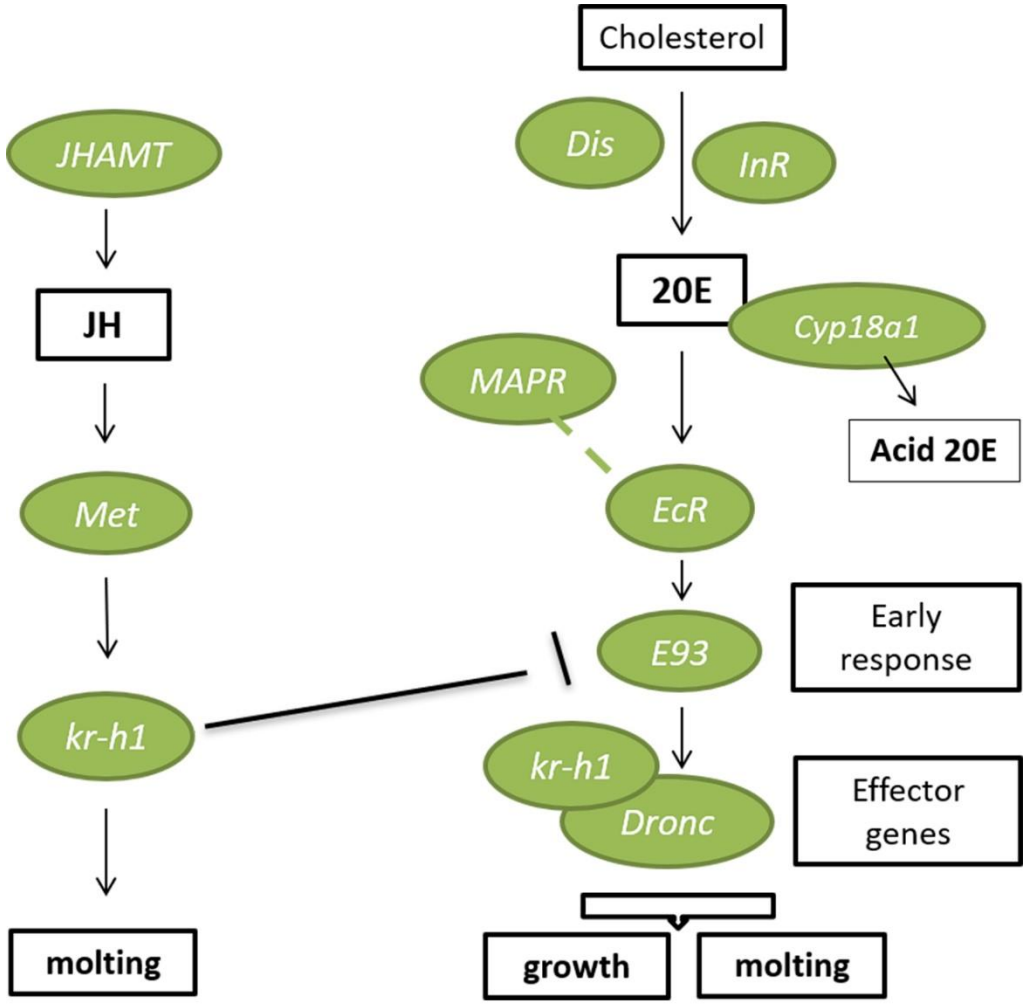

Figure 2. Endocrine system in invertebrates and the genes analyzed in the array.

Multiple xenobiotics demonstrated their capacity to alter this system in *Chironomus riparius*, demonstrating its importance as a target for chemical compounds. Plasticizers, triclosan, vinclozolin, or UVFs modify hormonal receptors expression on larvae or embryos (Aquilino et al., 2016; Martínez-Paz et al., 2017b; Ozáez et al., 2016 a,b; Park & Kwak, 2010).

Detoxification mechanisms

The detoxification mechanisms are the first barrier against harmful compounds, and traditionally they are divided into three phases.

Phase I involves chemical reactions that try to reduce the toxicity of the compound. The reactions can be diverse depending on the nature of the compound, and they are carried out by several types of enzymes such as hydrolases, reductases, peroxidases, or flavin-monooxygenases. However, the principal role is reserved for the cytochromes P450 oxidases (Snyder, 2000). CYP enzymes are heme-dependent oxidases, acting by molecular oxygen activation and introducing an oxygen atom into the substrate (Sharifian et al., 2019). CYP proteins constitute a widely distributed protein family present in all the organisms. They are classified in families according to their structure and then in subfamilies and members (Snyder, 2000). In insects, four clans have been described, including CYP2, CYP3, CYP4, and CYP12 (mitochondrial) (Feyereisen, 2006). They possess higher diversity in comparison to vertebrates, more in CYP3 and CYP4 family (Sezutsu et al., 2013).

CYPs insects participate in the metabolism of endogenous compounds, like ecdysone and juvenile hormone (Feyereisen, 2006), and exogenous xenobiotics degradation (Martínez-Guitarte, 2018; Martínez-Paz et al., 2012; Wu et al., 2019). Besides, it is known the implication of CYP genes to insecticide resistance (Liu et al., 2015; Seong et al.,2019).

Phase II is focused on increasing the solubility of the compound by conjugation with different molecules, for example, to electrophilic compounds as glutathione (GSH) by thioether linkages (Hayes et al., 2005) via glutathione-S-transferase (GST). Other enzymes like sulfur, methyl or

acetyltransferases, and UDP-glucuronosyltransferases also participate in this phase. GSTs are a crucial group of enzymes for detoxifying endogenous and exogenous compounds (Board & Menon, 2013). Besides, GSTs belong to the antioxidant enzyme families acting against reactive oxygen species (ROS) and oxidative stress (Enayati et al., 2005). Similar to CYPs, GST is a superfamily of proteins classified in cytosolic, microsomal, or mitochondrial based on their distribution. In insect, the main class is the cytosolic, subdivided into 6 subclasses: delta, epsilon, omega, sigma, theta, and zeta (Shou-min, 2012). The major classes in insects are epsilon and delta, representing more than half of the GSTs (Ortelli et al., 2003). Eleven GST members belonging to cytosolic classes were characterized in *C. tentans* (Li et al., 2009). Certain GSTs can conjugate specific types of xenobiotics. For example, delta and epsilon classes have been related to resistance and detoxification against pesticides (Li et al., 2007). Nevertheless, sigma is involved in protection against oxidative stress (Shou-min, 2012).

Phase III is the last step in the detoxification mechanisms and is removing the conjugated compound out of the cell. The responsible for it is a family of proteins known as ATP-binding cassette transporters (ABC transporters) that enhance the transport capacity of different compounds across the membranes for their final excretion (Xu et al., 2005). ABC transporters are present in all living organisms (Jin et al., 2019). They are classified into eight subfamilies named A to H (Dermauw & Van Leeuwen, 2014). Each transporter is formed by two nucleotides binding domains (NBDs) and two integral transmembrane domains (TMDs) (Bretschneider et al., 2016). In arthropods, a considerable number of them have been discovered, higher than in humans (Dean et al., 2001). They possess a double role, participating in

transport, and detoxification response, similar to CYPs and GSTs, several of them have been related to insecticide resistance (Pignatelli et al., 2018; Rösner & Merzendorfer, 2020).

The array designed for the analysis covers the three phases of detoxification by including four genes coding CYPs, four GSTs, and two ABC transporters as are shown in the figure 3. The sequences code for Cyp 4 delta 2 (*Cyp4d2*), Cyp 6 beta 7 (*Cyp6b7*), Cyp 9 beta 2 (*Cyp9b2*), Cyp 12 alfa 2 (*Cyp12a2*), GST epsilon 1 (*GSTe1*), GST delta 3 (*GSTd3*), GST omega 1 (*GSTo1*), GST theta 1 (*GSTt1*), ABC C 1 known also as multidrug resistance protein 1, (*MRP-1*) and ABC B6 (*ABCB6*).

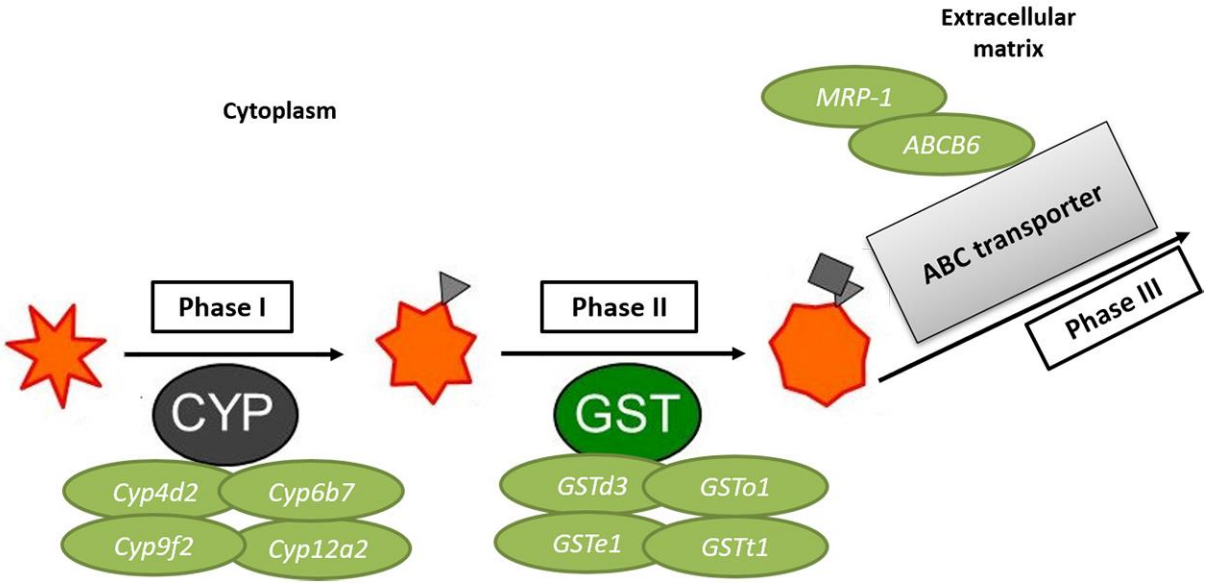

Figure 3. The detoxification response mechanism and the genes employed in the array.

Stress response

The changing environment and external damages can disrupt the homeostasis of organisms and initiate a cellular stress response aimed to decrease the possible damages. The process is mainly characterized by heat shock proteins (HSPs) synthesis modulating stress tolerance and adaption, which is essential in insects (King & MacRae, 2015; Zhao & Jones, 2012). They are one of the most conserved protein groups, initially identified through their induction by heat; however, many other stressors stimulate their response, being defined as biomarkers for stressful conditions (Xia et al., 2017; Yang et al., 2019). HSPs are grouped into five main families according to their molecular weights, homology, and functions: Hsp90, Hsp70, Hsp60, Hsp40, and small heat shock protein (sHSP) (Gupta et al., 2010). In addition to stress response, they are essential chaperones for transport, folding, and refolding of proteins (Clerico et al., 2015). Moreover, HSPs participate in signal transduction or the immune system, thus have been used as a therapeutic target (Kryeziu et al., 2019). Furthermore, the HSPs overexpression provoked cryoprotection (Bidmon-Fliegenschnee et al., 2015), proving the importance of these proteins in the regular life of an organism. Figure 4 summarizes the five groups of HPSs and their role in the cell.

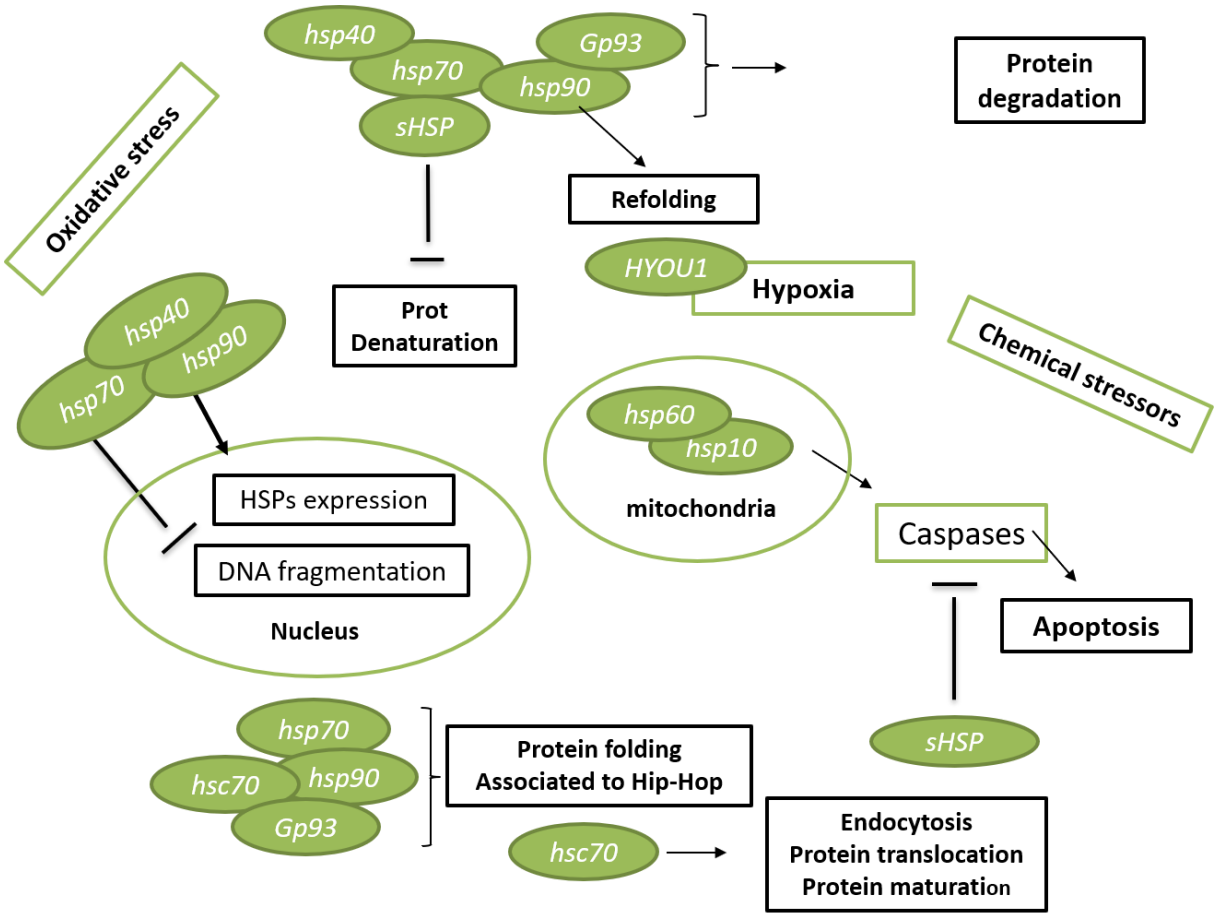

Figure 4. The stress response in invertebrates and the genes analyzed in the array.

Hsp90s are cytosolic HSPs abundant in eukaryotes, whose structure is formed by three domains M, N, and C terminal (Harris et al., 2004). Hsp90 members are indispensable for diverse functions (Radli & Rüdiger, 2018). Hsp90s control stability and correct folding of proteins by interactions to other chaperones as hsp70 and hsp40, besides, regulate immune response, cell cycle, nuclear receptors maturation or thermal tolerance (Eckl & Richter, 2013; Radli & Rüdiger, 2018). Hsp90 has been determined as a marker in diapause and crucial in the correct development in insects (Tachibana et al., 2005). Also, it is capable of stimulating detoxification genes as Cyps or GSTs in response to stress environments (Guo et al., 2017). Recently *hsp90* has been employed as a target for clinical treatment design. Different inhibitors have been used successfully in the treatment of tumors (Kryeziu et al., 2019).

hsp90 induces its expression by temperature changes and other stressors as xenobiotics or bacteria altering its transcription pattern (Liu et al., 2016; Zhang et al., 2009). In invertebrates, *hsp90* is a useful biomarker for aquatic toxicology evaluation in crustacea (Chiasson & Taylor, 2017; Park & Kwak, 2019) or mollusks (Aquilino et al., 2019; Morales et al., 2018). Finally, in *C. riparius*, *hsp90* has been characterized by Park & Kwak, 2018, opening the opportunity to analyze this essential protein in this base organism in the trophic chain.

Another member of this family is the Gp93; it was identified in *Drosophila* as an ortholog of mammalian GPR94-Gp96 (Maynard et al., 2010). Gp93 is a chaperone involved in the innate immune system in

Drosophila through its interaction with Toll-like receptors (TRLs) in the Toll pathway (Morales et al., 2009). Moreover, it is necessary for the correct development in insects due to its action on midgut regulating the nutrient absorption, hence the growth (Maynard et al., 2010). Finally, it participates in the morphogenesis and the survival of invertebrates (Rutherford et al., 2007). It has hardly been used for invertebrate's toxicity evaluation, with only two studies in *C. riparius*. They showed different results with upregulation by Vz and no affected by metals (Aquilino et al., 2016; Martín-Folgar & Martínez-Guitarte, 2017).

The HSP70 family is the most known, so three members of them have been selected to be analyzed (constitutive hsc70, hsp70, and Hypoxia upregulated protein (HYOU1)). This family represents the central axis of the stress proteins, being essential for the role of hsp70. Its primary function is focused on proteins avoiding their misfolding and aggregation and sending to degradation when it is required, both in vertebrates and invertebrates (King & MacRae, 2015; Zhang & Zhang 2012). The protein homeostasis is a precise process that requires many resources and coordinated action with other chaperones such as hsp40 or hsp90 generating complexes (Alderson et al., 2016; Doyle et al., 2019). The *hsp70 gene* has demonstrated its role in stress response against heat and multiple xenobiotic compounds (Alqarni et al., 2019; Liu et al., 2015). Moreover, *hsp70* induction has been related to immune response, detoxification, and thermotolerance (Liu et al., 2015; Zhou et al., 2010). Therefore, *hsp70* has been proposed as an essential biomarker for oxidative stress and thermal tolerance (El Golli-Bennour & Bacha, 2011; Hassan et al., 2019).

According to that, it is one of the most widely used stress biomarkers in aquatic toxicology for heat and xenobiotics exposure in bivalves (Nie et al., 2017; Xia et al., 2017), crustacea (Xu et al., 2019), fish (Mitra et al., 2018) or snails (Morales et al., 2018). In *Chironomus riparius*, *hsp70* has demonstrated be a useful tool for aquatic pollution, being altered by many different conditions as heat and pollutants like plasticizers, metals, antibiotics or UVFs (Aquilino et al., 2016; Arambourou et al., 2020; Herrero et al., 2015a; Ozáez et al., 2016a; Park & Kwak, 2018).

HYOU1 codes for a chaperone with an hsp70 domain, working in oxygen deprivation events; therefore, its expression is stimulated by ischemia and hypoxia processes being a useful indicator of damage and related to cancer diseases (Arnouk et al., 2010; Stojadinovic et al., 2007). So HYOU1 has been validated as a protective member facilitating the adaptation to oxygen depletion environments hence avoiding the start of apoptosis and senescence events in mammals (Krętowski et al., 2013; Montesi et al., 2016). Hypoxia is defined as a decrease in available oxygen. In vertebrates, the O_2 limitation has been related to defects in embryonic development, weight, brain, and cardiovascular development showing the crucial need for oxygen (Sharma et al., 2006; Sugishita et al., 2004).

During the last years, primarily due to the anthropogenic contribution to the severe effect of climate change, the oxygen availability in oceans decreased (Breitburg et al., 2018). In aquatic environments, the O_2 depletion could be more limiting due to the energy is mainly obtained by aerobic processes, then the metabolic rate is compromised (Nelson, 2016). Therefore, some essential functions as growth or reproduction and even survival can be affected (Thomas et al., 2019). Only a few studies in fishes have studied *HYOU1* expression after xenobiotics exposure (De Felice, 2012; Mitra et al., 2018). However, so far this gene has not been studied in aquatic invertebrates, despite the increasingly common hypoxia events in marine environments. So *HYOU1* can be applied as a valid gene to know the capacity of xenobiotics in altering oxygen levels in the cell.

hsc70 gene is constitutively expressed in the cells in normal conditions. However, some studies have observed altered expression by pesticides (Xing et al., 2013) and manifested tissue-specific expression in response to heat shock in oysters (Jackson et al., 2011). Similar to hsp70, it has typical functions for

protein homeostasis, transport, or degradation (Kose et al., 2005). Despite many similarities to hsp70, hsc70 has specific interactions and functions. For example, recently has been discovered its role in immune response through the Toll pathway (Lv et al., 2019). Moreover, hsc70 has proven therapeutic potential for cancer, cardiovascular or neurological disorders (Nirdé et al., 2010).

In *Chironomids, hsc70* has been evaluated against multiple chemical compounds showing different expression patterns. In *C. riparius*, upregulation by UV filters and pesticides has been described (Lencioni et al., 2016; Martín-Folgar et al., 2018). Additionally, *hsc70* has been observed as a marker for the correct development by *EcR* analysis (Planelló et al., 2015). In other Chironomids, the expression was variable according to the stressor condition or xenobiotics (Bernabòl et al., 2011; Lee et al., 2006).

The next family, Hsp40, is divided into three groups defined as type I, II, and III, according to the domain-containing. Similarly to the hsp70 family, they are well conserved. Their main functions are focused on protein folding, refolding, and regulation, as well as participate in gene expression and translation processes (Qiu et al., 2006). One important group is the type III that contains the J domain, commonly named by it as DNAJs. They are involved in apoptosis, tumor development, and cancer, and these hsp40 have been used in the study of antibiotic resistance (Wu et al., 2017). The J domain is crucial in the hsp40/hsp70 function, facilitating the interaction with hsp70 and controlling its ATPase function. The domain is also relevant for the interaction of hsp90 to form the hsp40/hsp70/hsp90 macro complex for protein regulation (Li et al., 2009). On the other hand, the hsp40 family acts in the immune response developing antibacterial and antiviral functions (Knox et al., 2011; Li et al., 2011). According to their stress response are usually overexpressed after temperature alterations or xenobiotic exposure (Alqarni et al., 2019; Wang & Bag, 2008). Besides, they can minimize the oxidative damage mainly through hsp70 ubiquitination. All the response is focused in maintain the protein homeostasis hence the survival of the organisms (Kim et al., 2008).

Recent studies in aquatic invertebrates have demonstrated its role in heat stress response in mollusk (Wang et al., 2019; Xu et al., 2019). In *C. riparius*, *hsp40* has shown a compound-specific pattern with up, down-regulation and even no effects by different xenobiotics like UV filters or plasticizers (Herrero et al., 2018; Herrero et al., 2015; Martín-Folgar et al., 2018)

The Hsp60 binds to co-chaperone hsp10, and both belong to mitochondrial molecular chaperones working coordinately to form hsp60/hsp10 complex (Cappello et al., 2008; Höhfeld & Hartl, 1994). These proteins present high similarity between groups like humans, insects, or crustacea and possess a specific and unique structure that determine their action mode (Stevens et al., 2019). Hsp60/hsp10 develops different actions in the cells, mainly acts in the native proteins folding and stimulates the protein damaged degradation by the proteasome, being essential for mitochondria functions (Hartl et al., 2011; Maisonneuve et al., 2008). The complex develops a particular mechanism, encapsulates the unfolded polypeptide inside, and the folding is done along time. All the process is ATP dependant (Horwich, 2007). Furthermore, the complex hsp60/hsp10 also is involved in other processes, like the immunity response in vertebrates and invertebrates and heat shock response (Gao et al., 2019; Zhou et al., 2010). Besides, it has been employed to avoid antibiotics resistance and used as a target in cancer treatment (Corrao et al., 2010).

In aquatic invertebrates, xenobiotics and other stressors were capable of altering the *hsp60* and *hsp10* expression (Xia et al., 2017; Xu et al., 2014). The effects were related to the immune system in crabs (Yang et al., 2013) or as an adaptation to adverse conditions such as temperature or salinity (Xu et al., 2014). In insects, concretely *C. riparius*, previous studies showed alteration in *hsp10* after plasticizer

and pesticide exposition (Herrero et al., 2015a; Lencioni et al., 2016). Only one study has been evaluated the effects on both *hsp10* and *hsp60* with no effects by UV filters (Martín-Folgar et al., 2018).

The most extensive and heterogeneous family of HSPs, in structure and functions, is the sHSPs. Thus, this family has not yet been fully characterized (Morrow & Tanguay, 2012). The small HSP family contains proteins with molecular weights between 15 and 30 kDa (Gupta et al., 2010). Small HSPs are present in the majority of Eukaryotes and Prokaryotes organisms (Singh et al., 2017); all members possess a common α-crystallin domain. However, there is still significant diversity within the family (Liu et al., 2014). In invertebrates, small HSPs have considerable variability in the number of these proteins present within each organism (Dou et al., 2017). Because of the differences in the terminology used, it is difficult to compare results between species from the same phylum.

sHSPs play a role in disease and infection processes (Bolhassani & Agi, 2019; Zhu & Reiser, 2018) they also regulate critical biological processes, including growth, apoptosis, and diapause (Aruda et al., 2011; Bakthisaran et al., 2015; Kamradt et al., 2005). sHSPs are involved in the response against bacterial infections, heavy metals, UV radiation, or chemical exposure stressors (Aquilino et al., 2016; Martín-Folgar & Martínez-Guitarte, 2017; Simoncelli et al., 2019). As stated, the diversity of sHSPs challenges to establish the homology between the different proteins of each species. In *C. riparius*, seven members of sHSPs were characterized (*hsp17, hsp21, hsp22, hsp23, hsp24, hsp27,* and *hsp34*) (Martín-Folgar et al., 2015; Martínez-Paz et al., 2014). Previous studies in this organism demonstrated its variable expression pattern. For example, all of them react differently to heat shock and cadmium metal exposure (Martín-Folgar et al., 2015; Martín-Folgar & Martínez-Guitarte, 2017; Martínez-Paz et al., 2014). Some members, as *hsp24*, were upregulated by heat shock, Vz, and cadmium (Aquilino et al., 2016; Martín-Folgar et al., 2018, 2015). A similar pattern was shown in *hsp27* after BPA and cadmium exposure (Martínez-Paz et al., 2014). Therefore, they seem to respond fast, being valuable biomarkers for xenobiotic pollution.

Immune system

The immune system plays an essential role in preventing microbial infection and protecting the body. Historically, the immunity on invertebrates had been considered much less complicated than that observed on vertebrates. However, research has found multiple immunity mechanisms such as melanization, phagocytosis, adhesion cells, or pro-inflammatory cytokines (González-Santoyo & Córdoba-Aguilar, 2012), showing a complex picture.

Insects possess innate immunity, divided into two types of response: the humoral and the cellular, although both work complementary (figure 5). The cellular response is based on phagocytosis and encapsulation processes mediated by hemocytes. Once hemocyte, acting as a recognition molecule, detects the pathogen, activates the cellular response (Strand, 2008). The hemocytes are classified according to their morphology or function (Gupta, 1985). One example is the crystal cells in *D. melanogaster*. The hemocytes belong to the cellular response but also stimulate and express components related to the humoral response, such as Phenoloxidase (PO) (Strand, 2008).

The humoral response represents the primary immunity response in insects. It consists of the synthesis and the releasing of soluble effector molecules such as antimicrobial peptides (AMPs) or enzymatic cascades components like PO. The Prophenoloxidase (proPO) cascade is the primary mechanism of response. It is based on multiple enzymatic and non-enzymatic reactions mainly developed by serine proteinases, which end with the synthesis of melanin to carry out nodules production to trap invaders (Marmaras & Lampropoulou, 2009). proPO, the inactive PO form, is synthesized and stored in the hemocytes (cellular response). Upon activation, PO is liberated to the hemolymph stimulating the

cascade. The entire process is heavily regulated to prevent the excessive stimulation of the cascade from damaging the system (Wang et al., 2017). The main regulators are proteinases as serpentine. Twenty-nine were characterized in *Drosophila* acting as inhibitors in the proPO pathway for the adequate melanization process in insects (Gooptu & Lomas, 2009; Tang et al., 2008).

Another humoral process is mediated by antimicrobial peptides (AMPs), a family of cationic peptides with alpha or beta conformation present in all kingdoms. In Diptera, five types have been identified as cecropins, defensins, attacins, and diptericins (Liu et al., 2017, 2018). AMPs are synthesized in the fat body in holometabolous insects as Chironomids, and then they are released to the hemolymph to be transported throughout the body (Wang & Lai, 2010). AMPs can attack bacteria, viruses, or fungi. Its action mode consists of high production and releases to invade the pathogen and thus destroy it, helping to decrease stress on the immune system (Soares et al., 2014). Defensin is an AMP composed of six cysteine residues that act against bacteria or fungi infections, but many of them identified in different insects are related to bacteria (Islam et al., 2016).

Immunity has two representatives in the array. The Prophenoloxidase (*Proph*) and the defensin (*Def*) genes cover the humoral response and provide information that can be combined with the enzyme activity assay of PO to evaluate the impact on the immune system. The immune system in insects and the genes employed are shown in figure 5.

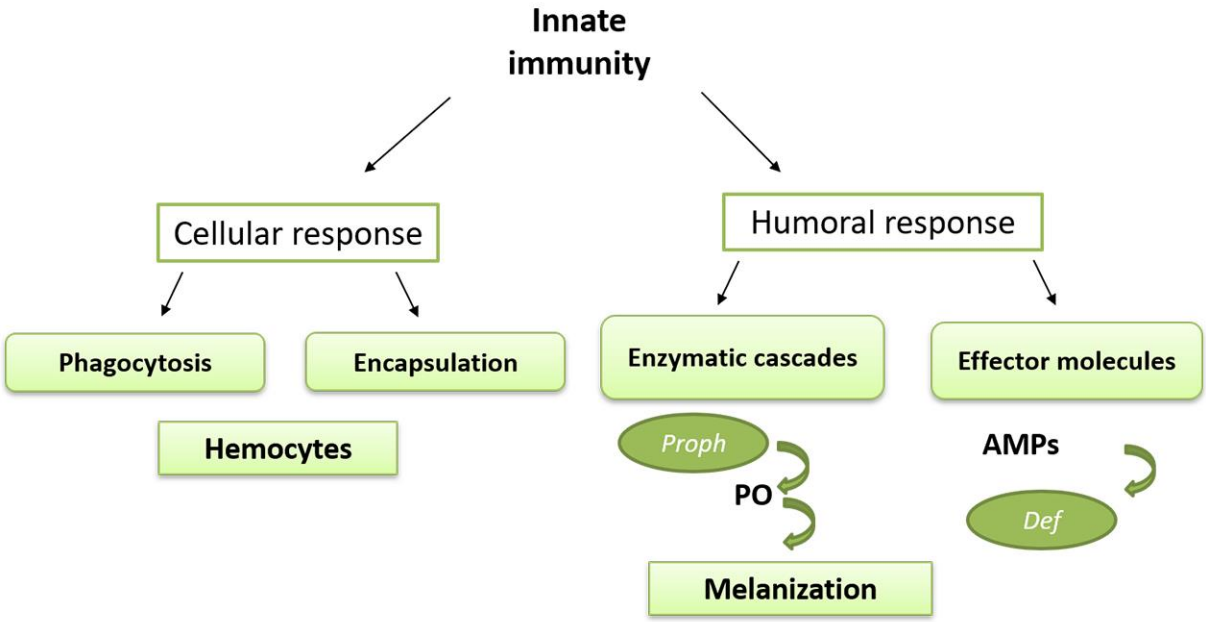

Figure 5. Immune system response in insects and the genes employed for the array.

DNA repair

DNA, as a transmitter of genetic information, possesses a vital function in organisms and, therefore, must maintain its integrity and stability. However, DNA is continuously threatened by endogenous and exogenous alterations (Caron et al., 2019). Some typical endogenous factors are the ROS species generated by stress conditions or the accumulation of errors during replication. On the other hand, physical and chemicals agents, among others, are classified as exogenous factors (Jackson and Bartek, 2009).

There is a wide variety of DNA damages such as simple base modifications, base mismatches, protein-DNA crosslinks, intrastrand crosslink (ICL), and the most known DNA single-strand break (SSB) and double-strand break (DSB) (Roos et al., 2016). Each type of damage can generate a specific response and then a different final effect on the cell. In normal conditions, the cells can respond activating their DNA repair mechanism; however, when the damage is increased or if the cell was previously injured, cell death or senescence program is activated (O'Connor, 2015).

Usually, the DNA damage response (DDR) involves the transcriptional regulation of genes activated in response to unfavorable conditions. Abnormal DDR function can be related to diseases like cancer (Jin & Oh, 2019). Mostly, the cells employ a variety of mechanisms once the DNA has been damaged, such as cellular repair mediated by polymerases, translesion synthesis (TLS), or replication lock through repair and recombination. All of them lead to cell survival. Nonetheless, if the forks fall-down, blocking the replication, or the transcription is locked, the cell death processes are activated (Roos et al., 2016).

Some of the most studied damages are SSB and DSB, partly due to the strong effect provoked on the cell if they persist. The SSB reparation is developed by DDR sensors liberation like checkpoint kinase1 (Chk1) or replication protein (RPA) acting as substrates for lesion repair (Burgess & Misteli 2015; Lord & Ashworth, 2017). When it fails is transformed into DSB causing various harmful defects (Abbotts & Wilson, 2017). To solve DSB damages, different mechanisms can be activated, but the most used and effective are the non-homologous end joining (NHEJ) and the alternative end-joining (HR) (Ceccaldi et al., 2016; Samadder et al., 2016).

Many xenobiotics have been defined as genotoxic compounds being essential to recognize the level of damage on the genetic material. Hence, in this work some DDR mechanisms (figure 6) are analyzed through the study of the genes coding for Ataxia telangiectasia mutated (*ATM*), Poly(ADP-Ribose) Polymerase (*PARP*), Nemo Like Kinase (*NLK*), X-ray repair cross-complementing protein 1 (*XRCC1*) and the Death executioner caspase (*Decay*) as components of DDR system.

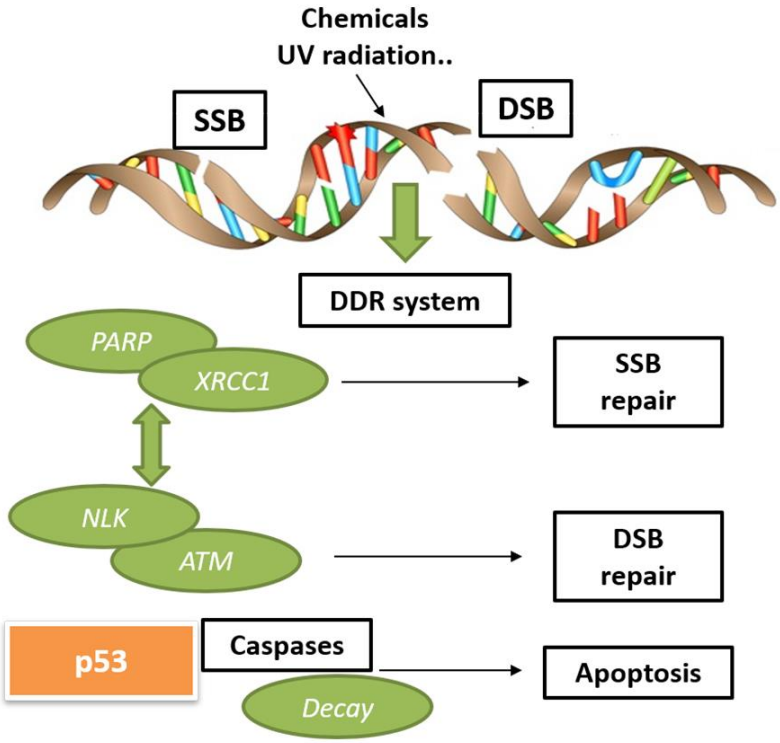

Figure 6. DNA repair mechanism and the genes evaluated in the array.

Poly (ADP-Ribose) Polymerase (PARP) encodes a protein involved in both SSB and DSB repair mechanisms (Chaudhuri & Nussenzweig, 2017). PARP binds to the damaged strand generating poly-ADP-ribose (PAR), which promotes chromatin relaxation as well as the accumulation of X-ray repair cross-complementing protein 1 (XRCC1) and other proteins at the chromatin site (Gao et al., 2017). PARP is also related to transcription repression in response to DSB (Caron et al., 2019) and repair during DNA replication (Hanzlikova et al., 2018).

ATM belongs to the phosphatidylinositol 3-kinase family and is involved in DSB repair. Also, it participates in the cell cycle, metabolic regulation, migration, and chromatin remodeling (Stracker et al., 2013). It is also expected that DSB activates the expression of other genes related to the repair process (Burgess & Misteli, 2015). It was demonstrated that ATM and NLK work together in concert to activate nuclear factor enhancing the kappa light chains of activated B cells (NF-kB), one protein complex in the cytoplasm, in response to DNA damage, acting as genotoxic stress alarm (McCool & Miyamoto, 2012; Wu et al., 2018). It is because NLK is a post-transcriptional regulator of the NF-kB transcription factor.

Finally, Decay is an effector caspase that is expected to operate where the cell fails to repair the damage stimulating the apoptosis process. Decay has a vital role during the translation being capable of eliminating defective transcripts (Guan et al., 2006). It is a crucial issue in telomeres avoiding disease development (Isken & Maquat, 2008). Its role in the DDR system was discovered by the modulation of the HR process, Decay controls RAD55 protein, as key in the HR reparation (Janke et al., 2016).

Biochemical biomarkers

Enzymes activity: GST, PO, and AChE

The combination of mRNA levels studies and biochemical biomarkers as enzymatic activity provides a more complete assessment of chemical stressors. The use of biomarkers has proven to be very useful in defining the quality or condition of aquatic environments (Dalzochio et al., 2016). In this thesis, three critical enzymes related to the essential mechanisms of the detoxification response, the immune system, and the nervous system were selected. Enzyme activity is a useful tool for xenobiotic exposure evaluation, from the past to nowadays, due to their fast response and their capacity to detect effects in early exposure. The enzymes selected were Glutathione-S-transferase (GST), Phenoloxidase (PO), and Achetilcolinesterase (AchE).

GSTs, as previously mentioned, belong to the detoxification response at Phase II level. One advantage of using the enzymatic activity is that we evaluate all the GST classes as a whole at the same time, offering a more global vision than if we evaluated a single class gene, for example, delta or epsilon alone. GST activity is an easy and fast response method for the possible activation or damages on detoxification response widely used in aquatic organisms (Cikcikoglu & Yaman 2018). GST enzymes are too related to oxidative stress response and insecticide resistance, increasing their utility as biomarkers (Hellou et al., 2012; Pavlidi et al., 2018). Consequently, multiple studies on invertebrates have used GST as a target from diverse xenobiotics in mollusk (Vranković et al., 2020), earthworms (LaCourse et al., 2009), or crustacea (Awali et al., 2019; Masteling et al., 2016). Previous studies in *Chironomus riparius* demonstrated the high value of GST activity as a biomarker in this organism. GST was modulated by insecticides (Campos et al., 2016; Monteiro et al., 2019a; Saraiva et al., 2017), UVFs (Campos et al., 2017b), or drugs (Xie et al., 2019).

The PO is an oxygen oxidoreductase (EC1.14.18.1) with a molecular mass of around 60–70 kDa. It intervenes in melanogenesis to repair tissues in defense against pathogens like bacteria or fungi, and

encapsulate multicellular pathogens (Cerenius et al., 2008). Notably, toxic compounds are released during the cascade, capable of killing the bacteria (González-Santoyo & Córdoba-Aguilar, 2012). The PO has a double role in the insects by activating the melanogenesis and acting against the pathogens (Cerenius & Söderhäll, 2012). Therefore, the employment of PO as a biomarker represents a useful tool for assessment of immune response against chemical and other stressors evaluation. PO activity has been used to evaluate the effects of pesticides and its relation to resistance in insects like *Culex* (Cornet et al., 2013) and *C. riparius* (Bordalo et al., 2020) even in crabs (Hong et al., 2019). Besides PO alterations has been related to diseases in bivalves (Luna-Acosta et al., 2017) and temperature and other compounds (BPA or TiO2 nanoparticles) can modulate the PO activity in crustacea or Diptera (Gnatyshyna et al., 2019; Mastore et al., 2019).

The acetylcholinesterase (AChE) is a type B esterase enzyme, involved in the acetylcholine hydrolysis. The process facilities the neurotransmission at the central nervous system level mediated by motor neurons (Edward & Martyn, 2019). It is a relevant target for multiple pesticides and insecticides but mainly for organophosphorus compounds like chlorpyrifos (Li et al., 2010). Its altered activity is associated with damage to the nervous system (Fukuto, 1990). In insects, it is coded by a unique gene with two subunits, as was detected *in Drosophila melanogaster* (Fournier et al., 1988), which facilitates AchE evaluation. Many pesticides, including insecticides, act on target as well as non-target organisms by inhibiting AchE, generally by phosphorylation (Ghorab, 2015). It decreases acetylcholine, and as a result, inefficient nerve transmission occurs due to uncontrolled stimulation of the nervous system (Pham et al., 2017).

A critical issue in the AchE biomarker's useful application is related to the effects of multiple xenobiotics, mostly pesticides, on non-target organisms as aquatic fauna. AchE was altered in fishes (Caliani et al., 2019; Ma et al., 2017), crustacea (Pham et al., 2017), or insects (Somparn et al., 2017). Due to the essential importance of the nervous system for the neurotransmission defects in it can lead to movement failure or neurological disorders such as Parkinson's (García-Ayllón et al., 2011).

Moreover a battery of biochemical biomarkers was developed for an specific study on polyethylene MPs developed in the Aveiro University. Besides the the GST and AChE activity, the catalase (CAT) was evaluated to determine the possible oxidative damage complemented to the lipid peroxidation (LPO) analysis. For detoxification response the GST activity was joined to the total gluthatione reductase (TGSH). Finally, the energy reserves through carbohidrates, lipid and protein content and the energy consumption employing the electron transport system (ETS) were analyzed. This battery of biomarkers has proven their effectiveness for *C. riparius* toxicity evaluation exposed to diverse xenobiotics (Campos et al., 2016, 2017; Monteiro et al., 2016 a, b, c).

Invertebrates for toxicology evaluation: Chironomids

In the last decades, there has been a growing tendency for the development of alternatives in toxicology testing, among them, the use of invertebrates for complement and even replace the vertebrates (Lagadic & Caquet, 1998). Invertebrates have a significant presence in the environment with a wide range of habitats as well as the occupation of key positions in the food webs, regulating the trophic transfer of nutrients, being a food source for vertebrates. The use of invertebrates in toxicology offers many advantages, short life cycles, their maintenance and handling culture in the laboratory is cheaper, in addition to the absence of bioethical implications, which tend to hinder and make the study in vertebrates more expensive. Finally, they have great utility in relating the effects that occur at the individual level and the consequences for higher levels of organization (Forbes, 1996).

The Organization for Economic Co-operation and Development (OECD) and others as US EPA establish and regulate the testing of chemicals by the design of the guidelines to assess the potential effects of multiple xenobiotics on the organisms and ecosystems. The tests can include acute or chronic exposure, life cycle assessment, or reproductive effects. Due to the continuous development of new compounds, these guides are regularly renewed, leading to an increase of guidelines for invertebrate species. Some examples of invertebrate's guidelines are for the crustacea *Daphnia magna* with two OECD test 211 and 202 (OECD 2012,2004), annelida like *Lumbriculus* test 225 (OECD 2007) and the eathworms *Eisenia* test 222 (OECD 2016).

Chironomidae is an insect family belonging to the Diptera order, also known as non-biting midges. They are the most widely distributed and frequently most abundant insects present in lotic and lentic freshwater systems. Moreover, plays roles in the ecosystem equilibrium through recycling organic matter (Péry & Garric, 2006). Because they can interact with human and aquatic life in many ways, chironomids have been used from the past until nowadays as a food source for the fish, both naturally and for fish farming, being of vital importance in countries like China (Sahandi, 2011). Due to their ubiquity, they have been employed in multiple monitoring programs for quality water determination in lotic and freshwater ecosystems throughout the world (Kim et al., 2006; Stoian et al., 2009). In Finland, one study established the direct relationship between water quality variables and distribution, diversity, and abundance of chironomids from fifty-one different lakes showing their importance as a bioindicator for water quality evaluation (Luoto, 2011).

Chironomids are holometabolous insects whose life cycle comprises a complete metamorphosis regulated by complex hormone signals, with four stages (figure 7) (egg mass, larva, pupa, and adult). Three of them are developed in water-sediment media, emerging the adult to the air. The larval stage is the longest and has to undergo four molts (I, II, III, and IV instar larvae) before it transforms into a pupa. From the pupa emerges the adult, which is short-lived and orientated to the reproduction functions.

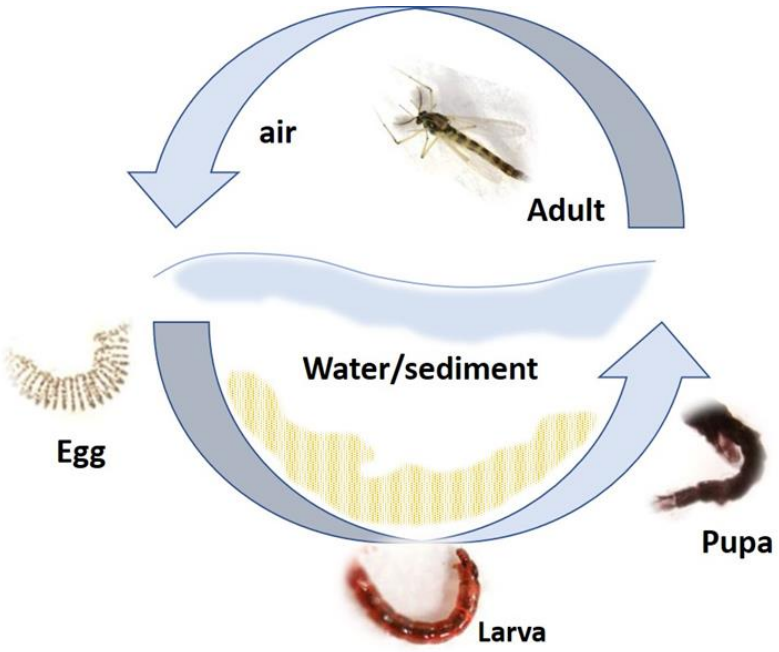

Figure 7. The life cycle of Chironomid, the case of *C. riparius*.

The chemicals can enter to aquatic environment by different routes, such as runoff, drainage, and spray drift. The exposure of sediment-dwelling organisms as chironomids can occur via the overlying water, the pore water, and the sediment. Therefore, different exposure scenarios can be investigated by the use of acute and chronic chironomid tests.

In summary, all these characteristics increased the value of the chironomids as ecotoxicology organisms. Therefore, in the present study, one specie has been selected to evaluate the effects of xenobiotics with multiple origin and nature, *Chironomus riparius*.

Chironomus riparius for freshwater toxicology

Chironomus riparius (Meigen, 1804) is one of the most known Chironomid species and usually employed for aquatic toxicology. Four different OECD guidelines have been designed for chemical toxicity evaluation, test 218, 219, 233, and 235 (OECD 2004a, 2004b, 2010, 2011). Test 218 & 219 assess development, emergence rates, survival, and EC values in acute and chronic conditions in water and sediments. Test 235 evaluates immobilization and, finally, Test 233 is focused on assessing the effect on the life cycle, including the first and early second-generation.

C. riparius has become one of the most widely used aquatic invertebrates in toxicity studies. One of the main advantages is its short cycle and its easy culture in the laboratory. Multiple works have studied the effects of various xenobiotics such as plasticizers, UV filters, pharmaceuticals, pesticides, or microplastics on this species. They were mainly focused on development, reproduction or biomarkers (Campos et al., 2017, 2019; Lee & Choi, 2007; Monteiro et al., 2019a, b; Nieto et al., 2017; Silva et al., 2019; Xie et al., 2019). Fewer studies evaluated alterations at the transcriptional level, although in recent years they have been increasing (In et al., 2019; Martín-Folgar et al., 2018; Martín-Folgar & Martínez-Guitarte, 2017; Martínez-Paz et al., 2017, 2019; Ozáez et al., 2013, 2016; Xie et al., 2019).

OBJECTIVES

2. OBJECTIVES

The general objective of the thesis is to improve the knowledge of the cellular and molecular responses that allow the aquatic invertebrates to manage the present multi-stress environmental scenario of pollution and climate change. Furthermore, to reach this goal, an additional methodological objective involves designing and validate an array of genes to study the response in five relevant processes in response to toxicants. To achieve these objectives, six different compounds and PE microplastics are used as representatives of different emerging pollutants, and *Chironomus riparius* is selected as experimental organisms.

The specific objectives to be achieved are:

1. Design and validate a specific array with forty genes (plus six of reference) for evaluating five essential metabolic pathways in invertebrates.

2. Define the response at the cellular and the molecular levels of binary and ternary mixtures of toxicants.

3. Evaluate the influence of temperature on the toxicity of a UV filter and the putative consequences that climate change can have in response to toxicants.

4. Analyze the effects and the response to emerging contaminants and microplastics by combining the transcriptional profile and the enzyme activity to establish the impact that they can have at the cellular and the molecular level and the ability of *Chironomus riparius* to cope with them.

MATERIAL & METHODS

3. MATERIAL & METHODS

Biological Material

Chironomus riparius

The organism selected for the studies was *Chironomus riparius*, a freshwater insect from Diptera Order. The animals belong to a natural population from Massamagrell (Valencia, Spain). They have been maintained for several generations in the laboratory of Environmental Toxicology and Biology group (UNED) according to the standardized toxicity test (OCDE 2010). The culture was done in a temperature-controlled room 19 ± 1 °C, continuously aerated and light-dark period 16:8 h, allowing regularity in the conditions of growth and development. The cultures were started from egg masses disposed of in individual aquariums with culture medium and a grind of dried leaves of nettle. Besides, cellulose paper was added to promote the construction of the tubes where they evolve through the four different larval stages. The larvae were fed with commercial fish food (Tetramin) two times a week, and new egg masses were collected two days a week. The adults were collected to a stock tank to allow the crossing between males and females (Morcillo et al., 1993).

IV Instar larvae

Chironomus riparius has a short life cycle based on following different life stages from egg masses to adults. The more critical stage, in water, is larval with four instars: I, II, III, and IV. The OCDE guidelines, concretely, OCDE 218, 219 (2004,2004b), 233 (2010), and 235 (2011), define the conditions and instructions for the toxicological test in Chironomids. For the acute test, IV instar larvae are defined as an optimal organism.

Reactives

- *C. riparius* culture media: $CaCl_2$ 0.5 mM, NaCl 1mM, $MgSO_4$ 1mM, $NaHCO_3$ 0.1 mM, KH_2PO_4 0.025 mM.
- PBS: NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.8 mM, Na_2HPO_4 10 mM. 1 tablet in 200 ml water and adjust pH
- Electrophoresis loading buffer: bromophenol blue 0.25%, xylene cyanol 0.25% and glycerol 50%.
- TBE buffer: Tris-borato 40 mM, EDTA 0.5 M.
- Protein extraction buffer (10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EGTA pH 8, 0.5 mM DTT, 5% glycerol and 0.5 mM PMSF)

Chemical compounds

Three UVFs, 2-Ethylhexyl 4-(dimethylamino) benzoate (OD-PABA), octocrylene (OC) and benzophenone 3 (BP-3), one plasticizer bisphenol A (BPA), the ibuprofen as a non-steroidal anti-inflammatory drug (NSAID), one insecticide and acaricide Endosulfan, and finally, low-density polyethylene (LDPE) microplastics were selected to evaluate their effects on *C. riparius*.

The chemical properties of organic UV filters were defined in table 1. For the UVFs OD-PABA and OC exposures, stock solutions were made in absolute ethanol and stored in the dark at 4 °C. The stock solution concentrations were 50 and 100 mg/mL for single and binary mixtures, respectively.

For OD-PABA, OC, and BPA treatments, the solutions were made in absolute ethanol, at 50, 100, and 150 mg/L as stock solutions for single, binary, and ternary mixtures exposures. The plasticizer BPA is a known endocrine disruptor whose chemical characteristics were defined in table 2.

OD-PABA	
Name (INCI nomenclature)	2-Ethylhexyl 4-(dimethylamino)benzoate
Synonym	Padimate O
Abbreviate	OD-PABA
Empirical formula	$(CH_3)_2NC_6H_4CO_2CH_2CH(C_2H_5)\ (CH_2)_3CH_3$
CAS No.	21245-02-3
Molecular weigth (g/mol)	277.40
Density (g/cm^3)	0.995
Log Kow	5.412
Purity	≥ 98%
Solubility (g/L)	4.7×10^{-3}
Octocrylene	
Name (INCI nomenclature)	Octocrylene
Synonym	2-Ethylhexyl 2-cyano-3,3-diphenylacrylate
Abbreviate	OC
Empirical formula	$(C_6H_5)_2C{=}C(CN)CO_2CH_2CH(C_2H_5)\ (CH_2)_3CH_3$
CAS No.	6197-30-4
Molecular weigth (g/mol)	361.5
Density (g/cm^3)	1.055
Log Kow	6.88
Purity	≥ 97%
Solubility (g/L)	3.6×10^{-4}
Benzophenone 3	
Name (INCI nomenclature)	Benzophenone-3
Synonym	2-Hidroxy-4-metoxybenzophenone Oxybenzone
Abbreviate	BP-3
Empirical formula	$HOC_6H_3(OCH_3)\ COC_6H_5$
CAS No.	131-57-7
Molecular weigth (g/mol)	228.24
Density (g/cm^3)	1.201
Log Kow	3.79
Purity	≥ 98%
Solubility (g/L)	0.10

Table 1. UV filters chemical properties.

Finally, for the UV filter BP-3, a stock solution (50 mg/mL) was prepared in absolute ethanol and stored in the dark at 4 °C until use. All the chemicals were provided by Sigma-Aldrich. Dilutions from the stock solution were done in ethanol at 1/10, 1/100 and 1/1000 for the three studies.

Bisphenol A

Name (INCI nomenclature)	Bisphenol A
Synonym	2,2-Bis(4-hydroxyphenyl)propane, 4,4'-Isopropylidenediphenol)
Abbreviate	BPA
Empirical formula	$(CH_3)_2C(C_6H_4OH)_2$
CAS No.	80-05-7
Molecular weigth (g/mol)	228.29
Density (g/cm³)	1.2
Log Kow	3.3
Purity	≥ 99%
Solubility (g/L)	0.3

Table 2. BPA chemical properties.

Ibuprofen was provided by Sigma-Aldrich; its properties were shown in table 3. The stock solution was prepared with methanol at 1 g/L. Experimental solutions were prepared from this in 1/100 and 1/10000 diluted in methanol. The solutions were maintained in the dark at 4 °C.

Ibuprofen

Name (INCI nomenclature)	2-[4-(2-methylpropyl)phenyl]propanoic acid
Empirical formula	$C_{13}H_{18}O_2$
CAS No.	15687-27-1
Molecular weight (g/mol)	206.28
Log Kow	3.97
Purity	≥ 98%
Solubility (g/L)	0,021

Table 3. Ibuprofen chemical properties.

The action mode of the insecticide is determined by its chemical properties, for the endosulfan are shown in table 4. A stock solution was prepared from an initial stock of 250 mg/mL (PESTANAL®, analytical standard), for a final concentration of 10 mg/L in acetone and store at 4 degrees.

Endosulfan

Name (INCI nomenclature)	endosulfan
Empirical formula	$C_9H_6Cl_6O_4S$
CAS No.	1031-07-8
Molecular weight (g/mol)	422.92
Density (g/cm³)	1.745
Log Kow	3.83
Purity	≥ 99%
Solubility (g/L)	0.32

Table 4. Endosulfan chemical properties

Low-density Polyethylene (LDPE) microplastics of 40–48 µm (average size, ultra-high molecular weight powder, CAS No. 9002-88-4) were provided by Sigma-Aldrich. The PE microplastics was shaken by vibration to generate smaller PE particles and then filtered using mesh pore-sizes of 45 and 32 µm, to obtain particles of 32-45 µm and <32 µm. These particles are employed for sediment contamination.

Acute toxicity test: Experimental set-up

The experimental design was focused on the evaluation of diverse chemical compounds and stressors on IV instar larvae, mimicking the current multi-stress environmental situation. All the treatments were developed at nominal concentrations. Besides, polytetrafluoroethylene vessels (100 mL) were used for all the treatments to avoid absorption of the chemicals to vessel walls, except for the PE MPs exposure, done in another laboratory following the conditions set out below. In all the cases, non-treated larvae (control) were exposed to 0.02% of solvent concentration that does not affect the survival of organisms. Furthermore, three independent biological replicates were performed. For short term exposures, no sediment, cellulose tissue, or food was added. On the other hand, for the medium-term exposure (96h), the media was renewed to avoid chemical degradation, and the larvae were fed at 48h (25 mg of Tetramin per larva). The set up was run at 19 ± 1°C and 16:8 h light: dark photoperiod (OCDE, 2010) with continuous aeration.

Endpoints analyzed

Survival

The survival test for ibuprofen and for benzophenone-3 plus temperature was performed with thirty IV instar larvae exposed to the selected xenobiotic concentrations diluted in 50 mL of culture medium for 96h. Then, the mortality was checked every 24h, checking the mobility and aspect of the larvae. The media was changed, and the larvae were fed at 48h, according to the previous instructions.

Gene expression and enzymatic activity evaluation

Fourth instar larvae were exposed to the selected xenobiotic concentration diluted in 30 mL of culture medium for the exposure time selected in each experiment.

In all the mixtures (UVFs and UVFs + BPA), the stock solution was prepared with equal concentration for each compound (1:1, 1:1:1). Besides, to prevent the biodegradation of UVFs, the exposure was carried out by protecting the top of the vessel with aluminum foil. The rest of the exposures were done without special conditions. The control treatment in all the cases was done according to 0.02% of the solvent. Three independent experiments were performed for each set of conditions using larvae grown from different egg masses. For each condition, one experiment provided three larvae for analysis, so the total number of larvae per condition was n=9. Subsequently, each larva was considered as a biological replicate. Larvae were individually frozen and stored at -80 °C for RNA and protein extraction.

The experiments developed were:

1. Effects of a single exposure and binary mixtures of ultraviolet filters OD-PABA and OC on gene expression.
2. Effects of a single exposure, binary and ternary mixtures of two UV filters (OD-PABA and OC), and the plasticizer, BPA.
3. Combined effects of BP-3 and temperature (18.5 and 23 °C) on survival, gene expression, and enzymatic activity.
4. Effects of Ibuprofen on survival, gene expression, and enzymatic activity
5. Persistent pesticides: effects of endosulfan on gene expression and enzymatic activity.
6. Acute exposure of Polyethylene microplastics on the biochemical and transcriptional levels in *C. riparius*.

The exposures were done according to the instructions previously defined, the specific conditions for each exposure are presented in table 5. The experiment 6, was conducted during the international leave, and the display employed is detailed in the next paragraph.

Exp	Time (h)	Concentrations	Larvae /replicate	Type of analysis
1	24	0.1, 1, 10 mg/L	5	Gene expression
2	24, 96	0.1, 1 mg/L	8	Gene expression
3	24, 48, 72, 96	10,100, 1000, 10000 µg/L	30	Survival
3	8, 24	10, 100 µg/L	15	Gene expression and enzymatic activity
4	24, 48, 72, 96	0.1, 1, 10,100 µg/L	30	Survival
4	24, 96	0.01, 1,100 µg/L	15	Gene expression and enzymatic activity
5	24	0.1, 1,10 µg/L	9	Gene expression and enzymatic activity

Table 5. Summary of the exposure conditions used for each experiment in *C. riparius*.

Acute exposure of Polyethylene microplastics on the biochemical and transcriptional levels in *C. riparius*. Collaboration with the Biology Department and the *Centro de Estudios do Ambiente e do Mar* (CESAM, *Universidad de Aveiro*, Portugal) and UNED.

The acute test was developed using the IV instar larvae of *C. riparius* exposed for 48h. Polyethylene microplastics particles from two different sizes (<32 and 32-45 µm) previously prepared, were weighted and added to inorganic sediment, previously burnt (500 °C for 4 h) to obtain final nominal concentrations of 0.025 and 2.5 g/Kg of PE. Once contaminated, the sediment and ASTM (ASTM, 2005) were maintained in the crystal vessel for 48h to equilibrate. The experiments were done in a crystal vessel containing 25 larvae, 1.5 cm layer of contaminated sediment, and 150 mL of ASTM. The control group consists of larvae exposed to pure sediment and ASTM. Fifteen larvae as pool were used for biochemical biomarkers analysis and three individual larvae for gene expression analysis for each condition and replicate. Seven and three replicates were carried out for the biochemical response and gene expression analysis, respectively. No fed was added during the exposure. Larvae were individually frozen in liquid nitrogen and stored at -80 °C for RNA extraction. The pool larvae for protein extraction were frozen in liquid nitrogen and stored at -80 °C.

Nucleic Acid Extraction

The RNA extraction was done following two different commercial solutions, TRIzol reagent and RNAzolRT reagent. The first one is based on a single-step method (Chomczynski & Sachi, 1987; Chomczynski, 1993). The second one provides higher yield and quality of the final RNA than the previous methods based on a single-step. It is because it eliminates the Chloroform step, decreasing the possible interactions with the RNA.

TRIzol RNA extraction

The samples were frozen at -80 °C, mechanically homogenized in a 200 µl of TRIzol with a homogenizer for microcentrifuge tubes, and immediately frozen in dry ice. After that, the samples were thawed at room temperature (rt) for facilitating the disruption of nucleoproteins complex. Next, 0.2 volumes of chloroform were added. Then, the sample was mixed for 15 seconds and maintained 2-3 minutes at rt. A centrifugation step (for 15 min at 10000 rpm, 4 °C) was performed, and three phases were obtained. The lower Phaseis the organic phase; the interphase contains the proteins, and the upper aqueous Phasecontaining the RNA. This step removes the high molecular weight DNA, cellular

membranes, polysaccharides, and extracellular membranes. The upper Phaseis recovered, and the RNA is precipitated by adding 0.7 volumes of 2-propanol alcohol, incubating for 10 minutes at rt. The samples were centrifuged for 15 min at 10000 rpm and 4 °C. The supernatant was discarded. The RNA was visualized as a little white pellet on the bottom of the tube. The pellet was washed with one volume of ethanol 75%, vortexed, and centrifuged for 5 min at 10000 rpm and 4 °C. Once discarded the supernatant, the sample was spun for 45 seconds, and the remains of ethanol were removed by pipetting. The pellet was resuspended in Diethyl pyrocarbonate-treated water (diethylpyrocarbonate 0.1% v/v, DEPC, Sigma), avoiding to dry it since it decreases the RNA solubility.

RNAzol RT RNA extraction

The samples were homogenized with 200 µl of reagent. Then, 0.4 volumes of water were added and mixed strongly for 15 seconds. The samples were incubated for 10 min at room temperature and centrifuged for 15 min at 12000 g and 4 °C. This step removes DNA, proteins, and polysaccharides. RNA was recovered in the aqueous phase and precipitated with 0.7 volumes of 2-propanol alcohol (v/v) for 10 min at rt. A centrifugation step for 10 min at 12000 g and 4 °C precipitated the RNA. The supernatant was discarded, and the pellet was washed with 0.4 volumes of ethanol 75%. After it was centrifugated for 3 min at 8000 g and 4 °C, the supernatant was discarded, and spin was done for 45 seconds. The ethanol was removed with a pipette, and the RNA resuspended in DEPC water.

DNAase treatment

Samples were treated with RNAase-free DNAase I (Roche) to eliminate any DNA, improving the quality and yield of the retrotranscription. The RNA was incubated with 90 µL DEPC water, 10 µL of 10X DNAase buffer, and 1 µL of RNAase-free DNAase I for 45 min at 37 °C. Later, the enzyme was removed by organic phenol-chloroform-isoamyl alcohol extraction using Gel Phase Lock Light tubes (5Prime). The sample was centrifuged for 5 min at 10000 rpm and 4 °C. The aqueous Phasecontaining the RNA was collected to a new microcentrifuge tube, precipitated with 0.7 volumes of 2-propanol, and washed with one volume of 75% ethanol. Finally, the RNA was resuspended in 25 µL DEPC water and stored at -80 °C.

RNA quality and quantification

The quality of the RNA was checked by agarose gel electrophoresis. The gel was prepared at 1.5%, the recommended percentage for RNA, with 1.5 g of agarose dissolved in 100 mL of 1X TBE buffer (Tris-Boric acid-EDTA). The mixture was heated to solve the agarose. Then was cooled down to about 60 °C, pour on a tray with a comb, and allowed to solidify at room temperature. The samples were loaded in the gel using a 6X loading buffer. As a reference, a 1kb DNA marker was loaded in the first well. The electrophoresis was run at 60-80 V for 1h. Once the run is finished, the gel was dyed in a water-ethidium bromide solution by shaking it for 1 hour. The gel was visualized in a Chemigenius 3 gel documentation system (SynGene – UK).

The quantification was done by spectrophotometry (Biophotometer, Eppendorf). One microliter of each sample was dissolved in 99 µL DEPC water and measured at 260 nm. The quantity was defined in µg/mL. Two different indexes, 260/280 and 260/230, were determined to know the remaining proteins and organic solvent, respectively. As a reference, the indexes should be:

260/280: 1.7-2.1 (lower values indicate the presence of proteins)

260/230: 1.6-2.3

Reverse transcription

For Real-Time PCR, the RNA was transformed into complementary DNA (cDNA) by retrotranscription. The step was essential because the final signal obtained in RT-PCR was dependent on the efficiency of retro-transcription. The reaction was based on Valasek & Repa, 2005, that used a DNA primer to synthesize the complementary DNA strand of RNA by using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) enzyme. As stated by the manufacturer protocol, 1 µg of total RNA was mixed with 1 µL of oligonucleotide (poliT), 1 µL of 10 mM deoxynucleotides triphosphate (dNTPs), and DEPC water until reach 20 µL . The mixture was incubated for 5 min at 65°C and cool down on the ice. Next, after a spin, 4 µL of First-Strand Buffer 5X, 2 µL of 0.1 M Dithiothreitol (DTT), and 1 µL of M-MLV enzyme were added. The sample was mixed and centrifuged for 45 sec at 10000 rpm. After incubating the sample for 50 min at 37 °C, the reaction was stopped and inactivated by thermal shock for 15 min at 70 °C. The cDNA was stored at -20 °C.

Amplification by Real-Time PCR

The Real-Time PCR or quantitative PCR is a technique based on the polymerase chain reaction (PCR) with some variations. It is capable of amplifying and quantify the molecules of DNA or cDNA, generating more reliable and accurate data about the gene expression (Heid et al. 1996). The reaction is carried out in a thermal cycler coupled with a detector. In each PCR cycle, a beam of light with a determined wavelength is sent to each well, and the fluorescence emitted by the excited fluorochrome is detected. The fluorophore binds unspecified double-strand DNA and generates the light at a specific wavelength. The emitted light is used to calculate the rate of generation of the specific product throughout the PCR (Heid et al. 1996). This technique can generate very small amplicons (60pb) being ideal for detecting quantitative changes in the gene expression during pathological or experimental alterations. The reaction was performed using 25 ng cDNA. Primer stocks, forward and reverse, were at 5 or 2.5 µM. A 2X solution of dNTPs and reaction buffer was prepared and supplemented with 40 µL of DNA polymerase (Biotools) and 25 µL of EvaGreen® (Biotium). The reaction volume was 20 or 10 µL. The reactions were done in multiwell plates (Sarsdedt) and a CFX96 RT-PCR system (Biorad).

The amplification program employed is defined in table 6.

	Temperature (°C)	**Time (seconds)**
Polymerase activation	95	300
1.Denaturation	95	15
2.Annealing	58	15
3.Elongation	72	30
4. Reading	72	1
Melting curve	65-95	5/ °C

Table 6. Real-time PCR cycling program.

The steps 1 to 3 were repeated for 39 cycles to make a total of 40 cycles.

The melting curve is used to check the specificity of the primers because Eva Green is a non-specific DNA dye and can also bind non-specific products (such as primer-dimers). In this way, specific amplicons can be defined against non-specific products (Ririe et al., 1997).

Primers efficiencies and Real-time PCR data analysis

Firstly, the efficiencies of each primer pair must be obtained for the correct Real-time PCR analysis. The efficiency is defined as the reaction capacity to duplicate the DNA of the gene of interest in each cycle (Bustin & Nolan 2004a). It is calculated from the slope of the standard curve obtained from serial

dilutions (1/2; 1/4; 1/8; 1/16) of PCR products and using a negative (without product) as internal control (Pfaffl, 2004), employing the same PCR conditions of the experiments following ($R^2 > 0.98$ for all primers) and based on the equation:

$$E=10^{(-1/slope)-1}$$

Real-time PCR data were acquired when the amplification was initiating the exponential phase. It was determined by the identification of the number of the cycle at which the emission intensity of the fluorophore increases concerning background noise. This cycle number is called the cycle threshold (Ct) or quantification cycle (Cq). The Ct is more reliable than conventional endpoint measurements and is inversely proportional to the number of copies of the tempered target. So, the Ct was selected for the subsequent analysis.

The analysis of the data can be carried out as absolute or relative quantification. The second method was selected in these studies. The measuring of the changes in the expression of a specific gene is obtained by comparing it to one or more reference genes, previously selected (Pfaffl, 2004). The selection of optimal reference genes is essential for reliable results analysis. These genes have a regular expression in the selected organisms. The so-named "housekeeping" genes are generally used (Ambion, 2008). The reference genes employed were Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), ribosomal protein 11 and 13 (*rpL11, rpL13*), RNA polymerase (*RNA pol*), Phosphofructokinase (*PhFk*), and TATA-binding protein (*TBP*). The best reference genes for each analysis were selected using the **BestKeeper tool** for Excel. The calculus was based on Ct values comparison using the efficiency of PCR reaction as a correction factor. Biorad CFX Manager 3.1 and CFX Maestro software were used to determining the total mRNA levels of normalized gene expression ($2^{-\Delta\Delta CT}$).

Sequence identification

Genes were identified from a transcriptome project for *Chironomus riparius* from a database (Sequence Reading Archive, NCBI). The sequence identification was performed by downloading all assembled sequences of the SRX147945 project (Marinković et al., 2012) from the Transcriptome Shotgun Assembly (TSA, NCBI) sequence Database. The sequences were compared against the GenBank database by employing Blastx with the Blast2GO tool (Conesa et al., 2005). Blastx searches translated nucleotide databases using a translated nucleotide query. It was used with no redundant database, and the Blast Expect Value was fixed in 10^{-3}. Once the sequences were identified as proteins of interest, the open reading frame (ORF) was identified using SnapGene software (from GSL Biotech, available at www.snapgene.com). The homology of ORFs from the proteins was confirmed by making a comparison by using Blastp. Those sequences identified as proteins of interest were used for primer designing and, therefore, to real-time PCR (RT-PCR).

Primer design

The database was constructed from the GenBank database with the reference proteins from *Drosophila melanogaster*. Afterward, the validation of the genes was performed by PCR as previously defined.

The primer pairs used in this study fall into two categories. Some of them have been designed previously in the laboratory or from published references. The rest of the primer pairs were designed for this work, the sequences are shown in the annex section. The primers pairs were designed employing the Primer-Blast software, following as requirements:

- Primer length: 18-25 pb

- GC content between 40-60%
- Primer Melting Temperature (Tm): in the range of 55-65 °C
- Locations close to 3´extreme of mRNA
- Avoid 3´ terminal T
- Avoid secondary structure or complementary at 3´ends

The sequence primers and efficiencies of the genes evaluated in this thesis are presented in table 7.

Gene	Forward	Reverse	Efficiency (%)
GAPDH	5´GGTATTTCATTGAATGATCACTTG	5´TAATCCTTGGATTTGCATGTACTTG	**94.1**
rpL11	5´TGGCGAAAGCTACCGTCGTA	5´TCACCAGATTCTCGACGCA	**106.8**
rpL13	5´ACCAGCTAGAAAGCACCGTC	5´ATGGGCATCTGACGATTGGG	**97.3**
PhFK	5´CAAGGTGGCTCCATCACCA	5´GTGTGCATTAACCTTTCCGTCCT	**104.5**
RNA pol II	5´TTCGCCTTCGTCACCATCTT	5´CGTCGGCGAATAAGGACTG	**99**
TBP	5´GCAGCAAGGAAATACGCTCG	5´TGCAATGTGTGAGAACAAGTCC	**95.3**
Dronc	5´GAAATGTCACAGATTTCAGTGCC	5´GTGAATATCGTAAGCATGTTCTGC	**99.3**
JHAMT	5´AGAAAATGCTACGGGAAAGTGG	5´CAGGCATTAGCTTCAAGCAAGG	**104.6**
EcR	5´TCTTCTCACGGCCATCGTCA	5´GCTGCATCTTGTTTCGCCAC	**102.9**
Met	5´GAGTTCAGTACTTAAGCGCACTC	5´GTGGAGGCTGTTGTGGCACTC	**97.3**
InR	5´CGGAAGTTTCGGTATGGTTT	5´GACAAATATTGGCTGGCCTT	**108.4**
MAPR	5´GGACCGTATGCAGCTTTTGG	5´TTGTTGCCAGGCCTTAACA	**94.6**
Cyp18a1	5´GTTTCACTCGAGACGATCCA	5´TTTAGCGGCTTGAAATGTTG	**104.5**
Kr-h1	5´CCCTCGAGCTAACTCTCACCC	5´GCTGCAATGTTTGACTGGTT	**109.7**
E93	5´CGAGAACCGAAACCACAGCC	5´GCGCTGCCATTGATGCATGATC	**107.1**
Dis	5´GAGGCATCCATACAATTCATTC	5´CAAAATCCTTCCAACTCCAATAG	**101.3**
Cyp4d2	5´AGCACCGATCATCGCAAGAC	5´ACAAACTCGTCTGGCTTGTCA	**108.8**
Cyp6b7	5´AGCGTGAGCAGGAAAAGTCA	5´TGCTGCTGGTTTCATAGCCT	**103**
Cyp9f2	5´GTTCTCTACGCTCCATTCGGT	5´GGTTGCCTCGAAGCTGAAGT	**99**
Cyp12a2	5´TGGCTTTGGAAGCAGGGTTT	5´CGCCAGCCTTGAACTTTAGG	**111.1**
GSTd3	5´TGGTTGAAACGAGAGCACCA	5´TCGGATATAGAGTGCCAGCATCG	**106.7**
GSTd6	5´GCCTGGAGGATGTGGATTGT	5´GCTTGAAGAATGCTGCTGACT	**104.2**
GSTe1	5´CTCCGGCGAGCATAAAAGTG	5´CAGTCGCGTACCGTTCTACT	**98.2**
GSTo1	5´TGAGCTTGATAAGGTGCGCT	5´TAATCAGGTGTGCCTGCGAG	**96.8**
GSTt1	5´CGCAGACATTTTGGCAGCAT	5´TTCGTCGCATCCCTTACTCG	**99.7**
MRP-1	5´TGGCCACAAAGTGGAATTGT	5´CTTTCCAGCTCTGTTCGTC	**102.2**
ABCB6	5´GCTTTAGCTCGAGTTTGTAGCA	5´TCGTGCTTTCCTCGCTCTAC	**99.2**
hsp70	5´ACTTGAACCAGTTGAGCGT	5´TTGCCACAGAAGAAATCTTG	**102.8**
hsc70	5´TGGCGGTGTCTTTACACGTC	5´TGCCATTTCACGTTCGCCTT	**103.0**
HYOU1	5´CGCTACTATGGGCGCAGTTT	5´TGGTGCCACTTTCACGTTCA	**110.3**
hsp90	5´AGGCTGAAGCTGACAAGAATG	5´TCATGCGATAAATGCGAGCAG	**108.1**
Gp93	5´ACCCCATGTGTACTCGTTGC	5´CGTGGATTAAATTCGAGAGC	**100.9**
hsp40	5´TGATATTCGAGGTGCCAACA	5´AATGCCACCAAGATCACC	**105.0**
hsp60	5´TGCTGTCCTTAAAGTCGGTGG	5´TCCACCACC-CAACGATTC	**95.5**
hsp10	5´CGTCCAAAGAGCTGAGGCAT	5´CTCCTGGACCGCAAGCAATA	**102.6**
hsp17	5´GATGGCTTCTTTCGGACCGC	5´CCTTTTCATCATGAGCGGCAG	**100.7**
hsp21	5´CGAGACAACTTCAAGTTCCACA	5´CCTTATTATCTCCATCATCTTGG	**105.9**
hsp22	5´CCACCACCAGCAATCTCTG	5´GACAGCAGGTGATTTTCC	**99.8**
hsp23	5´GAAACTTCGACCAGAAATTGAAG	5´CTTCTTTATCTTCCTGACCGTCG	**108.2**
hsp24	5´TCACTTAATGACTGGATATCG	5´GAATCCATCCTTGCCGAAATGC	**100.0**
hsp27	5´TCAACACACAGGACCG	5´ATCCTTTATTGGTGATTAATTAT	**94.4**
l(2)efl	5´TCTCAAGGCACTTCACACGA	5´ACGTGATGGTCCAACGTGAG	**113.3**
Decay	5´AAAGTGTTCCGATTATGGC	5´TTCACACCAGTTAAAATCC	**97.9**
XRCC1	5´GACGATTTGCATTGGATAGT	5´ATCAACATATCGCCATCAG	**109.7**
NLK	5´CATCTCACCAGATCGTCTCT	5´GAATTTATTTGATTATGCGGC	**98.3**
ATM	5´ACATTTGGCGTAGATCAGGCA	5´ACGAGATGCATCAAATCATGC	**102.0**
PARP	5´ATTTGAGTTAATTAAGCGTTACGTT	5´GAGACGACTTCCGTGCCATA	**98.3**
Proph	5´CTCGAACAGCACCTTTGTCTG	5´CCATGAATCGTTCACGCCATC	**108.0**
Def	5´CGCACTTGCATTCGCTAATCC	5´ACACCATCCACCTCTAAATCC	**113.9**

Table 7. Primers sequences and their efficiencies.

To help the reader to follow the different cellular processes analyzed, it is presented a summary of the genes used in the present study grouped by their metabolic pathways (Table 8).

Process		Gene
Endocrine system	20-E	*Dis*
		EcR
		Cyp18a1
		E93
		Dronc
		Kr-h1
	JH	*JHAMT*
		Met
		InR
		MAPR
Detoxification response	Phase I	*Cyp4d2*
		Cyp6b7
		Cyp9f2
		Cyp12a2
	Phase II	*GSTd3*
		GSTo1
		GSTe1
		GSTt1
	Phase III	*MRP-1*
		ABCB6
Stress response	Hsp70	*hsp70*
		hsc70
		HYOU1
	Hsp40	*hsp40*
	Hsp90	*hsp90*
		Gp93
	Mitocondrial	*hsp60*
		hsp10
	sHSPs	*hsp17*
		hsp21
		hsp22
		hsp23
		hsp24
		hsp27
DNA repair	SSB	*ATM*
		NLK
	SSB/ DSB	*PARP*
		XRCC1
	Apoptosis	*Decay*
Immune system	PO system	*Proph*
	AMP	*Def*

Table 8. PCR-array for C. riparius classified by the metabolic pathway.

Enzymatic activity evaluation:

Protein extraction

Proteins were extracted following the protocol described for *Drosophila* (Zhong et al., 1996) adapted for *C. riparius*. Each frozen larva was homogenized in a microcentrifuge tube with extraction buffer (reactives section), frozen on dry ice, thawed, and frozen again on dry ice. Later, the samples were maintained for 15 min on ice and centrifuged for 10 min at 10000 rpm and 4 °C. Finally, the supernatant containing the proteins was recovered, aliquoted, and maintained at -80 °C until use. Protein concentration was quantified using the Bradford method (Bradford, 1976) in a microplate reader (Multiskan Go, Thermo USA) with Bio-Rad Bradford solution. The method is based on the protein quantification by colorimetric assay using a specific reactive, Bradford reactive (Bio-rad). A standard curve was done with serial dilutions of a Bovine Serum Albumin (BSA) solution. The standard curve allows determining protein concentration. The initial stock, BSA 2 mg/mL, was diluted according to table 9.

Final concentration (µg/mL)	BSA volume (µL)	Water volume (µL)
0	0	20
125	2.5	37.5
250	5	35
500	10	30
750	15	25
1000	20	20
1500	30	10
2000	20	0

Table 9. Standard curve preparation for protein quantification.

Enzymatic Activity

Three different enzymatic activities were analyzed: Glutathione-S-transferase (GST), Achetilcolinesterase (AChE), and Phenoloxidase (PO).

GST activity was assessed by measuring 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) conjugate based on the Habig et al., 1974 method. The assay cocktail was prepared with Phosphate Buffered Saline (PBS) pH 6.5, 100 mM CDNB, and 200 mM GSH. The blank consisted of this cocktail without the protein sample. The reaction was developed by mixing 188 µl of the cocktail with 12 µl of the homogenate protein. Absorbance was measured in a plate reader (Multiskan, Thermo USA) at 340 nm for 5 min, and the activity was calculated according to:

$$(\Delta A340)/\text{min} = \frac{\text{A340 (final read)} - \text{A340 (initial read)}}{\text{reaction time (min.)}}$$

$$\frac{(\Delta A340)\ /\text{min} \times V\ (\text{ml}) \times \text{dil}}{\varepsilon_{mM} \times V_{enz}\ (\text{ml})} = \mu\text{mol min}^{-1}\ \text{mg}^{-1}\ \text{of protein},$$

Where: dil is the dilution factor of the original sample, εmM (mM^{-1} cm^{-1}) is the extinction coefficient for the CDNB conjugate at 340 nm [5.3 mM^{-1} cm^{-1}], V is the reaction volume, and V_{enz} is the volume of the protein added. The final GST activity is divided by the quantity of protein and then expressed as µmol/min/ mg of protein.

For AChE activity, a previously described method (Freitas et al., 2014) was used with modifications. The technique employed the artificial substrate acetylthiocholine iodide (ATCI), which is broken in acetyl and thiocholine by the action of AchE present in the sample. The measure is based on the reaction between thiocholine with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) that produces a yellow-colored product. The product is measured by spectrophotometric detection, and the activity is calculated with the known extinction coefficient of the product. The assay was performed by mixing 100 µL of 8 mM DTNB diluted in PBS pH 8.0 supplemented with 0.75 mg/mL $NaHCO_3$, 50 µL of protein homogenate, and 50 µL of 16 mM ATCI diluted in PBS pH 8.0. The blank was 50 µL of PBS pH 8.0, plus ATCI and DTNB. The reaction was measured at 405 nm for 10 min with intermittent shaking every 30 sec. The activity of the enzyme in µmol min^{-1} mg^{-1} protein was by the following equation.

$$\text{Activity} = \frac{(\Delta OD/min)}{(MEC \times C)}$$

Where ΔOD/min is the variation of optical density in the time, MEC is the molar extinction coefficient of the colored product at 405 nm, 8160 L/mol.cm, and C is the protein extract concentration in the assay (mg/L).

PO activity was detected with dihydroxyphenylalanine (L-DOPA) as the substrate. The method was modified from Lilley et al., 2012. Ten microliters of protein extract were mixed with 200 µL of 10 mM L-DOPA diluted in PBS pH 7.5, and the reaction was measured at 495 nm for 30 min at 20 °C at 1 min intervals. The blank was the L-DOPA solution without a sample. PO activity is expressed as the total change in optical density per mg of protein (ΔOD/mg protein).

Biochemical biomarkers for microplastics evaluation

This work was developed in the Biology department and Centro de Estudios do Ambiente e do Mar (CESAM, Universidad de Aveiro, Portugal).

Protein extraction

Larvae for biochemical studies were picked up and dried on filter paper, weighed, and frozen in liquid nitrogen for the store at –80 °C until use. The pool of larvae was homogenized in 1600 µL of Milli-Q water by the tissuelyser method. The homogenization was carried out using bead mills with 5 mm of diameter, at a speed of 15 Hz for 30 seconds employing the Tissuelyser II (QIAGEN, Cat nº 85300).

The total homogenate was divided into two. For lipid peroxidation, 150 µL of homogenate were mixed with 4 µL of BHT 4% (2,6-Dieter-butyl4- methyl phenol). The remaining volume was mixed with K-phosphate buffer (pH 7.4, 0.2 M) and centrifugated at 10000 × rpm for 20 min at 4 °C. The supernatant (PMS) was collected in aliquots to analyze the glutathione-S-transferase (GST), catalase (CAT), and acetylcholinesterase (AChE) activities, the total glutathione (TGSH), the energy reserves (sugar, lipid, and protein content), the energy consumption by electron transport (ETS) and for protein quantification.

Lipidic peroxidation (LPO)

LPO was determined in the homogenate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird & Draper, 1984; Ohkawa et al., 1979). The reaction included a mixture of 150 µl homogenate (previously prepared 12.2.1), 500 µl of 12% (w/v) trichloroacetic acid sodium salt, 50 µl of 0.73% (w/v) 2 - thiobarbituric acid and 400 µl of 60 mM Tris-HCl with 0.1 mM diethylenetriaminepentaacetic acid. The mixture was incubated for 60 minutes at 100 °C, centrifuged

for 5 minutes at 11500 rpm, without light, and the absorbance was read at 535 nm. Results were expressed as nmol TBARS per g of wet weight using an $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ as molar extinction coefficient.

Enzymatic Activity: GST, CAT, and AChE

Glutathione-S-transferase and AChE activity was measured employing the PMS fraction following the methodology previously defined, although the activities were expressed as nmol/min/mg of protein.

CAT activity was assessed with PMS fraction through measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm (Clairborne, 1985). The reaction was done using a mixture of 135 µl of K-phosphate (pH 7.0; 0.05 M), 150 µl of 30% H_2O_2, and 15 µl of PMS. Absorbance was read at 240 nm for 2 min. The results were expressed as µmol minute/mg of protein employing the extinction coefficient ($\varepsilon = 40\ M^{-1}\ cm^{-1}$).

Total glutathione content (TGSH)

TGSH was measured from PMS fraction according to the recycling reaction of reduced glutathione supplemented with 5,50-dithiobis-(2-nitrobenzoic acid) in the presence of glutathione reductase excess (Baker et al., 1990). TGSH was quantified by adding 250 µl of the reaction solution and 50 µl of PMS, following the method described by Baker et al. (1990). The reaction solution was prepared with 18 ml of Na-K phosphate buffer (0.2 M; pH 8.0), 3 ml of adenine nicotinamide dinucleotide and adenine 2 beta-phosphate reduced the tetrasodium salt, 6 ml of 10 mM 5.5' -dithiobis (2-nitrobenzoic acid), and 1.5 ml glutathione reductase (25 µl stock with 1 U/ml). Absorbance was read at 412 nm for 3 minutes after the reduced glutathione recycling reaction in the presence of excess glutathione reductase. Total glutathione levels expressed as µmol per mg protein were calculated using a standard curve emloying L-GSH.

Energy reserves (lipids, carbohydrates and proteins contents) and energy consumption (ETS activity)

The methodology was developed according to De Coen & Janssen (1997) and modified by Rodrigues et al., 2015.

For Lipids: 300 µL homogenate were pre-treated with 500 µL of chloroform and 500 µL of methanol, mixed and then centrifuged for 5 minutes at 10000 rpm. 100 µL from the supernatant of each sample were collected to a new glass tube, 500 µL of sulfuric acid were added and incubated at 200 °C for 15 min. After, the samples were cooled at room temperature and mixed with 1500 µL ultrapure water. Finally, the absorbance was read at 375 nm employing the tripalmitine (palmitic acid triglyceride) as the standard.

For Carbohydrates and proteins: 300 µL of homogenate were mixed with 100 µL 15% (w/v) trichloroacetic acid and incubated at -20 °C for 10 minutes. Then, the samples were centrifuged, 10000 rpm 5 min, to obtain two phases. The supernatant contains the carbohydrates, 200 µL was collected from each sample and transferred to a glass tube. The volume was supplemented with 200 µL of 5% phenol and 800 µL of sulfuric acid. The mixture and standard curve with known glucose concentrations were incubated for 30 min at room temperature. The absorbance was read at 492 nm.

The pellet containing the proteins was suspended with 500 µL of NaOH, incubated at 60 °C for 30 min, and lastly, 280 µL of HCl was added. Total protein content was measured following Bradford's method (Bradford, 1976). The absorbance was read at 592 nm after 30 min of incubation using γ- globulin as a

standard. The energetic values were calculated based on the energy of the combustion of the different fractions (De Coen & Janssen, 1997).

The lipid, carbohydrates, and protein content were expressed as millijoules per milligram tissue.

ETS activity as energy consumption measurement was done from 300 µL homogenate mixed with 150 µL homogenization buffer (0.3 M Tris base; 0.45% (w/v) Poly Vinyl Pyrrolidone; 459 mM MgSO4; 0.6% (v/v) Triton X-100 at a pH of 8.5), centrifuged at 4 °C during 10 min (1000 rpm). 50 µL of supernatant was collected and 150 µL of buffered solution was added (0.13 M Tris base containing 0.27% (v/v) Triton X-100; 1.7 mM NADH; 274 mM NADPH) and 100 µL of INT solution (p-iodonitrotetrazolium; 8 mM). Finally, the absorbance was read at 490 nm for 3 min. The oxygen consumption rate was calculated based on the stoichiometric relationship (2 mmol of INT-formazan generate 1 mmol of oxygen $M^{-1}cm^{-1}$). Finally, defining of the caloric values was done by ETS consumed calculation and using the Lambert-Beer formula with ε= 15900 M^{-1} cm^{-1} activity using specific oxy enthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 kJ/mol. ETS is expressed as (millijoule/Planck's constant)/milligram tissue.

Statistical analysis and figure design

All the data collected from survival, gene expression, and enzymatic activity were evaluated employing the SPSS 25 software (IBM, USA). Firstly, it was checked whether the data accomplish the criterion of normality and homogeneity of variances through the Shapiro-Wilk and Levene tests, respectively. In case that the data follow a normal distribution, analysis of variance (ANOVA) test was selected. Within each ANOVA, a two-sided Dunnett's test was used to assess which treatment groups showed transcriptional activity significantly different from the control group at the $p \leq 0.05$ level. To evaluate the differences between treatments, Tukey, and Games-Howell tests were used. The non-parametric Kruskal-Wallis test was used for non-normal data. In all the cases, a significance level was fixed as (α =0.05). For figure construction and design, GraphPadPrism8 was employed.

ULTRAVIOLET FILTERS

4. ULTRAVIOLET FILTERS

Description, characteristics, and uses

The UVFs are compounds used to prevent damages generated by UV radiation (Serpone et al., 2007). They are inorganic and organic chemical components acting as a barrier to UV radiation, encompassing UVA:315-400nm; UVB:280-315nm. The inorganic filters (titanium oxide or zinc oxide) act by reflecting radiation, they have been used to a lesser extent, increasing their usage along the last years. Contrarily, organic filters absorb UV radiation and are the most employed UVFs in the industry, therefore they has been selected for this thesis.

UVFs are present in PCPs, like shampoos, lipsticks, and sunscreens (Li et al., 2007). The USA Food and Drug Administration (FDA) authorized the use of 16 different UV filters in the composition of PCPs, being 27 (25 organic and 2 inorganics) according to EU Scientific Committee on consumer products (European Parliament, 2009a). The formulation for sunscreens is based on maintaining the balance between the skin protection factor (SPF), UVA radiation absorption, and the optimal values of water resistance (Lionetti & Rigano, 2017). Many different combinations of filters variable in number, usually three, and one right stabilizer can be developed (Lionetti & Rigano, 2017). Besides their use as cosmetics, there many other applications for industrial products, food packaging, plastics, paints, textiles, and detergents, among others (Cadena-Aizaga et al., 2020). For all this, these compounds have been defined as CECs.

Organic filters are classified following their chemical structure and properties. Typically, they are divided among eleven families including benzophenone derivatives (composed by two benzene rings joined by a carbonyl group), p-aminobenzoic acid derivatives (containing one benzene ring substituted with an amino group), camphor derivatives (composed by terpenoid structure), and crylene derivatives (aromatic acrylates). The chemical properties determine their way of interacting with the environment and the organisms. It is expected their accumulation in sediments and biota because of their low water solubility and high log Kow, being considered persistent in the environment (Gago-Ferrero et al., 2012; Hopkins & Blaney, 2016).

Ultraviolet filters in the environment and biota

Nowadays, exposure to solar radiation, especially to UV radiation, has increased and has been related to skin damage, injuries, and diseases, even in the development of cancers such as melanoma (Osterwalder et al., 2014). It has led to a significant concern about human health, magnifying the consumption of UVFs mainly in lotion on spray format during the last years; the sales has reached values close to 161 882 779 bottles per year in the US between 2011-2016 (Teplitz et al., 2018). Hence, their presence was extended to all the environmental compartments being notable in water resources.

In part, due to the variable degradation efficiency, ranging 11% to 96% in the WWTPs then returning to the water (O'Malley et al., 2020). The UVFs were detected in the 95% of effluents from WWTPs in Korea and Australia in ranges of 120-2800 ng/L (Ekpeghere et al., 2016; O'Malley et al., 2020) besides from Europe (Magi et al., 2013; Rodil et al., 2012). As a consequence of their use associated with recreational activities, such as sunbathing, the presence of these compounds is seasonal (Ekpeghere et al., 2016). It has been reported in seawater, for example, beaches in Italy or France with values between 30 to 155 ng/L (Labille et al., 2020; Rainieri et al., 2017) or even in American Virgin Islands showing 1395 ng/L of BP-3 (Cadena-Aizaga et al., 2020).

Besides, in German and Japanese rivers and lakes, UVFs were detected 20 to 250ng/L and from no detected to 4928 ng/L, respectively (Kameda et al., 2011; Rodil & Moeder, 2008). Finally, they have also been detected even in drinking water (Rodil et al., 2012; Díaz-Cruz et al., 2012).

On the other hand, derived from their persistence and lipophilicity, UVFs are capable of entering in tissues and bioaccumulate in the organisms. Multiple studies detected the presence of UV filters in animal tissues in vertebrates reaching from 20 to 182 ng/g d.w in fishes from China or Norway (Cunha et al., 2018; Langford et al., 2015), even in humans with values in placenta tissues similar to 11.79 ng/g (Valle-Sistac et al., 2016). Due to the elevated presence in the water environment, aquatic organisms, especially invertebrates, are the main objective containing UVFs in their tissues; with 112 ng/g in clams tissues from Antarctic coast (Emnet et al., 2015), in mussels ranging 150 ng/g lipid of EHMC or 418 µg/kg d.w of 4MBC (Picot Groz et al., 2014; Vidal-Liñán et al., 2018) or corals (He et al., 2019; Tsui et al., 2017).

UVFs toxicity

The UVFs as EDCs alter estrogen, androgen, or non-nuclear receptors in vertebrates (Wang et al., 2016b). Benzophenones and camphor filters, provoked an estrogenic response in fishes (Kunz & Fent, 2006; Rodríguez-Fuentes et al., 2015), Xenopus (Kunz et al., 2004), and humans (Jiménez-Díaz et al., 2013; Molina-Molina et al., 2008). In invertebrates, camphor compounds demonstrated to affect endocrine activity (Schmitt et al., 2008), besides different UVFs altered receptors related to the endocrine system in *C. riparius* at the embryo or larval level (Ozáez et al., 2013, 2014; Ozáez et al., 2016). In line with this, the reproduction system, an essential key for the maintenance of the populations was altered by these compounds. Multigeneration exposure to BP-3 demonstrated defects in the fertility of *Chironomus riparius* (Campos et al., 2019), besides EMC provoked snail's reproduction toxicity (Kaiser et al., 2012)

Moreover, the toxicity of UVFs has been evaluated in aquatic organisms at different levels as survival, growth, or sub organismal level (gene expression, enzymatic activity, or biochemical response). Firstly, survival was affected by 4-MBC, increasing the mortality in snails (Schmitt et al., 2008), clams with an LC50 value of 7.71 µg/L (Santonocito et al., 2020), and the crustacea *Daphnia magna* with an EC50 of 0.80 mg/L (Sieratowicz et al., 2011), even increased bleaching events in corals (Danovaro et al., 2008). Physiological parameters as feeding and growth, among others, were also modified by different UV filters exposure (Campos et al., 2017, 2019).

At the sub-organismal level, UVFs were capable of modifying the biochemical response in insects and clams (Campos et al., 2019; Santonocito et al., 2020), altering the detoxification response or activate apoptosis or cellular stress response. UV filters in single or binary mixtures generated altered gene expression on different essential pathways in invertebrates as earthworms (Novo et al., 2019) or *Chironomus riparius* (Martín-Folgar et al., 2018; Ozáez et al., 2014, 2016).

In the present work, three compounds have been selected as representatives of the UVFs Ethylhexyl 4-(dimethylamino) benzoate (OD-PABA), Octocrylene (OC), and Benzophenone 3 (BP-3) (figure 8).

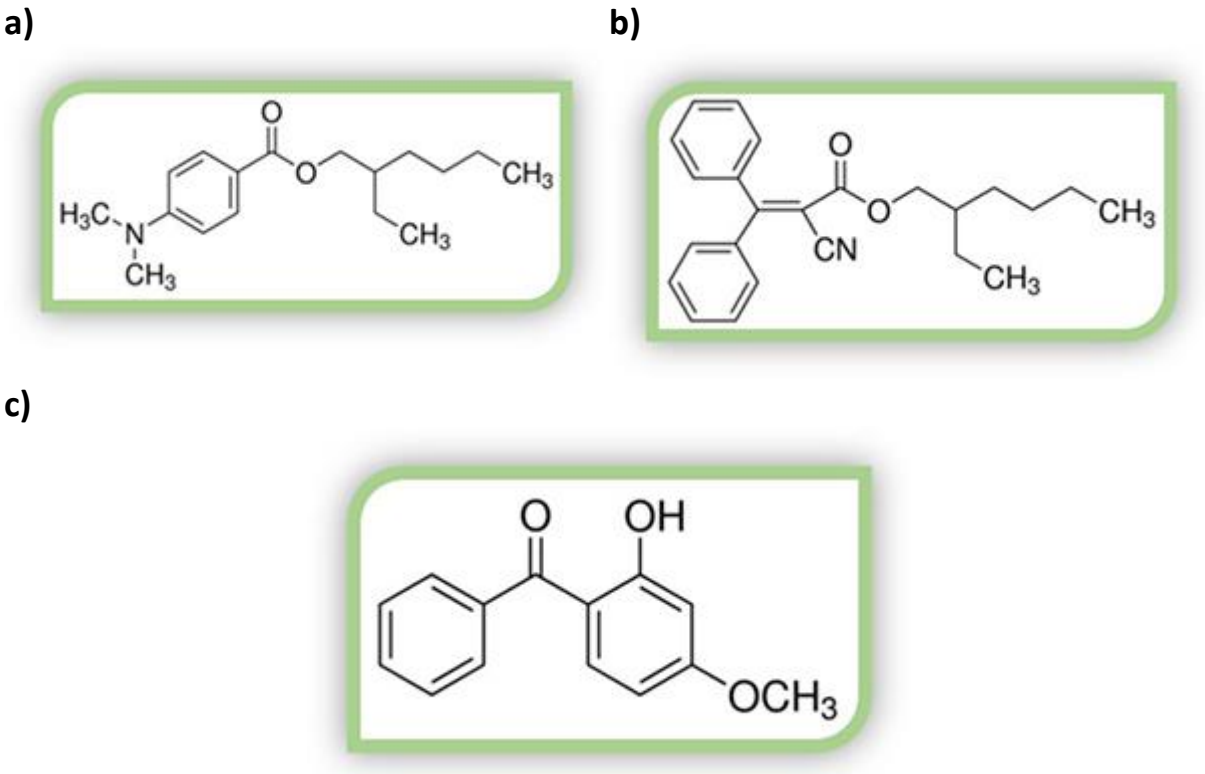

Figure 8. UV filters chemical structure, a) OD-PABA, b) Octocrylene, and c) Benzophenone-3.

OD-PABA, Octocrylene, and Benzophenone-3

OD PABA

OD -PABA is an UVF capable of absorbing the UVA radiation. It belongs to the p-aminobenzoic acid derivatives family, being a photo-sensible compound fast degraded by sun radiation (Rodil et al., 2009), determining their interaction with the media and biota. It was one of the components most used in sunscreen; however, due to EU legislation in 2008, it started to be replaced by other UVFs.

No efficient degradation treatment has been implemented in WWTPs to remove it (Tsui et al., 2014). Therefore, it has been detected in surface water and tap waters in the range near to 2 to 100 ng/L (Díaz-Cruz et al., 2012; Magi et al., 2013; Ma et al., 2016), in lakes around 5 ng/L (Rodil & Moeder, 2008), and sediments from non-detected to 5.2 ng/g (Gago-Ferrero et al., 2011). In farmed fish and wild mussels, it can accumulate up to 24.1 ng/g (dry weight) (Sang & Leung, 2016). In humans was detected in urine or placenta (Jimenez-Díaz et al., 2013). However, low information is available about the effects of OD-PABA, although its estrogenic power was demonstrated in humans (Schlumpf & Lichtensteiger, 2001) and in the invertebrate *C. riparius* (Ozáez et al., 2013). Moreover, alterations on biochemical biomarkers were detected in fishes (Ma et al., 2017).

Octocrylene

OC belongs to the Crylene derivatives family, used in most sunscreens' formulation due to their efficient UV radiation absorption properties. It is more photostable, refractory, and hydrophobic than other UV filters (Rodil et al., 2009). Because of it, it is detected in multiple environmental compartments. It appears in WWTPs effluents in variable ranges according to the elimination efficiency (68-88%) (Magi et al., 2013; O'Malley et al., 2020). Using UVFs increases in spring-summer, so OC was

detected in seawater, mainly on beaches around the world from 30 to 1324.9 ng/L (Labille et al., 2020; Tsui et al., 2015), and lakes or rivers (Li et al., 2007; Rodil & Moeder, 2008). Besides, it tends to accumulate in tissues of aquatic organisms as mammals (Gago-Ferrero et al., 2012) or fishes (Langford et al., 2015). In invertebrates, it has been reported in a variable range between 256 to 7112 ng/g (Bachelot et al., 2012).

Despite its extensive use, there is a lack of studies evaluating its effects on organisms. Some estrogenic effects were found in fishes (Kunz & Fent, 2009) and *Saccharomyces* (Balázs et al., 2016). The most relevant study on OC effects was done through transcriptomic evaluation in fishes, concluding that OC alters the expression of genes related to developmental processes in the brain and liver as well as metabolic processes in the liver (Blüthgen et al., 2014). On the other hand, it showed no effects in reproduction or development on relevant invertebrates as *Chironomus riparius*, *Lumbriculus variegatus*, *Melanoides tuberculata*, or *Potamopyrgus antipodarum* (Kaiser et al., 2012). At the molecular level, low information is available in invertebrates one study on *C. riparius* showed no alteration on the transcriptional level of the ecdysone receptor, estrogen-related receptor, or ultraspiracle (Ozáez et al., 2016b).

Benzophenone-3

BP-3 is a broad-spectrum UV absorber, possesses high lipophilicity, light stability, and bioaccumulation (Fent et al., 2010). As one of the most widely used UV filters, it has been detected in different environmental samples mostly in waters like wastewater effluents ranging from non-detectable levels to 153 ± 121 ng/L (Archer et al., 2017; Kim & Choi, 2014; Molins-Delgado et al., 2017), with variable removal efficiencies depending on the countries, 68-96% in Australia (O'Malley et al., 2020) and 56% in Spain (Molins-Delgado et al., 2017) even detected in sediments (Molins-Delgado et al., 2017; Tsui et al., 2015). In surface water as lakes or beaches, BP-3 was detected in values 22-118 ng/L (Downs et al., 2016; Tsui et al., 2017).

Similar to other UV filters, estrogenic activity has been described in humans and fishes (Blüthgen et al., 2012; Watanabe et al., 2015). BP-3 acts as a xenobiotic compound showing toxic reactions in corals and fishes from reef bleaching to mortality (DiNardo & Downs, 2018; Kim & Choi, 2014). Even in *Chironomus riparius*, it generated a decrease in larval growth, delayed development of the females, and a significant decrease in the average weight of males were observed at 0.75 and 3.41 mg/kg of BP-3, respectively (Campos et al., 2017b). At the molecular level, BP-3 alter endocrine receptors and stress response genes (Ozáez et al., 2014, 2016b).

Moreover, in the multi-stress context, these two UVFs were also evaluated combined with one commonly used plasticizer, Bisphenol A (BPA). Due to its widely used in the plastic containers as employed for PCPs and the risk to be liberated to the sunscreen's lotion.

Bisphenol A

Bisphenol A, 2,2-bis(4-hydroxyphenyl)propane), is a synthetic compound belonging to phenols widely used in polycarbonate and epoxy resins manufacturing (Jalal et al., 2018), its chemical structure is exposed in figure 9.

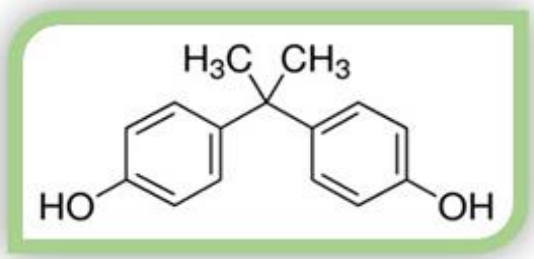

Figure 9. BPA Chemical structure**.**

It was one of the most used plasticizer compounds with millions of tons of production around the world (Jalal et al., 2018). Some of its applications are for kitchen utensils, packaging, bottles, inner coating of canned foods, and paper products (Geens et al., 2012; Rubin, 2011). However, the concern about the BPA has increased in the last years due to its proven endocrine disruption activity on humans and animals (In et al., 2019; Novo et al., 2018; Wang et al., 2019). Therefore, it was classified as EDC and CEC (Tijani et al., 2016), so its use has decreased in food packaging being replaced with other analogs as bisphenol P or S (Chen et al., 2016; Yu et al., 2015) but it still used for other applications.

The fact that BPA leaches out from the food and beverage containers (Almeida et al., 2018b) joined to the transmission by ingestion of seafood was demonstrated being present in human urine, blood, milk or placental tissue and umbilical cord blood (Foster et al., 2019; Kuruto-Niwa et al., 2007). Conversely, alteration of androgenic and estrogenic activity was observed (Delfosse et al., 2012; Okada et al., 2008) with reproduction effects (Galloway et al., 2010; Li et al., 2011). Moreover, BPA exposure was capable of altering the nervous system and has been related to different chronic diseases (Jiang et al., 2020b; Rezg et al., 2014).

Due to its ubiquitous nature and widespread use, it is detected in sediments and water resources around the world. A review of sewages from WWTPs showed values ranging in 49 to 1960 ng/L in Spain or China and even reaching up to 84110 ng/L in Brazil (Hu et al., 2019). In sediment, the values are about 2-144 µg/kg in Taiwan (Lee et al., 2020) and 97 ng/g in Italy (Pignotti & Dinelli, 2018). Finally, in freshwaters and tap water, BPA was detected in levels from 24 to 714 ng/L (Ashfaq et al., 2018; Jin & Zhu, 2016). BPA is also present in aquatic organisms' tissues from fishes (0.2-13000 ng/g), crustacea, and mollusks, converting in a hazardous compound for aquatic organisms (Corrales et al., 2015).

On the one hand, it was a proved EDC altering the endocrine system in fishes (Huang et al., 2017; Wang et al., 2019), crustacea (In et al., 2019; Nagato et al., 2016), mollusks (de Andrade et al., 2017; Morales et al., 2018), and insects (Park & Kwak, 2010). On the other hand, BPA induced defects in development and alteration on the stress response in different invertebrates as mussels and the insect *C. riparius* (Balbi et al., 2016; Lee et al., 2018; Stanković et al., 2020). All this supports the importance of assessing the effects of BPA on invertebrates, as the presence of the compound can be biomagnified through the food web reaching to the humans.

Results

The use and production of UV filters for personal care products and sunscreens has been increased the last years, partly due to growing concern about the effect of solar radiation (Watson et al., 2016). They have been detected in various environmental matrixes being notable in surface waters becoming a relevant problem on aquatic organisms (Campos et al., 2020; O'Malley et al., 2020; Santonocito et al., 2020), doing mandatory a better knowledge of mechanisms of action. Diverse studies have evaluated their toxicity on the aquatic biota but focused on single exposure. Moreover, a deeper analysis is requerided to know the effects at environmental realistic conditions.

The effects of UV filters on the gene expression of the fourth instar larvae of *Chironomus riparius* have been evaluated. To this end, different scenarios have been defined.

Firstly, larvae have been exposed to single and binary UVFs mixture. Then, a scenario combining one typical plasticizer such as BPA with these UVFs in single, binary, and ternary mixtures has been performed. As sunscreen's formulations contain a mixture of organic filters, the employ of binary and ternary mixtures aims to get closer to real conditions. Finally, due to the current context of climate change, the influence of temperature on a UV filter toxicity has been addressed.

Transcription profile alterations by the single exposure and binary mixtures of ultraviolet filters OD-PABA and OC

In this study, two of the typical organic UV filters used in sunscreens formulation were selected, OD-PABA and OC, for single and binary exposure. The aim was to evaluate the acute response (24h) of both UVFs at 0.1, 1, and 10 mg/L, by comparing the effects of single and combined exposure. The concentration and time were defined according to the laboratory's previous studies.

The selected endpoint was the expression profile of seventeen genes related to four different pathways, endocrine system, detoxification response, stress and immune response. The transcriptional activity was analyzed by Real-Time PCR and the results are shown in the figures 10, 11, and 12.

The results for the genes related to the hormonal system (*MAPR*, *EcR*, *Cyp18a1*, *JHAMT, Kr-h1*) showed no statistical differences in any of the treatments used (fig. 10), although some tendencies were observed.

For 20-E related genes, *EcR* showed a trend to decreased expression respect to the control by both UVFs treatments but contrary respect to the mixture. However, in *Cyp18a1,* it was contrary, with increased expression in all the cases being notable at 10 mg/L OD-PABA, but for *MAPR*, the expression was more or less maintained. Focusing on the genes related to JH, *JHAMT* presented a slight trend to decrease except in the case of OC 10 mg/L and the mixture at 0.1 and 1 mg/L. Finally, *Kr-h1* showed a general irregular expression, tending to increase its expression respect control with some exceptions.

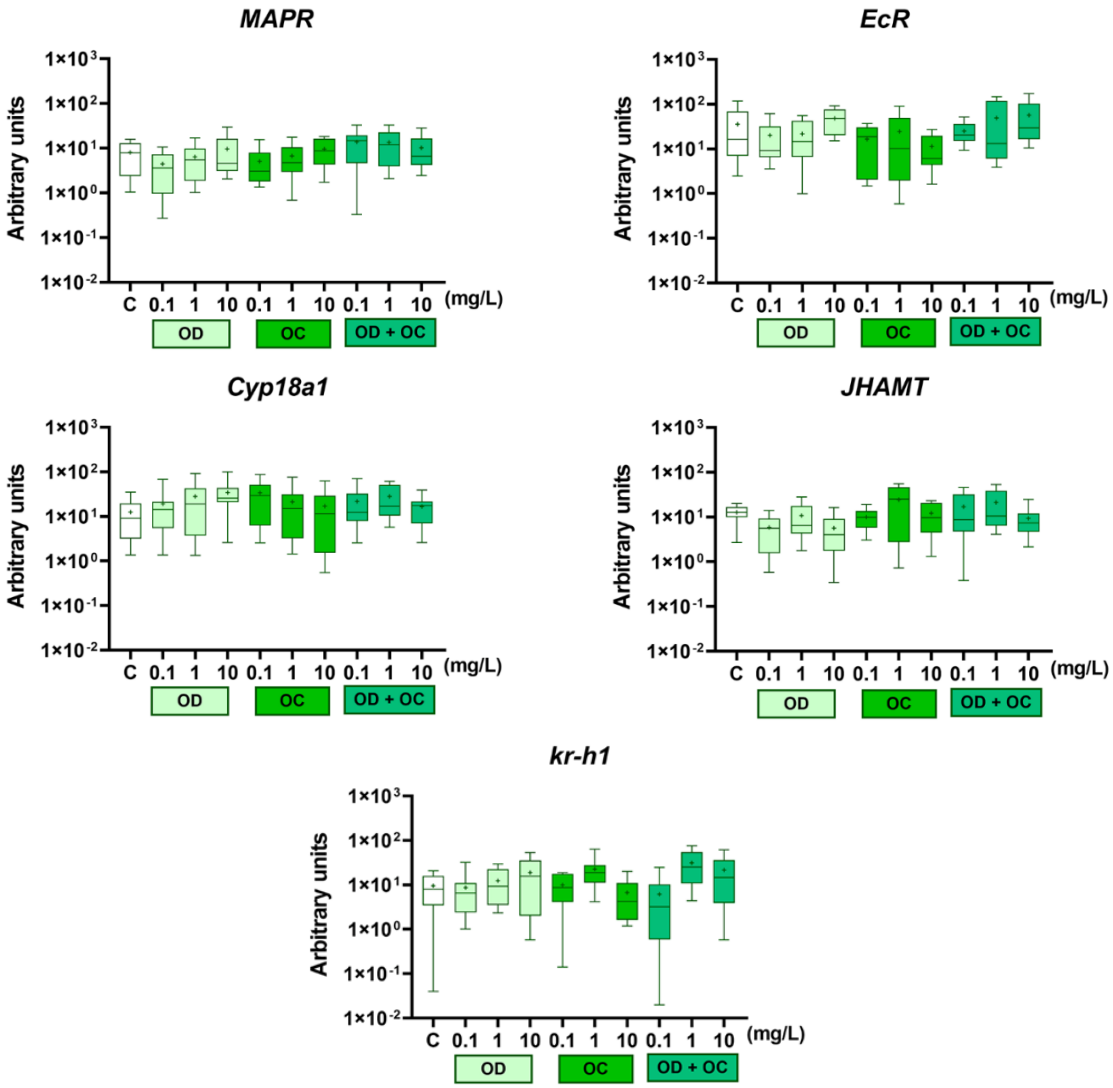

Figure 10. Expression of genes related to the endocrinesystem (*MAPR*, *EcR*, *Cyp18a1, JHAMT,* and *Kr-h1)* in fourth instar *C. riparius* larvae after exposure to 0.1, 1, or 10 mg/L OD PABA and OC in the single and binary mixture for 24 hours. mRNA levels were normalized using *GAPDH*, *rpL13*, and *TBP* as reference genes. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. The differences were defined according on ($p < 0.05$).

Detoxification response was studied employing eight genes. Four of them, *Cyp4d2, Cyp6b7*, *Cyp9f2*, and *Cyp12a2*, were related to the Phase I and the other four, *GSTd3, GSTo1, GSTe1,* and *GSTt1*, to Phase II (fig. 11). Similar to the endocrine system, no effects were detected in any of the genes evaluated from this system. However, some tendency was observed in their pattern expression.

For Phase I, *Cyp4d2* and *Cyp12a2* showed an irregular expression pattern with increased by OD-PABA 10 mg/L and decreased modulated by the higher concentrations of OC, but the mixtures seem to have a weak effect. The other two genes showed a more stable expression only with notable increased by OD-PABA at 1,10 mg/L in *Cyp9f2*. For the Phase II genes, spite no statistical differences, similarly to the CYPs genes, OD-PABA at 10 mg/L showed an increased tendency in all the GSTs. Moreover, the

expression starts to decrease by the OC exposure until returns to control similar levels at the mixture exposure

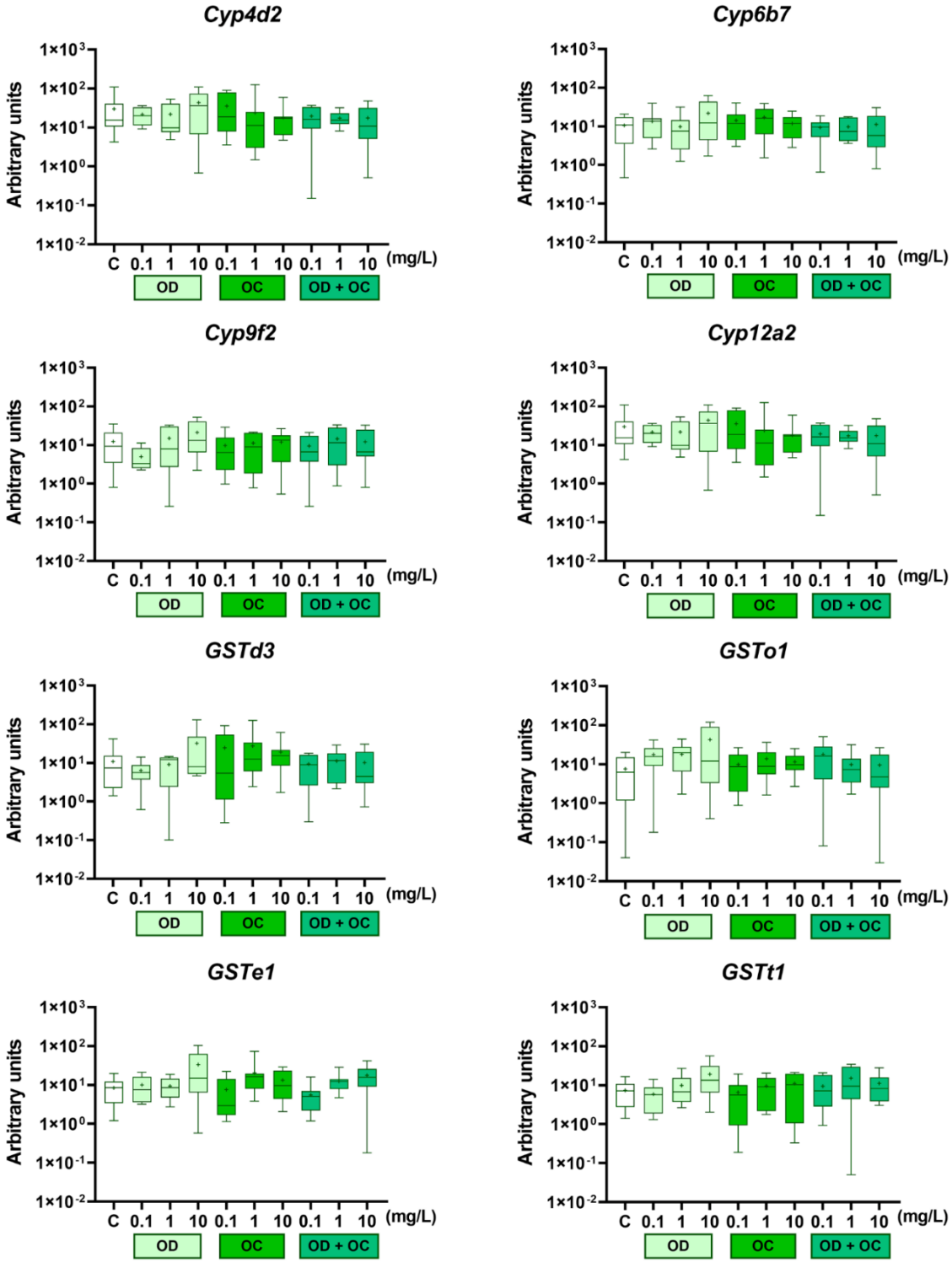

Figure 11. Expression of *Cyp4d2*, *Cyp6b7*, *Cyp9f2*,*Cyp12a2*, *GSTd*3, *GSTo1*, *GSTe*1, and *GSTt1* related to the detoxification response, in *C. riparius* larvae after in vivo exposure to 0.1, 1, or 10 mg/L OD PABA and OC in the single and binary mixture for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend, in all the cases ($p < 0.05$).

Two genes were selected for the study of the stress response; those that code for the Hypoxia upregulated protein 1 (*HYOU1*) and the Lethal 2 essential for life protein (*Le2fl*). Besides, the immunity was evaluated through two genes related to the humoral response, the Prophenoloxidase (*Proph*) and the AMP defensin (*Def*). In both cases, the results are shown in figure 12.

The stress response is the primary mechanism to maintain homeostasis in the cells against changing environmental conditions. *HYOU1* belongs to the Hsp70 family and is related to hypoxia events. In this study, it was altered at the higher concentration of OD-PABA respect to the control even differences respect to OC but biologically irrelevant. The other gene analyzed, *L2efl*, is a protein with the typical alpha-crystallin domain of sHSPs that upregulated at 1 and 10 mg/L of mixture but respect to 0.1 mg/L being not biologically relevant. Invertebrates immunity involves cellular and humoral responses, being crucial the second to repel the pathogens. The two genes tested concerning immunity were not modified, although appear to developed contrary tendencies with increased by OD-PABA on *Proph* and by OC on *Def*. However, no statistical differences were detected, so it seems that the immune system was not altered.

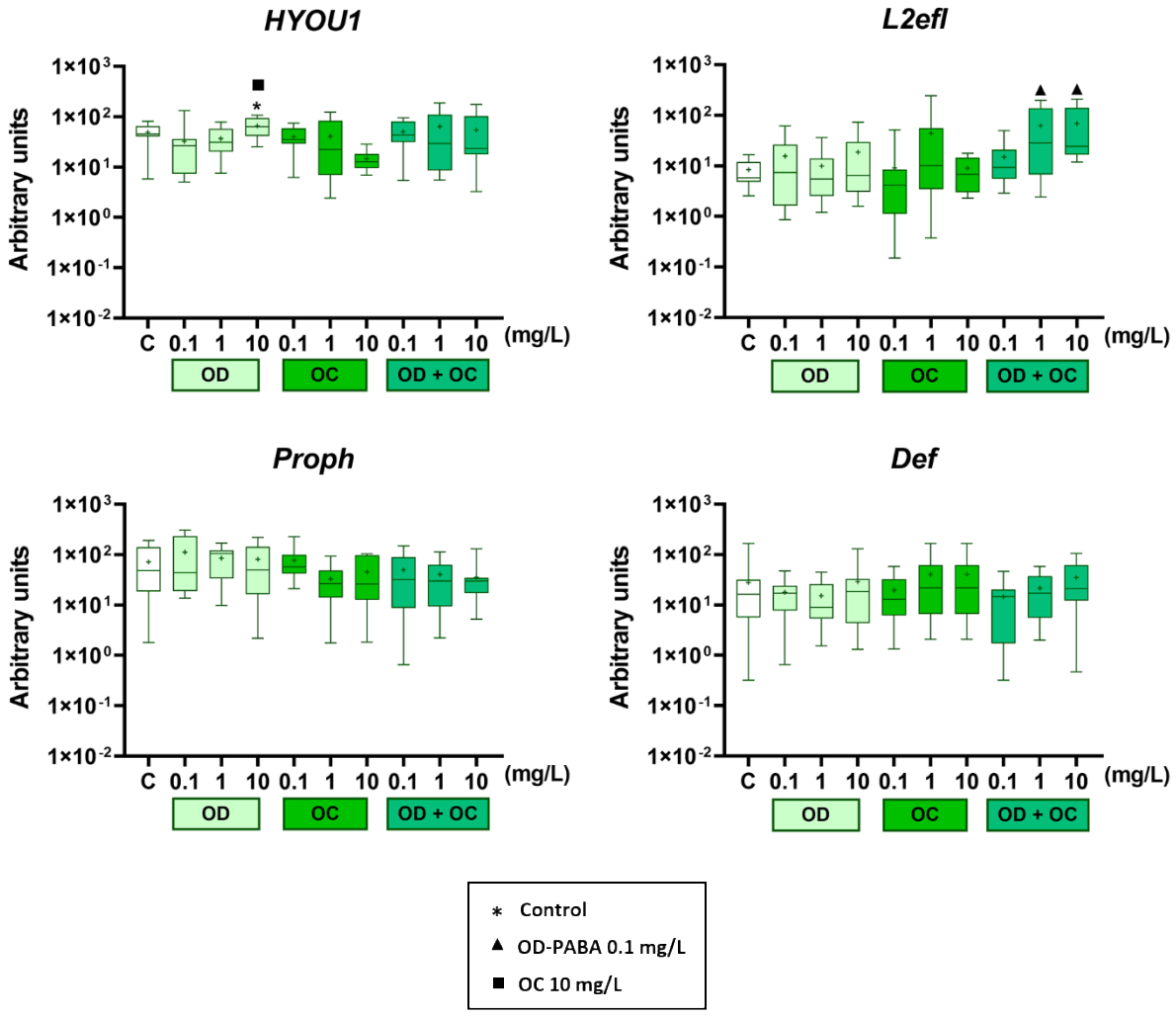

Figure 12. Expression of *HYOU1*, *L2efl* related stress response; *Proph*, *Def* related to the immune system, in fourth instar *C. riparius* larvae after exposure to 0.1, 1, or 10 mg/L OD PABA and OC in the single and binary mixture for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend, in all the cases ($p < 0.05$).

SUMMARY

- **No alterations are observed for the genes related to the hormonal system.**
- **Detoxification response were not modified at Phase I or II.**
- ***HYOU-1* was upregulated by OD-PABA 10 mg/L.**
- ***L2efl* mRNA levels are altered at mixture 1 and 10 respect to 0.1 mg/L OD-PABA.**
- **Immune system was not altered at these conditions.**

Effects of a single exposure, binary, and ternary mixtures of UV filters OD-PABA and OC, and the plasticizer BPA.

In line with the previous study, the combination of both UV filters with the plasticizer BPA was proposed and evaluated due to its wide application in multiple containers such as those used in PCPs. The octanol-water partition coefficient of the BPA suggests a putative transferring to the sunscreen inside to the bottle.

Therefore, this study evaluates the effects on gene expression of fourth instar larvae exposed to BPA, OC, and OD-PABA at 0.1 and 1mg/ L in single, binary, and ternary mixture exposure for short and medium-term exposure (24, 96h). In this case, an array employing 40 genes from five essentials routes in invertebrates was used. In this complex scenario, multiple comparisons were developed to know the relations between single and mixture exposure at both times, the combinations used are shown in table 10.

Comparison	Time
96h Vs. 24h	
Treatment Vs. control	24, 96h
BPA Vs. mixtures	24, 96h
OC Vs. Mixtures	24, 96h
OD-PABA Vs. mixtures	24, 96h
OC+OD-PABA Vs. BPA+OC-OD-PABA	24, 96h
BPA+OC Vs. BPA+OC+OD-PABA	24, 96h
BPA+OD.PABA Vs. BPA+OC+OD-PABA	24, 96h

Table 10. Comparisons used for the mixture's statistical analysis.

Because of the complex analysis, multiple figures were obtained (Annex 1), so the results were summarized in tables 11, 12, 13, 14, and 15. Five different systems were evaluated by using the designed array. The endocrine system was studied using ten genes (*MAPR, Dis, InR, EcR, Cyp18a1, E93, Dronc, JHAMT, Met, Kr-h1*) related to 20-E and JH, among other processes. The detoxification response was evaluated through ten genes for analysis of Phase I (*Cyp4d2*, *Cyp6b7*, *Cyp9f2*, *Cyp12a2*), Phase II (*GSTd3*, *GSTo1*, *GSTe1*, *GSTt1*) and Phase III (*MRP-1*, *ABCB6*). Five families of HSPs were evaluated to study the effects on stress response employing fourteen genes (*hsp70, hsc70*, *HYOU1*, *hsp40*, *hsp90*, *Gp93*, *hsp60*, *hsp10*, *hsp17*, *hsp21*, *hsp22*, *hsp23*, *hsp24*, *hsp27*) while immunity was represented by two genes (*Proph*, *Def*). Finally, the DNA repair system due to its essential role in the correct DNA maintenance was evaluated through five genes (*Decay, XRCC1*, *NLK*, *ATM*). As indicated, all the figures from those genes are in Annex 1.

Firstly, the expression between times was analyzed, showing a general downregulation at 96h respect to the short exposure at 24h, as is observed in table 11. Fifteen genes maintained their pattern

expression unaltered. For endocrine system (*Kr-h1*, *JHAMT*, and *InR*), for detoxification response (*Cyp9f2*, *Cyp12a2*, *GSTd3*, and *GSTo1*). For the stress response seems that more genes were unaltered (*hsc70*, *hsp40*, *hsp90*, *Gp93*, *hsp60*, *hsp17*, and *hsp21*). The rest of the genes showed decreased expression being notable for the DNA repair and immune system genes, with downregulation at all the conditions evaluated.

			Vs. 24h													
			96h													
Treatment			C	BPA		OC		ODPABA		OC+OD		BPA+OC		BPA+OD		BPA+OC+OD	
Concentration (mg/L)				0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1
Endocrine system	Ecdysone	*EcR*															
		E93															
		Kr-h1															
		Dronc															
		Dis															
		Cyp18a1															
	JH	*Met*															
		JHAMT															
		InR															
		MAPR															
Detoxification	Phase I	*Cyp4d2*															
		Cyp6b7															
		Cyp9f2															
		Cyp12a2															
	Phase II	*GSTd3*															
		GSTe1															
		GSTo1															
		GSTt1															
	Phase III	*MRP1*															
		ABCB6															
Stress response	HSP70	*hsp70*															
		hsc70															
		HYOU-1															
	HSP40	*hsp40*															
	HSP90	*hsp90*															
		Gp93															
		hsp60															
		hsp10															
	sHSPs	*hsp17*															
		hsp21															
		hsp22															
		hsp23															
		hsp24															
		hsp27															
DNA repair		*Decay*															
		XRCC1															
		NLK															
		ATM															
Immunity		*Def*															
		Proph															

Table 11. Summary of 96h expression results in respect to the 24h. (Red= Down-regulation)

Secondly, the differences in respect to the control at each time were evaluated. Focused on 24h low response was observed (table 12) for all the routes similar to the previous results with the OD-PABA and OC mixture. Only three genes from endocrine, detoxification, and stress response respectively were modified with upregulation in all the cases. First, the *Cyp18a1* at OC 1mg/L, the *GSTt1* was altered with increased expression at 24h at OC 1 mg/L, OD-PABA, and OD-PABA+OC for both concentrations and BPA+OC 0.1 mg/L. The other gene *hsp70* showed upregulation in all the cases from OC 0.1 mg/L to BPA+OC+OD-PABA 1 mg/L.

			Vs. Control													
			24h													
Treatment			BPA		OC		ODPABA		OC+OD		BPA+OC		BPA+OD		BPA+OC+OD	
Concentration (mg/L)			0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1
Endocrine system	Ecdysone	*EcR* *E93* *Kr-h1* *Dronc* *Dis* *Cyp18a1*														
	JH	*Met* *JHAMT*														
		InR *MAPR*														
Detoxification	Phase I	*Cyp4d2* *Cyp6b7* *Cyp9f2* *Cyp12a2*														
	Phase II	*GSTd3* *GSTe1* *GSTo1* *GSTt1*														
	Phase III	*MRP1* *ABCB6*														
Stress response	HSP70	*hsp70* *hsc70* *HYOU-1*														
	HSP40	*hsp40*														
	HSP90	*hsp90* *Gp93*														
		hsp60 *hsp10*														
	sHSPs	*hsp17* *hsp21* *hsp22* *hsp23* *hsp24* *hsp27*														
DNA repair		*Decay* *XRCC1* *NLK* *ATM*														
Immunity		*Def* *Proph*														

Table 12. Summary of 24h expression results in respect to the control. (Green = Up-regulation)

However, turning the attention on the 96h, the response compared to the control was strongly affected by many disruptions on the expression from all the routes evaluated (table 13). In all the cases, a higher alteration was observed in mixtures respect to the control, although OD-PABA in single exposure also generated diverse alterations. From the endocrine system, the *MAPR, InR, Cyp18a1, JHAMT, Met and Kr-h1* were downregulated; besides, the mRNA levels of *EcR, E93, Dis,* and *Dronc* were upregulated. In the case of the detoxification genes (*Cyp6b7*, *Cyp9f2, GSTo1*) showed decreased expression and *Cyp12a2*, *GSTe1*, and *ABCB6* were upregulated. In comparison to control, mRNA levels were downregulated for *hsp40*, *hsp17,* and *hsp24,* although increased activity was observed for *hsp10*, *hsp21, hsp22,* and *hsp23*. Moreover, combined alterations with up and downregulation were observed for *hsp70, hsp90, Gp93,* and *hsp27.* According to the immune genes, only *Def* showed decreased expression by BPA+OC and BPA+OD-PABA. From the genes related to DNA repair, increased expression

was observed on three genes (XRCC1, NLK, and ATM) by OC, OD-PABA, or mixtures but with the exception for the fourth gene *Decay with* decreased expression at BPA 0.1 mg/L.

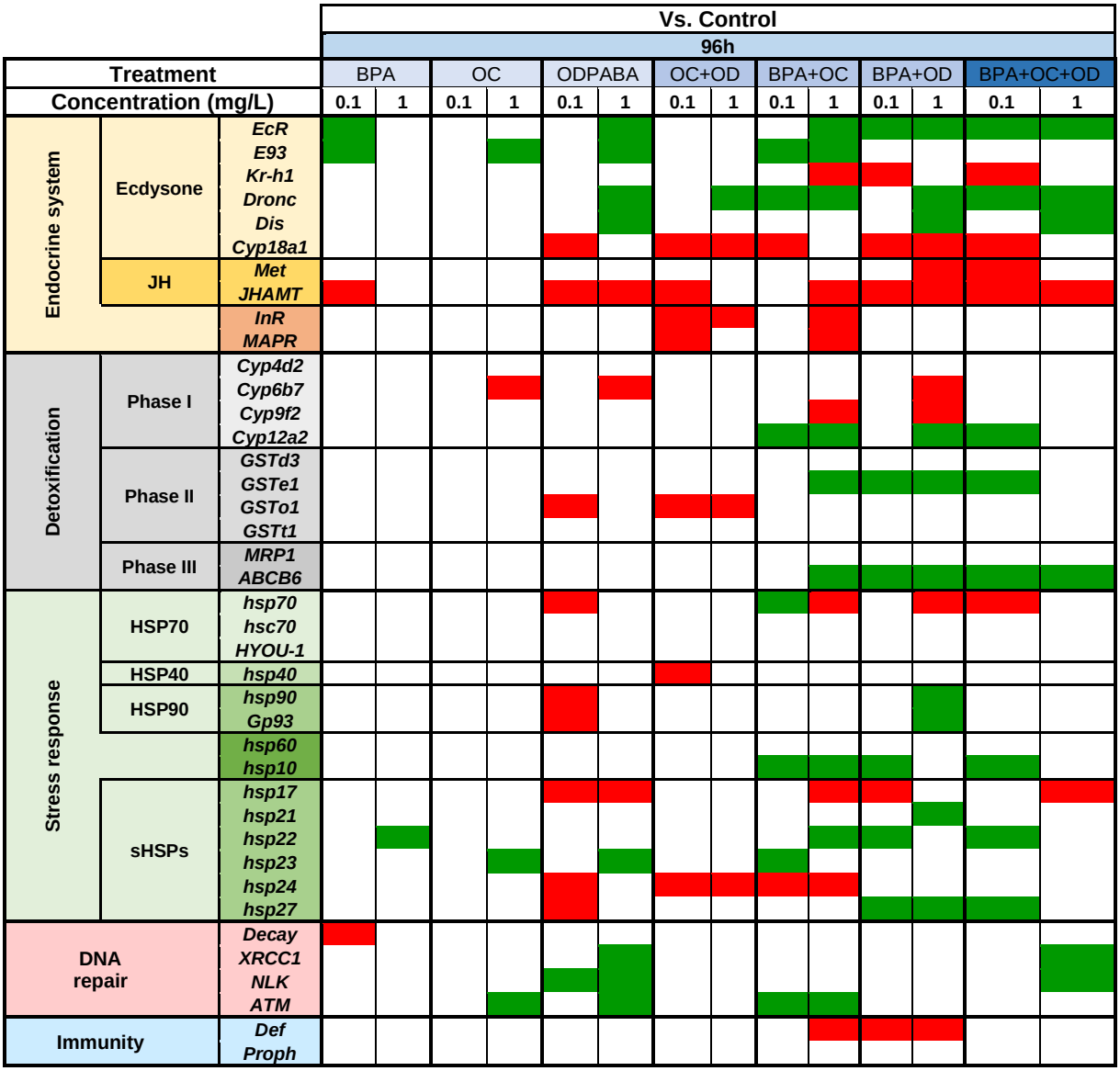

Table 13. Summary of the expression results from 96h respect to the control. (Green = Up-reulation, Red= Down-regulation).

Next, the single exposure respect to the mixtures was evaluated at each time. At 24h, no effects were observed in any of the genes studied, so no summary table is shown. However, many differences were detected by medium-term exposure in all the mixtures respect to the single exposure (table 14).

Respect to BPA, BPA+OC showed upregulated for *EcR*, *Dronc, GSTe1*, *ABCB6, Gp93, hsp10, hsp23, Decay, and ATM* while *MAPR*, *Cyp18a1, Cyp6b7, hsp70, hsp17, hsp24, Def* were downregulated. For BPA+OD-PABA, the genes with increased expression were *Dis, EcR, Dronc, GSTe1*, *ABCB6, hsp40*, *hsp90*, *Gp93*, *hsp10*, *hsp27, Decay, and XRCC1.* Contrary *Cyp18a1, JHAMT, Cyp9f2b, hsp70, hsp17, Def were* decreased. For BPA+OC+OD-PABA, increased expression was observed on *Dis*, *InR, EcR, hsp40*, *hsp10*, *hsp27, Decay, XRCC1, NLK, and ATM.* However, *E93*, *Cyp18a1, Met, Kr-h1*, *hsp70*, *hps17,* and *hsp23* showed decreased expression.

Respect to OC, the mixture of OC+OD-PABA showed increased expression for *MAPR, and Decay* but downregulation on *Dis*, *InR, JHAMT, GSTo1, ABCB6*, *hsp70*, *hsp40*, *hsp17, hsp24*, *hsp27, XRCC1, NLK, and ATM.* For BPA+OC, *MAPR, Cyp12a2, GSTe1, and hsp10 were* upregulated *while Dis, Cyp18a1, JHAMT, Cyp9f2*, *hsp17, and XRCC*1 were downregulated. For the BPA+OC+OD-PABA mixture, the genes *EcR, InR, Cyp12a2*, *GSTe1*, *ABCB6*, *hsp10, hsp21, and hsp22* were up-regulated; but *Kr-h1 JHAMT,* hsp70 and *the sHSPs 17, and 23* were down-regulated.

The comparison to OD-PABA showed, for OC+OD-PABA, only upregulation on *GSTt1* while *InR*, *EcR, hsp70*, *hsp22 XRCC1*, *NLK*, and *ATM were* downregulated. BPA+OD-PABA mixture increased mRNA levels for *MAPR*, *Cyp12a2*, *GSTt1, ABCB6, hsp40, 90, 10, 21 and 27* was observed. Besides *Met, Cyp9f2*, *GSTo1*, *hsp17*, *23*, *24*, and *ATM* were down-regulated. For the BPA+OC+OD-PABA, the mRNA levels of *MAPR*, *GSTt1*, *ABCB6*, *hsp40*, *17*, and *21* were increased while *Kr-h1*, *Met*, *GSTo1*, *Gp93*, *hsp24*, and *NLK* were down-regulated.

			Vs. BPA						Vs. OC						Vs. OD-PABA					
			96h						96h						96h					
Treatment			BPA+OC		BPA+OD		BPA+OC+OD		OC+OD		BPA+OC		BPA+OC+OD		OC+OD		BPA+OD		BPA+OC+OD	
Concentration (mg/L)			0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1
Endocrine system	Ecdysone	*EcR*																		
		E93																		
		Kr-h1																		
		Dronc																		
		Dis																		
		Cyp18a1																		
	JH	*Met*																		
		JHAMT																		
		InR																		
		MAPR																		
Detoxification	Phase I	*Cyp4d2*																		
		Cyp6b7																		
		Cyp9f2																		
		Cyp12a2																		
	Phase II	*GSTd3*																		
		GSTe1																		
		GSTo1																		
		GSTt1																		
	Phase III	*MRP1*																		
		ABCB6																		
Stress response	HSP70	*hsp70*																		
		hsc70																		
		HYOU-1																		
	HSP40	*hsp40*																		
	HSP90	*hsp90*																		
		Gp93																		
		hsp60																		
		hsp10																		
	sHSPs	*hsp17*																		
		hsp21																		
		hsp22																		
		hsp23																		
		hsp24																		
		hsp27																		
DNA repair		*Decay*																		
		XRCC1																		
		NLK																		
		ATM																		
Immunity		*Def*																		
		Proph																		

Table 14. Summary of 96h expression results, comparing mixtures respect to the single exposure. (Green = Up-regulation, Red= Down-regulation).

Finally, the binary mixtures were compared to the ternary mixture BPA+OC+OD-PABA, the sum-up of

Finally, the results are presented in Table 15. Respect to OC+OD-PABA, the ternary mixture showed upregulation on *EcR, Dronc, Cyp18a1, Cyp12a2*, *GSTo1*, *GStt1*, *ABCB6, hsp40*, *hsp21*, *hsp22*, *hsp24, hsp27, XRCC1* and *NLK* genes. Although *E93, Kr-h1, Met, Cyp9f2, Gp93,* and *hsp23* were downregulated at this condition. Compared to BPA+OC, the genes with increased mRNA levels were *MAPR*, Dis*, Cyp18a1, Dronc, Met, Cyp6b7, GSTt1, hsp21, 24, 27*, and *XRCC1*. Besides *E93, Kr-h1, Met,* Cyp*12a2, GSTo1, hsp90, and 23* showed decreased expression. Respect to the BPA+OD-PABA, the BPA+OC+OD-PABA showed upregulated expression only on *Met* and *NLK*, while downregulated was observed on *Cyp12a2, Gp93, hsp10, and XRCC1.*

			Vs. OC+OD		Vs. BPA+OC		Vs. BPA+OD	
			96h		96h		96h	
Treatment			BPA+OC+OD		BPA+OC+OD		BPA+OC+OD	
Concentration (mg/L)			0.1	1	0.1	1	0.1	1
Endocrine system	Ecdysone	*EcR*						
		E93						
		Kr-h1						
		Dronc						
		Dis						
		Cyp18a1						
	JH	*Met*						
		JHAMT						
		InR						
		MAPR						
Detoxification	Phase I	*Cyp4d2*						
		Cyp6b7						
		Cyp9f2						
		Cyp12a2						
	Phase II	*GSTd3*						
		GSTe1						
		GSTo1						
		GSTt1						
	Phase III	*MRP1*						
		ABCB6						
Stress response	HSP70	*hsp70*						
		hsc70						
		HYOU-1						
	HSP40	*hsp40*						
	HSP90	*hsp90*						
		Gp93						
		hsp60						
		hsp10						
	sHSPs	*hsp17*						
		hsp21						
		hsp22						
		hsp23						
		hsp24						
		hsp27						
DNA repair		*Decay*						
		XRCC1						
		NLK						
		ATM						
Immunity		*Def*						
		Proph						

Table 15. Summary of 96h expression results, comparing the ternary mixtures respect to the binary mixtures**.**

SUMMARY

- **The comparison at 96h respect to 24h showed decreased mRNA levels for *MAPR, Dis, EcR, Cyp18a1, E93, Dronc, Met, Cyp4d2, Cyp6b7, Cyp9f2, GSTe1, MRP-1, ABCB6, hsp70, HYOU1, hsp90, hsp10,* sHSPs *(*17, 22, 23, 24, 27*) Proph, Def, Decay, XRCC, NLK,* and *ATM.***
- **Low effects were observed for 24h exposure respect to the control with upregulation only on *Cyp18a1, GSTt1,* and *hsp70.***
- **For 96h respect to the control the results showed:**
  - **Increased expression for Dis, *EcR, E93, Dronc, Cyp12a2, GSTe1, ABCB6, hsp10, hsp21, hsp22, hsp23, XRCC1, NLK, and ATM.***
  - **Decreased expression for *MAPR, InR, Cyp18a1, JHAMT, Kr-h1, Met, Cyp6b7, Cyp9f2, GSTo1, hsp40, hsp17, hsp24, Decay* and *Def.***
  - **Combined up and downregulation was observed for *hsp70, hsp90, Gp93, and hsp27.***
- **The comparison between single and mixtures exposure showed altered expression for all the genes at 96h except for *Cyp4d2, GSTd3, MRP-1, hsc70, HYOU1, hsp60, and Proph.***
- **The differences on ternary mixtures respect to the binary showed altered expression for 96h except for *InR, JHAMT, Cyp4d2, GSTd3, GSTe1, MRP- 1, hsp70, hsc70, HYOU1, hsp60, hsp17, Decay, ATM, Proph,* and *Def.***

Combined effects of BP-3 and temperature (18.5 and 23 °C) on survival, gene expression, and enzymatic activity.

Climate change has been determined as one of the critical problems to face in our decade, being the altered temperature pattern one of the most representative affections on ecosystems and modifying the toxicity assessment (Landis et al., 2013). Xenobiotics behavior as toxicokinetic changes with temperature (T) variations (Leon, 2008; Marchand & Haddad, 2017). On the other hand, T affects the organism's response at different levels (Collier & Gebremedhin, 2015; Wojda, 2017). In aquatic organisms, being the majority poikilotherms, the temperature variations can alter the metabolism actively (Lim et al., 2017). Some studies have evaluated this complex scenario in different organisms (Guo et al., 2018; Kimberly & Salice, 2014), but with low information at the molecular level and mainly focus on T (Dölling et al., 2016; Ikeda et al., 2017; Liu et al., 2018). BP-3 was the selected because its extensive presence in surface waters (Downs et al., 2016) besides its toxicity was demonstrated for aquatic organisms as *C. riparius* among others (Campos et al., 2017b; DiNardo & Downs 2018; Ozáez et al., 2014, 2016).

The aim was to analyze the effect of T on physiology by exposing the animals to different concentrations of BP-3 at two T (18.5 and 23 °C) and two times, 8 and 24 hours. Gene expression profile and enzymatic activities were selected as parameters at the molecular level. Furthermore, the survival was tested until 96h of exposure. 8 hours of exposure were selected to elucidate the rapid response to BP-3 and test whether the temperature causes an accelerated response at the gene expression level. According to Ozáez et al., 2014 that observed BP-3 response in minutes.

Survival effects

No previous studies have been found evaluating survival for BP-3 plus T in *C. riparius*. Therefore, a survival study was done to know the effects and to define the adequate concentrations for molecular

studies. The survival curve included five different BP-3 concentrations (0, 10, 100, 1000, and 10000 µg/L) that were analyzed at 18.5 and 23 °C. Death larvae were counted every 24 hours, and the results are shown in Table 16. The survival in the culture temperature, 18.5 °C, showed statistically significant differences at 72 h for 10, 1000, 10000 µg/L, and at 96h for 100, 1000 and 10000 µg/L. In the temperature mimicking the climate change, 23 °C, altered survival compared to control treatment was observed at 24h (10, 100 µg/L), 72h (10, 100, 10000), and 96h (10 µg/L). Moreover, comparison among both temperatures rendered differences for 10 µg/L (96h), 100 µg/L (24, 72h) and 10000 µg/L (72h). According to these results, the concentrations selected to perform the molecular analysis were 10 and 100 µg/L.

		Benzophenone 3 (µg/L)				
T(°C)	**Time (h)**	**0**	**10**	**100**	**1000**	**10000**
18.5	**24**	98.89 ± 1.57	93.33 ± 4.71	90.44 ± 4.43	85.55 ± 4.16	96.66 ± 2.72
	48	96.66 ± 0.00	90 ± 2.72	87.77 ± 4.16	83.32 ± 2.72	90 ± 2.72
	72	94.44 ± 1.57	86.66 ± 1.93^{C}	83.33 ± 4.71	76.88 ± 3^{C}	84.44 ± 4.16^{C}
	96	92.22 ± 1.57	85.55 ± 3.14	77.78 ± 3.14^{C}	74.44 ± 4.16^{C}	82.22 ± 4.16^{C}
23	**24**	96.66 ± 0.00	92.22 ± 1.57^{C}	90 ± 1.57^{C*}	94.44 ± 4.16	93.33 ± 0.00
	48	91.11 ± 3.14	88.22 ± 1.37	85.55 ± 1.57	90 ± 4.71	86.66 ± 2.72
	72	90 ± 2.72	81.11 ± 3.14^{C}	77.55 ± 1.57^{C*}	86.67 ± 2.72	79.99 ± 4.71^{C*}
	96	85.55 ± 3.14	75.55 ±1.57^{C*}	75.55 ± 4.16	73.33 ± 2.72	75.55 ± 3.66

Table 16. Survival percentages of *C. riparius* fourth instar larvae exposed at BP-3 and two temperatures. The **C** correspond to differences with control, and the ***** define differences respect to the same concentration at 18.5 °C. All the differences were fixed according to ($p < 0.05$).

Gene expression profile

As a first approximation to this unexplored multi-stress scenario, the study was performed with a limited number of representative genes from different relevant cellular pathways. Firstly, the endocrine system was analyzed by studying *EcR, InR*, and *Met* genes. The results are shown in figure 13. At 8h and 18.5 °C no effects were dectected. Compared to those levels at 8 hours, there were differences at 24 hours for the three genes at both concentrations tested, but not for the control, with a significant effect derived from higher temperatures. Focusing on each gene, *EcR* showed down-regulation at 8 hours, the temperature modifies the response; since those samples with BP-3 modified their transcriptional activity compared to control at 23 °C, but also when compared to their counterparts at 18.5 °C. When turned the attention to 24 hours, 10 µg/L of BP-3 significantly downregulated the transcriptional activity, respect to the control at 23 °C. Besides, the results also showed that, compared to 8 hours of treatment, there were changes for 18.5 °C and 23 °C.

The other two hormone receptor genes studied showed a less complicated pattern. On the one hand, mRNA levels of BP-3 exposed larvae were increased for *InR* at 24h respect to the counterparts at 8h in both temperatures, being notable for the treatment with 10 µg/L at 18.5 °C. On the other hand, *Met* showed a similar result when mRNA levels at 8h and 24h were compared. However, additionally, a downregulation is observed for 10 and 100 µg/L compared to control in the treatment at 8h and 23 °C.

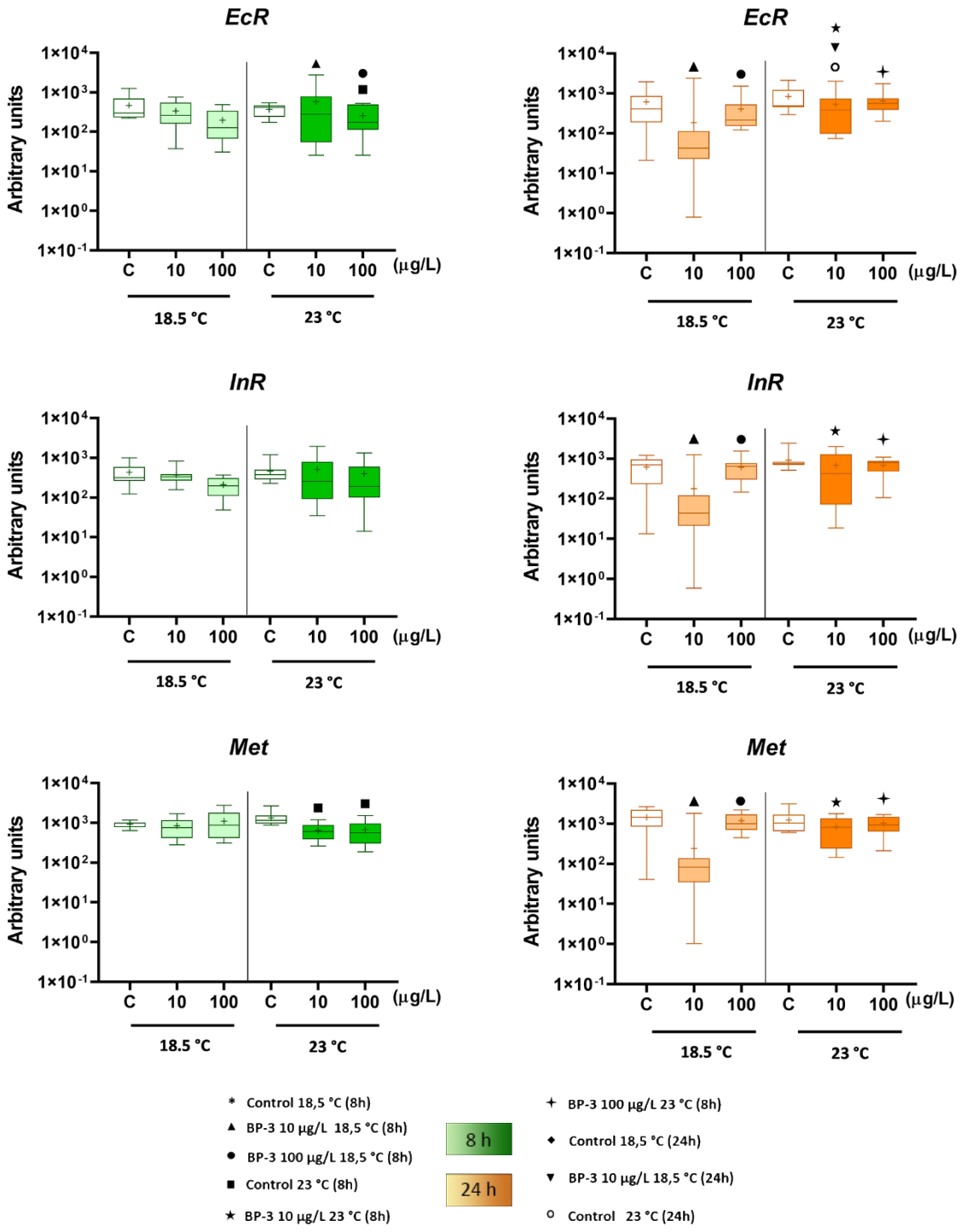

Figure 13. Expression of genes from the endocrine response (*EcR*, *InR*, and *Met*) in fourth instar *C. riparius* larvae after exposure to BP-3 (10 or 100 µg/L) at 18.5 and 23 °C for 8, 24h. mRNA levels were normalized using *GAPDH*, *rpL11,* and *rpL13* as reference genes. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. All significant differences respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases ($p < 0.05$).

The next cellular process evaluated was detoxification mechanisms. As in the previous point, Phase I and Phase II genes were analyzed. Here two representatives of each Phase were selected: *Cyp4d2* and *Cyp6b7, and GSTd6,* and *GSTo1*. All genes were affected by any of the conditions tested. The results are shown for Phase I genes (fig.14) and for Phase II (fig.15). At 8h, no effects were observed on CYPs genes, but at 24h, both genes disrupted their expression at both temperatures with a substantial decrease compared to the counterpart at 8h but without differences respect to the control.

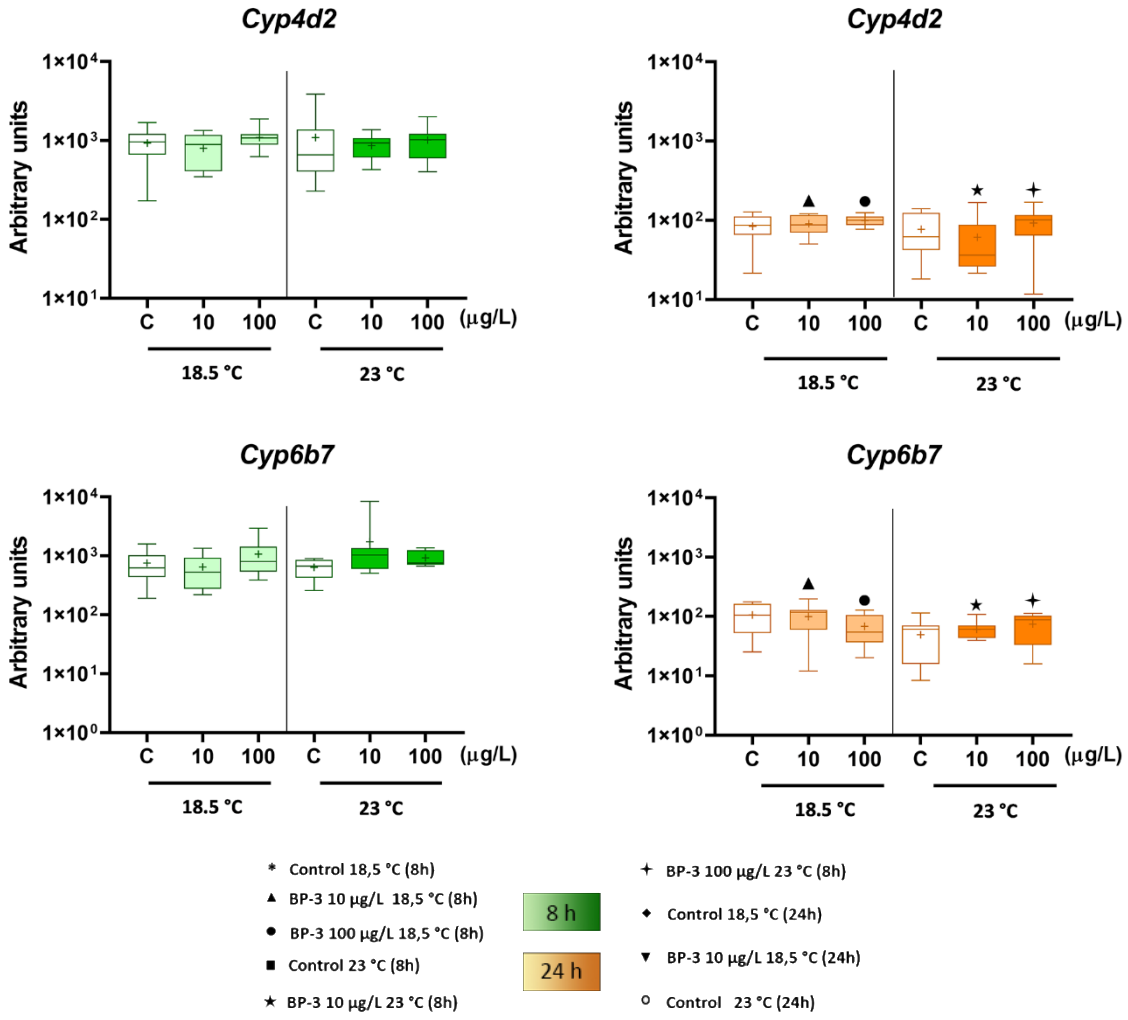

Figure 14. Expression of *Cyp4d2*, and *Cyp6b7* related to Phase I detoxification response in *C. riparius* larvae after exposure to BP-3 (10 or 100 µg/L) at 18.5 and 23 °C for 8, 24h. All significant differences respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases ($p < 0.05$).

For Phase II, only *GSTd6* was altered at 8h. BP-3 causes alteration at the medium concentration at 18.5 °C, respect to control. Also, the comparison among temperatures showed that for 10 µg/L, there is an increase at 23 °C compared to 18.5 °C. More complexity is observed at 24h. For both temperatures, the two genes modified the transcription compared to the counterpart with at 8h, with a decreased expression. Besides, *GSTo1* showed significant differences at 100 µg/L respect to the control (18.5 °C),

besides at 10 µg/L respect to the 18.5°C. As happened with the endocrine-related genes, no changes were detected between controls at different temperatures or time.

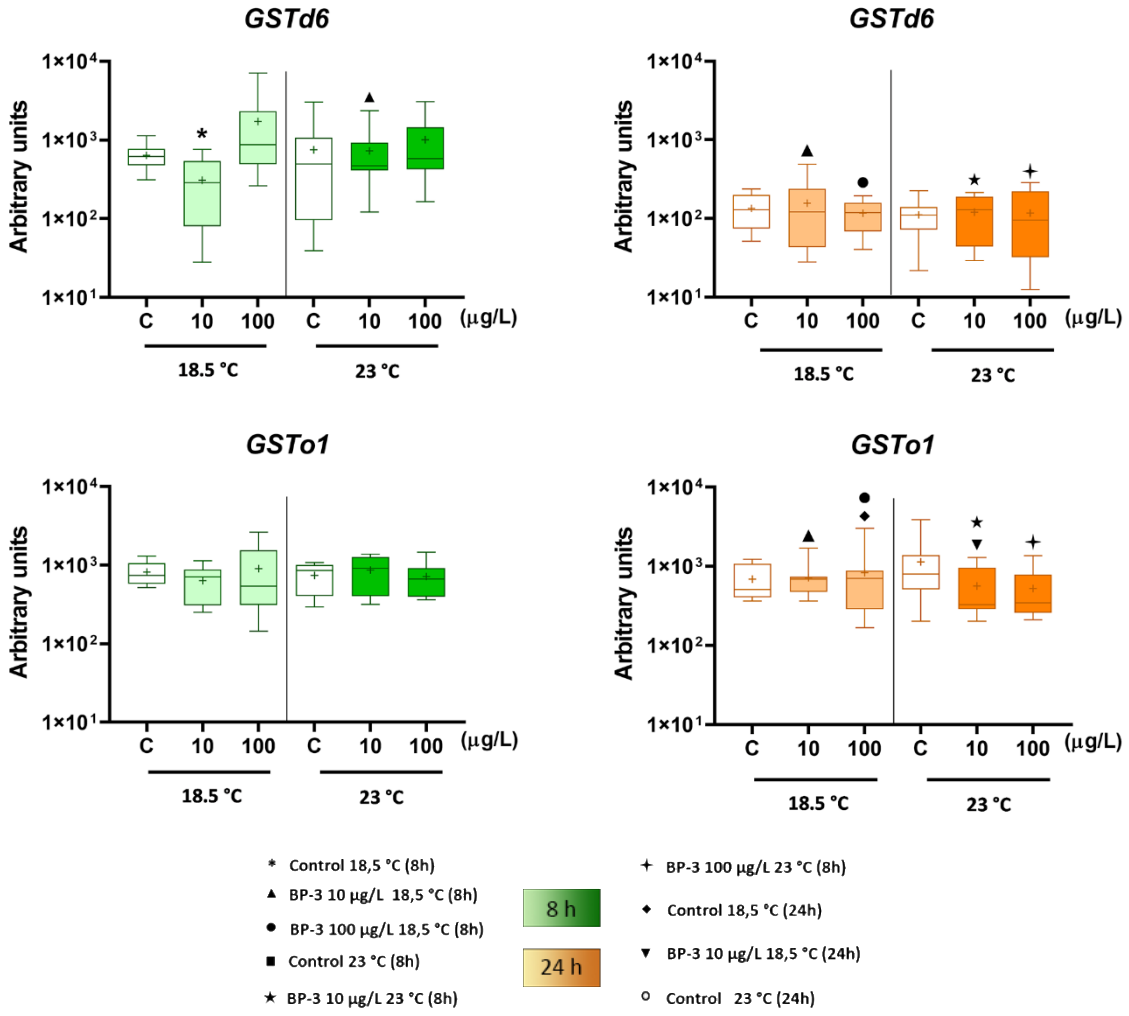

Figure 15. Expression of *GSTd6*, and *GSTt1* related to detoxification response in the fourth instar *C. riparius* larvae after exposure to BP-3 (10 or 100 µg/L) at 18.5 and 23 °C for 8, 24h. All significant differences respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases ($p < 0.05$).

Due to their fundamental role in the stress response, five genes (*hsp70, HYOU1, Gp93, hsp22,* and *hsp27*) belonging to three different families HSP70, HSP90, and sHSPs respectively, were analyzed. The results are shown in Figures 16 and 17.

At 8h and 18.5 °C, only *HYOU1* was altered at 10 µg/L with respect to the control. Besides at this time increased expression was stimulated by 23 °Cat both concentrations. On the other hand, at 24h exposure, the three genes were modified at both T compared to the counterparts at 8h (fig. 17). Besides, *hsp70* showed upregulation respect to the control at 100 µg/L and 18.5 °C; at 23 °C the 10 µg/L was downregulated respect to the control temperature at the same time. For the sHSPs, at 8h and 18.5 °C, both genes were altered with respect to the control for *hsp27* at 10 µg/L and for *hsp22* at both concentrations tested. Conversely, at 23 °C, the sHSPs, *hsp22*, and *hsp27* were altered compared to control, showing down and up-regulation respectively. Besides, when compared between

temperatures at 8h, the transcriptional activity of both genes were altered. On the other hand, at 24h exposure, the two genes were altered at both T compared to the counterparts at 8h.

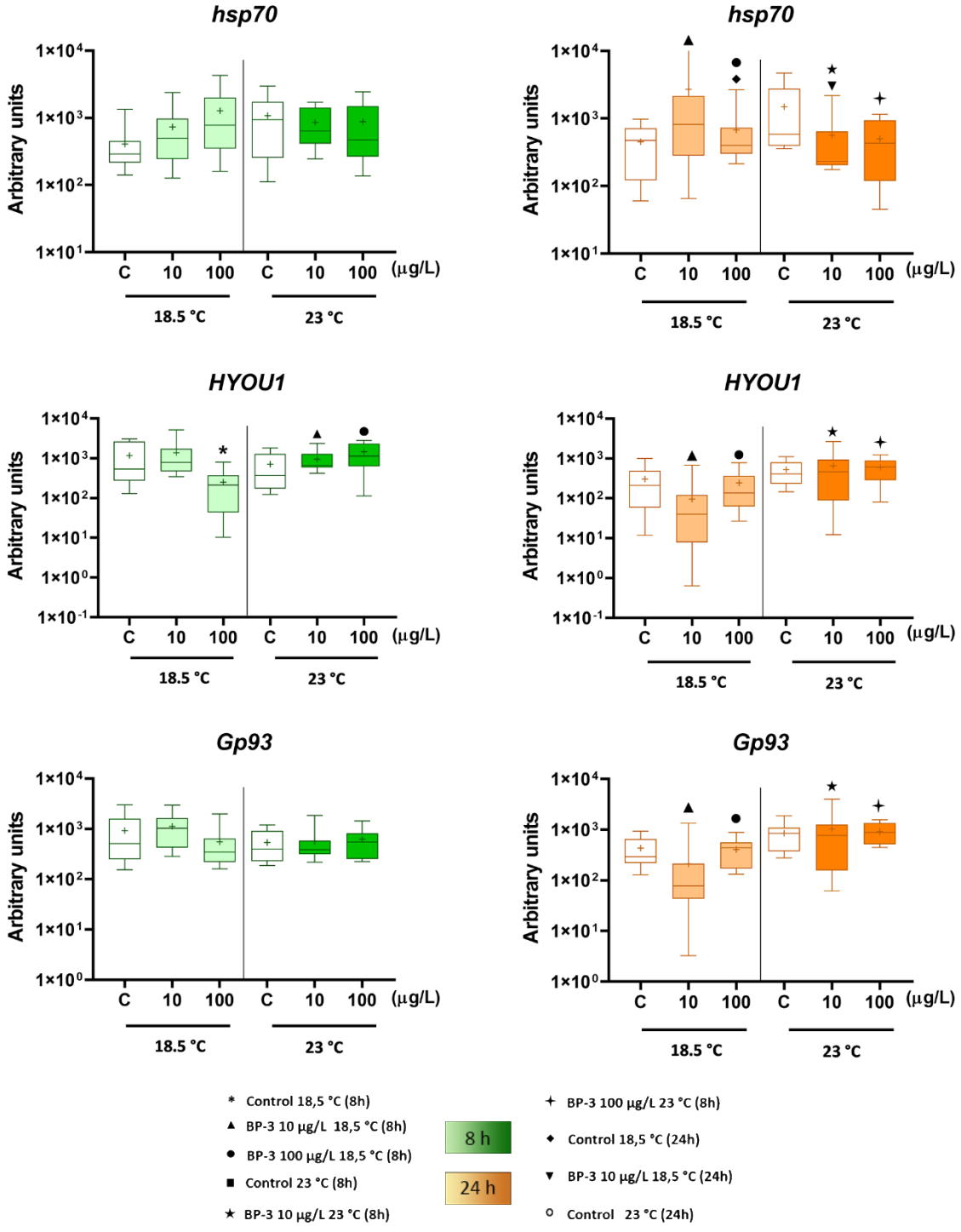

Figure 16. Expression of genes from stress response (*hsp70*, *HYOU1,* and *Gp93)* in *C. riparius* larvae after exposure to BP-3 (10 or 100 µg/L) at 18.5 and 23 °C for 8, 24h. All significant differences respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases ($p < 0.05$).

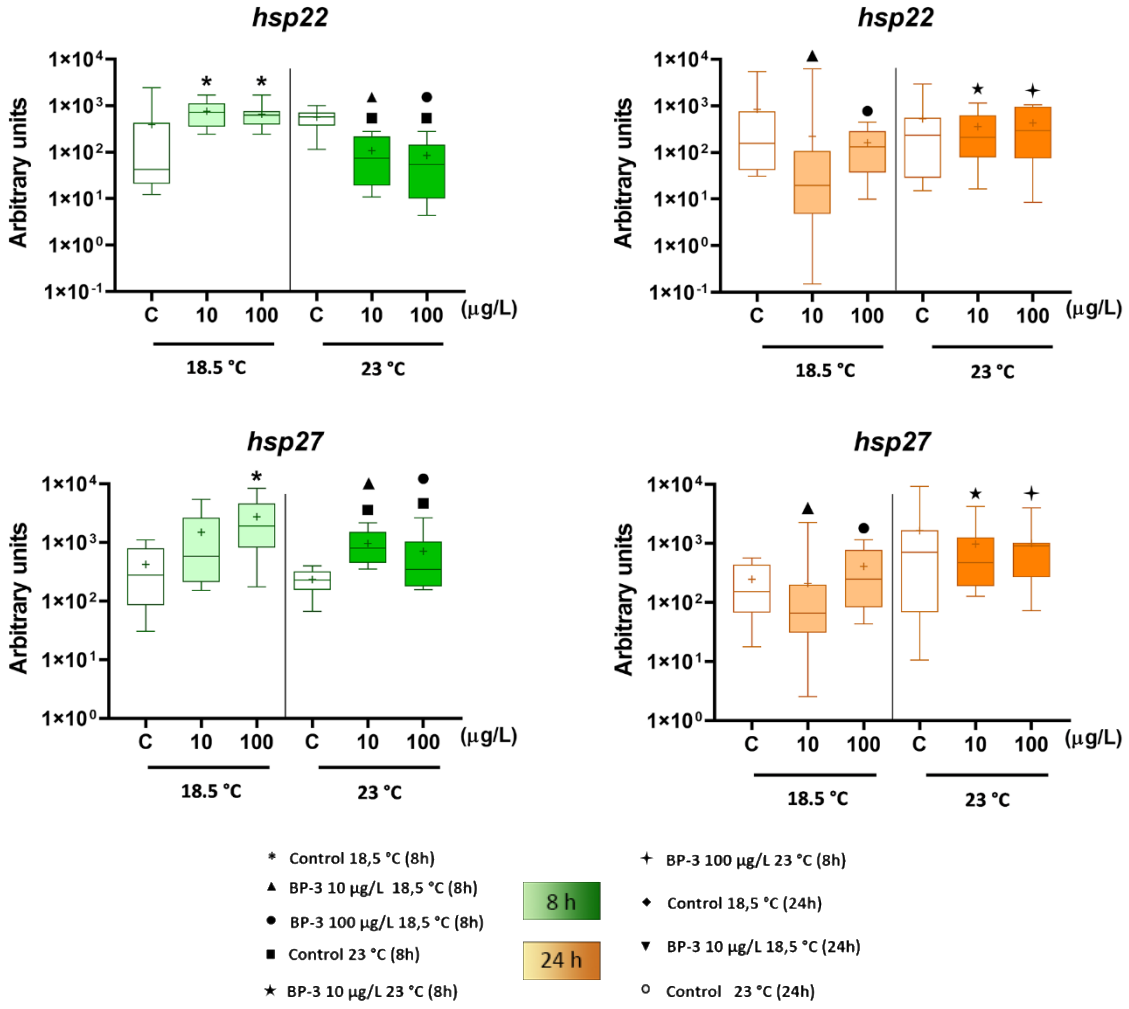

Figure 17. Expression of *hsp22* and *hsp27* related to stress response in the *C. riparius* larvae after exposure to BP-3 (10 or 100 µg/L) at 18.5 and 23 °C for 8, 24h. All significant differences respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases ($p < 0.05$)

Finally, three different enzymatic activities were evaluated (figure 18). GST belongs to the detoxification response, Phenoloxidase (PO) for immune response, and finally, Achetilcolinesterase (AChE) related to the nervous system. The activities were not altered for any of the conditions at 8h.

However, at 24h, statistically significant differences were observed with respect to 8h for PO and AChE at 18.5 °C for 100 µg/L. Besides, PO showed upregulation respect to the control. Finally, PO presented altered transcriptional activity for 10 µg/L in comparison to the same concentration at 18.5 °C. In contrast, both BP-3 concentrations at 23 °C showed increased activity compared with their counterparts at 8 hours.

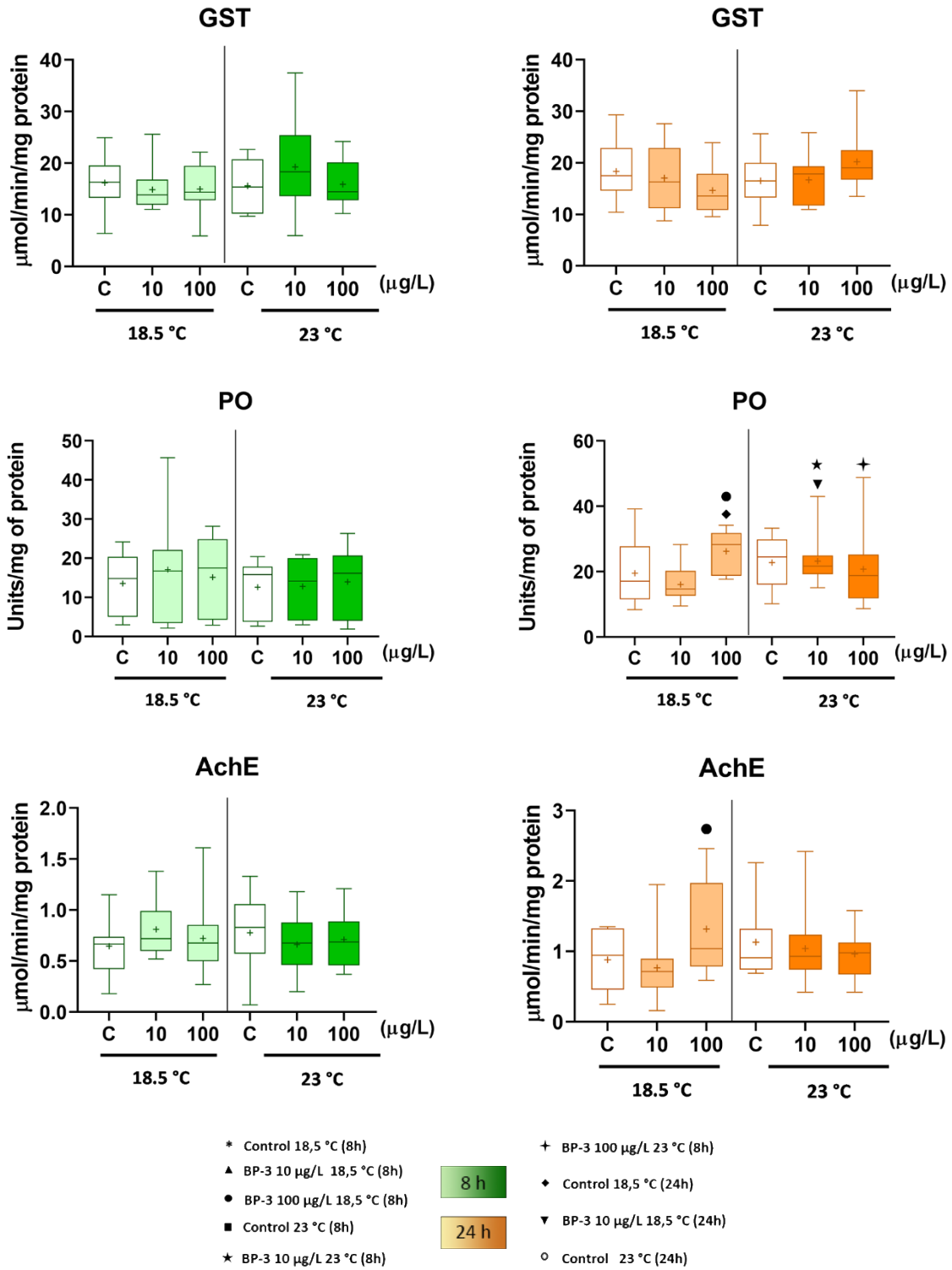

Figure 18. Enzyme activity of GST, PO, and AchE in the *C. riparius* larvae after exposure to BP-3 (10 or 100 µg/L) at 18.5 and 23 °C for 8, 24h. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. All significant differences respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases ($p < 0.05$).

SUMMARY

- **Survival was altered at different conditions with higher effects by 23 °C.**
- **24h exposure showed more effects on gene expression and enzymatic activity.**
- ***EcR* and *Met* were altered at 8, 24h, *InR* only was affected at 24h.**
- ***Cyp4d2, Cyp6b7, GSTd6*, and *GSTo1* were modified at all the conditions by 24h, besides at 8h for *GSTd6*.**
- **HYOU1, *hsp22*, and *hsp27* modified its expression at 8h respect control and between temperatures.**
- **The five HSPs were altered at 24h even with differences between time and T.**
- **Only AchE and PO activities were altered at 24h, notable in PO at 23 °C.**

Discussion

UVFs mixtures

Following the objective of studying the multi-stress response, the first approach involved exposure to mixtures. Two different experiments were done. The first one was the analysis of the single and binary mixture of two UVFs, OD-PABA and OC, at 24h. A second experiment was designed by including both UVFs and BPA, a plasticizer common in the recipients containing sunscreens, and two times. The initial experiment was performed with a limited number of genes that covered the endocrine system, the detoxification mechanisms, the stress response, and the immunity. The last experiment, however, was more ambitious, using an array with a more extended number of genes covering these processes and also the DNA repairing mechanisms. Because of the complexity of the data, the discussion concerning UVFs is performed for each experiment separately.

It is known the role of UVFs as EDCs on diverse organisms, from vertebrates to invertebrates (Kaiser et al., 2012; Rodríguez-Fuentes et al., 2015). Previous data on *C. riparius* confirmed the role of EDCs in this species (Ozáez et al. 2013, 2014, 2016 a, b, c), so *EcR*, *Kr-h1*, *JHAMT*, *Cyp18a1*, and *MAPR* were evaluated in the binary mixture of OD-PABA and OC. Strikingly, none of them were altered by the conditions tested. It seems then that the ecdysone response pathway, the ecdysone degradations, and the juvenile hormone synthesis were unaffected by single and binary mixtures.

The EcR is the receptor for 20-E initiating all the ecdysone signaling pathway. Previously was showed that It is modulated by OD-PABA but not for OC (Ozáez et al., 2016c), so it was an unexpected result that none of the conditions tested altered its expression. Some trends are observed, and the experiment was performed with some technical differences, so it could be argued that the different results could be due to them. Downstream to *EcR*, *Kr-h1* intervenes in molting regulation and connects ecdysone and JH response pathways (Qu et al., 2018; Yamanaka et al., 2013). The absence of response to this gene has also been observed by vinclozolin (Ozáez et al., 2016). Concerning UVFs, no previous data on it exist, but *HR38*, another late response gene, was not affected by UVFs (Ozáez et al., 2016c).

The 20-E is degraded by *Cyp18a1*, controlling its concentration in the cell, so the lack of response suggests that 20-E catabolism is working correctly. Although there is no previous data for UVFs, it is modulated by other toxicants such as BPS or polycyclic aromatic hydrocarbons (Abolaji et al., 2015; Herrero et al., 2018). The result obtained with *JHAMT* provides information about the synthesis of JH (Qu et al., 2018). This hormone works coordinately to 20-E for correct molting and development, so the absence of response suggests that the conditions tested were not acting on the levels of JH. However, it cannot be discarded effect on JH degradation. Although no data are in the literature

evaluating *JHAMT* concerning UVFs, its transcriptional activity is modulated by insecticides (Liu et al., 2018; Muema et al., 2017). It indicates that JH can be a target for toxicants, and *JHAMT* could be a new xenobiotic biomarker.

The last gene analyzed was *MAPR*, which codes for an orphan receptor. Identically to 4-MBC and BP-3 UVFs (Ozáez et al., 2016c), *MAPR* did not show modification for the single and binary mixture. *MAPR* belongs to the CYB5 family (Ryu et al., 2017) and, despite the absence of any known role in the ecdysone response, it could be a candidate to connect the steroid hormones with cytochrome P450s pathways acting on the detoxification response.

Hence, although UVFs are defined as EDCs, it appears that the conditions tested for OD-PABA and OC are not enough to trigger their endocrine disruptor potential. The lack of response in binary mixture suggests, additionally, that either have no interaction or that it is so weak that it is not able to induce a more substantial effect and response from the cell in *C. riparius*. Additional research is required to elucidate the real impact in the hormonal system, which should include longer exposure times and additional genes to evaluate other pathways and additional responses described on insects.

The cell has several mechanisms to manage environmental changes. One of them, with an essential role in chemical stress, is that formed by the detoxification enzymes. It is essential against toxicants modulating the biotransformation to minimize the number of toxicants and to remove it from the cell. The detoxification process is divided into three phases, named Phase I, Phase II, and Phase III. In this experiment were evaluated eight genes related to Phase I (CYPs) and II (GSTs). No changes were observed for any of the compounds, suggesting that there is a specific pattern for each UVF. Moreover, the lack of alterations suggests that the interaction between OC and OD-PABA is not enough to produce any relevant response in the mixture.

The cyp450 family is extensive and contains a vast number of CYPs responding to several xenobiotics, as has been observed previously in invertebrates (Park et al., 2018; Seong et al., 2019; Wang et al., 2016). Similarly to that observed here, it has been described that BP-3 did not modify transcription of *Cyp4d2*, *Cyp6b7*, *Cyp9f2*, and *Cyp12a2,* but downregulation can be detected for 4MBC (Martínez-Guitarte, 2018). The different pattern suggests that the involvement of CYPs on UVFs detoxification is differential and could also encompass other enzymes, such as carboxylesterases since there are carboxyl ester residues in some UVFs. Additionally, it cannot be discarded the participation of other CYP members due to the great variety of this family on aquatic invertebrates (Han et al., 2019; Hatfield et al., 2016a).

On the other hand, a similar picture can be proposed for GSTs. The pattern of response is different from that observed with other UVFs, with BP-3 upregulating *GSTd3* and *GSTe1* and 4MBC downregulating *GSTo1* (Martínez-Guitarte, 2018). The different pattern observed here suggests that it is specific for each compound. However, other enzymes, like methyltransferases or sulfotransferases, could also have a role in Phase II for these compounds (Hodges & Minich, 2015). Several GSTs genes have been described in *C. riparius*, which are sensitive to metals or nanoparticles (Nair et al., 2011, 2013) while decrease their activity levels by plastics and pesticides (Herrero et al., 2015a; Rodrigues et al., 2015). Nevertheless, it cannot be dismissed that additional members of the GST family, not analyzed in this work, would be involved in detoxification of OD-PABA and/or OC.

To sum up, the OD-PABA and OC in single or binary mixture did not alter the detoxification response showing a response different to that described for other UVFs. Extensive research employing more members from both phases could help to determine how the cell biotransforms these compounds and provide additional clues about the putative products generated in each step of the process.

The stress response allows the cell to maintain the homeostasis when it is submitted to any stress. It is triggered by a wide variety of stresses, including chemicals, heat, or hypoxia. Two stress-related genes were evaluated, showing a weak response. *HYOU1* codes for a protein from the endoplasmic reticulum (ER) belonging to the HSP70 family involved in the protein folding (Kozlov et al., 2017; Ozawa et al., 1999). *HYOU1* was upregulated by OD-PABA, suggesting some effect on the ER. Although there are no previous studies on invertebrates, altered mRNA levels were observed on fishes by diclofenac and POPs mixture (De Felice, 2012; Mitra et al., 2018). The changes in the mRNA levels could reflect a direct effect which impacts on the folding process at the chaperone level. However, additionally, it should be considered that an indirect consequence of the effect could be an alteration in response to hypoxia events modulating the sensitivity to it and other stressors. The returning to control levels showed by the mixture suggests that, although OC is not producing any change, it someway counterbalances the effect of OD-PABA. It reassures the different toxicity mechanisms implicated for each UVF in the cell.

On the other hand*, l(2)efl* is a protein member of the sHSPs family. It did not show any biological relevant change. However, specific trends were observed for mixture, with higher means than single exposures. It could be the start of an interaction that might be relevant at longer exposure time. More research would provide additional data to elucidate this possibility. Similar to other small HSPs, *L2efl* has multiple functions in the cell on differentiation, cell division, or growth (Jagla et al., 2018), besides control stress and development (Landis et al., 2012; Wojtowicz et al., 2015). The present study is the first time that *l(2)efl* is analyzed concerning chemical stress. Since it is poorly known on invertebrates, the absence of alteration could be explained because it is a non-inducible stress gene, similar to *hsc70* or other sHPSs previously reported in *C. riparius* (Martín-Folgar et al., 2015; Martín-Folgar & Martínez-Guitarte, 2017). Due to its role in the development process, future studies to elucidate its behavior on stress can be focused on the use of other stages as the embryo or first instar larvae.

The immune system modulates the response to infection. It was evaluated employing *Proph* and *Def*, both related to the humoral response as part of the primary immune response in insects. In both cases, no effects were detected at any of the conditions. Until now, only one study evaluated the effects on these immune genes in *C. riparius* by Cd/Cu in a single and binary mixture with identical results to the obtained for UVFs (Martín-Folgar & Martínez-Guitarte, 2019). Taking into account the results obtained, the impact on the immunity of those UVFs is low, especially in comparison to other xenobiotics. Microbial insecticides were able to increase PO activity (Bordalo et al., 2020) while *Def* was upregulated in honey bees by fungicides (Cizelj et al., 2016). Furthermore, it could be proposed that the ability of the larvae to respond to infection was not compromised either single or a mixture of these UVFs.

The analysis of OD-PABA and OC at the molecular level showed that both are weak inducers of the processes studied. These results are in agreement with previous analysis (Ozáez et al., 2013, 2016b) that show low toxicity, even among the UVFs. The mixture results suggest that the interaction between both compounds is weak or irrelevant, at least at these conditions. However, additional experiments at longer times and including additional genes will provide a better picture of the actual effect of OD-PABA and OC on *C. riparius*. Pursuing this goal, it was designed a second experiment, which included an additional compound.

To evaluate a more complex scenario, closer to the real environmental conditions, both UVFs were used in an experiment that added a third chemical, Bisphenol A. It is a plasticizer present in multiple plastic containers, including the PCPs bottles. The UVFs are usually provided as an oily solution so, due to the log Kow value for BPA, a possible transfer to the sunscreen contained in the bottle can arise, reaching to the aquatic environment as a mixture. To get the kinetics of the response, two times of

exposure (24 and 96h) were selected, and single, binary, and ternary mixtures were studied. The analysis also involved a higher number of genes, covering in this way the same pathways plus the DNA repairing mechanisms. In some of these pathways, additional genes filled some gaps, such as the JH receptor, the insulin-peptide receptor, or Phase III. The total number of genes was forty.

As stated, the study was performed at two times. There was previous data for the three compounds at 24 hours, and, for the genes previously analyzed, the results were mostly coincident. Only three of the genes were altered in some way when compared to control, suggesting that in the conditions tested, the three compounds had a weak response. Then, it could be suggested that the fourth instar larvae of *C. riparius* can manage the compounds in the short-term, with weak responses that affect only *Cyp18a1*, *GSTt1*, and *hsp70*. It means that a slight effect on ecdysone synthesis, Phase II of detoxification, and the stress response is produced. The damage observed for mixtures suggests some kind of interaction between the compounds, although it is still too weak to be evident.

Cyp18a1 showed modulation, reinforcing the activity of those compounds as endocrine disruptors. It participates in the catabolism of ecdysone. The upregulation indicated an activation of the ecdysone degradation, possibly by the role of the compounds as EDCs. Then, they stimulated the response and tricked the cell, which triggered the usual response to high hormone levels. In this way, the compounds would be mimicking the action of the ecdysone. It is also clear that the response probably is more complicated since other related genes did not show changes. The reason could be an early response, similar to that observed for BP-3 (Ozáez et al., 2013;2016), but also an indirect consequence of UVFs and BPA exposure that needs to be explored.

GSTt1, as a coding gene of a Phase II enzyme, provides information about the detoxification mechanisms. An earlier study employing other UVFs did not generate effects on this gene (Martínez-Guitarte, 2018), so it is a specific modulation. Since the GST family is vast, it can reflect the involvement of theta members on the biotransformation of those compounds. The result, combined with that observed in the first experiment and those present in the literature, supports the idea that an array with the genes coding for several families of CYPs, GSTs, and other enzymes involved in biotransformation will elucidate the putative role of these genes and how the compounds are processed.

Finally, *hsp70* is, probably, the most known member of the HSP70 family, and it is continuously used as a stress biomarker. A strong response was observed in all the conditions except for BPA exposure. This gene has been evaluated in *C. riparius* against multiple chemical compounds showing similar pattern with increased expression (Herrero et al., 2015a; Martínez-Paz et al., 2017a; Morales et al., 2011) even this response was detected by BP-3 and 4-MBC (Ozáez et al., 2016). Joined to the results from detoxification at this time, it can be suggested weak toxicity that starts to activate both protective systems. Therefore, similarly to the first experiment, it is needed more time to induce a substantial alteration at the transcriptional level in *C. riparius*, even for the mixtures.

One of the weaknesses of the molecular analysis is usually the lack of kinetic studies that involve different times. Sometimes, it is hard to know the progress of the changes in the cell during the exposure since only a fixed picture is taken focused on one time. Then, it is elusive the information because it is not possible to know where in the curve of response is the organism: is it in the responding section of the curve or the recovering section of the curve? In this thesis, it has been analyzed the response at two times. Comparing both times helps to understand what is happening in the cell and the trend of the genes. The results can be summarizing in a general downregulation at 96h, respect to 24h, for twenty-five genes. The stable genes were *InR, JHAMT*, *Kr-h1*, *Cyp9f2*, *Cyp12a2*, *GSTd3*, *GSTo1*, *GSTt1*, *hsc70*, *hsp90*, *Gp93*, *hsp40*, *hsp60*, *hsp17* and *hsp21*. These genes belong to the endocrine

system, the detoxification mechanisms, and the stress response. However, it does not mean that these processes were not affected because other genes involved in them were altered.

The observed repression in many genes could be explained as part of the normal development process in *C. riparius* due to its short life cycle. It is important to remember that during the fourth instar larvae stage, many processes are happening simultaneously, inducing a transformation of the metabolism. The molting process is regulated by the 20-E and JH levels, so the larvae start to transform in pupae when simultaneously JH decrease, and ecdysteroids are stimulated (Truman, 2019). In this sense, the results agree to that with downregulation on genes related to the 20-E response and synthesis at 96 hours. While *JHAMT* and *Kr-h1* involved in juvenile hormone pathways and synthesis were typically expressed. It suggests a faster molting since no increase of transcription in genes related to the juvenile hormone was observed.

The detoxification also followed a decreasing transcriptional pattern because of the lower levels for Phase I (*Cyp4d2*, *Cyp6b7*), Phase II (*GSTe1*), and Phase III (*MRP1*, *ABCB6*) involved genes. Similarly, the decreased expression affected to the stress response (*hsp70, HYOU1,* and the sHPSs*: hsp17, hsp22, hsp23*), the DNA repairing (*Decay, XRCC1, NLK, ATM*), and immunity (*Def and Proph*), being stronger the disruption in these both last systems. All these genes possess roles in many other cell processes, from molecule synthesis to oxidative stress response. Therefore, the altered pattern expression could be part of normal cell behavior (Bakthisaran et al., 2015; Board & Menon, 2013; Rewitz et al., 2006). The absence of previous results comparing times emphasizes the lack of information on the mixture at the molecular level being this one of the first studies showing the alterations at gene expression level on different cellular responses on invertebrates along the time.

Turning the attention on 96h exposure, the analysis showed a more complex pattern expression with alteration in the majority of the genes analyzed. The analysis is complex since multiple comparisons were made, and not all of the statistically significant results had biological meaning. Only those results with biological meaning are discussed. Furthermore, it necessary to keep in mind that the analysis is performed comparing all samples to control and also to single and binary mixtures in the case of mixtures. The differences to control rendered that only eight genes (*Cyp4d2, GSTd3*, *GSTt1*, *MRP-1*, *hsc70*, *HYOU1*, *hsp60*, and *Proph*) maintained their expression stable. From these results, an evident metabolism disruption was in progress, being higher on endocrine, detoxification, and immune response. For single exposure, OD-PABA seems to be the more toxic compound altering all the routes except the immune response, in line with the results at 24h. The substantial alteration on the endocrine system coincides with the role of UVFs as EDCs. Besides, OD-PABA provoked an upregulation of DNA repairing except for *Decay,* which is logical because this gene is also related to apoptosis event (Dorstyn et al., 1999b). These results suggest some possible genotoxic effects of this compound similar to that observed by vinclozolin on this organism (Aquilino et al., 2018). However, the activation is diminished by the mixture exposure, reducing the OD-PABA toxicity by combination with the other compounds. For the mixtures, a high number of genes were altered with relevant effects derived to the combination with BPA in binary and ternary mixtures, suggesting some kind of positive interactions. However, the mixture of both UVFs showed lower activity respect for the single OD-PABA in line with the preliminary study. The disruption on the endocrine system was mainly focused on stimulating the 20-E synthesis and signaling. Additionally, the downregulation of *Cyp18a1* would reflect a decrease in the degradation of ecdysone, which was synchronous to the downregulation of genes related to JH. These data suggest a longer molting process that could delay the development. The results are in agreement with previous effects on invertebrates by BPA altering embryo development or molting on the larva stage (Balbi et al., 2016; Maria et al., 2019) besides the emergence was delayed on *Musca domestica* by this compound (Izumi et al., 2008).

Another essential point was the interaction between compounds that can be analyzed by comparing the mixtures against the single compounds and to the binary and the ternary mixtures. Interactions are usually identified by changes in the behavior of the endpoint analyzed, being positive or negative interactions. At the transcriptional level, the specific interaction is hard to elucidate since the transcription is a highly dynamic process, and the regulation of the genes is versatile. Then, the same gene may show different behaviors along the time being activated at the start of the exposure and repressed in later times. Molecular events can have short-, mid-, or long-term, so it necessary to be cautious about describing an interaction as antagonism or synergy. However, some clues and trends are drawn by the results.

Three binary mixtures were analyzed by doing all the putative combinations between the three compounds (OC+OD-PABA, BPA+OC, BPA+OD-PABA). Disruption was observed on all pathways evaluated, with a higher or lower effect depending on the compound used as a reference, although differences were more evident when compared to BPA. In general, the result is in line with the previous pattern, with higher toxicity for BPA mixtures. The impact on the different pathways could compromise the future of the population since there are affected relevant mechanisms involved in response to environmental changes. The alteration of the endocrine system could deregulate the physiology of the organism. A more in-depth analysis, including additional hormonal related genes, would provide a better understanding of the damage, even showing indirect damages produced by the EDCs. Most of the research is focused on steroid hormones, but there are appearing pieces of evidence that other hormones could also be affected. Detoxification and stress responses are expected to be modulated by the mixtures, mainly because they usually involve additional chemical stress with the presence of a second compound. However, it is also worthy to note that many of the genes code for proteins participating in other processes such as development, growth, or cell cycle. Then, the impact is not only in response to homeostasis alteration if not also could alter the primary events of the organism. More research is required to get a picture of the network of events that allow connecting the molecular changes with physiological and ecological consequences.

The changes in DNA repairing mechanisms and immune genes are also relevant in the future of the population. Possibly, both would be the most important in the long-term consequences. While they have direct damage by affecting the cell's ability to progress in the cell cycle and the organism's ability to respond to the infection, also they can affect the population's future indirectly by altering the ability to prevent DNA damage and the fixation of mutations and to survive the infection. On the other hand, another indirect effect of the pollutants can be magnified by the mixtures. Deregulation of the genes may deviate resources, so the cell requires more resources and energy to maintain homeostasis because they are suffering many different alterations at the same time. Thus, the cell tries to maintain the homeostasis by modulating their transcription activity, which modifies the number and quantity of transcripts hence altering all the metabolism. The consequence is an increased demand for energy and resources for homeostasis maintenance, which could produce an imbalance, reducing the amount of energy for other essential processes such as growth, among others. Additional research by combining molecular analysis with physiological endpoints could help to elucidate the critical events on the response to toxicants.

Finally, the newest and challenging scenario was ternary mixtures evaluation. It requires to perform a comparison to control, single compounds, and binary mixtures. The pattern is similar to binary mixtures with increased alteration respect to single compounds. Mostly, a trend to positive interactions between chemicals was observed. Focusing on each component, OC seems to be the less toxic because its addition to BPA+OD-PABA demonstrated lower effects that the observed by BPA-OD-PABA, or OC+OD-PABA when BPA was added. Besides, this issue was confirmed by the lower transcriptional alteration on a single exposure. As binary and ternary mixtures are not usually analyzed

at the transcriptional level, there is a lack of references in the literature. The mixtures studies were mainly focused on the toxicity effect by LC50 values or similar patterns. Only one study evaluated the effects of UVFs in the ternary mixture on fishes showing additives behavior higher than the expected and pointing attention to the complexity of the mixtures (Kunz & Fent, 2009). Besides, other ternary mixture employing metals or pesticides were evaluated on invertebrate's through acute exposure. Synergism was detected on *C. dilutus* by neonicotinoids for 96h and by antibiotics mixtures on *D. magna* (De Liguoro et al., 2018). In line with the fish results, additives' effects were detected on *D. magna* by metal mixture (Traud et al., 2017). Finally, more complex pattern with antagonism and synergism was observed by CECs mixtures on aquatic invertebrates (Di Poi et al., 2018).

BP-3 and temperature

Following the multi-stress scenario analysis, an approximation to know the effects on a typical UVF as BP-3 was done in combination with the most evident climate change effect, the increased global temperature. Firstly, the survival was analyzed for the combination of temperature and benzophenone 3. As a poikilotherm organism, *C. riparius* can be affected by climate change, modifying its growth, reproduction, stress response, or survival (Everatt et al., 2015; Wojda, 2017), while BP-3 is one of the most detected UV filters in surface waters (Archer et al., 2017). The experimental design included different concentrations and times. As a result, it could be suggested that a mild increase in the temperature could impact the survival of the animals since there is an increase in deaths at 23 °C compared to 18.5 °C. It is an acceleration of the damage by the compound and/or the temperature since the difference is in the moment when mortality is detected. In this sense, a previous study in *Chironomus riparius*, also known as *Chironomus thummi*, showed that the stress response was activated (Carretero et al., 1986). Besides, a previous study evaluating the effects of growing at 30 °C on *C. riparius* shown a decrease in survival (Park & Kwak, 2014), indicating that temperature alone can modulate the life of this organism. However, it is relevant to mention that *C. riparius* can acclimate to a higher temperature scenario because animals grown at high temperatures (26 °C) were able to survive and prosper (Foucault et al., 2017). The authors conclude that chironomids respond rapidly to climatic variation via adaptive mechanisms and, to a lesser extent, via phenotypic plasticity. It could be a relevant element to consider in the interaction with the pollutants. About other invertebrates, it could be said that it has been described the impact of temperature in survival, as happens on shrimps at 32, 38, and 41°C (Hoang et al., 2020).

Conversely, a previous study reported that BP-3 could decrease survival at 10000 µg/L after 24 hours of exposure at 18,5 °C with high mortality rates at later times (Ozáez et al., 2013). Here the mortality started later at this temperature, suggesting that also the genetic background of the larvae could modulate the sensitivity to BP-3. Regardless, the results indicate that probably the higher temperature is modifying in some way the response. It could be by accelerating the metabolism and/or favoring the entering of the compound in the cell. In this sense, *Chironomus riparius* showed less sensitivity to BP-3 ($LC50_{24h}$= 194 mg/L) in comparison to other invertebrates like corals (LOEC 1000 µg/L) or crustacea as *Daphnia magna* ($LC50_{48h}$=1.09 mg/L) (Du et al., 2017; He et al., 2019; Ozáez et al., 2016).

Hence it could argue that a mild temperature change produced by climate change could influence the toxicity of UVF BP-3, increasing it on *C. riparius*. However, the impact could be less than expected since a previous study suggested that chironomids could have the ability of rapid adaptation to new temperature conditions. Overall, the results obtained reinforce the importance of multi-stress analysis.

As stated, there was an alteration in the survival, so an additional analysis at the transcriptional level was performed. The study involved genes related to the endocrine system (*EcR, InR, Met*), the detoxification mechanisms (*Cyp4d2*, *Cyp6b7*, *GSTd6*, *GSTo1*), and the stress response (*hsp70*, *HYOU1*,

Gp93, *hsp22*, *hsp27*) evaluated at two different times (8, 24h). In summary, it can be said that most of the genes were altered by the temperature to any of the times. The first conclusion is that the temperature can modulate the impact of a compound, in this case, BP-3. It is a known EDCs, so alterations in the endocrine system were expected because they have been previously described in *C. riparius* at 24h (Ozáez et al., 2013; 2016c) at control temperature. What is new is the effect of temperature in the response, which seems to affect the transcription of *EcR* negatively. Besides, *Met* also showed downregulation, but *InR* was stable. An increase in temperature appears to impact negatively in those genes involved in molting, so a higher temperature of the water could translate the climate change in a more extended time of development of *C. riparius* larvae. More prolonged exposure combined with the use of organisms grown at different temperatures could help to establish the relevance of this finding. However, *C. riparius,* like other poikilotherms, can manage increases in water temperature by altering its metabolism or other mechanisms (Robinet & Roques 2010).

Lastly, the expression between times was also compared. Upregulation on the three genes was observed, which agrees with a previous study on *C. riparius,* combining a plasticizer and temperature (Park & Kwak, 2014). In this sense, it has been observed on fishes that increased temperature induces changes in growth rate (Eya et al., 2017). Therefore, the temperature is a factor to consider in the modulation of physiology. BP-3 shows a weak response at standard growth temperature, but an increase of four degrees is enough to modify the expression of some genes involved in the regulation of endocrine processes. In this thesis, it has been shown that the increase alters the transcription of two crucial genes (*EcR*, *Met*) in the response pathways to the main hormones of molting and metamorphosis. It could affect the development by altering the transformation between larval stages and, maybe, changing the timing to reach the later stages like pupae and adult. Therefore, more genes should be evaluated to elucidate the real effect on this system.

The Phase I detoxification mechanisms were not modified compared to each control. However, differences were observed comparing both times suggest some kinetic alterations with an irregular pattern. These results are in agreement with previous studies showing that, even within the same cytochrome family, a diverse response can be developed. For example, in *Bemisia tabaci*, *cyp6cm1* mRNA levels were modified depending on the temperature, with increased expression at 31 °C and decreased at 35 °C (Guo et al., 2018). Similarly, altered temperature provoked increased mRNA levels on CYPs in fishes (Vergara-amado et al., 2017).

Conversely, nano plastics exposure in *D. pulex* generates a variety of responses with no effect on *DpCYP4* but decreased expression on the other CYPs (Wu et al., 2019). Overall, the response of CYPs is variable and specific according to the family belonging and xenobiotic exposure. Moreover, it cannot be discarded the fact that other members of these families or even other CYP families can participate in the UVFs metabolism. It is worthy to note the high diversity of CYP families, and some of them have been related to drug and xenobiotics metabolism are the CYP2A or CYP1 (Hodges & Minich, 2015). However, it is also relevant to consider the specificity of families, which are associated with particular animal groups.

On Phase II, mainly controlled by GSTs, a similar picture was observed with changes at different times for both genes analyzed with a pattern combining up- and downregulation or vice-versa. It has been described as a daily variation on GSTs (Bagheri et al., 2016), so the changes observed could be related to the circadian rhythm. For *GSTd6*, increased expression was previously observed in another study in *C. riparius* by UVFs but with decreased tendency at 24h, although no significant differences were detected (Martínez-Guitarte, 2018). However, *GSTo1* was not previously upregulated in this condition, so maybe it can be related to experimental differences (Martínez-Guitarte, 2018). The GSTs analysis was complemented with a GST enzyme activity study, which shows the global response of these

enzymes. The lack of response backs the low alteration observed at the gene transcription level. The time used could be a handicap, and longer exposures may render some alterations. However, due to the wide variety of detoxification enzyme acting on Phase II, could also be possible that carboxylesterases could be responsible for detoxification due to the chemical structure of BP-3 (Hatfield et al., 2016a).

Contrary to the endocrine system, the increase in temperature had a low impact on the transcriptional activity to detoxification response. It is a contrast to that observed on *Bemisia tabaci*, where a combination of pesticide and temperature upregulated detoxifications genes, although were not identical GSTs, so it is challenging to establish a correlation (Guo et al., 2018). The differences could also be due to the nature of the toxicant since BP-3 usually is considered less toxic than pesticides. At these conditions, the temperature does not modulate the toxicity of BP-3. It could be explained in several ways. One of them is that the time exposure and the concentration of BP-3 are limiting the response, so there is not reached the threshold level that would trigger the response. Another putative explanation is that other enzymes perform the bioprocessing. In any case, additional experiments could help to put some light in the influence of temperature on UVFs toxicity.

For this experiment were selected different genes coding HSPs that cover the Hsp70 (*hsp70*, *HYOU1*), the HSP90 (*Gp93*), and the small HSPs (*hsp22*, *hsp27*) families. It would be understandable that a high level of transcription could be found in some of them. However, it is essential to note that not all the members of the heat shock protein group are inducible. So, it should be kept in mind that some of them can be transcribed regularly in the cell. Furthermore, the induction of the HSPs could be variable depending on the stress, and some that respond to heat could be not responsive to chemical stress. Nevertheless, they are frequently used as biomarkers because of their fast response against unfavorable conditions. On *C. riparius*, HSPs transcriptional activity of HSPs has been analyzed with multiple inductors, including temperature and diverse xenobiotics (Aquilino et al., 2016; Martínez-Paz et al., 2014; Martínez-Paz et al., 2017b; Xie et al., 2019 a,b). Although not alteration was observed at 8h for *hsp70*, it follows the response described previously for BP-3 (Ozáez et al., 2016b). The time comparison offered an increased transcription, similarly to other xenobiotics in this organism as vinclozolin, sulfadiazine, 4-MBC, or triclosan (Aquilino et al., 2016; Martínez-Paz et al., 2017b; Ozáez et al., 2016b; Xie et al., 2019b). Surprisingly, the temperature did not stimulate any effect on *hsp70*, contrasting to the typical HSP response against heat shock in this and other insects as *Bemisia tabaci* (Díaz et al., 2015). However, it is essential to be cautious about it because 23 °C may still be below the temperature that triggers the heat shock response.

The other member of the Hsp70 family, *HYOU1*, has a role in hypoxia events and has been related to development diseases. According to the results, it seems to develop a faster and opposite response than *hsp70*. There is sparse information about this gene, but the response was similar to that observed for OD-PABA at 24h. The consequence of the downregulation could be a less effective response to hypoxia and xenobiotics and besides can alter the correct protein folding, since it is a chaperone.

Gp93 belongs to the Hsp90 family, and it was only significantly altered when it was compared between both times. The increase at later times suggests a slow induction. Conversely, it is an ortholog of mammalian Gp96, which participates in the immune response as a chaperone interacting to cell-surface Toll-like receptors (TLRs) (Pockley & Henderson, 2018). The absence of a response could suggest that this mechanism of immunity was not activated during short BP-3 exposure. The data would agree with the lack of response observed for the PO activity at 8h.

Small heat shock protein genes have been previously analyzed in the presence of metals and xenobiotic exposure (Martín-Folgar et al., 2015, 2018; Martín-Folgar & Martínez-Guitarte, 2017; Martínez-Paz et al., 2014). Both genes studied, *hsp22* and *hsp27*, were modulated by BP-3 at both temperatures, but

with a different behavior to each one. While *hsp27* was upregulated, *hsp22* was firstly upregulated and later downregulated at the higher temperature. The overexpression by BP-3 is in agreement with the general HSPs response against xenobiotics exposure in *C. riparius* (Herrero et al., 2015a; Martínez-Paz et al., 2014; Xie et al., 2019 a,b). The decreased mRNA levels of *hsp22* by heat suggest that it is a sensitive marker for temperature. Previously has been observed that the sHSPs as a set could be a good source of biomarkers (Martín-Folgar et al., 2015). In this sense, the response is similar to that described for cadmium exposure (Martín-Folgar & Martínez-Guitarte, 2017). The trend was similar for *hsp27,* so both genes were responsive to the conditions tested, showing a fast response with detectable modifications as early as 8 hours.

On the other hand, the dynamic response shows modulation for both temperatures suggesting a reinforced stress scenario affected by chemical and heat stimulus for these genes. The lower mRNA levels could reflect the return to standard conditions after a peak in earlier times. Also, it could be an inhibition of the genes as a consequence of the combined stress. In any case, the reduction could affect the response to stress and the correct folding of the proteins. The specific pattern observed reinforces the goodness of sHSPs as biomarkers for toxicity. However, additional research with the rest of the genes coding for these proteins will help to understand their actual potential.

The analysis of the cellular response was complemented with three enzymatic activities. One of them, Glutathione-S-transferase, has been discussed previously with the transcription results of GSTs. The other two, phenoloxidase (PO) and acetylcholinesterase (AChE), were analyzed, finding that the PO was responsive at 24 hours with increasing levels. It could reflect the activation of the immune response but also the modulation of specific genes during the day (She et al., 2019). According to the results, the combined stress of BP-3 and T can stimulate PO activity so that both stressors may modify the innate immune response. The previous result obtained for *Gp93* could be related to it because of its relation to Toll-like receptors (TLRs). TLRs participate in the cellular response, hence taking together both results, the stress from the conditions used could induce innate activity but seems to have a weak effect on the specific response, at least at this time exposure. Presently, there are no previous studies in insects that analyze the effects of UVFs on immunity. However, it is crucial to evaluate their effects because it can compromise the ability of the population to resist infection.

On the other hand, AChE was employed to determine the possible damages to the nervous system as a target for drugs or insecticides (Thapa et al., 2017). A weak response was observed with only an increase at 24h in comparison to 8h for BP-3. Previously, no effects were reported by BP-3 in *C. riparius* (Campos et al., 2017b). However, the observed differences between times suggest a trend to stimulation at longer exposure time. Besides, the different experimental conditions could potentially justify this different response.

Nevertheless, the putative BP-3 effect on AChE activity could compromise larval survival by altering the nervous system. In summary, analysis of enzymatic activities suggested that BP-3 altered PO and AChE activities with some influence from temperature and time. Although, in general, the alterations are weak in both systems.

To sum up the results obtained from the UVFs analysis.

The molecular response to mixtures has been poorly studied, so there is an emptiness of knowledge. Hence, this study is one of the first to evaluate the cellular response in an aquatic invertebrate as *C. riparius* both in the short and medium-term. In summary, the acute response (24h) to OD-PABA, OC, and BPA showed either no effects or weak global effects on the different systems from *C. riparius* larvae. However, a substantial disruption was observed at more prolonged exposure (96h), being more evident by the mixture in comparison to the single exposure. The toxicity of the filters was higher for

OD-PABA, followed by BPA being the later the OC, as was demonstrated in the mixture's combinations. The main alterations were focused on the endocrine system confirming their role as EDCs, detoxification, and stress response.

So, the modulation of the endocrine system suggests some delays in the development, besides the detoxification and stress response with multiple roles in the cell could decrease the ability to respond to futures stressors. Moreover, spite the lower effects on DNA repair and the immune system, these alterations can lead to more severe problems for the population, such as defects in the cell cycle or repair as well as their responses to infections by bacteria, viruses, or fungi. Finally, the presence of multiple stressors as mixtures causes a greater need for energy and resources to maintain cellular homeostasis; thus, it is able to generate energy imbalances. Therefore, depriving energy in other essential processes. For the first time, binary and ternary mixtures were evaluated along the time on the cellular response in *C. riparius*. These data demonstrate the importance of mimicking the natural environment to improve our knowledge of the molecular mechanisms. Furthermore, these results showed the complexity modulation on the cell physiology derived from the multi-stress exposure and opening the need to more studies evaluating these conditions and including multiple stages to provide a better understanding of all mechanisms involved in these responses.

Moreover, the analysis combining two stressors on *C. riparius* fourth instar larvae showed that a mild increase in temperature can affect the response of the organism to the UVF BP-3. On the one hand, the temperature seems to increase the toxicity of BP-3 on this organism by decreased survival percentage. Moreover, the endocrine system was disrupted for both stressors, and the effects can be related to extended development. Detoxification mechanisms were altered, denoted by modified expression of genes involved in Phase I and Phase II metabolism. These changes could compromise the ability of the cell to manage the toxicant. On the other hand, the HSPs demonstrated their effective role as useful biomarkers being notable for the sHSPs *hsp22* and *hsp27* able to activate at short exposure time (8h). Besides, some of their classical functions as protein folding could be affected. Finally, the altered PO and AChE activities suggest damage to the immune response and nervous system, respectively. However, these damages appear to be weak. Altogether, the results propose that even a mild increase in the temperature due to global change can affect this insect's toxicant response. This study represents one of the first analyses related to multi-stress in this model organism combining different molecular biomarkers. Further research is required with additional genes from other pathways, other biochemical biomarkers or longer exposure times will help to elucidate the mechanism involved in response to BP-3, the ability of the organism to manage multiple stressors and the putative effect of global change on the survival of populations.

IBUPROFEN

5. IBUPROFEN

Description, characteristics, and uses

The consumption of pharmaceutical compounds, antibiotics, retroviral or anti-inflammatory drugs has raised around the world during last years (Groot & van't Hooft, 2016; UNas et al., 2015), with particular attention on antibiotics (Liu & Wong, 2013). One of the concerns is the absence of prescription requirements for their purchase and therefore increasing risk and use by the population, converting them into CECs (Tan et al., 2018). The pharmaceutical compounds and personal care products are classified as PPCPs (Yang et al., 2020). PPCPs are ubiquitous in environmental compartments due to the extensive use and inefficient removal in the WWTPs, increasing their risks on the biota (Ebele et al., 2017; Hopkins et al., 2016).

Pharmaceuticals are classified according to their target or type of affection they treat. Some of the most known are antihypertensives, antidepressants, analgesics, antibiotics, or anti-inflammatory (Afonso-Olivares et al., 2013) compounds. Each one possesses specific chemical properties. The vast majority are lipophilic, meaning that they are persistent in water and bioaccumulate in organisms (Chen et al., 2018).

Pharmaceuticals in the environment and biota.

More than three thousand different pharmaceutical substances have been detected in waters around the world (Boxall et al., 2012; Richardson et al., 2005). Animals and humans can excrete these chemicals as intact compounds or as metabolites derived from product degradation. They are released to the environment in direct or indirect ways (Richardson et al., 2005; Tan et al., 2018), highlighting the importance of the correct process in the WWTPs as vital to decrease the reinsertion into the water. Due to uncontrolled consumption, their presence in surface waters has been extensively evaluated to determine that compounds are predominant. The PPCPs were present worldwide from Uganda lakes (1-5600 ng/L) (Nantaba et al., 2020) or European rivers (122-640 ng/L) to drinking water (15-180 ng/L) (Padhye et al., 2014; Rodil et al., 2012). Pharmaceuticals were found in effluents around the world in variables ranges from 18 to 9965 ng/L in Asia, Europe, or USA (Nantaba et al., 2020; Papageorgiou et al., 2016; Su et al., 2020).

The PPCPs bioaccumulation capacity is directly related to their log Kow values, entering to biota tissues by selective uptake (Chen et al., 2018; Liu et al., 2018) and converting the aquatic organisms in a primary target. About thirty-four pharmaceuticals were detected in biota samples in the food web in vertebrates like fishes ranging to 174-269 ng/g (Xie et al., 2019c; Yang et al., 2020) even achieving 500 µg/L (Chen et al., 2018). The values in aquatic invertebrates were 311.8 and 159 ng/g in zooplankton and mollusks, respectively (Yang et at., 2020).

Pharmaceuticals Toxicity

Pharmaceuticals are ionizable molecules at environmentally relevant pHs; hence the ionization state in freshwater ecosystems can influence their potential toxicity on the organism. Biological effects were observed at different levels in the aquatic fauna. Endocrine disruption was checked through reproduction toxicity and development defects in fishes by nonsteroidal anti-inflammatory drugs (NSAIDs) exposure like diclofenac (Bickley et al., 2017; Yokota et al., 2015) besides defects in snail reproduction were produced by fluoxetine (Sánchez-Argüello et al., 2009). Paracetamol and acetylsalicylic acid demonstrated the activation of oxidative stress mechanisms in crustaceans and mollusks (Antunes et al., 2013; Piedade et al., 2020). Moreover, antidepressant compounds developed

its action through brain modulation, even damaging non-target organisms as *Daphnia magna* (Hossain et al., 2019; Yang et al., 2018) or mollusks with behavior alteration (Fong & Ford, 2014).

On the other hand, antibiotics as tetracycline are capable of modifying multiple routes such as endocrine, stress, and oxidative in invertebrates as *C. riparius* (Xie et al., 2019b). In summary, pharmaceutical compounds are a developing point of concern in the current context. Actually, from 2007, some members such as diclofenac or ibuprofen were identified as future emerging priority candidates by the European Union Water Framework Directive (Ellis, 2008). As a representative, ibuprofen was selected to evaluate its effects on the selected organism, *Chironomus riparius*.

Ibuprofen

Ibuprofen (figure 19) belongs to the nonsteroidal anti-inflammatory drugs (NSAIDs) employed for rheumatoid arthritis and musculoskeletal disorders treatment (Motov et al., 2019). It acts inhibiting the cyclooxygenase enzymes, COX-1 and COX-2, triggering the blocking of prostaglandin synthesis. Ibuprofen consumption has become widespread in recent years, being the most sold over-the-counter (OTC) medication in the world (Thorpe et al., 2013).

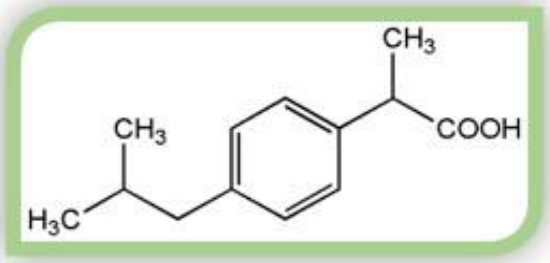

Figure 19. Chemical structure of ibuprofen.

Despite the high-efficiency elimination in the WWTPs, about 90% (Kermia et al., 2016), due to its lipophilicity and ubiquity, nowadays is present in the sediment, surface, and even drinking water (Ferrando-Climent et al., 2012; Santos et al., 2010). Effluents collected from Europe and Asia showed values in ranges from 3.08 - 140 µg/L to 341 ng/L, respectively (Kermia et al., 2016; Wiegel et al., 2004). Moreover, multiple studies detected ibuprofen in surface waters. For example, in Algeria, values between 341 to 431 ng/l were measured in tap and surface water (Kermia et al., 2016). In Uganda lakes, its presence was detected, ranging from 6 to 780 ng/L (Nantaba et al., 2020). Thus, it can affect the life and development of the organisms living in these waters.

Various authors have evaluated the effects of ibuprofen in vertebrates as fishes, altering the embryonic development or immune system even oxidative stress events were developed (Gutiérrez-Noya et al., 2020; Mathias et al., 2018). In the cases of invertebrates, the studies were mainly focused on mollusks and crustacea. In mollusks, the alteration in metabolic pathways as lipid peroxidation or detoxification was observed (André & Gagné, 2017; Milan et al., 2013). In the case of crustaceans, ibuprofen caused deformations as well as activation of detoxification mechanisms (GST, SOD, or CAT) so similar to vertebrates, the ibuprofen could develop oxidative damage (Grzesiuk et al., 2020; Wang et al., 2016). Besides, one study focused on behavioral effects, speed, or distance movement on *Diamesa zernyi* using different CECs like ibuprofen (Villa et al., 2018).

Results

Effects of Ibuprofen on survival, gene expression, and enzymatic activity

In the last times, the PPCPs have gained significant attention in aquatic toxicology due to the excessive consumption of pharmaceuticals reaching the environment, mainly to surface waters (Nantaba et al., 2020; Su et al., 2020). Ibuprofen is present in diverse surface water developing effects on aquatic organisms (Grzesiuk et al., 2020; Gutiérrez-Noya et al., 2020). In this thesis, the effects of ibuprofen employing environmental concentrations were evaluated in *C. riparius* fourth instar larvae for 24, 96h. Survival, gene expression, and enzymatic activity analyses were performed.

Survival effects

No previous survival studies have been published for ibuprofen on *C. riparius*, so five different concentrations were tested along 96h. The results are shown in Table 17. Survival was significantly altered respect to the control at 24h for 1, 10 and 100 µg/L besides at 48, 72, and 96h by all the conditions tested. In general, as it is expected, the survival was decreased along the time; however, low concentrations as 1 µg/L produced a survival percentage lower than the high concentration evaluated, and this pattern is repeated at all the times evaluated. The LC10 values for 24 and 48h were obtained by probit analysis ($LC10_{24h}$= 252.31 µg/L; $LC10_{48h}$= 0.024 µg/L), confirming the strong effect of Ibuprofen on C. riparius survival.

	Time (hours)			
Ibuprofen (µg/L)	**24**	**48**	**72**	**96**
0	100 ± 0	97,77 ± 0,32	95,55 ± 0,90	92,22 ± 0,93
0,1	97,78 ± 1,82	91,11 ± 0,32 **C**	81,33 ± 1,30 **C**	66,66 ± 1,93 **C**
1	91,11 ± 1,82 **C**	82,33 ± 2,89 **C**	75,55 ± 3,27 **C**	62,22 ± 1,92 **C**
10	95,55 ± 1,82 **C**	84,83 ± 1,01 **C**	75 ± 2,76 **C**	65 ± 2,76 **C**
100	91,11 ± 0,64 **C**	84,44 ± 2,05 **C**	81,10 ± 3,85 **C**	72,22 ± 4,58 **C**

Table 17. Survival percentages of C. riparius fourth instar larvae exposed at five ibuprofen concentrations. The **C** show the differences with control (0 µg/L), according on ($p < 0.05$).

Gene expression and enzymatic activity

In this case, eighteen genes were analyzed corresponding to four critical pathways in invertebrates, complemented with two enzyme activities. The results are shown in figures 20 to 27.

From the endocrine system, were selected seven genes (*EcR, Dronc, InR, Dis, JHAMT, Met,* and *Kr-h1*) belonging to the 20-E and JH synthesis or signaling pathways (figures 20, 21, and 22). Only three genes (*EcR*, *Dronc*, and *Met)* showed significant differences at 24h for all the conditions, with down-regulation respect to the control. However, at 96h, no effects were detected with respect to the control, although *EcR* and *Dronc* were upregulated respect to the control at 24h. Therefore, seems that the ibuprofen can alter the receptors for both enzymes (*EcR*, *Met*), besides the 20-E signaling pathway was modified through the downregulation on *Dronc*. The rest of the genes evaluated were not altered at any of the conditions employed, as shown in figures 21 and 22. The synthesis of ecdysone involves *Dis* and *InR*, direct and indirectly, respectively, so ibuprofen appears not to compromise the synthesis of this hormone.

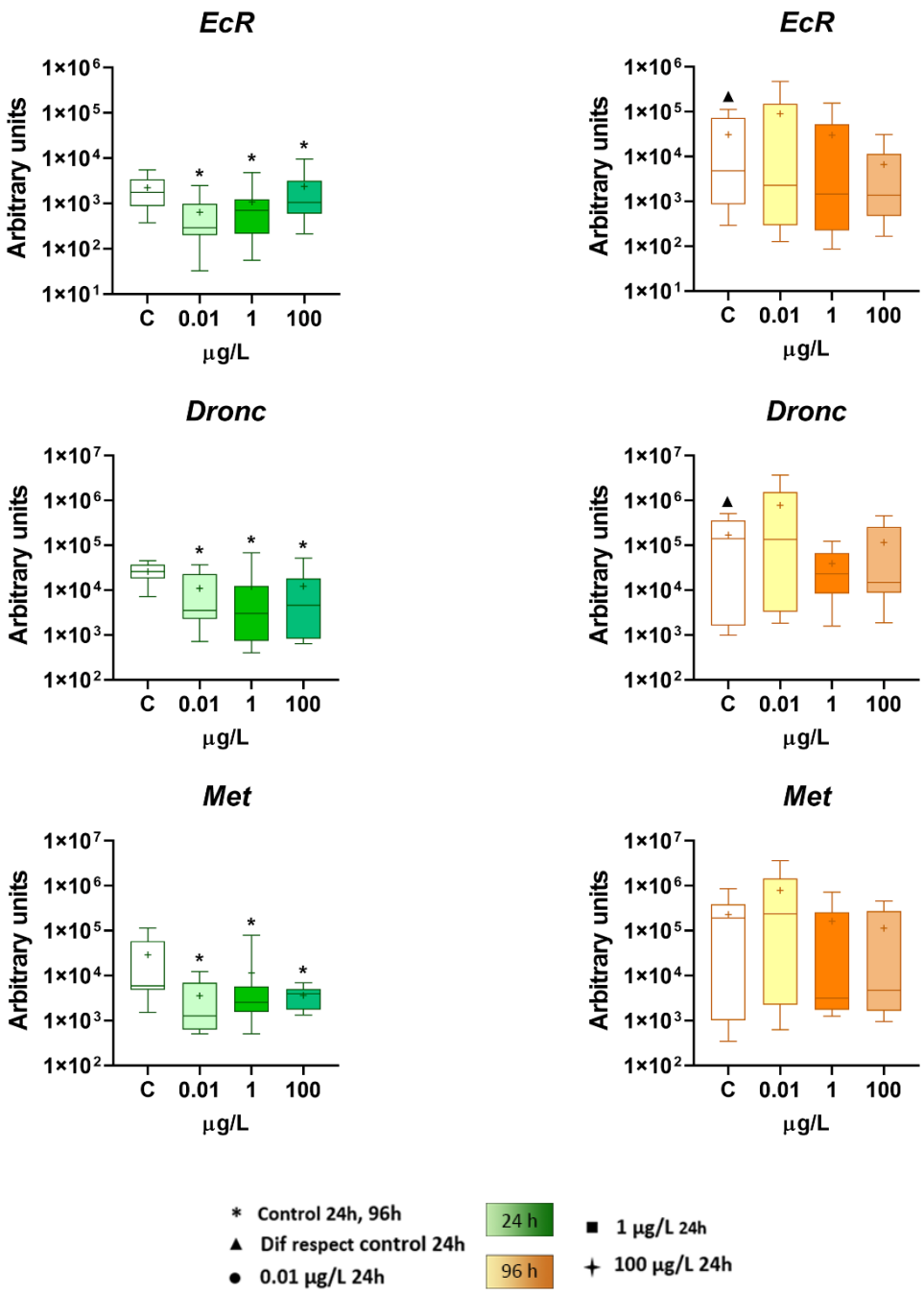

Figure 20. Expression of genes from endocrine system (*EcR*, *Dronc*, and Met*)* in the fourth instar *C. riparius* larvae after exposure to 0.01, 1, and 100 µg/L ibuprofen at 24, 96h. mRNA levels were normalized using *rpL11* and *rpL13* as reference genes. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. All significant differences in respect to the control and the comparisons between treatments and times are defined in the legend, in all the cases, according to ($p < 0.05$).

Finally, *JHAMT* regulates the synthesis of the JH, maintaining unaltered expression. An identical pattern was observed on *Kr-h1*, which acts on the signaling pathway of the hormone (fig. 22). In summary, ibuprofen showed a more substantial effect on 20-E that on the JH with the only effect on *Met*.

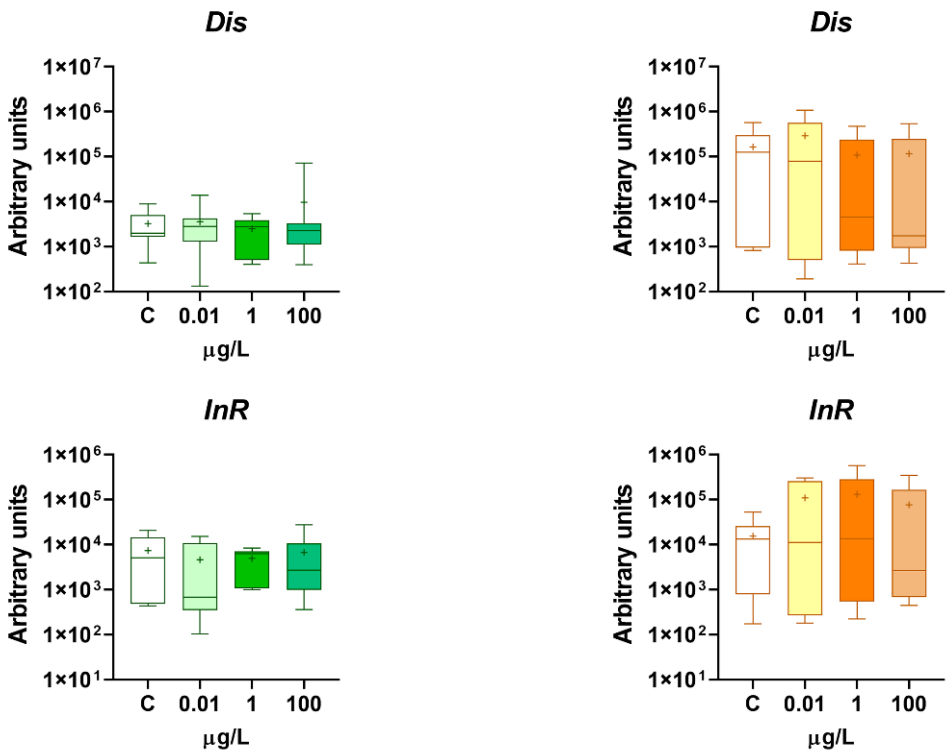

Figure 21. Expression of *Dis* and *InR*, from endocrine system in the *C. riparius* larvae after exposure to 0.01, 1 and 100 µg/L ibuprofen at 24, 96h. All significant differences, are according to ($p < 0.05$).

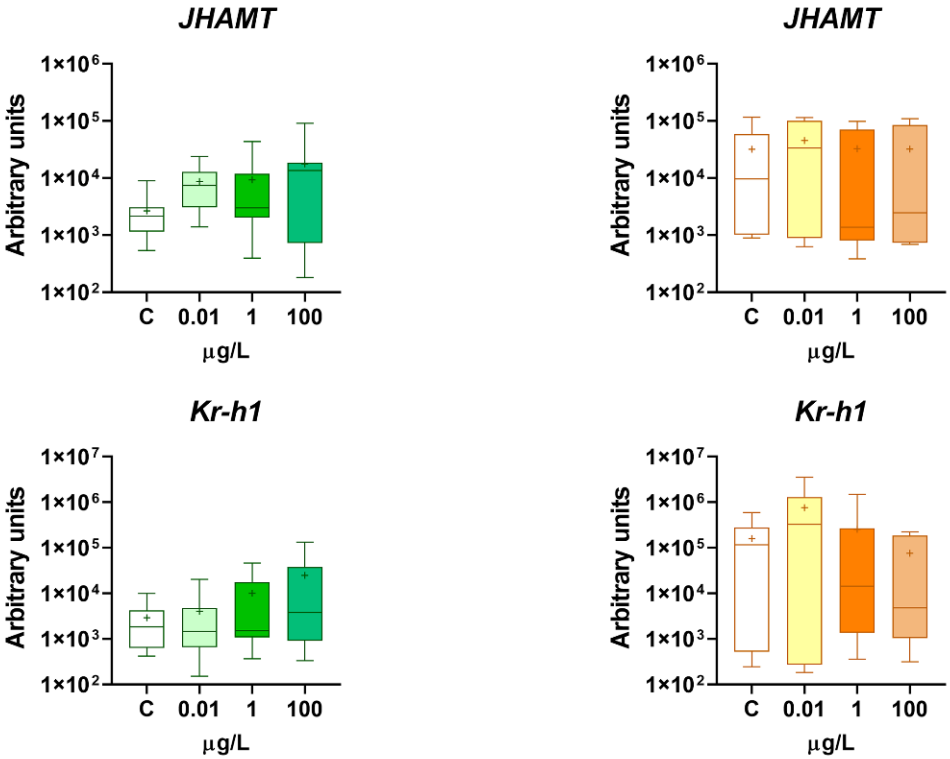

Figure 22. Expression of *JHAMT* and *Kr-h1* from JH, endocrine system in the *C. riparius* larvae after exposure to 0.01, 1, and 100 µg/L ibuprofen at 24, 96h. All significant differences, are according to ($p < 0.05$).

For the detoxification response five genes (*Cyp4d2, Cyp12a2, GSTd3*, *GSTo1,* and *ABCB6*) were analyzed (figures 23, and 24). No effects were detected in none of the genes evaluated at both times. However, the expression pattern trend for each time was different. In general, with lower expression at 96h, in line with the results observed on UVFs mixtures.

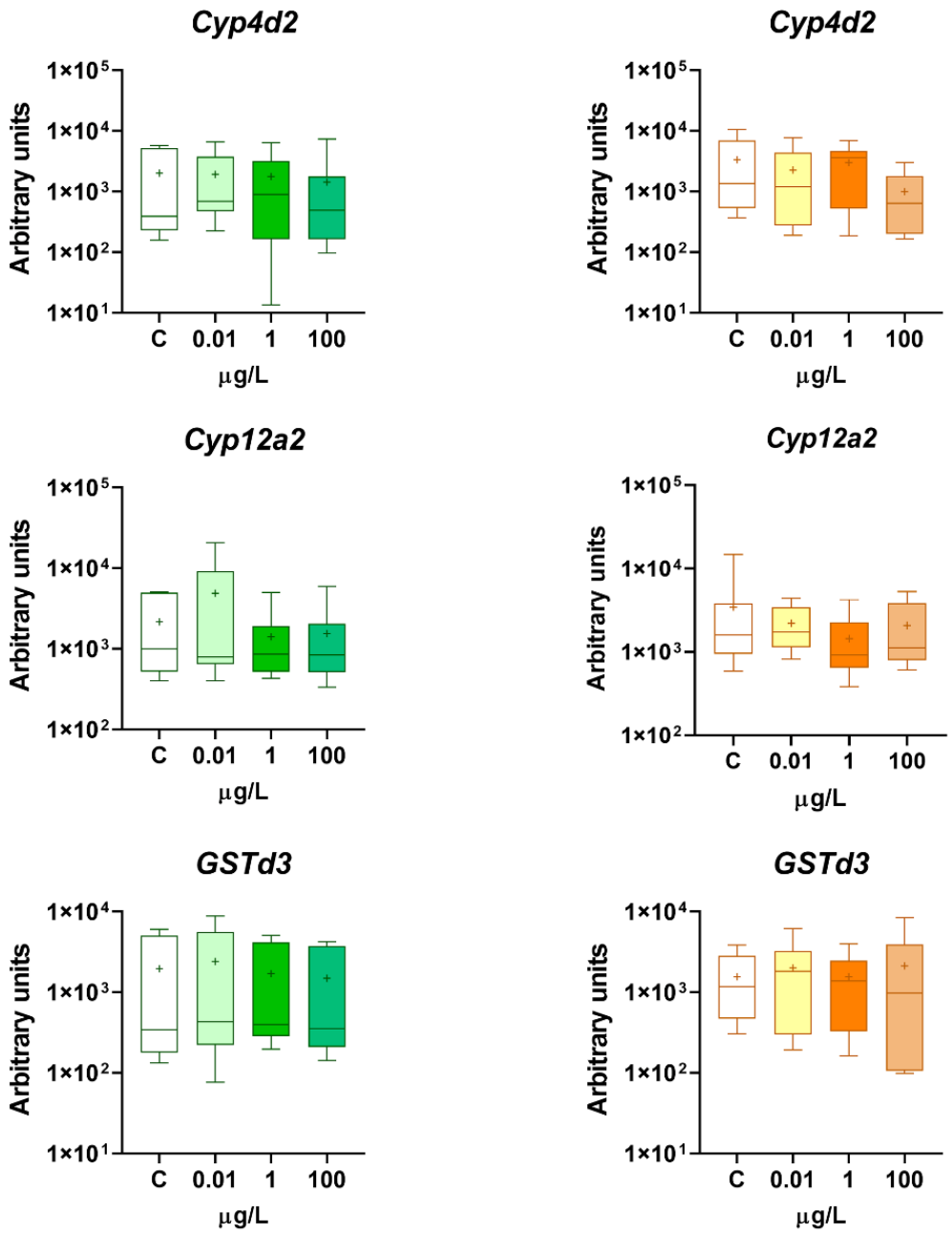

Figure 23. Expression of genes from detoxification response (*Cyp4d2*, *Cyp12a2*, and *GSTd3)* in the *C. riparius* larvae after exposure to 0.01, 1, and 100 µg/L ibuprofen at 24, 96h. All significant differences are according to ($p < 0.05$).

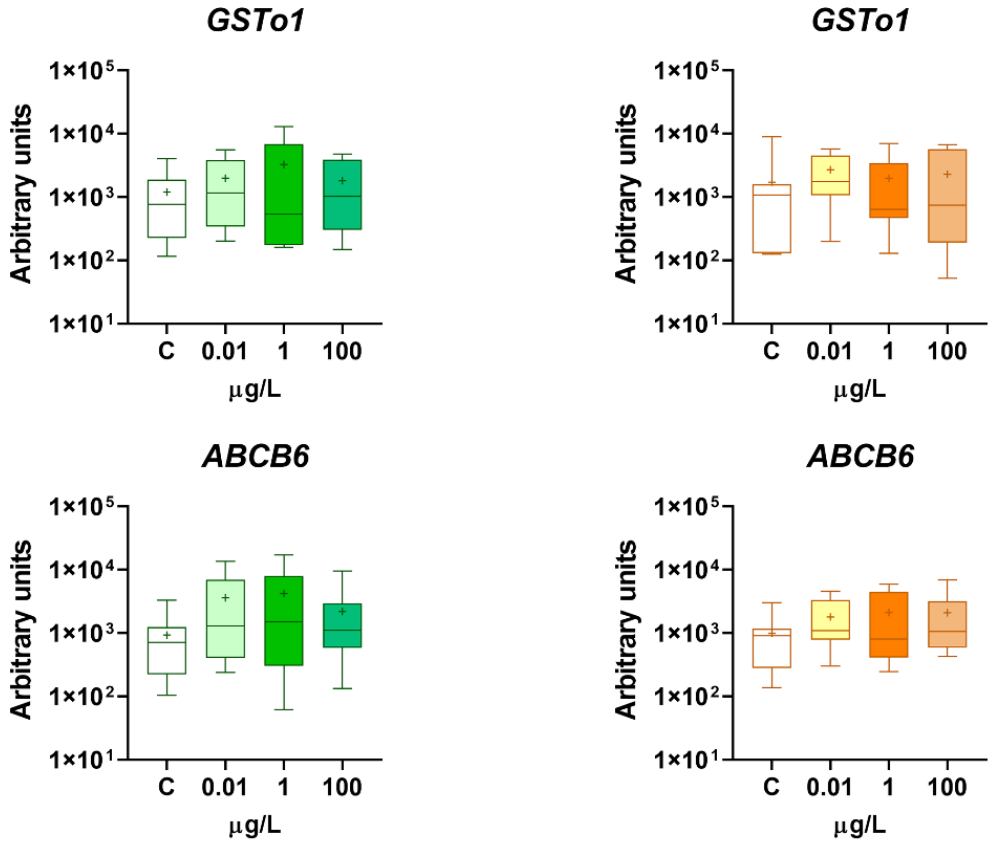

Figure 24. Expression of *GSTo1* and *ABCB6* related to detoxification response in the *C. riparius* larvae after the exposure to 0.01, 1, and 100 µg/L ibuprofen at 24, 96h. All significant differences are according to ($p < 0.05$).

Stress response was evaluated employing four genes, two from the hsp70 family (*hsp70* and *HYOU1*), and two for small HSPs (*hsp24* and *hsp27*).

Firstly, for the hsp70 family, compared to control treatment, no effects were detected at 24h. However, at 96h, only *hsp70* was altered after the low concentration exposure (fig. 25). So, the effects were low on Hsp70 family genes.

The sHSPs were altered at 24h exposure with decreased expression of *hsp24* at 1 and 100 µg/L and up-regulation at 0.01 µg/L for *hsp27*. Moreover, at 96h, *hsp27* showed differences in respect to the control at all the conditions tested (fig. 25).

By 96h, *hsp24* showed differences at control respect to the same condition at 24h. Additionally, *hsp27* showed differences for all the concentrations respect to 24h exposure. No effects were observed at 96h for the other genes.

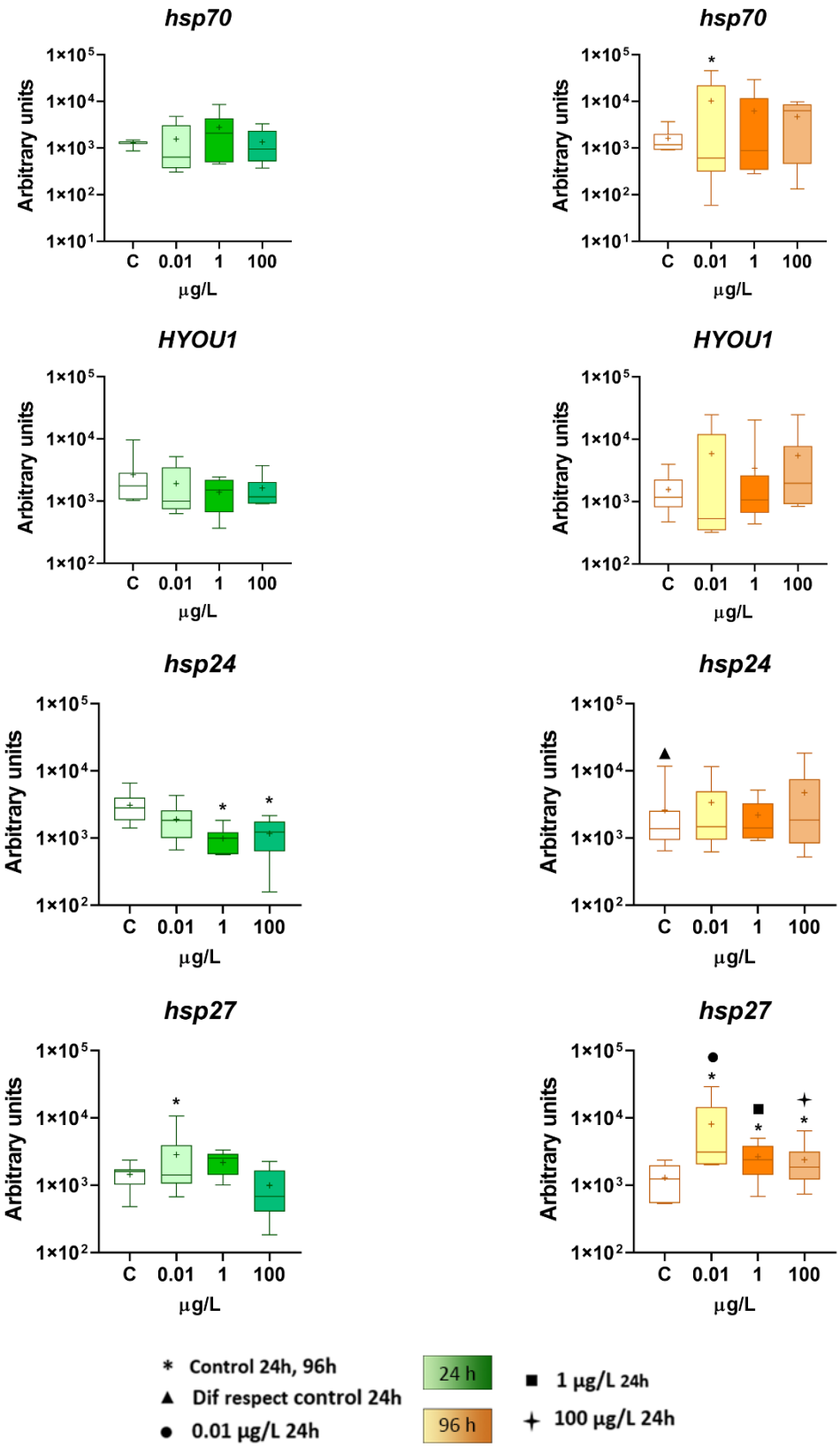

Figure 25. Expression of *hsp70*, *HYOU1*, *hsp24*, and *hsp27* related to stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.01,1 and 100 µg/L ibuprofen at 24, 96h. All significant differences are according to ($p < 0.05$).

The last system studied was the immune system, which was analyzed by employing two genes related to the humoral response (*Proph* and *Def*) in the figure 26. No effects were detected at 24h for gene expression. At 96h, significant differences in respect to the control were observed in *Proph* and *Def* with increased mRNA levels for all the conditions. Besides, significant differences were detected between times against all the concentrations.

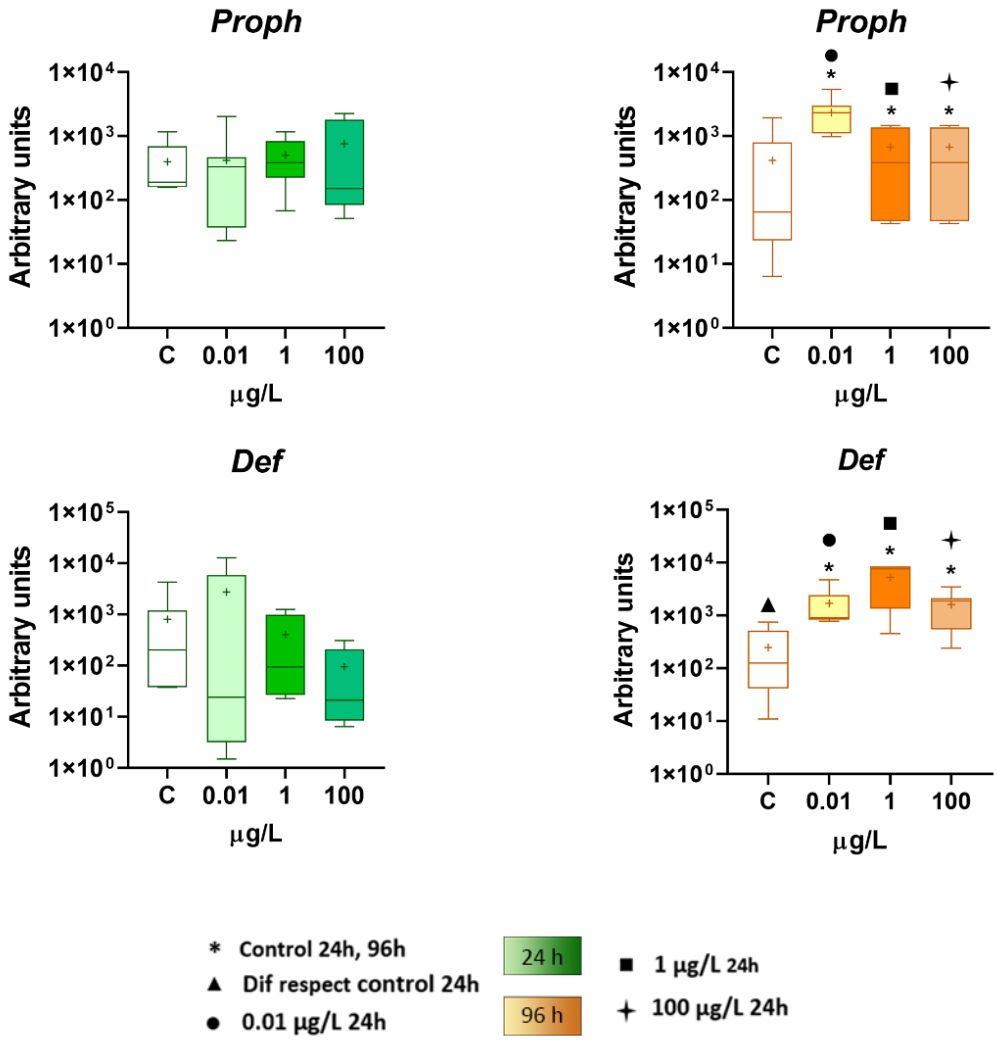

Figure 25. Expression of *Proph* and *Def* related to the immune system in fourth instar *C. riparius* larvae after in vivo exposure to 0.01, 1, and 100 µg/L ibuprofen at 24, 96h. All significant differences are according to ($p < 0.05$).

Additionally, two enzymatic activities were evaluated. On the one hand, the GST related to the detoxification response and, on the other hand, PO activity for the immune system (fig. 27). The GST activity was not altered at 24h but was increased at all the concentrations for 96h with significant differences respect to the control. Also, compared to 24h, higher activity was observed at 96h for all the conditions tested. Moreover, the PO activity was activated with significant differences concerning the control in both time exposure at all the conditions, and in 96h respect to the same conditions at 24h. Therefore, joined to the results on gene expression, ibuprofen was able to alter the immune response.

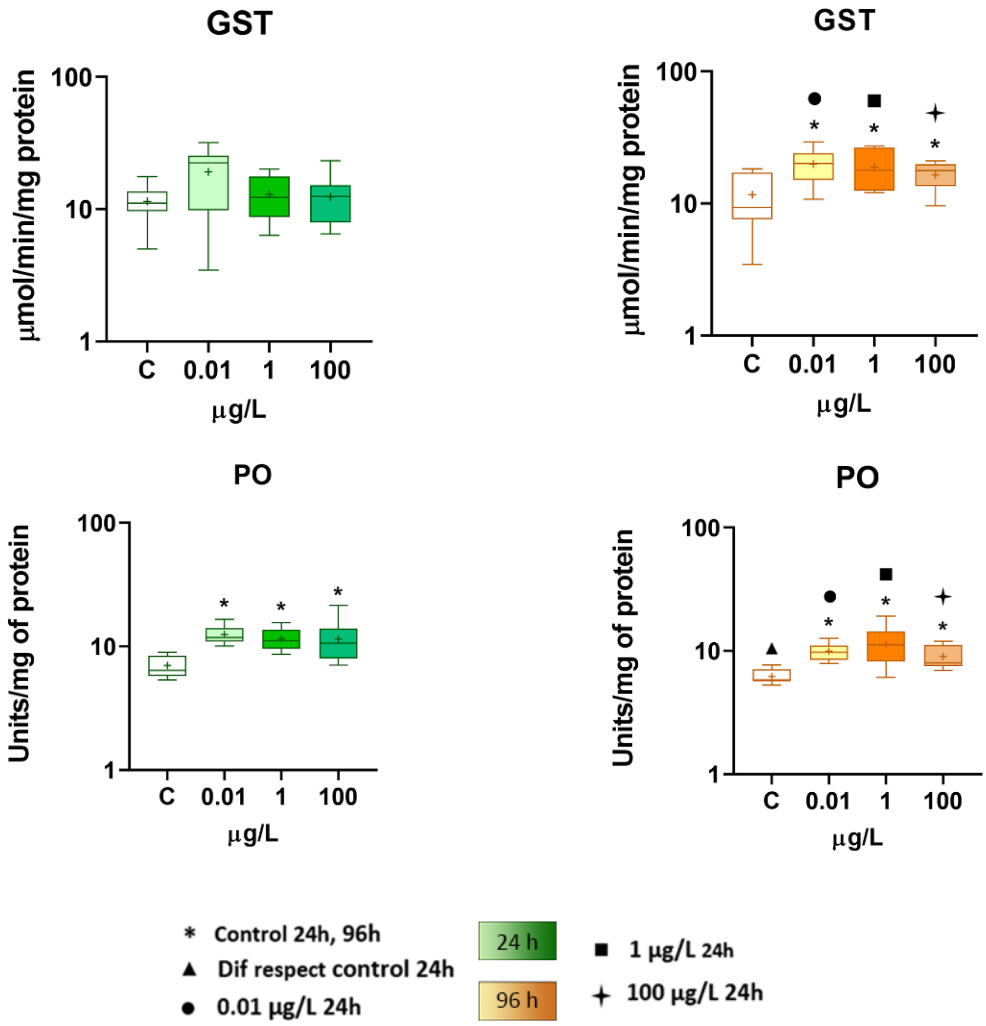

Figure 26. Enzyme activity of GST and PO in the fourth instar *C. riparius* larvae after in vivo exposure to 0.01, 1, and 100 µg/L ibuprofen at 24, 96h. All significant differences are according to ($p < 0.05$).

SUMMARY

- **Survival was significant altered at 1, 10, 100 µg/L for 24h and for all the concentrations at 48,72, and 96h.**
- ***EcR*, *Dronc* and *Met* were downregulated by all concentrations at 24h.**
- **No effects were observed on detoxification response at any of the phases**
- **At 24h *hsp24* was downregulated by 1, 100 µg/L and *hsp27* up regulated by 0.01 µg/L. Besides hsp27showed increased expression for all the conditions at 96h.**
- ***Proph* and *Def* were significant overexpressed by all the conditions at 96h.**
- **GST activity was significant increased by the three concentrations at 96h**
- **PO activity was increased at both times and all conditions.**

Discussion

Identical to other PPCPs derived from its chemical properties and extensively consumption, Ibuprofen has been detected in several compartments with mayor prevalence in surface waters at ng/L to µg/L ranges (Kermia et al., 2016; Nantaba et al., 2020).

Ibuprofen's effect on survival can be summarized as a strong impact at all the conditions tested with striking higher mortality for lower concentrations. Information on the toxicity of ibuprofen in invertebrates is scarce. In insects, only one report employing wild organisms defined the LC10 values at 24 and 48h (Cassa, 2018). Comparing those results to *C. riparius* ($LC10_{24h}$= 252.31 µg/L; $LC10_{48h}$= 0.024 µg/L), it is strongly affected and sensitive respect to them. It is evident in the case of *Pseudosiamesa branickii* ($LC10_{48h}$= 25340 µg/L), another chironomid. Similarly, other insects like *Baetis alpinus* ($LC10_{24h}$= 14800 µg/L) and *B. rondani* ($LC10_{48h}$= 18170 µg/L), sharing habitat conditions, also were more resistant to ibuprofen than *C. riparius*. When the focus is on other invertebrates, the pattern is identical with lower sensitivity than *C. riparius*. For example, low effects were observed on mortality in *Daphnia magna* for chronic exposure, showing effects only at 80 mg/L (Heckmann et al., 2007). In snails, acute exposure did not alter the survival at 24h only at 100 mg/L with 60% survival and reaching 0% at 48h (Pounds et al., 2008). Considering that *C. riparius* shows damage at a thousand times lower concentration, it is clear that this species is more susceptible to the Ibuprofen. Besides, the concentration needed to cause mortality in other invertebrates is above that found in the environment.

In short, ibuprofen, even in low concentrations as used in this study, can alter the survival of *C. riparius* on fourth instar larvae. It means that environmentally relevant concentrations could alter the habitat of this species. Parallel to the impact on survival also has been described alterations in behavior and morphology by ibuprofen in other invertebrates with morphological deformities in *D. magna* (Grzesiuk et al., 2020) and speed or movement modifications in *Diamesa zernyi* (Villa et al., 2018). Additional research is required to know the long-term impact of the Ibuprofen in those ecological relevant endpoints. However, the present work has raised the damage that could be done in the short-term to the population.

Ibuprofen, as PPCP, has been gaining special attention during the last years due to the growing presence in the freshwater environment, being classified as one of the leading CECs (Archer et al., 2017; Ebele et al., 2017). However, the information available concerning the toxicity on invertebrates is scarce, especially at the molecular level, hence transcriptional activity was analyzed to complement the survival information. PPCPs, similar to other xenobiotics, can act as EDCs mimicking the hormone action and potentially interfering with the hormonal system (Park & Kwak, 2010). Evaluation of the effects of ibuprofen as putative EDC was performed by studying seven genes involved in the hormone response pathway (*EcR*, *Met*, *InR*, *Kr-h1*, *Dronc*) and the synthesis (*Dis*, *JHAMT*). Globally, the effect as EDC of ibuprofen seems to be weak. Only some changes were detected at 24 hours for *EcR*, *Dronc*, and *Met*. These genes are related to the ecdysone and juvenile hormone response pathways. As the changes were downregulation in most of the cases, it could be proposed that ibuprofen shows a weak endocrine disruptor potential by repressing the activity of the hormone. However, there is no detectable effect on synthesis. The impact on the development could be significant in case the changes were maintained. However, there was a recovery to standard levels at 96 hours, suggesting a transient effect of ibuprofen. It would be interesting to know if this compound can be degraded or biotransformed, causing the lack of response at a longer time. In any case, the changes can alter the development in *C. riparius* because ecdysone and juvenile hormone pathways interact to control the molting and the metamorphosis (Jindra et al., 2013).

The 20E controls ecdysis and metamorphosis in arthropods by binding the EcR. Activated *EcR* regulates the activity of the response pathway (Yamanaka et al., 2013). Similar results were observed by bisphenol S another suspected EDC, in crustaceans (In et al., 2019). Besides, alterations on fish embryos development were detected by ibuprofen (Gutiérrez-Noya et al., 2020). Altogether, the result indicates that ibuprofen can show endocrine activity, but it is not by modifying the metabolism of the hormones. *Dis* belongs to the Halloween group genes, and it is responsible for catalyzing the last steps of ecdysone synthesis (Hentze et al., 2013), so the stable transcription suggests that ecdysone synthesis is unaffected at the conditions tested. Similarly, *JHAMT*, which oversees the final step in the synthesis by methylation (Shinoda & Itoyama, 2003), and was not modified. There are still few data about these genes in the literature, but *Dis* was not altered by the fungicide Vinclozolin (Aquilino et al., 2016).

The last changing gene, *Dronc*, is a so-called effector gene in the ecdysone signaling pathway. It means that it is not a transcription factor and performs actions related to the structural and physiological changes induced by the ecdysone. It is a caspase that participates in apoptosis in damaged cells in *Drosophila* (Khan et al., 2017). The activation could be related to the *EcR* so that it would be reflecting the activation of the ecdysone response pathway. Also, it may be related to some additional damage that activates apoptosis. The lack of response of genes that usually are activated earlier than *Dronc* opens the possibility of a hormone-independent activation. Previously, *Dronc* only has been evaluated in *C. riparius* after metal exposure (Martín-Folgar & Martínez-Guitarte, 2019), showing no changes. The diverse nature of the compounds used could justify a different result but also highlights the interest of analyses of a set of genes. The profile can be diverse depending on the nature of the chemical and, it is a possibility, these transcriptional profiles could add information about the mechanisms involved.

On the other hand, *Met* as a receptor of JH modulating its action, then the downregulation by ibuprofen could alter development on *C. riparius.* Previously alterations were detected according to the type of UVF used, so the response of this gene seems to be dependent on the compound. Decreasing transcriptional activity can hinder the action of JH because *Met* works as a transcriptional regulator, binding to JH target gene promotors and controlling the actions of JH (Ojani et al., 2016). The final physiological changes due to the modulation of nuclear receptors as *EcR* or *Met* depend on also the rest of the genes and processes involved. One example is the transcription factor *Kr-h1*, which takes over the anti-metamorphic effect of JH, being inducible by *Met* (Jindra et al., 2013) and is related to the ecdysone pathway, being a key piece in the regulation of metamorphosis. It is a connection between both hormone signaling pathways, and it seems that there is no effect. As the analysis has been included two times, it is hard to think that it is a transient response, and the modulation has been missing. In any case, additional research is needed to elucidate the processes that are altered by the ibuprofen.

In summary, it has been demonstrated that ibuprofen has potential as EDC. It works on the signaling pathway of two hormones involved in molting and metamorphosis, unaffecting their synthesis. However, it cannot be discarded other effects or indirect influences since some other hormone-related pathways and processes need to be studied. Nuclear receptors can be modulated in different ways depending on the chemical, as has been demonstrated here with ibuprofen and UVFs. Consequently, it can be proposed that chemical nature is a crucial element in the response of nuclear receptors. Moreover, ibuprofen acts blocking the prostaglandins synthesis through inhibitions of COX enzymes. Due to the role of this lipids on the reproduction system in insects, these alterations on the endocrine system could be also related to defects on the reproduction system, derived from the ibuprofen exposure.

The detoxification response for ibuprofen is still unknown in this species. As a starting point, five genes covering the three phases were selected; two of them were from Phase I (*cyp4d2*, *cyp12b2*), two from Phase II (*Gstd3*, *Gsto1*), and one from Phase III (*ABCB6*). Furthermore, the GST enzyme activity was studied. None of the genes showed changes in the transcriptional activity. The result could be explained in two ways. One possibility is that the genes selected were not good because none of them are involved in the biotransformation of ibuprofen. However, previous analysis with CYPs in other aquatic invertebrates, like *Daphnia magna* or *Ruditapes philippinarum,* exhibited altered expression by ibuprofen (Milan et al., 2013; Wang et al., 2016). It might be because other families were studied.

Phase II analysis also involved the GST activity, which was increased at 96 hours. GST activity has also been detected in the presence of ibuprofen for fishes and crustacea (Mathias et al., 2018; Wang et al., 2016), so the absence of response for *GSTd3* and *GSTo1* could be due to diverse GST classes (Atkinson & Babbitt, 2009). For example, theta and zeta classes are involved in protection against oxidative stress (Hayes & McLellan, 1999). Therefore, GST activity is activated, but it is unclear if the activation is a consequence of the detoxification or related to other processes. Previously it has been proposed that a second possibility could explain the results. It would be that *C. riparius* has tolerance to ibuprofen at the concentrations tested. Ibuprofen induced GST activity, suggesting the activation of detoxification mechanisms at the Phase II level. However, due to the role of GST as an antioxidant enzyme, this activation could be explained by oxidative damage because reactive oxygen species (ROS) generation derived from ibuprofen exposure. This ibuprofen toxicity mechanism has been confirmed in fishes (Gutiérrez-Noya et al., 2020). It would be necessary for the analysis of additional genes to confirm this possibility. Summarizing, ibuprofen could activate Phase II detoxification in *C. riparius,* but further research is required to discard the involvement of other cell processes that also activate the GST activity.

Phase III was studied by analyzing the response of an ABC transporter gene, *ABCB6*. There is no information on the literature about this gene related to toxicants on invertebrates. However, it has been observed that toxicants can activate ABC transporters, as happens with the *MRP-1* gene in *C. riparius* with UVFs (Martínez-Guitarte, 2018).

Another cell process analyzed was the stress response. Four genes covering the Hsp70 and the small HSPs families were selected. Both genes of Hsp70 family, *hsp70* and *HYOU-1*, showed a weak induction or not induction. On the contrary, *hsp24* and *hsp27* showed changes highlighting the utility of the small HSPs as biomarkers for toxicity. These data showed that the classical stress response is not fully activated. However, it cannot be discarded that a non-detected activation was in progress between 24 and 96 hours since the increase is observed in the lower concentration tested. It could mean that the cell is recovering the standard situation, and the dynamic is depending on the concentration. The *hsp70* transcriptional activity has been extensively studied, and different xenobiotics and organisms have modified it (Herrero et al., 2015; Liu et al., 2015; Morales et al., 2011; Xia et al., 2017). The other member of the Hsp70 family, *HYOU-1*, was unaffected. Although no changes were also observed for BP-3, it has been described that this gene can be induced in fish taken from a polluted river. The exposure was to a mixture of pollutants, so it is difficult to connect the effect to only one chemical (Mitra et al., 2018). Furthermore, pollution may alter the oxygen levels in the water so that the fish could be exposed to hypoxia. Thus, ibuprofen at environmentally relevant concentrations had almost no effect on the activation of Hsp70 family genes.

The other stress genes analyzed were the small HSPs. As indicated, both of the changed their levels by ibuprofen. The interesting fact is that *hsp24* was downregulated at 24h, while the trend for *hsp27* was to be upregulated at later times. In invertebrates, small HSPs are a diverse family with a specific number of genes that code to a variable number of proteins. The difficulty is that they are named upon

the molecular weight, doing complicated to know the homology between the species. Fortunately, there is some previous analysis in *C. riparius* with those proteins that show a complex response with variable patterns depending on the chemical (Aquilino et al., 2016; Martín-Folgar & Martínez-Guitarte 2017; Martín-Folgar et al., 2018). Ibuprofen is not different and shows a specific response. However, by the concentrations used and the response observed, it could be suggested that it is more effective than other compounds in the induction of sHSPs. An extensive analysis of sHSPs genes will provide a better picture, but these results confirm the goodness as biomarkers of this set of genes.

In summary, Ibuprofen seems to compromise stress response mechanisms and other essential actions that are regulated by sHSPs in *C. riparius*. Moreover, sHSPs could be interesting biomarkers for pharmaceutical compound evaluation because they can react even after limited exposure (24h).

The last analysis related to ibuprofen included two genes and one enzyme activity involved in immunity. Right now, there is no information about the effects of ibuprofen on the immunity of invertebrates. It is known that it produces oxidative stress in mussels, connecting the immune system with the ROS system and the oxidative stress (André & Gagné, 2017; Contardo-Jara et al., 2011; Stokman et al., 2017). The immunity was studied by testing two genes involved in the humoral response. Upregulation for both genes was observed at 96h. The enzyme activity analysis of the PO showed that it is increased yet at 24 hours, so it is activated as an early response. Then, there was an initial activation of PO and, later, the gene coding for the pro-enzyme was upregulated.

The *Proph* response is akin to observations of crabs after exposure to plasticizers (Park et al., 2019). The PO is a critical enzyme in melanin synthesis for wound healing and tanning of the cuticle. It has been observed stimulated by an herbicide in crabs (Hong et al., 2019) or BP-3 in *C. riparius*. Besides, it has been defined as an essential biomarker in crustacean and mollusks (Luna-Acosta et al., 2017). The induction of the enzyme is surprising since it is usually activated in response to pathogens, but it is not probable that the ibuprofen was detected as a pathogen. More research is needed, but it could be speculated that the activation is because ibuprofen acts on the transcription regulation, it is considered as a substrate by the enzyme, or it is producing some kind of hypersensitivity that could resemble allergic responses.

Another issue in this immunity is the detection of pathogens in the surface and the synthesis of antimicrobial peptides (AMPs) as a mechanism of host defense against pathogens like viruses, bacteria, or fungi (Parisi et al., 2015). The antimicrobial and anti-inflammatory properties of AMPs were recently observed on beetles. Besides, increased expression of AMPs has been linked to the resistance of pesticides in insects (James & Xu, 2012; Lee et al., 2019). Therefore, the increased *Def* expression by ibuprofen could be indicated that this compound is more toxic in *C. riparius* comparing to other xenobiotics, as UVFs that did not alter its expression. It also could be part of the anti-inflammatory response as part of a hypersensitivity episode. The last possibility is attractive since it would be one of the first time describing this kind of immune process.

Ibuprofen activates the immune system of *C. riparius* after short- and long-term exposure; hence seems to be toxic to this organism. The immune response is stimulated by damage and is a mechanism for avoiding and reducing harmful effects on the organism. Despite low concentrations, the immunity response seems to be especially sensitive. The activation could also have an indirect effect. Besides, because of the role of prostaglandins in the insect immunity, their inhibition synthesis by the ibuprofen could be derived in this immune system alteration. The immune system is energy-consuming because of the need to activate and synthesize many different molecules and regulators. Thus, the energy required in other essential processes, such as breathing and feeding, could be diminished, limiting the life of the organism and its future response against aggressions (Dolezal et al., 2019).

Ibuprofen, like many other PPCPs, is a CEC with potential effects on aquatic organisms that has been on the rise in recent years. For the first time, the potential effects of ibuprofen on survival, gene expression, and enzymatic activity were analyzed on the aquatic organism, *C. riparius*. Ibuprofen showed a substantial impact on survival in *C. riparius,* with LC10 values reaching up to 100 times lower than those observed in other invertebrates from insects to crustaceans. Hence, *C. riparius* appear to be more sensitive to ibuprofen than other invertebrates. It could compromise the future of the populations due to the concentrations assessed are similar to environmental.

At the transcriptional level, essential metabolic mechanisms were modified. The alterations on the endocrine system genes suggest that development and metamorphosis events could be disturbed by the negative impact on the nuclear receptors. The activation on Phase II through increased GST activity could be related to possible damage on *C. riparius*, derived from ROS species; however, it could not rule out that the detoxification process could be loaded by other Cyp or GST forms due to the vast number of different classes. Additionally, ibuprofen had a substantial impact on sHSPs, which could provoke defects in the stress response and standard protein folding. Finally, the activation of immune responses in the short and medium-term, due to its essential role in defense, supports the potent toxicity of ibuprofen in this aquatic insect. Moreover, the activation for the first time could be related to some hypersensitivity reaction.

Finally, system activation can indirectly compromise the energy balance due to the enormous resources required to function. Overall, ibuprofen affected all of the mechanisms studied, demonstrating that NSAID compounds, and specifically ibuprofen, provoke alterations on *C. riparius* metabolism. Because the prostaglandins are expressed along the insect's body like and their role in a different process like physiology. Ibuprofen seems to act similarly that on humans blocking its synthesis and then altering critical systems like endocrine or immune. Due to the environmental concentrations used here, these results emphasize the risk placed on aquatic invertebrates, particularly on *Chironomus* populations. Therefore, future studies should include a joint evaluation at both the organismal and sub-organismal level and should analyze molecular responses, including transgenerational evaluations, to confirm the tendency of ibuprofen to compromise the survival of these populations.

ENDOSULFAN

6. ENDOSULFAN

Description, characteristics, and uses

Pesticides are defined as any substance used to prevent, destroy, repel, control, or mitigate any pest in crops before or after the harvest. From the middle of the XIX century until today, the pesticide industry has developed multiple products for the maintenance of crops in optimal conditions. Global pesticide consumption is 11.8 % in Europe, 33.3 % in the USA, and about 51.3 % in Asia. Playing an essential socio-economic role in all the world; for example, about 184.6 million hectares are dedicated to land use in Spain and France (Pérez-Lucas et al. 2018). Pesticides can be classified, according to their uses, into three groups: fungicides, herbicides, and insecticides. Fungicides are mainly employed as prophylaxis treatment and represent about 29% of pesticides (Mojiri et al., 2020). Herbicides are the primary type, with 46% of the production and showing twenty-seven different modes of action. Finally, the insecticides are used for indoor and outdoor applications and represent about 17 % of the pesticides. Moreover, they are cataloged based on their chemical nature in ten groups: Benzoic acid, Carbamates, Dipyridyl, Organochlorines, Organophosphates, Phenoxy, Phenylamides, Phthalimides, Pyrethroids, and Triazines. The chemical nature and properties determine their impact, half-life, and persistence in the environment. According to its chemical nature, some of the most commonly used pesticides are the organochlorinated pesticides (OCPs), and then the study will be focused on them.

The organochlorines (OCPs) possess highly efficient insect pest control being applied from a half-century. They are hydrophobic, resistant, and most persistent pesticides (Taiwo, 2019). Some members, as endosulfan, belongs to the thirty listed POPs (Xu et al., 2013). Some of the best known OCPs are dithiothreitol (DDT), dicofol, dieldrin, endosulfan, heptachlor, lindane, and methoxychlor (Jayaraj et al., 2016). DTT and endosulfan were banned in many countries due to their toxicity for humans and ecosystems. However, they are still in use in developing countries.

Pesticides in the environment and biota.

Pesticides have long attracted public concern because of their potential environmental risks. When released into the soil, water, and atmosphere, pesticides can affect the health of non-target organisms, including humans, in different ways, such as direct contact, transformation, and migration via food webs. Derived from the extensive use and due to only 1 % of the pesticides applied to fields go directly to the target, they reach quickly to the environment where are frequently detected (Ali et al., 2019). The principal receptors of these xenobiotics are the soil and sediment, which can retain in their particles or release to water bodies, even polluting the drinking water.

Pesticides are environmentally persistent as they remain for a long time in soils and sediment (Carvalho, 2017). In Asia, agriculture lands showed the presence of OCPs pesticides in 84 % of the samples analyzed (Tan et al., 2020). However, in Europe, the values were higher near to 0.058 to 16.9 µg/Kg (Qu et al., 2019). They can reach the waters by runoff, stormwater, or from agriculture lands, having the ability to interchange between water reservoirs by spray drift, leaching, or subsurface drainage (Cosgrove et al., 2019). Pesticides in water have been assessed around the world; in South America, different pesticides were detected in ranges between 0.12 to 26.28 ng/L (Giordano et al., 2011; Grimalt et al., 2017). In China, twenty-nine different pesticides were detected in rivers reaching up to 103760 ng/L in winter (Xu et al., 2020). Finally, in Greece, 302 pesticides were quantified in values ranging from non-detected to 12.33 µg/L (Papadakis et al., 2018).

OCPs are lipophilic and are accumulated in fatty tissues on human or animal bodies and may be biomagnified along food chains (Taiwo, 2019). In humans, OCPs were detected in urine and hair

(Denghel & Göen, 2018; Lehmann et al., 2018) and in multiple fish species (Ernst et al., 2018; Pérez-Parada et al., 2018), even getting the higher value for endosulfan in India fishes (Arisekar et al., 2019).

Pesticides toxicity

Despite the benefits for the crops, the pesticides develop harmful effects on the environment. The damage generated vary according to the type of pesticide and its mode of action. Focusing on the OCPs as persistent pollutants that can have multiple effects on the organism, via direct or indirect through the food web. Its toxicity is mostly due to the presence of chlorine atoms, employing different vias as disruption of the endocrine system, oxidation stress, and epigenetic events. Scientific literature has evaluated the OCPs on humans showing various effects in the state of health as a neurological and immune defect, cancer disruption, and endocrine disruption (Jayaraj et al., 2016; Kumar et al., 2011; Pérez-Lucas et al., 2018;). Moreover, in fishes, endocrine disruption and neurotoxicity were proven with alterations on behavior and morphology (Martyniuk et al., 2020; Yu et al., 2017). In invertebrates, different pesticides as DEET or spinosad showed toxicity in *C. riparius* (Campos et al., 2016; Monteiro et al., 2019 a,b,c) and behavior alteration on honey bees (Villalba et al., 2020). Endosulfan was selected to study the effects of the OCPs on *C. riparius* (Figure 28).

a) **b)**

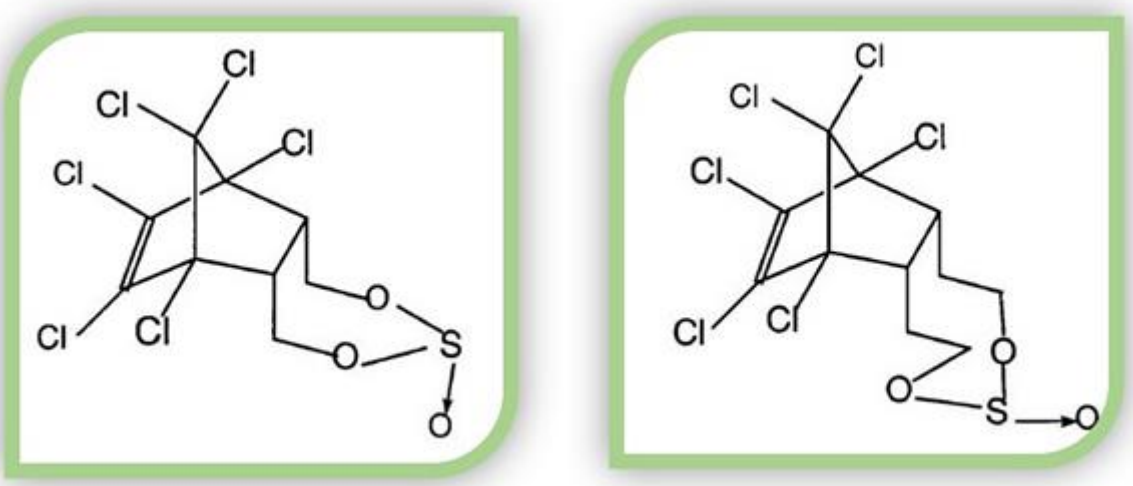

Figure 27. endosulfan a) alpha, b) beta chemical structure.

Endosulfan

Endosulfan, like OCP, has been used as an insecticide against pests and mites for the last five decades around the world (OSPAR 2004; Verma et al., 2011). It has been the most commonly used pesticide in the United States, India, Europe, China, and Australia for a variety of crops, including cotton, cereals, and fruits, as well as tea and coffee. It is commercialized as a mixture of α and β isomers in a proportion of 7:3, with the α isomer manifesting a longer half-life (about 157 days). It has been banned in many countries, although it is still used in developing countries due to its effectivity and low costs (Abbas et al., 2019).

Furthermore, due to its chemical properties (semi-volatility and high durability) like other OCPs is considered a persistent organic pollutant, hence a ubiquitous contaminant. Derived from its extensive use in agriculture is present in the air at 2.40 ng/m^3 (Fang et al., 2018) and soils with high values around 5 to 15 mg/kg in Europe and Canada (Daly et al., 2007; Růžičková et al., 2007). By runoff, it is disseminated and reach to water reservoirs in concentrations ranging from 0.036 to 62.3 µg/L in Africa and India (Arisekar et al., 2019; Mohammed et al., 2019).

Endosulfan residues were detected in organisms as birds or invertebrates (Fang et al., 2018), generating primary concern because of the possible biomagnification along with the food web and its possibility to reach humans via consumption. It was confirmed by its presence in blood, placenta, and other tissues in humans (Cerrillo et al., 2005; Ghosh et al., 2018).

Endosulfan, as other pesticides, is an extremely toxic and harmful compound to non-target organisms. In mammals, the nervous, immune, and reproduction systems were damaged by this pesticide (Lee et al., 2015; Milesi et al., 2015). In aquatic organisms, altered antioxidant and detoxifying enzymes, among other routes, were observed on fishes and clams (Kumari et al., 2017; Tao et al., 2013). Moreover, endosulfan modified microvilli and cuticle in earthworms (Shi et al., 2020) and being genotoxic to insects (Buhroo et al., 2016) showing their effects on invertebrates.

Results

Effects of endosulfan on gene expression and enzymatic activity

Pesticides have been a topic of importance throughout time due to their extensive use; there is a delicate balance between their advantages and disadvantages. Endosulfan spite banned in many areas is still used in developing countries (Abbas et al., 2019). Therefore nowadays has been detected in surface waters around the world (Arisekar et al., 2019; Mohammed et al., 2019). One of the main problems with this pesticide is its effects on non-target organisms like on aquatic organisms (Kim et al., 2018; Kumari et al., 2017).

The study was focused on the effects of endosulfan at the molecular levels in fourth instar larvae of *C. riparius* by using environmentally relevant concentrations of 0.1, 1, and 10 µg/L for 24 h exposure. The analysis was performed on the gene expression through five critical pathways in invertebrates, complemented with the study of the activity of three enzymes, GST, PO, and AchE.

Gene expression

The endocrine system was evaluated by ten genes (*MAPR*, *Dis*, *InR*, *EcR, Cyp18a1, E93, Dronc, JHAMT, Met,* and *Kr-h1*), and the results are presented in figures 29 and 30. The mRNA levels of five of the genes were significantly decreased in comparison to control (fig. 29). *Dis* was downregulated at all the concentrations, *MAPR* and *InR* at 10 µg/L, *E93* at 1 µg/L, and, finally, *Met* by 1 and 10 µg/L..

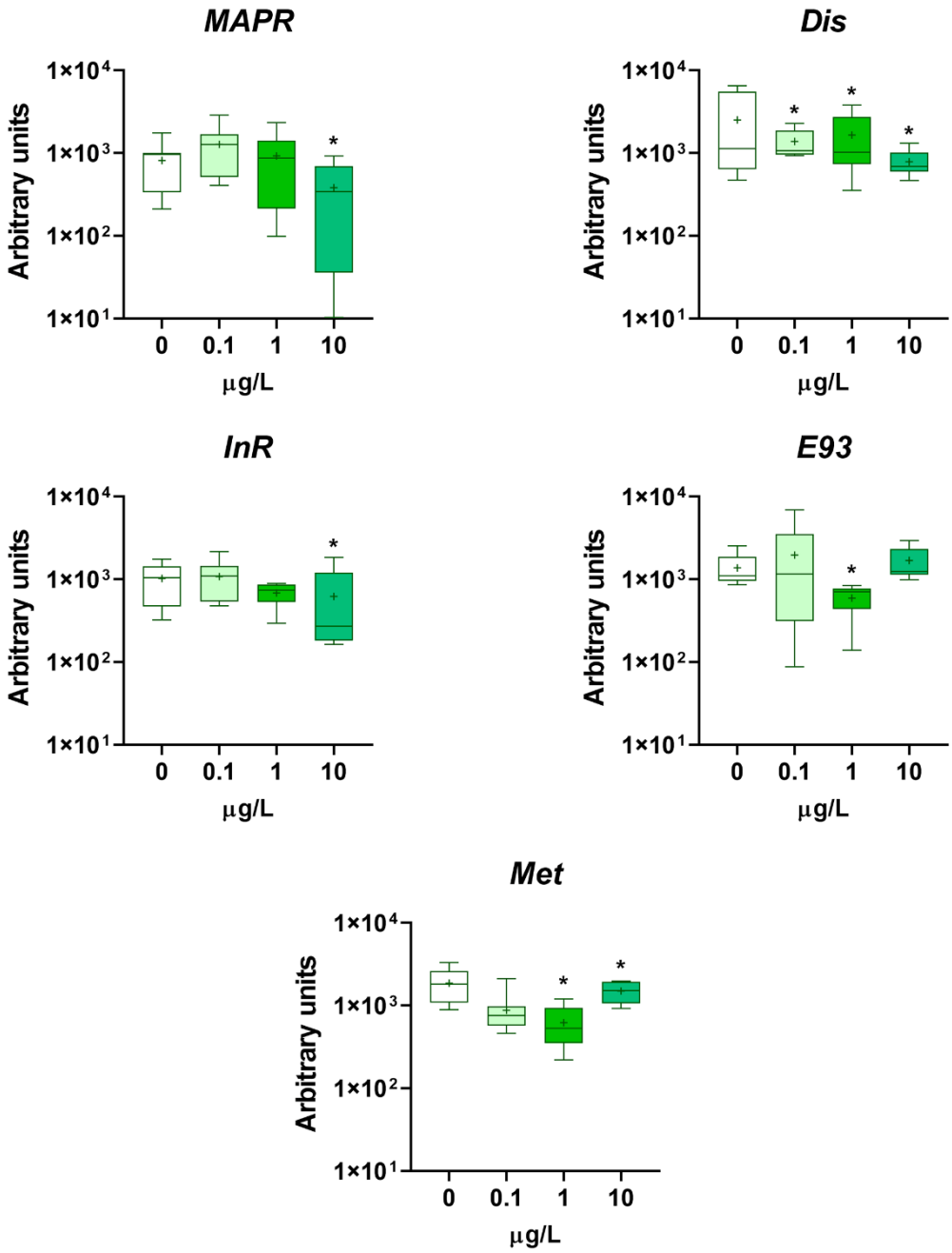

Figure 28. Expression of five genes from endocrine system (*MAPR*, *Dis*, *In*R, *E93*, and *Met)* in fourth instar *C. riparius* larvae after exposure to 0.1, 1, and 10 µg/L of endosulfan at 24h. mRNA levels were normalized using *rpL11*, *rpL13*, *PhFk*, and *TBP* as reference genes. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

However, the other five genes evaluated were not altered at any of the conditions, showing an irregular response on the endocrine system (fig. 30).

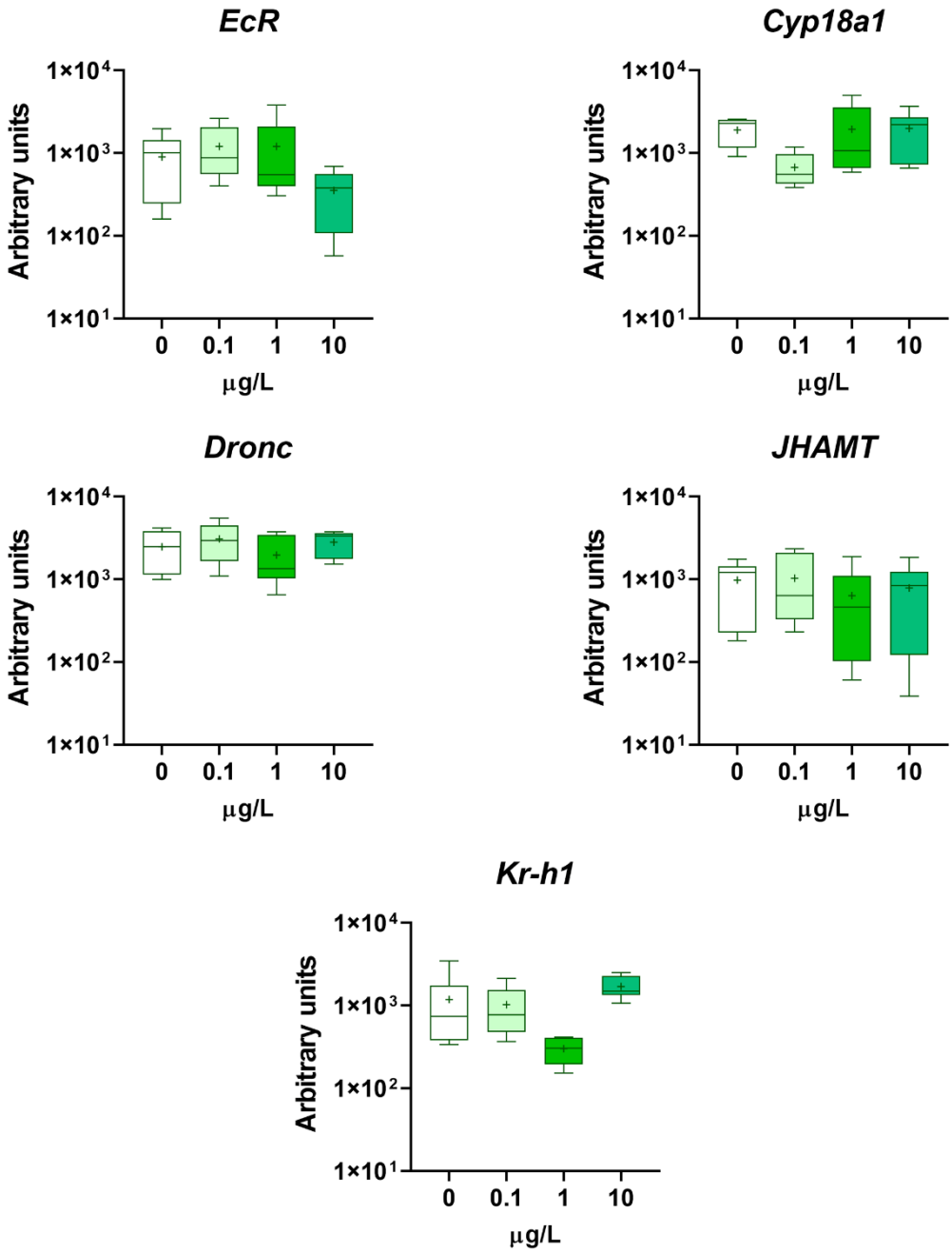

Figure 29. Expression of *EcR, Cyp18a1, Dronc, JHAMT* and *Kr-h1* related to the endocrine system in the *C. riparius* larvae after in vivo exposure to 0.1, 1 and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

The detoxification mechanisms were studied through ten genes (*Cyp4d2, Cyp6b7, Cyp9f2, Cyp12a2, GSTd3, GSTo1, GSTe1, GSTt1, MRP1,* and *ABCB6*) covering the three phases of response.

The results are shown in figures 31 and 32. CYP genes were not modified, *GSTt1* and *GSTd3* were downregulated respect to the control at 1 and 10 µg/L, respectively. Finally, Phase III was altered through a decrease of *MRP1* by 1 and 10 µg/L.

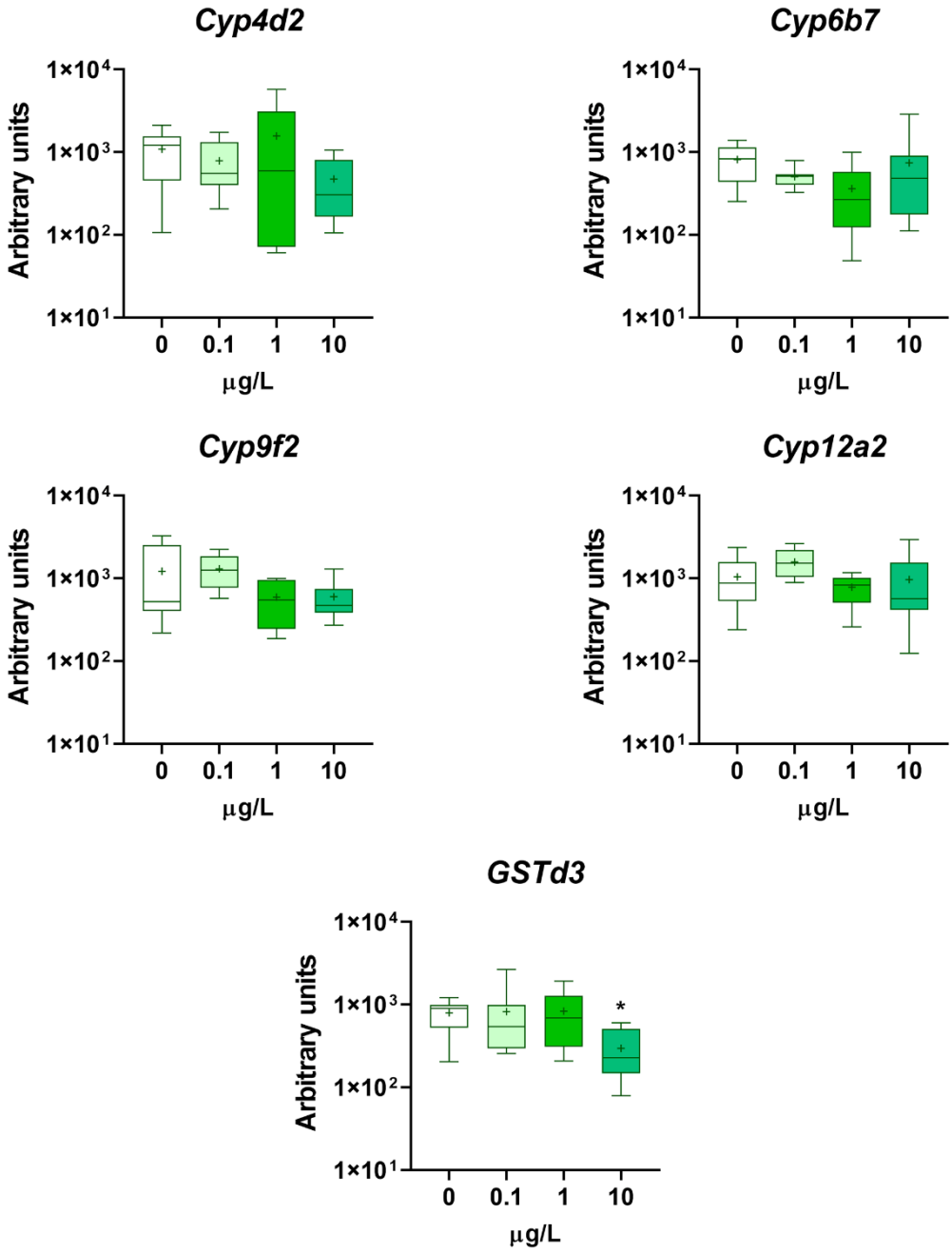

Figure 30. Expression of *Cyp4d2*, *Cyp6b7*, *Cyp9f2*, *Cyp12a2,* and *GSTd3* related to detoxification response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1 and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

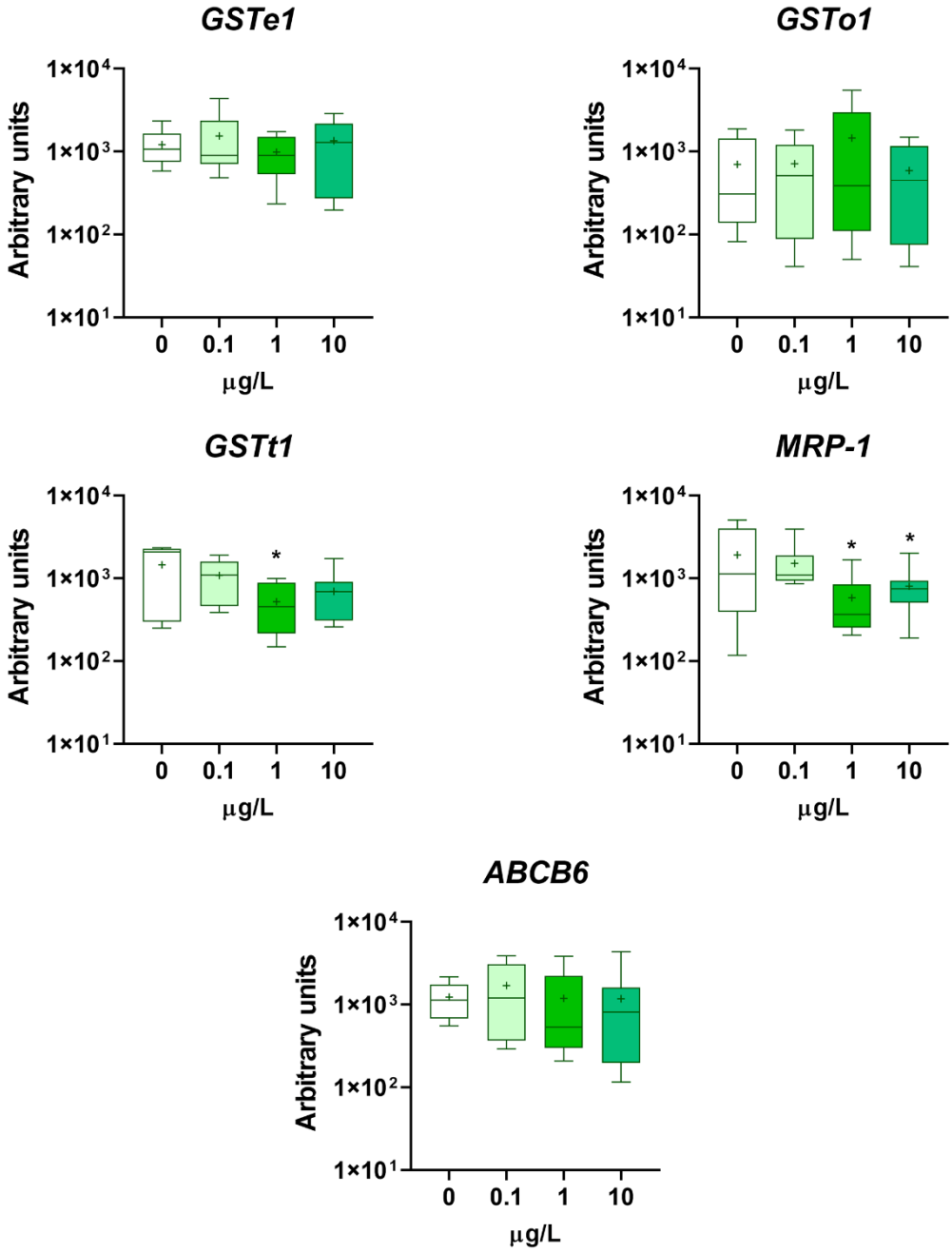

Figure 31. Expression of *GSTe1*, *GSTo1*, *GSTt1*, *MRP1*, and *ABCB6* related to detoxification response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1 and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

Thirteen genes (*hsp70, hsc70, HYOU1, hsp40, hsp90, Gp93, hsp60, hsp10, hsp17, hsp21, hsp24,* and *hsp27*) from five different families of HSPs related to the stress response were analyzed.

The results are shown in figures 33 and 34. Three genes showed altered expression pattern, *hsp70* decrease its expression at 1 and 10 µg/L, besides *hsp40* and *hsp24* were downregulated at 1 µg/L.

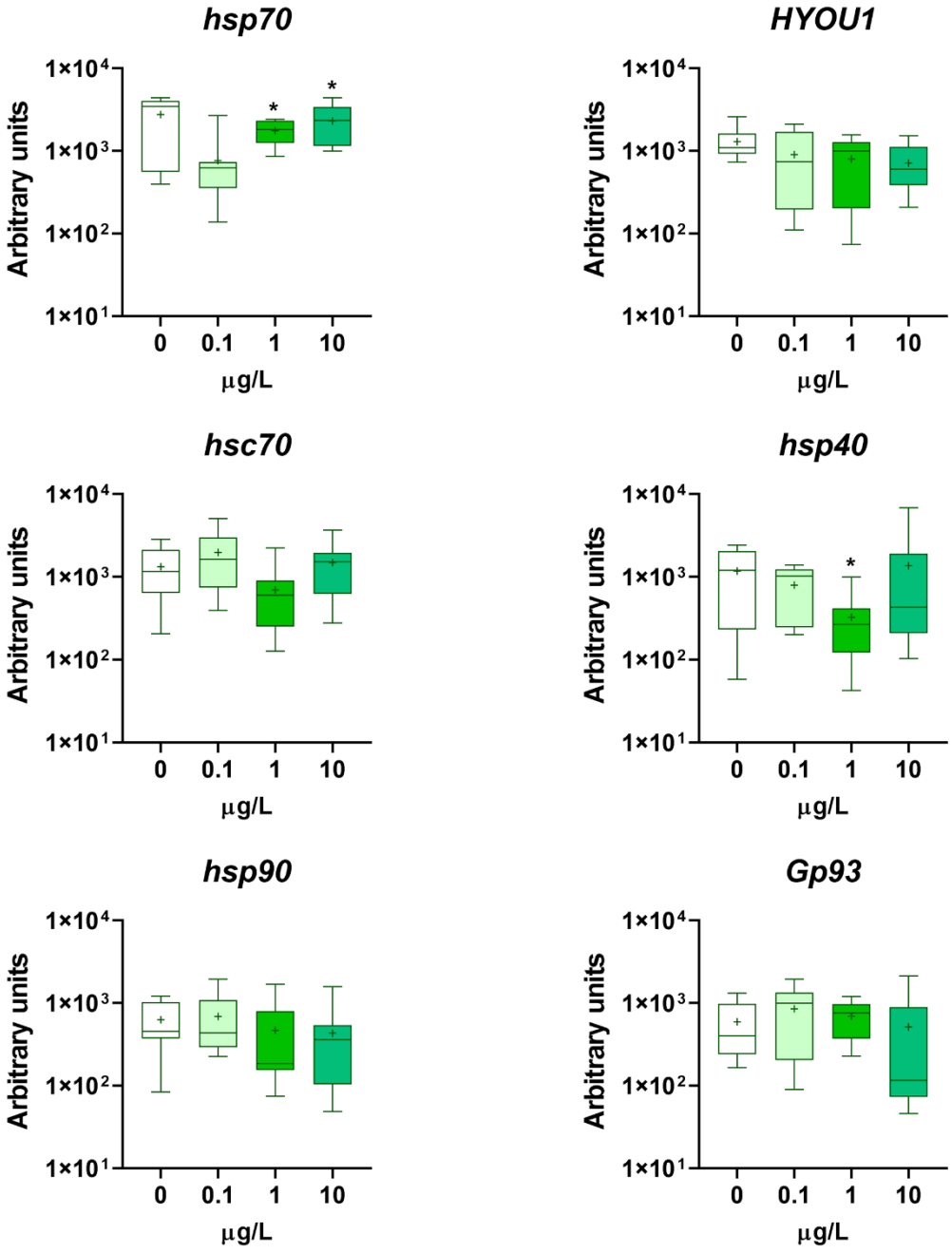

Figure 32. Expression of *hsp70*, *HYOU1*, *hsc70*, *hsp40*, *hsp90*, and *Gp93* related to stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1 and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

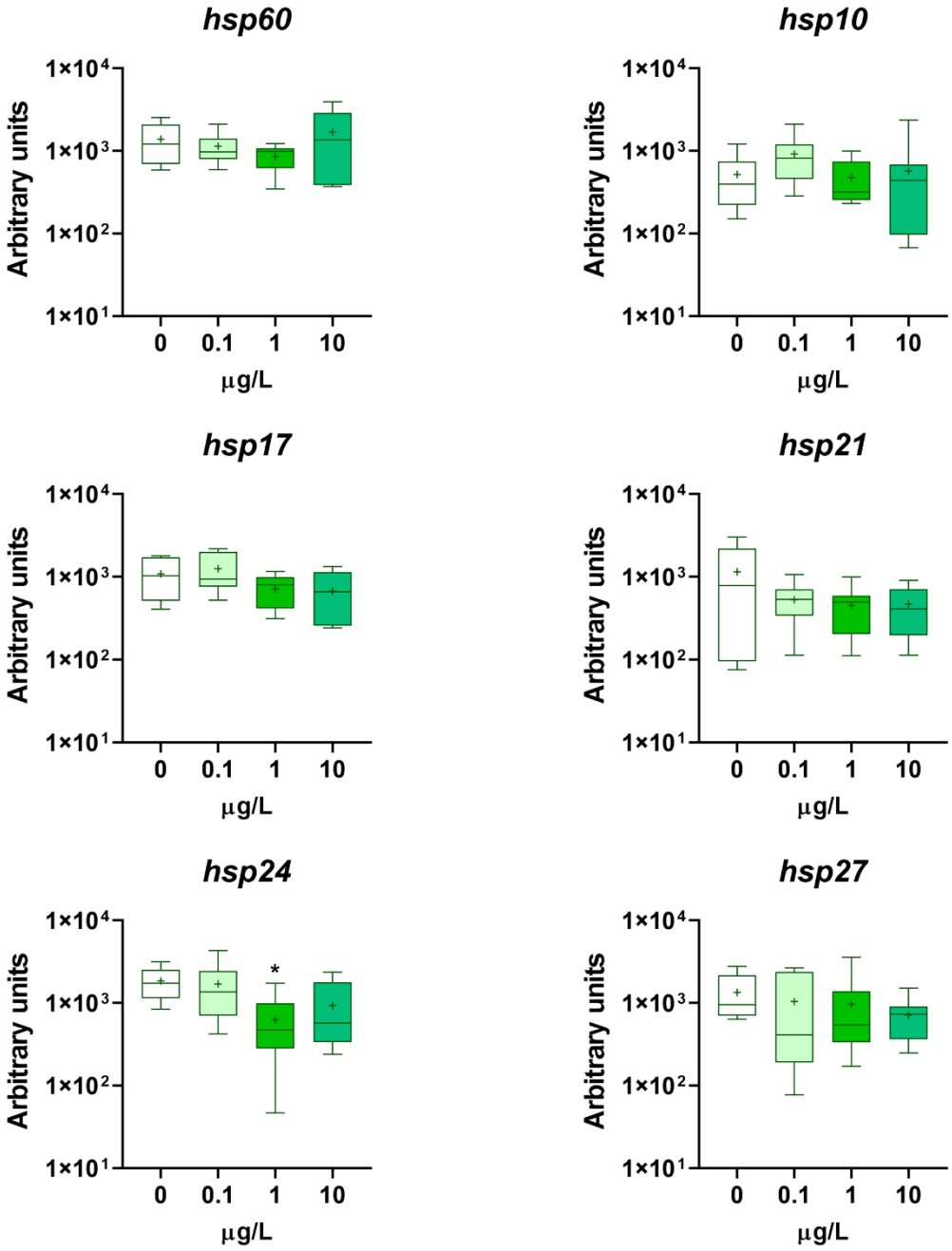

Figure 33. Expression of *hsp60*, *hsp10*, *hsp17*, *hsp21*, *hsp24*, and *hsp27* related to stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1 and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

Two genes from the humoral immune response, *Proph* and *Def*, were studied, and the results are shown in figure 35. Both genes showed decreased expression respect to the control at 1 µg/L, besides by 0.1 µg/L in *Proph*. Furthermore, in both cases, upregulation was observed at 10 µg/L.

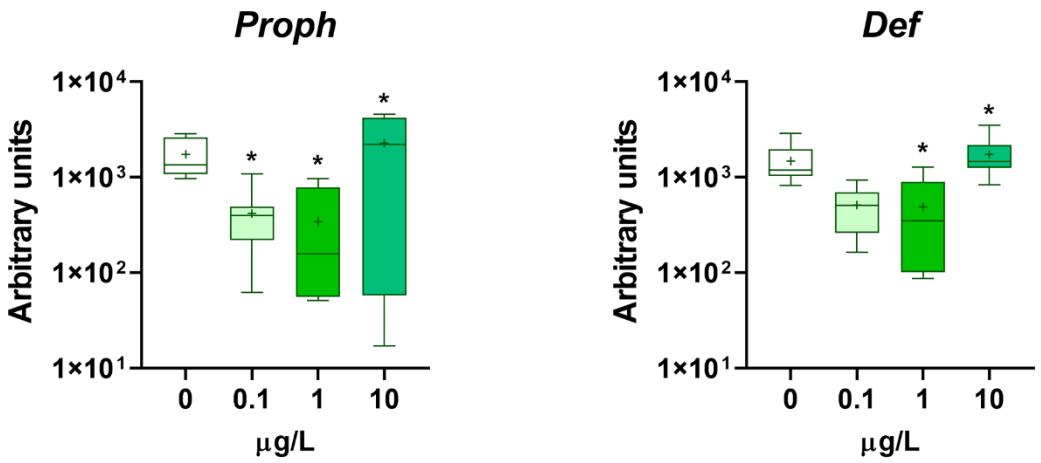

Figure 34. Expression of *Proph* and *Def* related to the immune system in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1, and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

Finally, the DNA repair was analyzed employing five different genes (*PARP*, *ATM*, *XRCC1, NLK, and Decay*). The results are presented in Figures 36 and 37. Only two genes showed statistical differences compared to the control, with an increased expression for *PARP* at 10 µg/L and *ATM* at 1 µg/L. The later gene showed downregulation for 0.1 and 10 µg/L (fig. 36).

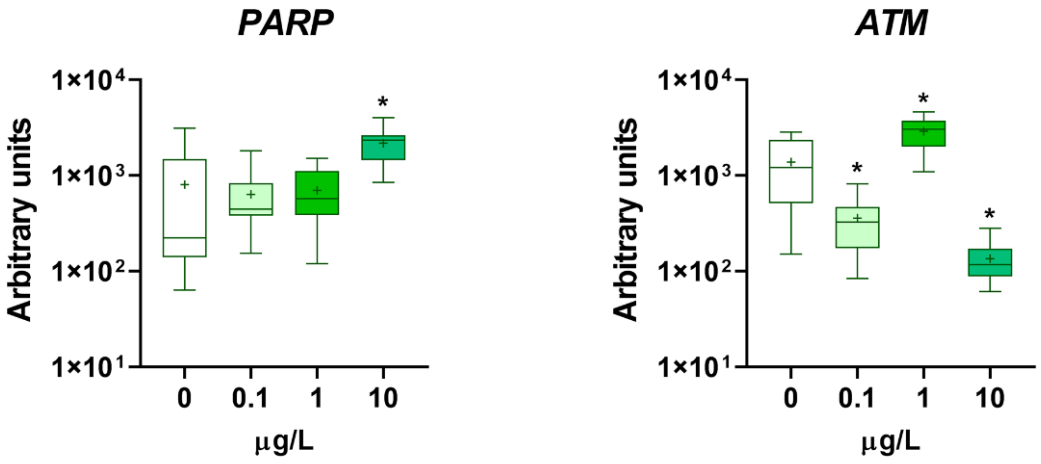

Figure 35. Expression of *PARP*, and *ATM*, related to DNA repair in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1 and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

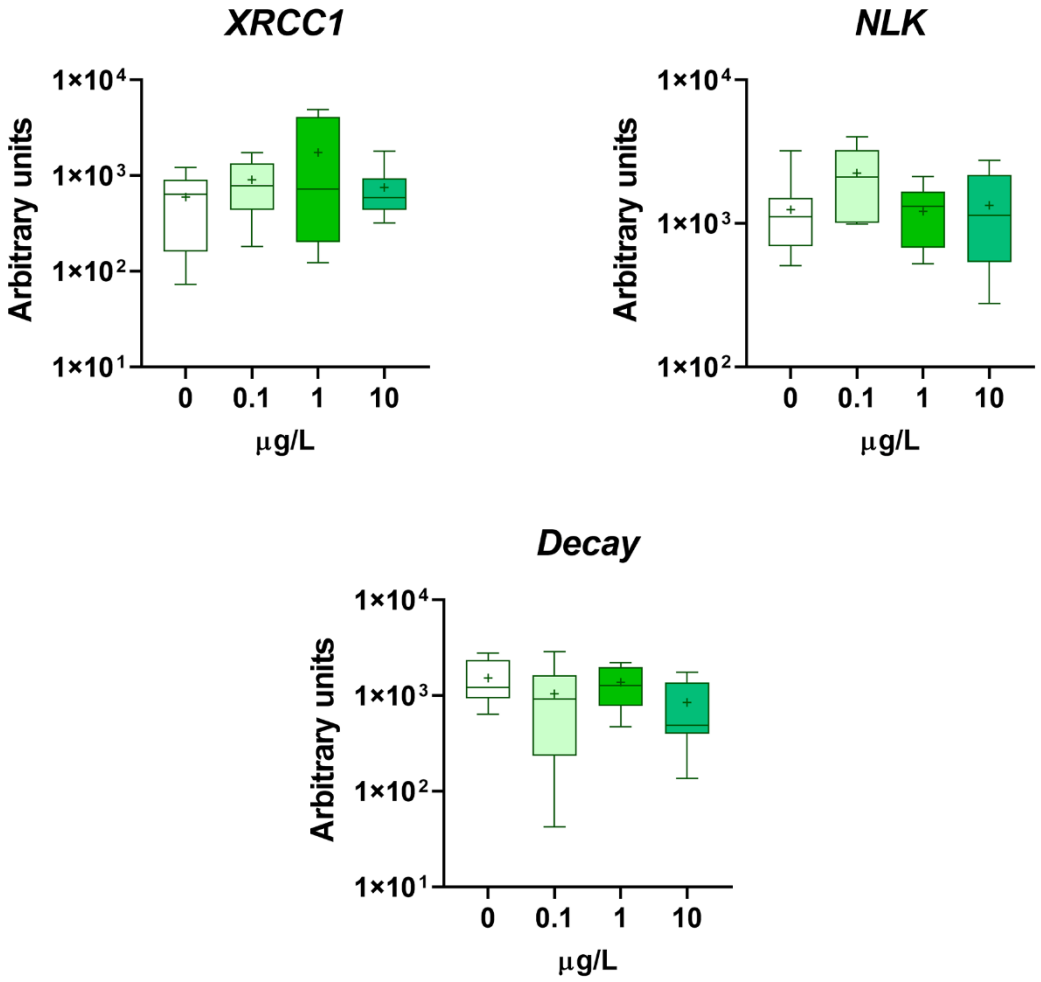

Figure 36. Expression of *XRCC1*, *NLK*, and *Decay*, related to DNA repair in the fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1, and 10 µg/L of endosulfan at 24h. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

Enzymatic activity

Three typical enzymatic activities were analyzed related to detoxification, immunity, and nervous system (GST, PO, and AChE), respectively. The results are presented in the figure 38. All of them were activated by endosulfan. GST stimulated its activity by all the conditions, while PO by 1 µg/L and, finally, AChE by 0.1 µg/L.

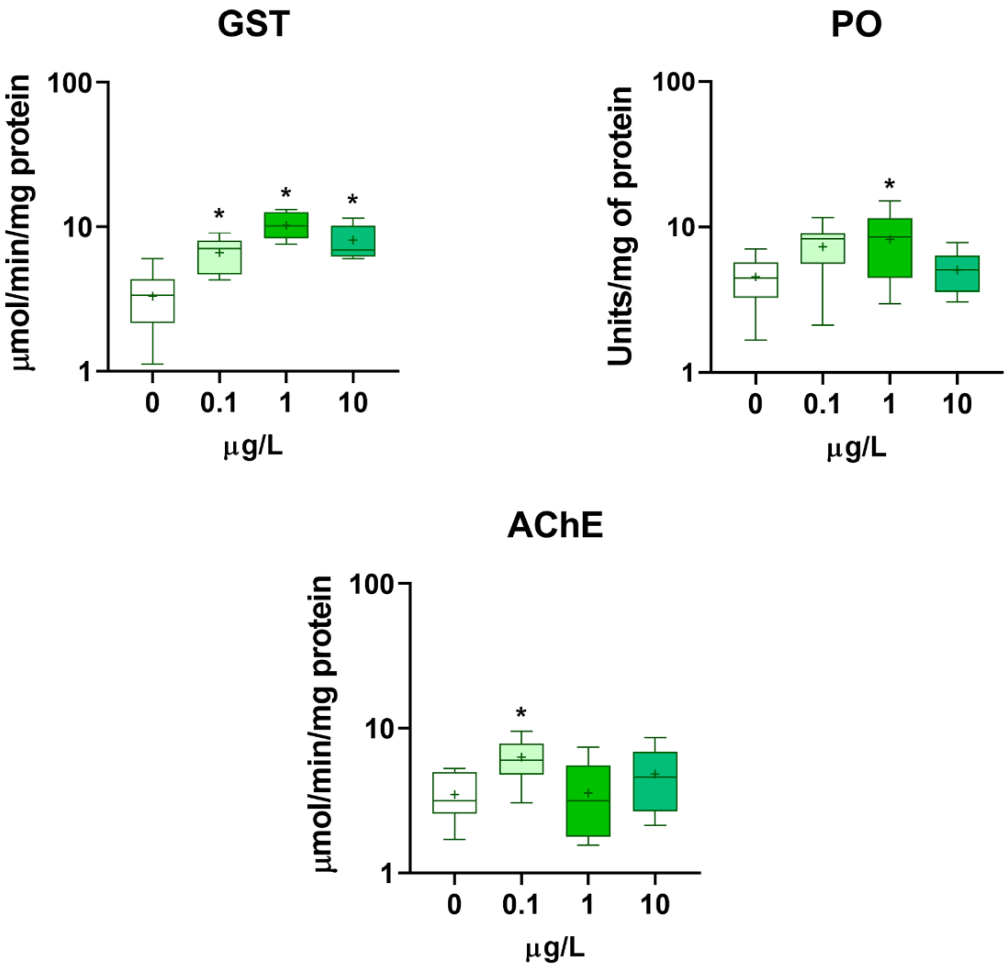

Figure 37. Enzymatic activity of GST, PO, and AChE in fourth instar *C. riparius* larvae after in vivo exposure to 0.1, 1, and 10 µg/L at 24h. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. All significant differences in respect to the control are marked with an asterisk (*), according to ($p < 0.05$).

SUMMARY

- **Endosulfan altered *InR* and *MAPR* expression at 10 µg/L, *Dis* at all the concentrations, *E93* at 1 µg/L and *Met* at 1 and 10 µg/L.**
- **Decreased expression was observed for *GSTd3* (10 µg/L), *GSTt1* (1 µg/L), and *MRP1* (1 and 10 µg/L) from detoxification response.**
- ***hsp70* was modified at 1 and 10 µg/L, besides *hsp40* and *hsp24* at 1 µg/L.**
- ***Proph* was altered by all the conditions tested while *Def* at 1 and 10 µg/L.**
- **Upregulation was detected for *ATM* and *PARP* at 1 and 10 µg/L, respectively, while mRNA levels decreased for *ATM* at 0.1 and 1 µg/L.**
- **Endosulfan activated the activity of GST for all conditions, PO (1 µg/L), and AChE (0.1 µg/L).**

Discussion

Endosulfan, as OCP pesticide, shows strong harmful effects on non-target organisms. Spite of banned in many areas, it is widely used in developing countries, increasing the risk to the environment (Martyniuk et al., 2020; Mohammed et al., 2019; Shi et al., 2020; Yan et al., 2019). Moreover, due to the chemical properties is persistent, so can be maintained along years in soils reaching to surface and groundwaters. Like many other pesticides, it has been classified as EDC, exhibiting alterations on estrogen receptors or defects on reproduction and growth both in vertebrates and invertebrates (Milesi et al., 2017; Palma et al., 2009 a,b; Shi et al., 2020). Ten genes related to the hormonal system were analyzed in this work, mainly focused on 20E and JH pathways. The genes cover the signaling response pathways and the metabolisms of both hormones. Additionally, also, *MAPR* and *InR* were analyzed. The results suggest that endosulfan did not distort the transcription of genes related to the ecdysone response pathway, but a gene that is connected with JH also, *E93*, was modulated (Gujar & Palli, 2016; Kayukawa et al., 2017). This is in agreement with the change observed for *Met*, the gene coding the receptor of JH, which decreases. Centering the attention in the metabolism, the gene related to the synthesis of JH was not altered, similarly to that involved in ecdysone degradation, *cyp18a1*. However, there was a reduced transcriptional activity of *Dis*, a Halloween gene that indicates an impact on ecdysone synthesis (Christiaens et al., 2010). Altogether, the results show that the endosulfan works as EDC in *C. riparius* could involve a disruption of the ecdysone synthesis and modulation of the JH response. It is necessary to elucidate if both effects are direct or one is a consequence of the other since both hormones work together to regulate the development.

On the other hand, JH and 20E work coordinately to control the correct development, sometimes acting as antagonists, as has been demonstrated in *Drosophila* (Liu et al., 2018). Hence, lower JH receptor availability might imply a reduced opportunity for JH to bind to it and exert its effects. In this way, ineffective control of the different transitions to other stages will occur, leading to metamorphosis failures. Previous studies at the molecular level detected changes after endosulfan exposure on genes related to development in both *D. melanogaster* and crustaceans, although the genes studied were different from those used in this work (Bauer et al., 2013; Sharma et al., 2012).

The *InR* and *MAPR* were downregulated, suggesting that endosulfan also impacts other hormonal processes. The insulin-like peptides (ILPs) are related to several processes as cell metabolism, behavior, or growth (Semaniuk et al., 2018; Géminard et al., 2009), then its downregulation might affect all levels of organization in the animal. The changes observed could be reflecting some interaction between ecdysone reduced synthesis and the effect of ILPs because it is known the relation between steroid regulation and insulin-response pathway (Hyun 2018). The consequence in those changes could be a reduced size, so additional research studying long-term response would help to confirm it. On the other hand, *MAPR* codes in invertebrates for a receptor belonging to the cytochrome b5 family. However, in vertebrates, it binds to progesterone, provoking steroidal effects (Mifsud & Bateman, 2002). According to that, it could be expected some relation with steroid hormones such as 20-E. It has been studied with other xenobiotics in *C. riparius,* but the data are still sparse (Ozaez et al. 2016c; Muñiz-González & Martínez-Guitarte, 2018) and very variable. Although there is no known ligand, the downregulation by endosulfan exposure might affect reproduction because of its possible role in progesterone in invertebrates (Stout et al., 2010).

Summarizing, endosulfan affects the synthesis of 20E and, therefore, might instigate defects on growth, development, reproduction, and maybe other physiological processes regulated by ecdysone (Park & Kwak, 2010). Moreover, the JH signaling was affected by the decreased expression of its receptor *Met*. Those effects exemplify the considerable potential to damage non-target organisms and populations by endosulfan. It is in line with previous studies on invertebrates as crustacea or

earthworms with altered growth, development, or reproduction (Palma et al., 2009 a,b; Shi et al., 2020).

The detoxification genes analysis showed that, surprisingly, the Phase I genes tested were stable. The result is similar to that found with BP-3 as well as with triclosan (Martínez-Guitarte, 2018; Martínez-Paz, 2018). Other compounds, such as 4MBC, were able to alter the expression of *Cyp4d2*, *Cyp6b7*, and *Cyp9f2* (Martinez-Guitarte, 2018). The absence of changes does not rule out the possible involvement of other Cyp P450 genes related to other families, such as other members of CYP6, overexpressed after triclosan exposure in *C. dilutus* (Zhang et al., 2020). Moreover, endosulfan could alter esterases gene expression, as was observed in *Galleria mellonella* against *Bacillus* (Grizanova et al., 2019).

Phase II was analyzed by studying genes coding GSTs belonging to the delta, omega, epsilon, and theta families. Endosulfan downregulated the expression of *GSTd3* and *GSTt1* in line with the effects on crustacea (Bauer et al., 2013) and in contrary to the response against UVFs in *C. riparius*, where no effects were detected (Martínez-Guitarte, 2018). The analysis of the enzyme activity performed rendered that it was increased. Combining both results, transcriptional and biochemical, the picture that arises is that in Phase II, intervenes any GST. Still, it is not one of the analyzed since they are decreasing the transcription levels. It cannot be discarded that the activity increase of GST was related to some other function of GSTs. However, GST activity was also elevated in fishes and invertebrates as crustacean or bivalves after exposure to endosulfan (Demirci et al., 2018; Kumar et al., 2016; Tao et al., 2013). Then, the effect of this compound on some GST genes could be specific and might compromise the ability of the cell to metabolize other chemicals. In conclusion, the data suggest that glutathione has a role in endosulfan biotransformation in *C. riparius*.

Phase III consists of removing the conjugate from the cell. The work is carried out by the so-called ABC transporters (ATP-binding cassette transporters) (Dermauw & Van Leeuwen, 2014). Two genes coding ABC transporters were analyzed, *MRP1* and *ABCB6*. Only *MRP-1* was downregulated. It encodes a P-glycoprotein that plays a role in multidrug resistance, besides associated with another process like cancer resistance, highlighting its importance in the cell (Shapira et al., 2011). In contrast with the decrease by endosulfan, it is upregulated in *C. riparius* and *Chlamys farreri* by UVFs and a benzopyrene mixture, respectively (Guo et al., 2017; Martínez-Guitarte, 2018). For *ABCB6,* there is no information on *C. riparius* since it is the first time that has been evaluated. However, a previous report in a cell line from *Ixodes ricinus* demonstrated that pesticides can induce it, although not all, because ivermectin did not change the *ABCB6* levels (Mangia et al., 2016, 2018). From the data, there are two conclusions. First, that endosulfan can reduce *MRP-1* transcription, showing specific damage to ABC transporters that can alter the future efficiency of removing toxicants from the cell. Second, for removing the biotransformed endosulfan, some other member of ABC transporter family or other mechanisms should be used.

To sum up, it can be said that endosulfan affects detoxification mechanisms by decreasing the mRNA levels of some GST members and *MRP-1.* However, stimulation on GST activity indicates that some other members of GST could be involved in the biotransformation of endosulfan. Nevertheless, the effects seem to be independent of the putative mechanisms that the cell activates to metabolize endosulfan, which probably involves other processes of Phase I, II, and III.

A primary response to chemicals is the activation of the stress response. A complete analysis of the response was performed by analyzing genes covering all the families of heat-shock proteins. The total number of genes was thirteen. Three of them, *hsp70*, *hsp40*, and *hsp24*, were in some treatment downregulated with no statistical significance for the other ten genes. The *hsp70* is the best known and sensitive to several stressors like heat, chemicals, or hypoxia (Martínez-Paz et al., 2017b; Nie et al., 2018; Xu et al., 2019). The downregulation observed by endosulfan contrasts with previous results

showing stimulated transcription by pesticides in *C. riparius* and fishes (Lencioni et al., 2016; Mitra et al., 2018). The result suggests a negative impact on *hsp70*, but some additional experiments would be required to elucidate the dynamic. It is worthy to note that *hsp70* is one of the first genes which is activated in stress but, also, it is one of the first genes in return to the normal levels. Often, this kind of response involves a wave that reaches some point below the control levels. In any case, endosulfan might compromise the ability to respond to the stress, besides other multiple processes controlled by this protein as correct protein folding, degradation of damaged proteins, developmental processes, and the immune response (Clerico et al., 2015; Gupta et al., 2010).

The lack of response of the two other Hsp70 family genes, *Hsc70* and *HYOU1*, contrast with a previous study. An analysis of the impact of Ganga river contamination on *Rita rita* fish showed an increase in *hsc70* and *HYOU1* (Mitra et al., 2018). However, the exposure was to a mixture of endosulfan, various metals, and other organic compounds. Therefore, it is difficult to establish a direct effect of endosulfan with the effects. For *hsc70*, it has been observed that some toxics such as pesticide mixtures may also induce it in *Cyprinus carpio* (Xing et al., 2013) and in some tissues in response to heat shock in invertebrates such as oysters (Jackson et al., 2011). *HYOU1*, on the other hand, codes for a protein involved in hypoxia, so it is a reference to know the possibility that the compound produces some kind of respiratory problem. The absence of response for both genes in *Chironomus riparius* suggests that endosulfan is not altering the respiratory process. As happens frequently, it cannot be discarded that the exposure to endosulfan in other times and levels could induce them, but they are irresponsive in the conditions tested.

The Hsp40 members work in combination with *hsp70* controlling multiple functions in the cells, mainly on correct protein folding, translocation, or assembly (Qiu et al., 2006). As *hsp70*, the mRNA levels of the *hsp40* analyzed were downregulated. The evidence is similar to previous studies against the antibiotic sulfathiazole in *C. riparius*, although the other tested concentrations increased *hsp40* expression (Park & Kwak, 2018). Moreover, augmented *hsp40* expression has been detected in clams exposed to heavy metals (Li et al., 2011). The downregulation of both *hsp40* and *hsp70* backs its coordinated action suggesting that *hsp40* lower mRNA levels could be due to the decrease in *hsp70*. Another possible explanation would be that the ATPase activity of *hsp70* was improved by another member of the family. Moreover, it is also feasible that *hsp40* could be the partner of *hsc70* due to a similar pattern that presents this gene, although without statistically significant differences. Further research with other members of the Hsp40 family could help clarify this possibility

Hsp90 is the most abundant cytosolic HSPs in eukaryotes. Decreased or absence on expression was related to a decrease in growth paired with higher mortality (Bansal et al., 1991). Besides, possess a fundamental role in the correct folding of proteins acting as a complex with *hsp70* and *hsp40*. Only one study evaluated the effects of endosulfan on this gene in a mixture in fishes with increased expression in contrast to this case without alteration (Mitra, Mahanty, Ganguly, Krishna, et al., 2018). Moreover, upregulation on *hsp90* has been observed in invertebrates by different xenobiotics, metals, or benzopyrene (Liu et al., 2015, 2016; Wang et al., 2017).

Similarly, the transcriptional activity of *Gp93* was maintained. It belongs to the Hsp90 family, so in addition to the classic functions from the family, it also participates in various processes. For example, in the immune response regulating the expression of proteins such as integrins or Toll-like receptors (Yang et al., 2007). Moreover, it is an orthologue of the mammalian GRP94 so, similarly, *Gp93* participates in the morphogenesis and survival in invertebrates. In *Drosophila, Gp93* is essential in the control of growth as well as the regulation of epithelial gut homeostasis (Maynard et al., 2010). Contrary to these results, metals and the fungicide vinclozolin increased its expression on *C. riparius* (Aquilino et al., 2016; Martín-Folgar & Martínez-Guitarte, 2019). The impact of endosulfan in the

transcriptional activity of both hsp90 members evaluated seems null. It is pending to define the putative role of these genes in case that conditions were different, with longer exposures or different levels of endosulfan. However, it would be expected some changes because the diminished expression in *hsp40* and *hsp70* might affect the shared functions between the three families of proteins.

Mitochondria effects are not usually analyzed in ecotoxicology. Two proteins, Hsp60 and Hsp10, are chaperones with a mitochondrial location so they could be suitable biomarkers to analyze damage on this organelle. Both mitochondrial proteins work coordinately for polypeptide folding (Dubaquié et al., 1998; Lund et al., 2003), besides both have been used for cancer diagnosis and treatment roles in cancer (Cappello et al., 2008; Corrao et al., 2010). As with other HSPs, they are involved in the response against diverse compounds, such as antibiotics and herbicides as well as against infections (Gao et al., 2019; Li et al., 2019; Yang et al., 2013). However, endosulfan did not alter the mRNA levels suggesting that the mitochondria were not affected, at least in the translocation of proteins, which is a critical role of these two HSPs.

The last family studied was the sHSP family. Five members were analyzed because of their heterogeneity. sHSPs are involved in the response against diverse stressors bacterial infections, heavy metals, UV radiation, and chemical exposure (Liu et al., 2019; Martín-Folgar & Martínez-Guitarte, 2017; Simoncelli et al., 2019). Concretely in *C. riparius*, previous studies showed that some sHSPs react differently to heat shock and cadmium so that each sHSP can have a specific response according to the conditions (Martín-Folgar et al., 2015; Martín-Folgar and Martínez-Guitarte, 2017; Martínez-Paz et al., 2014). Here, only *hsp24* was downregulated, contrasting the response against metal, fungicide, or heat shock where was upregulated (Aquilino et al., 2016; Martín-Folgar et al., 2015; Martín-Folgar & Martínez-Guitarte, 2017).

Because of the diversity of sHSPs, it is difficult to establish the homology between the different proteins from each species. In yeast, a protein *hsp24* overexpression was related to higher resistance against adverse conditions of salt, drought, and heat (Liming et al., 2008). However, as stated, it is not possible to ensure that it is homologous to the hsp24 of *C. riparius*. Centering the analysis in the impact at the cell physiology, the decrease in *hsp24* mRNA levels may affect the ability of *C. riparius* to cope with unfavorable situations and, consequently, compromise its survival. On the other hand, some studies have highlighted the induction of sHSPs by endosulfan in invertebrates as *Drosophila* (Sharma et al., 2012), while reduced *hsp27* expression was described in human cells (Skandrani et al., 2006). In fishes, the induction of *hsp27* was considered from rivers polluted with a mixture of compounds, including endosulfan (Mitra et al., 2018). All of them suggest significant variability in the endosulfan response, although it cannot be ruled out that these differences are related to difficulty in establishing structural homology between sHSPs. In any case, the lack of response for the rest of the sHSPs does not mean that endosulfan cannot affect them. There was already contrasting behavior on sHSPs depending on the exposure time and other conditions, as has been demonstrated in *C. riparius* for heat shock and metals (Martín-Folgar and Martínez-Guitarte, 2017; Martín-Folgar et al., 2015). Therefore, additional research employing other endosulfan concentrations and/or the longer time exposure would help to elucidate the response of this set of proteins because sHSPs are involved in many different cell processes.

In summary, the stress response in *C. riparius* was weak negatively affected by endosulfan through the downregulation of three HSP genes. This phenomenon may cause a decreased or inefficient stress response that compromises the survival of the cell. Thus, the organism, moreover, due to the other functions as growth, apoptosis, or diapause regulated for sHSPs, could be compromised or altered.

The genotoxicity of endosulfan has been previously observed in rats, fishes, and clams (Shao et al., 2012; Tao et al., 2013; Wei et al., 2017). The alterations are related to the reactive oxygen species

(ROS) production, as demonstrated in fishes (Dar et al., 2015). The DNA damage can result in a single nucleotide modification or to single or double-strand breaks (SSBs or DSBs), with the cell responding by activation of DNA damage response (DDR), so genes related to SSB, DSB, and apoptosis were evaluated after endosulfan exposure. Only *PARP* and *ATM* showed altered mRNA levels. *ATM* belongs to the phosphatidylinositol 3-kinase family participating in DSB repair, among other functions like cell cycle, metabolic regulation of chromatin remodeling (Stracker et al., 2013). The decreased expression at the lowest and the highest concentrations may reflect DNA damage and the derived inability of the cell to respond, respectively. In contrast, the upregulation detected at the medium concentration could indicate the DDR activation. In other words, the lowest endosulfan concentration could produce insufficient damage to activate the DDR, but it can affect the expression of the genes. The next concentration reflects that the damage at 24 hours is sufficient to activate DNA repair mechanisms, but this phenomenon remains in progress. Finally, the result with the highest concentration might reflect the inability of the cell to compensate for the damage, although it could also indicate the return to normal conditions once the response has been activated and the damage repaired. Alternatively, decreased mRNA levels on *ATM* might compromise the DDR due to the role of this protein in damaged strand recognition and the repair enzymes' coordination. Previously damages on DNA on human cells were detected through comet assay and changes in the ATM-p53 signaling pathway by this pesticide (Xu et al., 2018), so the effects are similar to those observed in *C. riparius*. Besides, DSB usually goes paired with the expression of other genes related to the repair process (Burgess & Misteli, 2015). Moreover, *NLK,* which participates with *ATM* in the factor NF-kB activation in the cytoplasm as a response to DNA damage, was not altered by endosulfan (McCool & Miyamoto, 2012). Therefore, it cannot be discarded that the ATM alteration is derived from the alteration of its functions due to the presence of the pesticide and not as a response to DNA damage.

On the other hand, *PARP* encodes a protein involved in SSB and DSB repair. Its mechanisms are based on binding to the damaged strand, poly (ADP-ribose) production promoting chromatin relaxation as well as the accumulation of X-ray repair cross-complementing protein 1 (XRCC1) and other proteins acting as scaffolds for the reparation (Gao et al., 2017). The upregulation on *PARP* suggests DNA damage and the activation of the DNA repairing in the cell. Contrary to the *ATM,* its expression might indicate the activation of SSB rather than DSB repair mechanisms. Nevertheless, the lack of response for *XRCC1* questions whether this protein is also involved in the regulation of SSBs (Abbotts & Wilson, 2017). The last gene studied, *Decay*, is an effector caspase that is expected to operate if the cell fails to repair the damage. The lack of response seems to suggest that at the analyzed concentrations and time, the cell has not yet entered into apoptosis.

The results were not conclusive because the gene expression did not show a clear picture. Nevertheless, endosulfan seems to cause some DNA damage and, therefore, the DDR is activated. DNA damage would involve DSBs and SSBs, but the exposure time may be too short of showing full DDR activation. More research is thus necessary to explore the response of other genes involved in later phases of DDR and to elucidate the ability of the cell to compensate for endosulfan damage.

The effect of endosulfan in the immunity was studied by using the genes coding for *Proph* and *defensin* and analyzing the activity of the PO. The activation of the PO system is by triggering a serine proteinase cascade that ends in the activation of the zymogen (proPO) to PO (de Eguileor et al., 2016). The enzyme plays a central role in melanization against microbe infection (Nakhleh et al., 2017). Results, as happened with other genes, were complex to be interpreted. There was a downregulation at the two lower concentrations, while at the highest concentration, the mRNA levels were increased. A reduced transcriptional activity might lead to increased sensitivity to infection and limit the quantity of PO. The low PO level was related to more risk for diseases in crustaceans and mollusks (Cerenius et al., 2012). However, the increase at the higher concentration may be because the cell cannot manage the damage

at this elevated endosulfan exposure. A similar response was detected on the antimicrobial peptide *Def*, with decreased expression but an increase at the highest concentration of endosulfan.

The pesticide alters both genes, but with an unclear mechanism. However, it is, apparently, specific, as indicated by the similar dynamics that both follow. The endosulfan could act directly or indirectly because there are many other genes involved in defensive mechanisms. The consequence of the modulation in the transcriptional activity of both genes could be an increased susceptibility to bacterial or fungal infections when they are lowered. However, the result with the enzyme activity also must be considered. There is an elevation of the activity of the PO at the medium concentration. Currently, it has been demonstrated that the declined PO activity was related to disease development in bivalves (Luna-Acosta et al., 2017), highlighting the importance of correct PO activity in the health of the organism. Moreover, the results are in line with the augmented PO activity observed in *Culex pipiens* after 24-hour endosulfan exposure, that it is related to insecticide resistance (Cornet et al., 2013).

Comparing the dose-response to that observed for the transcriptional study, it is similar but inverse. The lower concentrations showed higher activity that the highest concentration of endosulfan. It is a putative explanation that the PO is activated, and then, only when the turnover makes necessary, it is activated the transcription of *Proph*. It would be necessary to combine these results with additional studies, including other parameters and genes related to immunity. In any case, the impact of endosulfan on the immune system compromises the defense mechanisms, weakening the animal against the infection.

The last endpoint included evaluating the impact on the nervous system. Often, the pesticides and insecticides have their targets on the nervous system (Huynh & Nugegoda, 2012). One frequent target is acetylcholinesterase (AchE), so it was analyzed the activity that it showed when the larvae were exposed. Previous studies on *C. riparius* reported no significant differences for several compounds including spinosad, indoxacarb, and thiamethoxam (Monteiro et al., 2019 b,c; Saraiva et al., 2017), but with amitraz, an increased tendency was observed although it was not statistically significant (Monteiro et al., 2019). Yet, there is considerable inhibition by N-diethyl-3-methylbenzamide (DEET) and chlorpyrifos (Campos et al., 2016; Pérez et al., 2013). The endosulfan increased the AChE activity at the lowest concentration, with standard levels in the other two concentrations tested. Another invertebrate, *Gammarus kischineffensis*, exhibited a similar pattern (Demirci et al., 2018). The response can be interpreted in such a way that the moment of the response is being seen in the lowest concentration. While, in the case of the other two concentrations, a later point in the response is being observed, in which the organisms compensate the effects and returns to normal parameters. Nevertheless, in fishes, endosulfan repressed the AChE activity (Kim et al., 2018). Taking into account the literature and the results obtained, the AchE in *C. riparius* respond unequally according to the class and the specific pesticide employed. Therefore, the results suggest a mild effect in the central nervous system, although the effect at higher concentrations may occur early before returning to normal after 24 hours. The meaning of the induction remains unclear, as it suggests the increased activity of the CNS in the presence of low amounts of endosulfan

Endosulfan has demonstrated its harmful effects on non-target organisms from vertebrates to invertebrates. However, little information is available on its toxicity on insects. The present thesis simultaneously evaluated the effect of the pesticide endosulfan in five cell processes combining transcriptional and the enzymatic activity in *Chironomids*. The chemical altered the endocrine system, including response and hormone biosynthesis, detoxification mechanisms, stress response, DNA repair, and the immune system. As a consequence, the effects could generate failures in metamorphosis and reduced size due to the role of both 20-E and JH in the development. Endosulfan seems to be biotransformed by conjugation with glutathione. Besides this, it appears to generate

defects in genes related to Phase II and Phase III detoxification, compromising the future response against xenobiotics. Similarly, failures in protein folding, among other processes controlled by HSPs could be altered. A possible genotoxic effect of endosulfan was observed due to the alteration of genes involved with the DDR. The immune system modulates its response irregularly, but with an evident disruption by the pesticide. Finally, a weak effect on the nervous system was detected by AchE.

Altogether, the results suggest a complex mode of action that induces multiple types of damage at the cellular and molecular levels that can compromise the animal's survival, development, and response to other stressors. Hence, environmentally relevant concentrations of endosulfan reduce the ability of *C. riparius* larvae to manage acute environmental threats. Therefore, all this would jeopardize the future of the population due to the current context of global change marked by the increase in water pollution due to chemicals. Additional research at long-term exposure times—and even including more genes that cover other pathways—will provide a clearer picture of the global effects in the cell and the impacts for organisms.

MICROPLASTICS

7. MICROPLASTICS

Description, characteristics, and uses

Industrial development, parallel to the globalization process, has led to an exacerbated increase in the production and use of plastic compounds in our day-to-day. Plastics are defined as synthetic polymer compound, which contains many other chemicals to increase the efficiency performance as bisphenols or phthalates (da Costa et al., 2016) with an annual production around 335 million tons (PlasticsEurope, 2018). Their unique characteristics are allowing plastics to be employed in a range of different sectors and everyday applications. The most commonly used and abundant polymers are polyethylene (PE), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET) and plasticizers additives. They are employed in everyday products such as PCPs packaging, footwear, tires, constructions, among others. Plastics can be divided into macro-debris (20 mm diameter), meso-debris (5–20 mm), and micro-debris (<5 mm). One crucial point is if plastics are not properly disposed or recycled after use, often they are released into the environment, mainly in waters where they can persist for long periods and degrade into smaller pieces, called microplastics (Scudo et al., 2017). In particular, in recent years, there has been a growing interest in the presence and possible effects of these microplastics on ecosystems and organisms.

Microplastics (MPs) are tiny (< 5mm) solid particles composed of mixtures of polymers and functional additives, even containing residual impurities from manufacturing. Typically, they are classified into two types: primary and secondary. Primary are produced as proper products, for example, exfoliating beads in body scrubs or from the release of plastics dust from plastics products (Van Cauwenberghe et al., 2015). Secondary MPs are generated unintentionally from plastic degradation by the action of sun, temperature variations, waves, or marine fauna, being more massive than primary (Rochman et al., 2015). MPs can reach the environment from industrial and sewage effluents, airborne pollution, or sewages used in agriculture. There is growing awareness because of the impact of plastic and microplastics on natural environments, animals, and human health (Royal Society, 2019). Hence, different strategies try to improve the treatment of plastics (Penca, 2018; Tessnow-von et al., 2019).

Microplastics in the environment and biota and their toxicity

A critical consideration of these compounds is the fact that they are capable of drifting far away from the original release becoming an elaborate pollution factor. One problem for the MPs research is related to their quantification in the environment by the absence of standardized methods, generating an underestimation in the total amount of MPs. Besides the type (PE, PS, PVC), color, and sizes are characteristics that modulate the ingestion as well as the possible effects of the MPs (Xiong et al., 2019). The majority of the research was focused on marine waters. In the case of beaches, they usually receive extra pollution from human waste generated by leisure activities (Edo et al., 2019; Eriksen et al., 2014; Herrera et al., 2018).

However, the freshwater system (rivers and lakes) is an essential MPs pollution source with continuous MPs flows. Rivers and lakes around the world presented values of MPs from 24.8 to 84030 particles/m^2 (Hurley et al., 2018), higher values were detected in Asia, being the most freshwater polluted areas (Liu et al., 2020; Luo et al., 2019). MPs were even detected in remote areas like the deep sea or arctic sea (Jamieson et al., 2019; Woodall et al., 2014), generating great concern about the MPs.

Regarding the plastic toxicity, it should be taken into account not only the microplastic itself but also the additives it contains that can be released into the water and develop toxicity (Ke et al., 2019). MPs represent a double danger for aquatic organisms because are easily ingested by the organisms, from vertebrates (fishes) to invertebrates (Canniff & Hoang, 2018; Collard et al., 2018; Herrera et al., 2019; Windsor et al., 2019), reaching humans by the food chain (Backhaus & Wagner, 2019). Also, they may act as vectors for the chemical transfer of organic pollutants (Bakir et al.,2014), even altering their toxicity (Bellas & Gil, 2020; Ziajahromi et al., 2019).

Consequently, there is a growing concern about the possible plastic transfer from aquatic organisms to humans through the food chain, as was demonstrated by the detection of plastics in fishes and seafood destined to human consumption (Azevedo-Santos et al., 2019; Saturno et al., 2020). It is described that plastic exposure causes alterations in human cells (Pérez- Albaladejo et al., 2017; Xu et al., 2019).

Plastic evaluation in *C. riparius*: LDPE MPs

PE is made by the addition of radical polymerization of ethylene (olefin) monomers and is considered one of the most primary MPs produced (Conkle et al., 2018). LDPE is a semi-rigid and translucent polymer with multiple applications for manufacturing containers, dispensing bottles, wash bottles, tubing, plastic bags for computer components, and various molded laboratory equipment. Polyethylene particles represent environmental relevance (Rodrigues et al., 2018); for example, LDPE was the most detected microplastic in Indian lakes (Sruthy & Ramasamy, 2017).

Some studies observed the direct damages generated by MPs exposure on the behavior of aquatic organisms in fishes and crustacea (Blarer & Burkhardt-Holm, 2016; de Sá et al., 2018). In the case of PE MPs, reproduction and immune system were modified in fishes (Espinosa et al., 2019; Mak et al.,2019). Moreover, PE provoked alteration in aquatic invertebrates like mussels (Avio et al., 2015; Green et al., 2019) or corals (Lanctôt et al., 2020). Finally, in chironomids, PE was capable of altering growth and emergence (Silva et al., 2019; Ziajahromi et al., 2019). However, there is an information gap regarding the effects of PE MPs at transcriptional level with few studies in copepods and fishes (Granby et al., 2018; Heindler et al., 2017; LeMoine et al., 2018). Due to their presence in the environment and the proven harmful effects on aquatic organisms, one polymer (LDPE) has been selected to evaluate its effects on *Chironomus riparius* (figure 39).

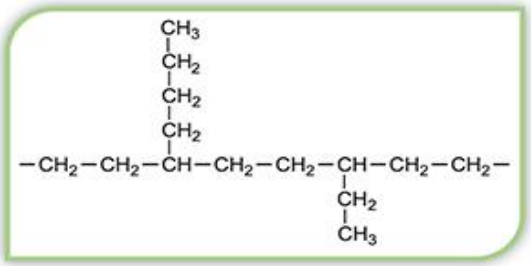

Figure 38. Chemical structure of the LDPE.

Results

Alterations at the molecular level by LDPE microplastics in *C. riparius*

Plastic is as one main pollution problem in the last century, being notable the concern about the MPs with a high presence in surface waters around the world (Eriksen et al., 2013; Sruthy & Ramasamy, 2017). Concretely, PE is the most produced plastic-type and developed harmful effects on aquatic

organisms. In the present study, fourth instar larvae of *C. riparius* were exposed to two LDPE particle sizes (<32 and 32-45 µm) at two different concentrations (0.025 and 2.5 g/Kg) and were evaluated by employing biochemical biomarkers and gene expression after 48h exposure.

Biochemical biomarkers

Firstly, a complete biochemical biomarker analysis evaluated the energy reserves (carbohydrates, lipid, and protein content), the ETS, the LPO, and the enzymatic activities of CAT, AChE, and GST, and the total glutathione by TGSH. All the results are presented in Table 18. Statistical differences were detected in lipid content, TGSH, GST, CAT, and AChE respect to the control. Besides, differences between MPs sizes were observed for carbohydrates content, TGSH, GST, CAT, and AChE at a high concentration of MPs. At last, in GST, there were differences between both sizes at 0.025 g/kg.

		<32 µm		**32-45 µm**	
Biomarkers	**Control**	**0.025 g/kg**	**2.5 g/kg**	**0.025 g/kg**	**2.5 g/kg**
Carbohydrates (mJ /mg tissue)	181,84 ± 16,59	165,34 ± 18,78	189,33 ± 24,61	130,94 ± 15,23	144,67 ± 19,22●
Lipids (mJ /mg tissue)	508,02 ± 36.91	937,64 ± 80.43 **c**	611.56 ± 45.28	870.37 ± 63.05**c**	507.01 ± 51.50
Proteins (mJ/mg tissue)	265,07 ± 8,21	256,08 ± 12,51	253,61 ± 12,42	238,26 ± 11,63	267,12 ± 5,79
ETS (mJ/h/mg)	33,84 ± 0,69	34,83 ± 1,01	32,50 ± 0,43	32,90 ± 1,69	35,45 ± 0,96
LPO (nmol/g wt)	114,73 ± 4,89	111,41 ± 2,22	120,71 ± 4,21	133,21 ± 14,00	129,81 ± 7,98
TGSH (µmol/mg)	13,58 ± 0,50	13,17 ± 0,98	13,02 ± 0,54	14,90 ± 1,90	26,20 ± 2,70 **c**●
CAT (µmol/min/mg)	33.37 ± 3,89	38.34 ± 2,61	39.31 ± 2,50	64.02 ± 8,50 **c**	80.84 ± 4,07 **c**●
GST (nmol/min/mg)	32,70 ± 0,66	32,58 ± 1,20	36,26 ± 1,46	60,02 ± 8,91 **c**▲	77,85 ± 3,35 **c**●
AChE (nmol/min/mg)	6,81 ± 0,42	7,37 ± 0,87	7,58 ± 0,36	11,68 ± 1,48	17,21 ± 1,37 **c**●

Table 18. Biochemical biomarker results in the fourth instar *C. riparius* larvae after LDPE MPs exposure at 48h The values are presented as mean ± SEM, the significant differences respect to the control are marked with **c** , to 0.025 g/kg < 32 µm with ▲ and to 2.5 g/kg <32 µm with ● . In all the cases the differences are according on ($p < 0.05$).

Gene expression analysis

The endocrine system was studied employing ten genes (*MAPR*, *Dis*, *InR*, *EcR, Cyp18a1, E93, Dronc, JHAMT, Met,* and *Kr-h1*), with the results on figures 40 and 41. Firstly, mRNA levels were altered respect to control in *InR*, *Dis, Dronc,* and *Met* with upregulation in all the cases and with downregulation for *EcR*. Besides, the two LDPE sizes were compared, and differences were detected at 0.025 g/kg in *EcR* and 2.5 g/kg in *Dis*, with decreased expression respect to the lower size particles (fig. 40).

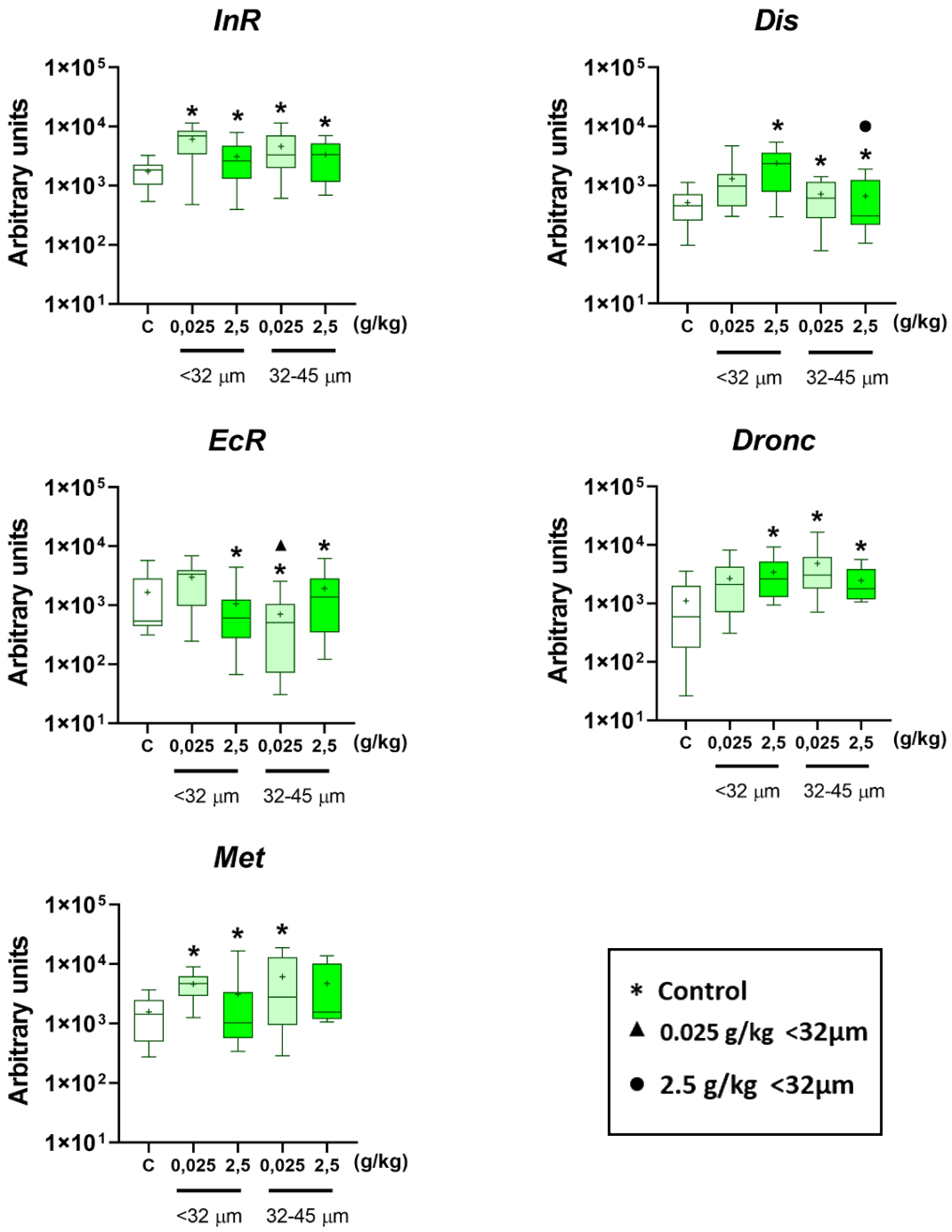

Figure 39. Expression of *InR*, *Dis*, *EcR*, *Dronc*, and *Met* related to the endocrine system in fourth instar *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 µm) for 48h. mRNA levels were normalized using *rpL13*, *RNApol*, and *TBP* as reference genes. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. The differences respect control and between treatments are defined in the legend. All significant differences according on ($p < 0.05$).

The other five genes analyzed were not altered at any of the conditions (fig. 41).

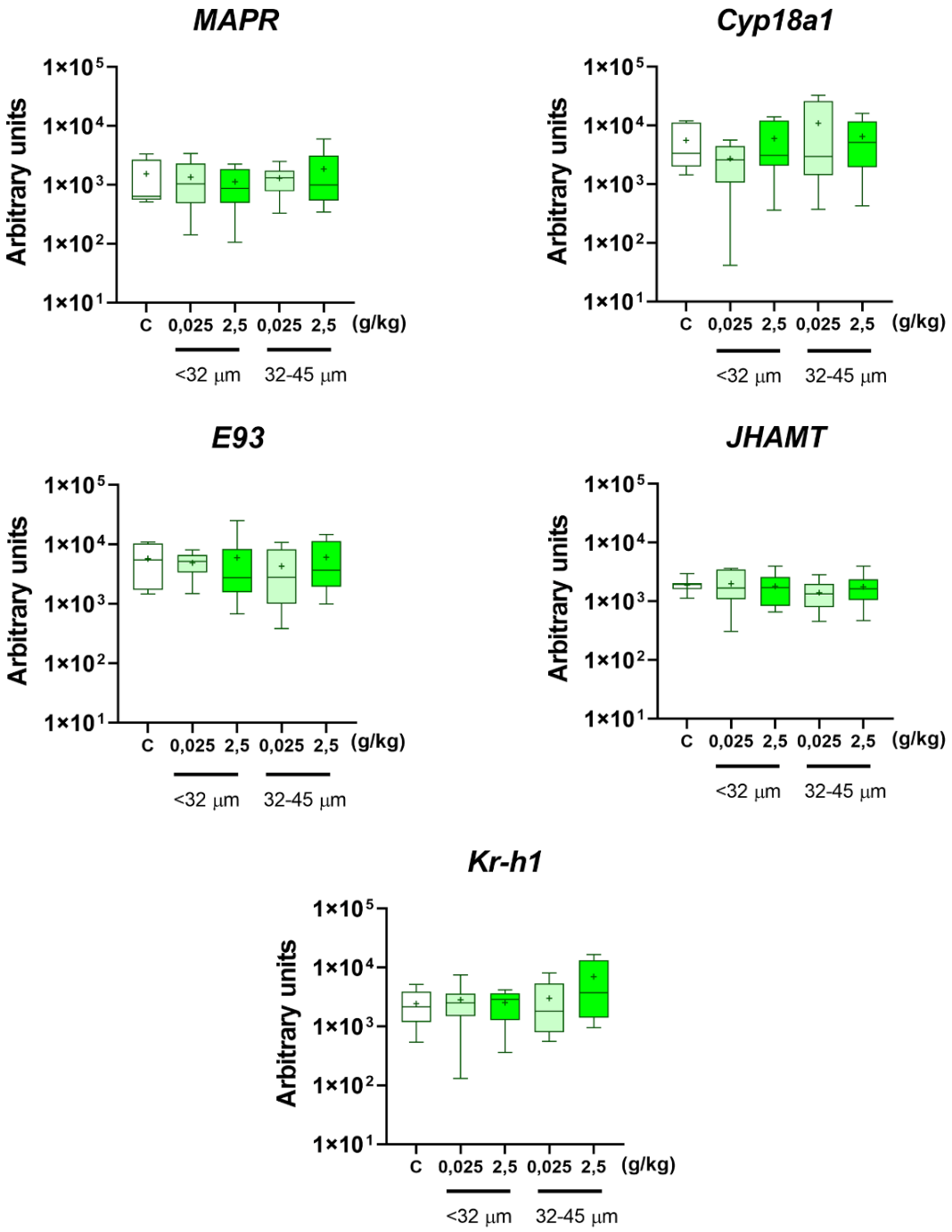

Figure 40. Expression of genes from endocrine system (*MAPR*, *Cyp18a1*, *E93*, *JHAMT,* and *Kr-h1)* in the *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 μm) for 48h. All significant differences according on ($p < 0.05$).

Detoxification mechanisms were analyzed by selecting ten genes (*Cyp4d2, Cyp6b7, Cyp9f2,* and *Cyp12a2*; *GSTd3, GSTe1, GSTo1, and GSTt1*; *MRP1* and *ABCB6*). No effects were detected in any of them, as was showed in figures 42 and 43.

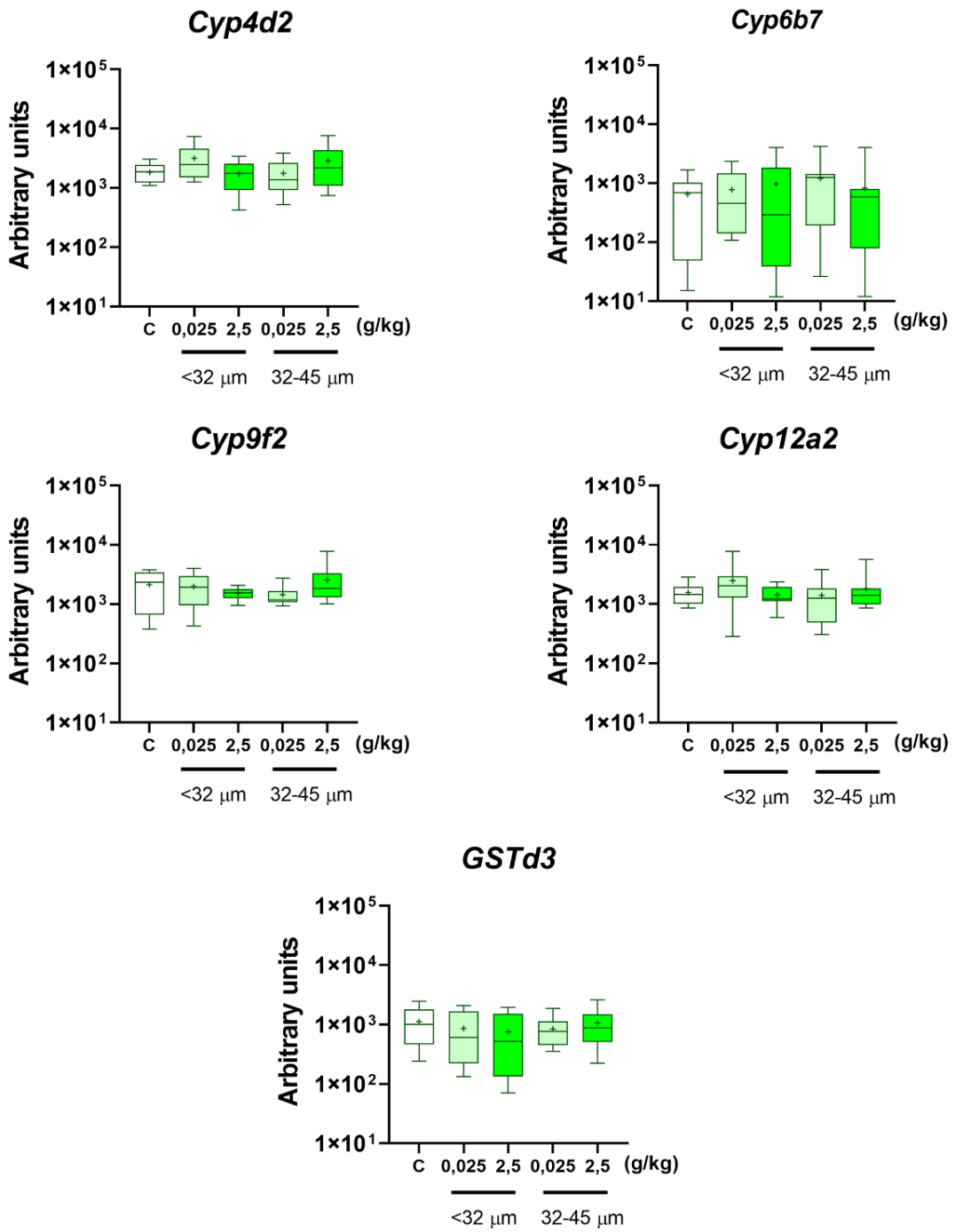

Figure 41. Expression of *Cyp4d2*, *Cyp6b7, Cyp9f2*, *Cyp12a2*, and *GSTd3* related to detoxification response in the fourth instar *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 μm) for 48h. All significant differences according to ($p < 0.05$).

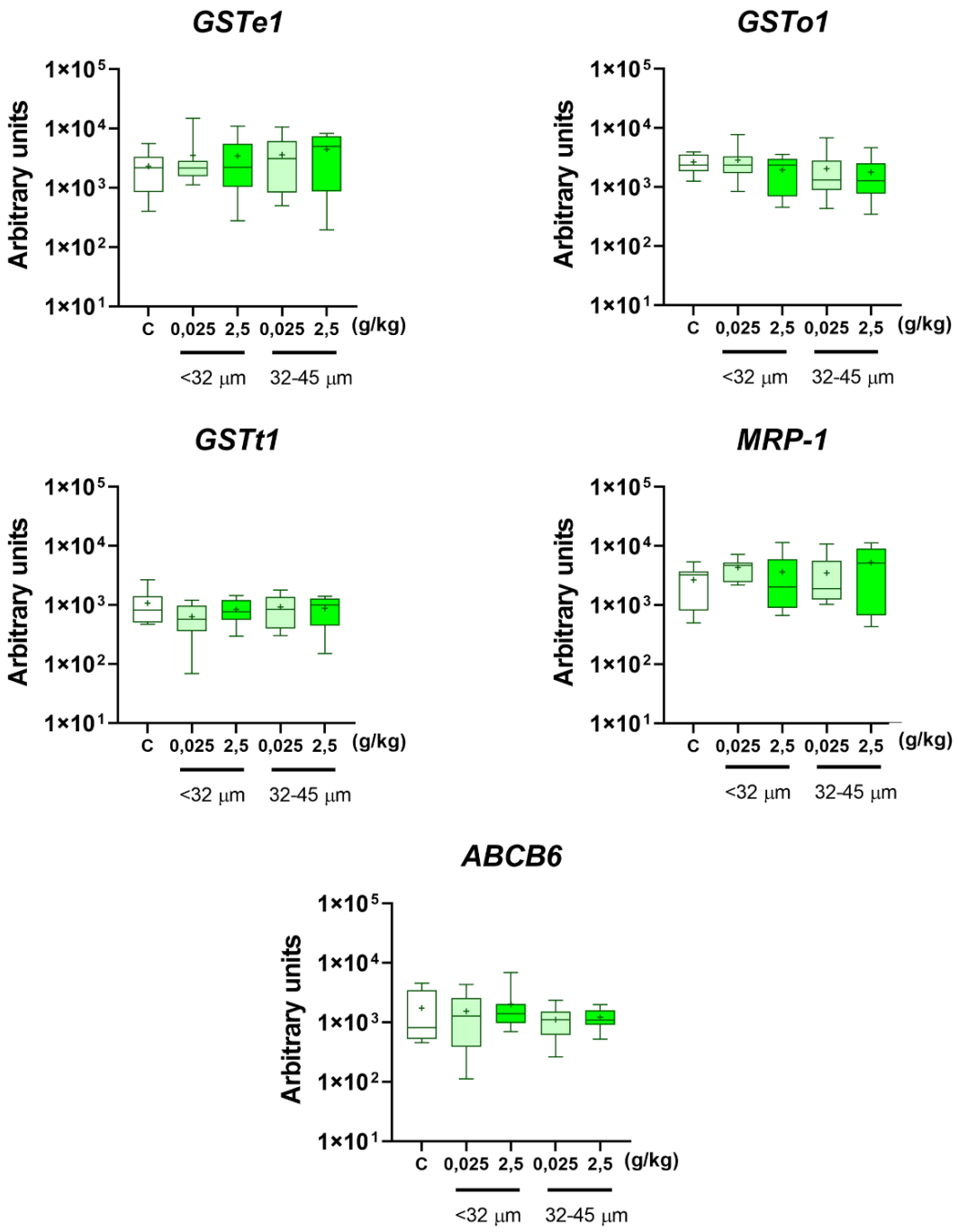

Figure 42. Expression of *GSTe1*, *GSTo1*, *GSTt1*, *MRP1*, and *ABCB6* related to detoxification response in fourth instar *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 µm) for 48h. All significant differences according on ($p < 0.05$).

Fourteen HSPs (*hsp70, HYOU1, hsc70, hsp40, hsp90, Gp93, hsp60, hsp10, hsp17, hsp21, hsp22, hsp23, hsp24,* and *hsp27*) were selected for stress response evaluation after MPs exposure (figures 44, 45, and 46). Only two genes were altered *Gp93* was upregulated at 2.5 g/kg respect to the small size, and *hsp60* showed differences in respect to the control at 0.025 g/kg for 32 µm (fig. 45).

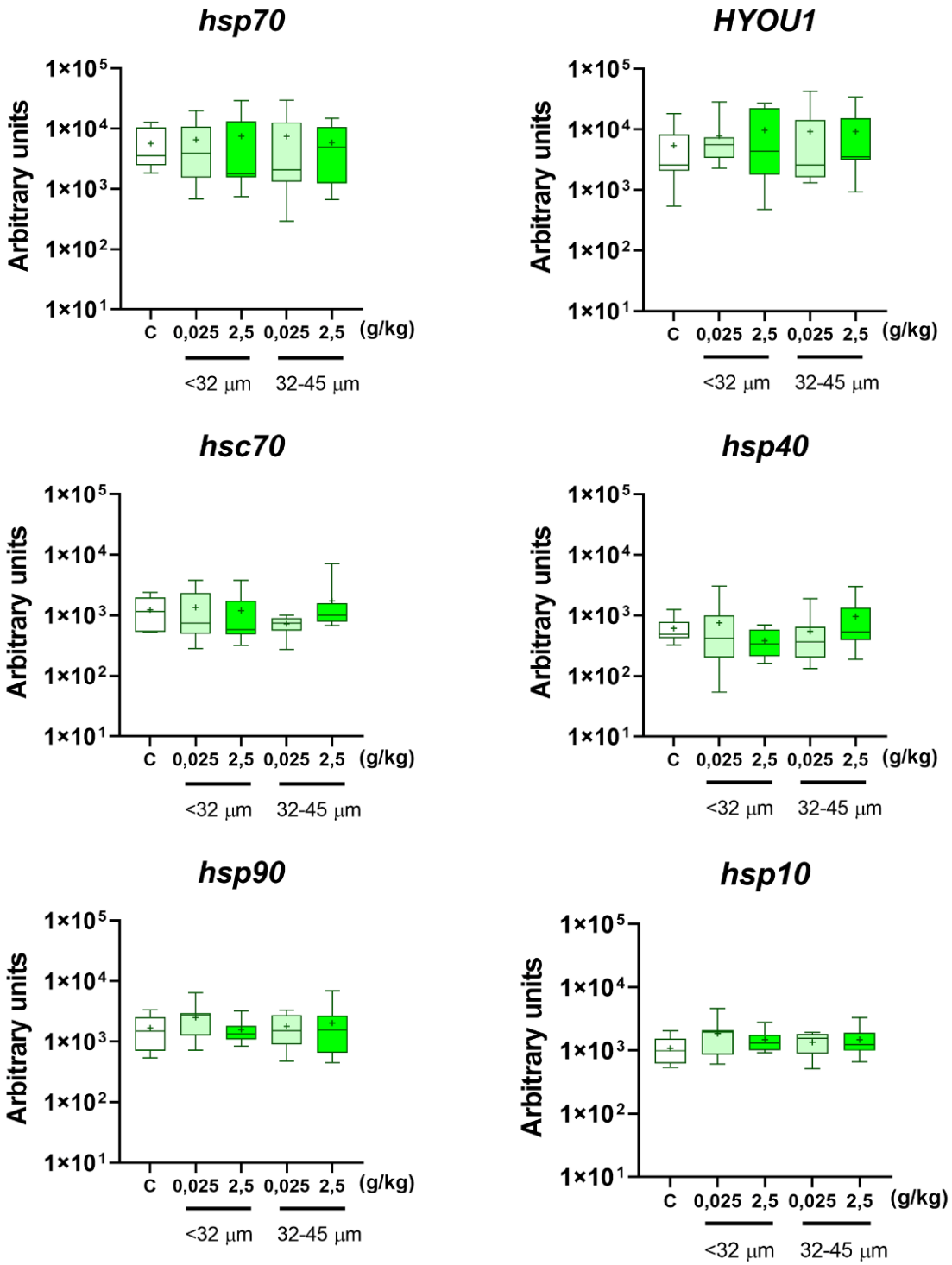

Figure 43. Expression of genes from stress response (*hsp70*, *HYOU1*, *hsc70*, *hsp40*, *hsp90*, and *hsp10)* in fourth instar *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 μm) for 48. All significant differences according on ($p < 0.05$).

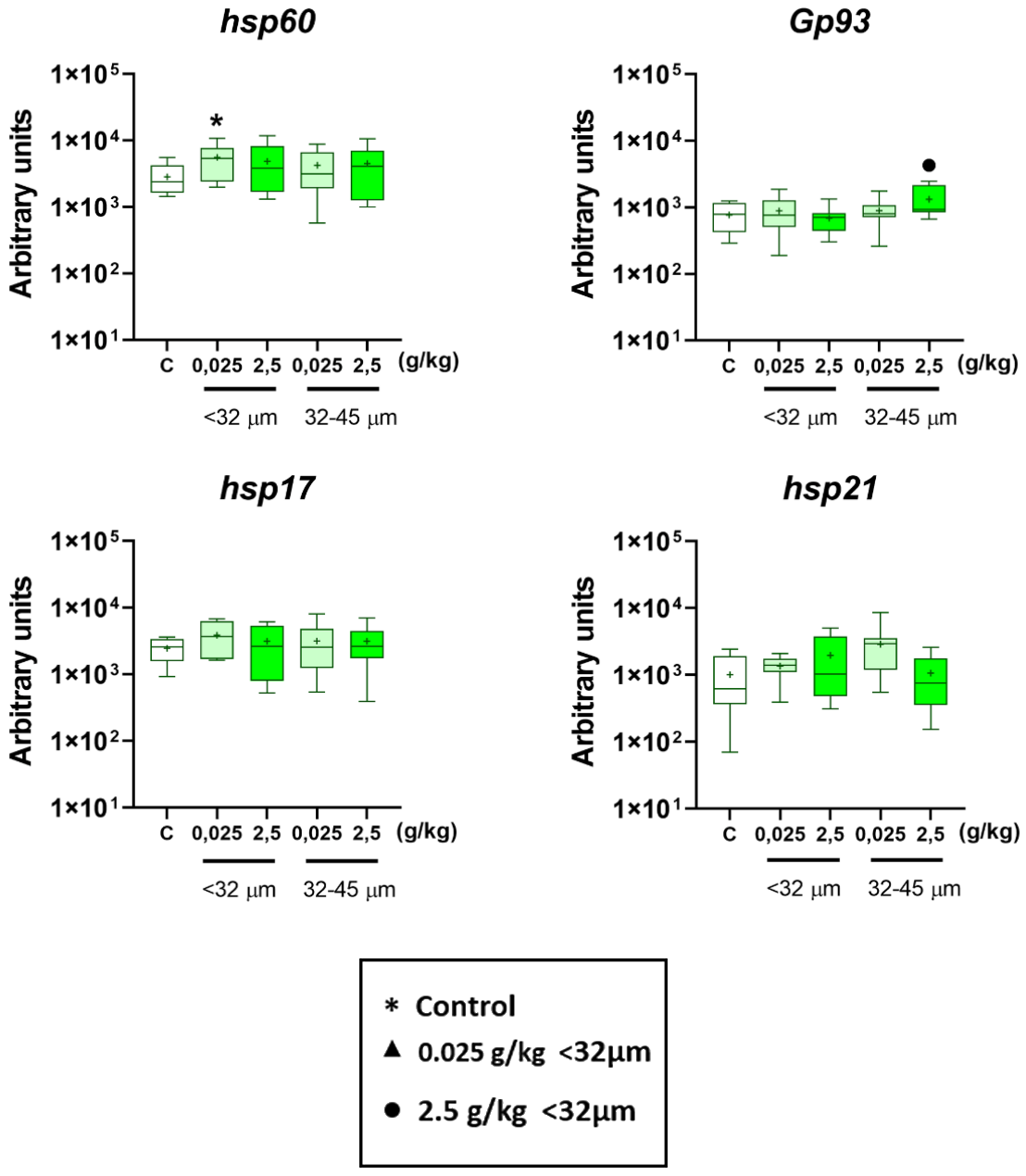

Figure 44. Expression of *hsp60*, Gp93, *hsp17*, and *hsp21* related to stress response in the fourth instar *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 µm) for 48. All significant differences according on ($p < 0.05$).

The immune response was analyzed through two genes, *Proph* and *Def*, related to the humoral response (fig 46). Only *Def* was upregulated by the smaller particle (<32 µm) at both concentrations analyzed.

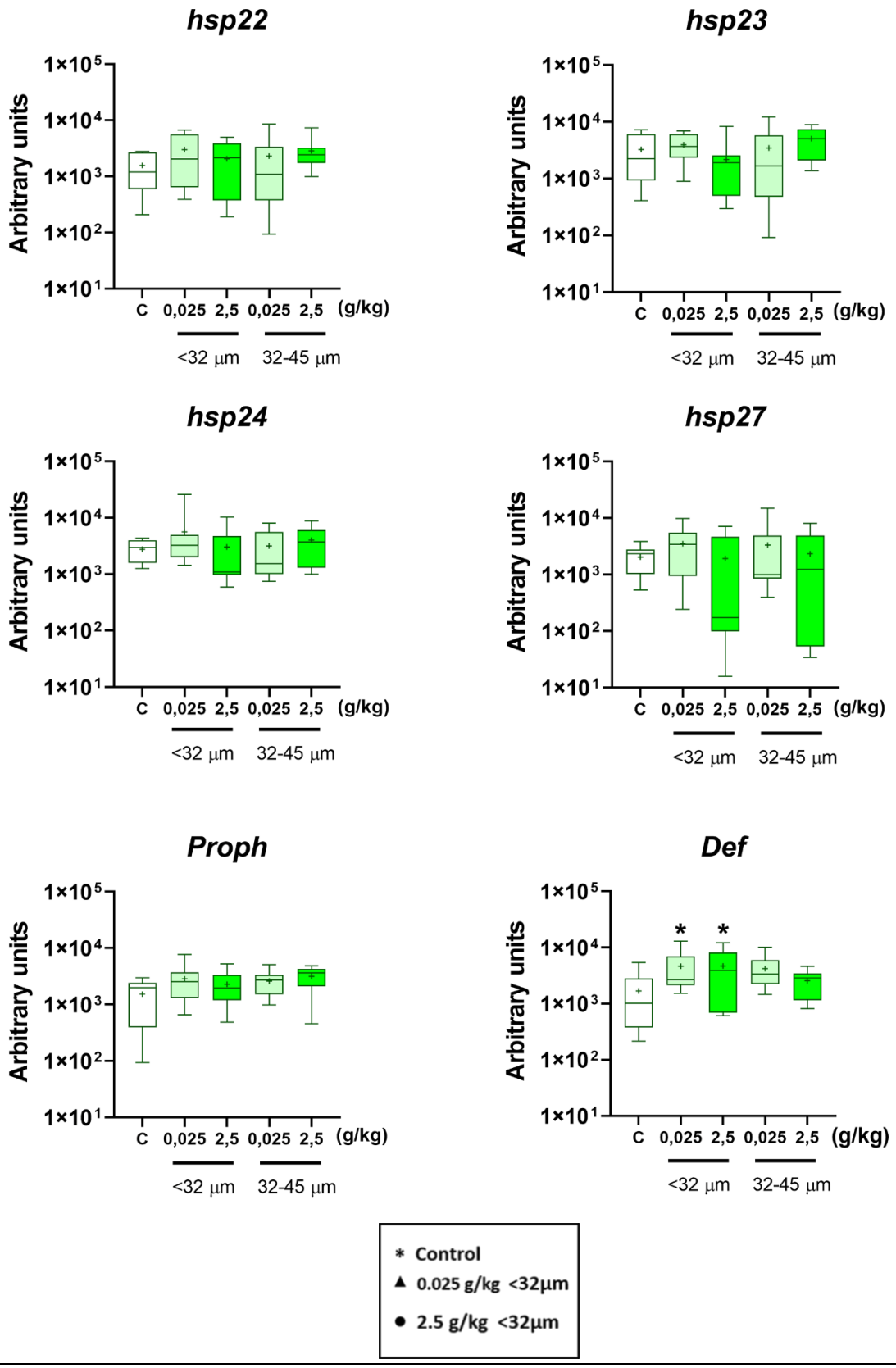

Figure 45. Expression of *hsp22*, *hsp23*, *hsp24*, and *hsp27* related to stress response, and *Proph* and *Def* related to the immune response in the *C. riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 µm) for 48. All significant differences according on ($p < 0.05$).

Finally, the DNA repair mechanisms were studied employing five genes (*PARP*, *ATM*, *XRCC1,* and *NLK*) related to single and double-strand breaks and *Decay* related to the apoptosis events (figure 47). *PARP*, *ATM,* and *NLK* showed increased expression respect to control at all the conditions, besides for *Decay* at both concentrations of the larger particle size. Moreover, differences between sizes were detected in *ATM* and *Decay* at low and high concentrations, respectively.

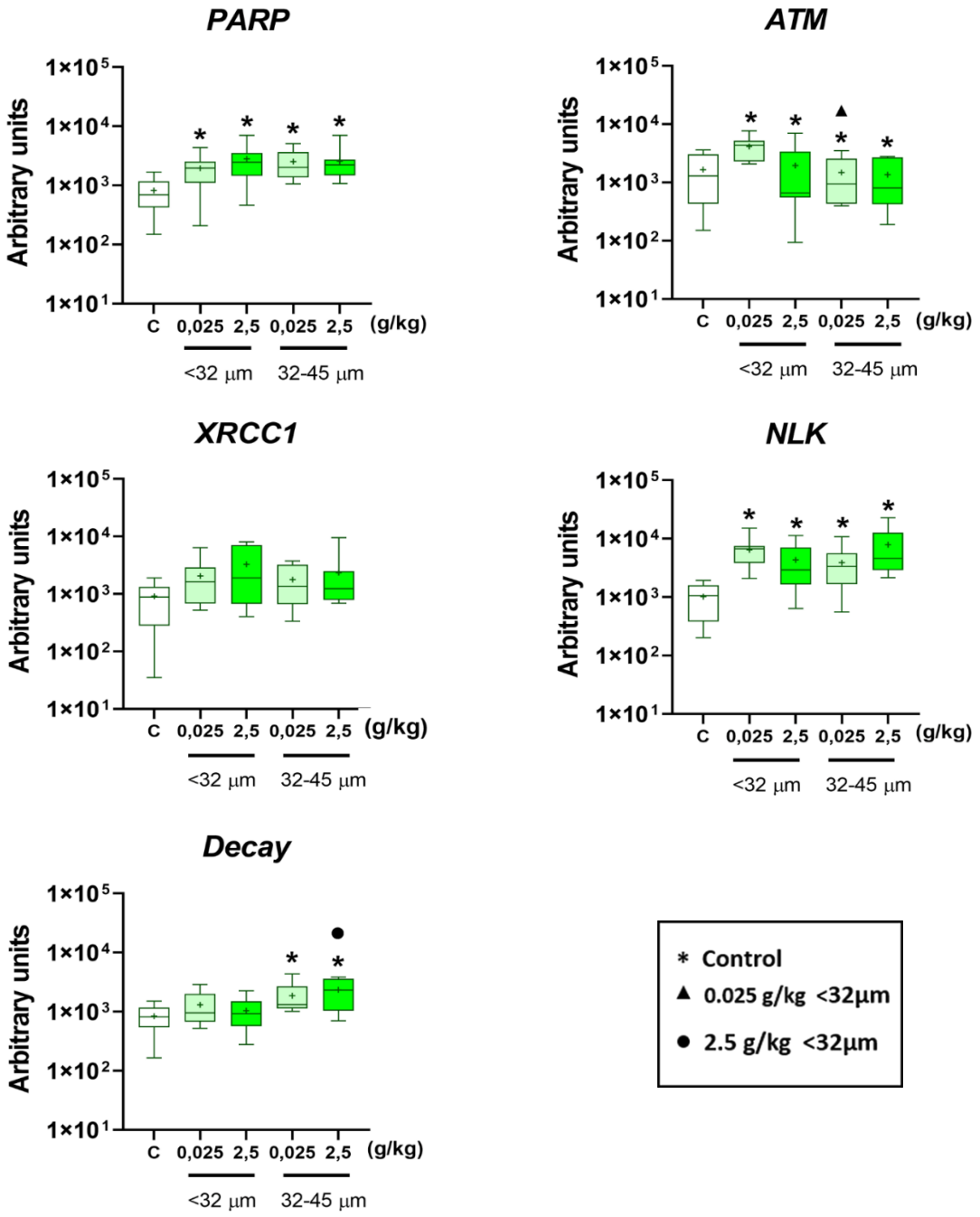

Figure 46. Expression of genes from DNA repair (*PARP*, *ATM*, *XRCC1*, *NLK*, and *Decay)* in the C. *riparius* larvae after exposure to LDPE MPs (0.025, 2.5 g/kg for <32 and 32-45 µm) for 48. All significant differences according on ($p < 0.05$).

SUMMARY

- **Lipid and carbohidrates content were modified.**
- **GST complemented with TGSH was altered by 32-45 µm; besides at high concentration CAT and AChE activity were increased.**
- ***InR, Dronc, Dis*, and *Met* were altered by LDPE MPs with upregulation respect to control. Besides, differences between sizes on *EcR* and *Dis* were observed.**
- ***Gp93* and *hsp60* were modified as stress response representatives.**
- **No effects were detected in detoxification response.**
- ***Def from* immune system was altered by both concentrations of 32 µm.**
- ***PARP, ATM, Decay* and *NLK*, from DNA repairing mechanisms, were altered with increased mRNA levels.**

Discussion

The last part of the work of the thesis was focused on the analysis of the newest emerging contaminants, the microplastics. The intensive consumption of plastics every day has increased the concern on the possible damages and alterations on the environment and the organisms. PE plastics belong to the most produced and employed polymers around the world, therefore reach easily to the environment. Both primary and secondary PE microplastics naturally produced by erosion in the environment have been detected in surface water, can be ingested by aquatic organisms and become a severe risk factor for wildlife (Sathish et al., 2020; Sruthy & Ramasamy, 2017; Xu et al., 2020). The toxicity of PE microplastics has been demonstrated in aquatic organisms from altered growth, reproduction, or immune response among others (Green et al., 2019; Lanctôt et al., 2020; Mak et al., 2019; Sıkdokur et al., 2020). Taken advantage of the designed array, it was used to study the effect of low-density polyethylene particles (LPDE) on biochemical biomarkers and transcriptional activity employing two different concentrations (0.025, 2.5 g/kg) and two particles size according to on the range ingested by *C riparius* (<32, 32-45 µm).

The biochemical parameters analyzed can be divided into two sets. One of them includes those that are related to structural information and physiological processes. The second set is focused on enzymatic activities. Lipids and carbohydrates content offer a picture concerning reserves in the presence of the toxicant. LPDE elevated the lipids content for the low concentration of both sizes. The response is similar to that observed in algae with increased lipid reserve after nano plastics exposure (González-Fernández et al., 2020). There is still a lack of information about microplastics, but other toxicants have been studied. Tadpoles exposed to herbicides increased their lipids content (Freitas et al., 2019), but the freshwater caddisfly *Sericostoma vittatum* exposed to UVFs maintained its lipid reserved unaltered (Campos et al., 2017). Therefore, stimulation on lipid content can be dependent on the compound and its toxicity. However, there is an additional factor to consider for microplastics. The physical properties can influence the effect. It is possible, for example, that the particles stay in the digestive tract difficulty the progress of the food. It would retain it more time and allow better digestion of the lipids, which usually are more slowly processed. There is also the possibility that increased lipid reserve could be derived to stress induced by the microplastic exposure as has been previously observed (Granby et al., 2018) besides the lipid accumulation is a typical energy reserve for future actions in the cells. The differences between concentrations could be assigned to the putative additional response that occurs in the cell and the organism at the same time. The higher pressure of the toxicant would require an increased investment of resources to maintain homeostasis, for example, by deriving the energy to the detoxification mechanisms (De Coen & Janssen 2003). In this

sense, it is essential to remember that microplastics are formed with polyethylene but also have additives and plasticizers that can affect the individual if released.

For carbohydrates content, some difference was observed at high concentration between both sizes. The 32-45 µm microplastics showed a lower amount of carbohydrates than <32 µm particles, in line with *Sericostoma vittatum* response to organic ultraviolet filters (Campos et al., 2017). The difference might be assigned to a higher demand of the cell to compensate for the changes due to the microplastics. Still, also it could be possible that the particles block the digestive tract preventing an adequate absorption of the nutrients. The activation of the GST and the TGSH value support the putative increase in the use of the energy reserves. However, the ETS was not modified, suggesting that there is not an increased production of energy. A similar conclusion is obtained when are compared both parameters to know the cellular energy allocation (CEA). To sum up, microplastics are inducing some change in the content of macromolecules. Nevertheless, it is not clear if they are related to the energy reserve use or can be an indirect effect due to the physical damages produced by them.

Lipid peroxidation (LPO) is an indicator of oxidative damage. In this experiment, no modification in the LPO was observed. It has been described that ROS production and activations of antioxidant systems occurs by single and mixture exposure to PE in oyster and earthworms (Chen et al., 2020; Revel et al., 2020). The CAT activity is increased in the higher concentration of the 32-45 µm particles, suggesting a similar situation in *C. riparius*. Then, the particles would be producing oxidative damage that is compensated by the activation of CAT, involved in the decreased or neutralization of ROS effects in the organisms. It is a useful indicator for toxicology evaluation in *C. riparius* showing stimulation by the presence of UVFs and nanoparticles (Campos et al., 2017b; Gopalakrishnan et al., 2015; Nair et al., 2011,2013).

Other of the enzymes analyzed, the GST, are related both to oxidative stress and detoxification. Coupling with it would be the total GSH since it is the base for the GST functioning. The 32-45 µm microplastics showed higher levels than the control for both, suggesting the activation of the detoxification response. The activation is in agreement with previous studies employing the same MPs on earthworms and mussels and even by PS microplastics in clams (Green et al., 2019; Ribeiro et al., 2017; Rodríguez-Seijo et al., 2018).

Taken together, the results with the enzymes define a picture with an increased ROS production and, probably, a detoxification mechanism activation. However, the analysis of the transcriptional activity of genes from Phase I, Phase II, and Phase III points towards that detoxification mechanisms are not activated, at least those involving the genes analyzed. The fact that also the DNA repairing mechanisms were activated, as suggested by the genes that code for proteins that do it, backs the idea that it is possible ROS production that produces some kind of damage in DNA. In summary, several data support the idea that ROS production could be a relevant mechanism to produce damage by microplastics. Additional analysis should elucidate the extreme and define more clearly the chain of events that happens in the cell and the organism.

The last enzyme analyzed was AChE. It was analyzed to define the putative damage that microplastics could produce in the nervous system. The AChE was increased for 32-45 µm particles, similar to the response to LDPE on *Eisenia fetida* (Chen et al., 2020). The question arises when the physiological role of AChE is considered. It is an enzyme that intervenes in the transmission of the nervous impulse, so hardly is possible that particles reach that point. It is a traditional nervous system biomarker that is usually repressed by different xenobiotics, mainly pesticides (Pereira et al., 2012; Pérez et al., 2013; Pham et al., 2017). So, at this point, it is crucial to keep in mind that microplastics have a double effect. First, as themselves by mechanical damage. Second, the chemicals which are part of them can act as any other compound from inside the organism because they are transported by the particle. So, the

activation of AChE might be a consequence of the induction by some of the chemicals that form the particles. Also, it may reflect an increase in the activation of the nervous system as a response to some motor problems or other damage produced by the mechanical action of the microplastics. Finally, it cannot be discarded with some other pathway that alters the nervous system indirectly.

As stated before, transcriptional activity was analyzed by using the same array as in previous chemicals. It was designed with genes covering five processes with relevance in the toxicity and the organism. Most of the genes were unresponsive to the microplastics, with *hsp60*, *def*, some related to the endocrine system (*Dronc*, *InR*, *Dis*, *EcR*, and *Met*) and those involved in DNA repairing upregulated. It is worthy to note that it is the first time that so detailed analysis is performed for microplastics on invertebrates as *C. riparius*, improving the knowledge about the putative targets at the subcellular level and the cellular response to them.

Surprisingly, none detoxification related gene was altered. As indicated previously, the GST activity was increased for the 32-45 µm particles, but none of the GST members analyzed was modulated. It probably is reflecting that another GST family is responsible for the increase. Furthermore, the lack of response of Phase I and Phase III support the idea that higher levels of GST activity were more related to the antioxidant role than to the biotransformation function. However, several MPs showed the capacity to modulate the GST expression with upregulation on fishes and crustacea (Choi et al., 2019; Granby et al., 2018; Xia et al., 2020). Besides, Cyp genes also have been described altered by MPs in larvae and adult fishes (Granby et al., 2018; Xia et al., 2020). The different experimental settings would explain the disparity in the results. These studies were performed at 30-40 days, while in this thesis, the exposure was for two days.

Similarly, the results obtained for the stress response were also striking. Only *hsp60*, coding for a mitochondrial protein, was upregulated. The induction of *hsp60* was also observed on crustacea after exposure to a MPs mixture, including PE. The lack of changes in the proteins of the hsp70 family (*hsp70, hsc70,* and *HYOU1*) contrasts with previous studies where PE MPs in single and ternary mixture produced a decrement in the expression of *hsp70* in fishes and *D. magna* (Espinosa et al., , 2019; Imhof et al., 2017). The same can be applied to *hsp90*, which has been described induced by PS nano plastics on *D. magna* (Liu et al., 2019, 2020). There is no previous reference for small heat shock proteins with microplastics. The studies with some additives have shown changes. It is not unexpected the lack of response to LPDE because the time of exposure is short to get enough additives resealed from them.

According to the data, the cell did not activate the response, so microplastics were not affecting the standard cellular procedures, at least to the extreme of inducing the activation of the stress proteins. It is a possibility that the cell response was focused on other mechanisms since the size of the particles could be too large to get the inner cell, and the number of additives was too low to induce any response. The change in the *hsp60*, since it is in mitochondria, might, in some way, be related to the energy reserves mobilization. It can be proposed that the alteration on protein folding in mitochondria can disturb the ATP obtaining and the respiratory process (Stevens et al., 2019). Future research should try to elucidate the correctness of the idea.

Another gene that was upregulated was *defensin*. In both concentrations of <32 µm particles, the levels were above that found in control larvae. One explanation could be that the organisms identify the microparticles and react to them activating some of the defense mechanisms. It would be interesting to get more in-depth about the idea. Supporting it, HDPE altered the immunity on blue mussels with overexpression of immune proteins like cytokines, macrophages, and related to complement (Green et al., 2019). Another study on crabs observed increased activity on acid phosphatase and PO, among others, and modified mRNA levels of hemocyanin and lysozyme (Liu et al., 2019). Moreover, in fishes,

disruption on immune response was detected by PE and PS microplastics exposure, confirming the strong effect in aquatic vertebrates (Espinosa et al., 2019; Wan et al., 2019).

The activation of the immune system can be linked to the existence of oxidative stress by LDPE microplastics. The release of ROS has been related to the activation of the immune response, specifically at the inflammatory response level (Mittal et al., 2014). Besides, the ROS liberation by MPs exposure was detected in copepods (Choi et al., 2019). On the other hand, the immune response demands a considerable amount of energy and resources, helping to decrease the energy reserves available for other cellular functions.

The alterations in the endocrine system affected the ecdysone synthesis (*Dis*), the juvenile hormone receptor (*Met*), 20-E signalling (*EcR*) and the insulin signaling (*InR*). Furthermore, *Dronc* was altered, but it is a caspase gene, so the activation of the transcriptional activity might depend on the apoptosis. It would be in agreement with the increased transcriptional activity of *Decay*, another caspase that is related to the response to DNA damage. The first conclusion that can be obtained is the putative role of microplastics as EDCs. It would be necessary to elucidate the cause, the polymer, or the additives. Still, there was a modulation of the synthesis of ecdysone, besides the decreased *EcR* can produce a defective 20-E signalling pathway that could develop changes in the development and molting. The impact on *Met* also suggests an alteration in the juvenile hormone response, adding more supporting evidence to the role of endocrine disruptors.

Nevertheless, the changes observed may be indirect consequences of the mechanical effects of microplastics. The insulin-receptor gene is also upregulated, and it is very related to growth and development. The induction could be produced by some chemical response but also could be a physiological response to the presence of microplastics in the digestive tract. Their accumulation could reduce the processing of the nutrients, and, as a response, the organism activates the physiological response that occurs when fasting. It would involve the increase of insulin-signaling to modulate the energy reserve use. It would also be affecting to the development since it depends on the disponibility of resources for molting and the metamorphosis. A preliminary study on *C. riparius* employing LDPE showed alteration on growth and delayed emergence mainly by 32-63 µm particles (Silva et al., 2019). Similar results were observed in another chironomid, *Chironomus tepperi*, by PE microplastics (Ziajahromi et al., 2018). Both studies are in line with the endocrine picture painted in this thesis.

The endocrine system is involved in the regulation of short- and long-term events in the organisms. The changes that happen in a few minutes can also show an effect later. Furthermore, hormones form a network of interrelated processes that regulate cellular metabolism. The consequence of that is a complex scenario that can be unbalanced by chemicals but also by physiological events that alter the homeostasis. The time of exposure and the size of the particles would determine the impact. It is because the time could be short of producing damage by the additives and the size of particles too big to enter the cells.

All the genes involved in DNA repairing, except *XRCC1*, were altered. The results suggest, then, that early DNA damage is produced. The question that arises is how it can be produced. It is hard to accept that it is a direct impact of the particles on DNA. It could be a consequence of the additives, but 48 hours seems to be a short time to induce extensive damage. Moreover, the data of the other pathways do not support damage by chemical additives. The study is performed by extracting the mRNA from the whole animal, so the data may reflect the response of the digestive tract cells, which are directly affected by the particles. A study separating the tissues would provide some light about this possibility.

However, it can also be produced indirectly by oxidative stress due to ROS liberation, as previously detected for nanoparticles. Oxidative damage can induce the breaking of the strands.

On the other hand, a detailed analysis of the gene roles could help to elucidate what is happening. *PARP* codes a protein related to the DNA damage response, and its inhibition causes alterations in the SSB that can degenerate into further damage such as DSB (Maltseva et al.,2015; Wang et al., 2019). It acts by binding to the damaged strand, inducing chromatin relaxation, allowing other factors, such as *XRCC1*, the access to repair the DNA (Gao et al., 2017). However, the absence of alteration on *XRCC1* suggests a partial activation of the SSB mechanism, so the type of damage could be a double-strand break. It is backed by the stimulation of *ATM* and *NLK* with the smaller particles, mainly related to the DSB reparation (Wu et al., 2018). *ATM* is a core component of the DDR activated when damage is detected and is involved in the homologous recombination (HR) repair pathway for DSB. Just like *PARP* and *XRCC1, ATM* interacts with *NLK* (a posttranscriptional regulator of NF-κB), forming a complex for DSB repair. Therefore, there are induced genes to repair damaged DNA and promote survival via NF-κB.

However, when the mechanism fails, *ATM* joins to p53 activating the apoptosis processes. The activation of *Decay* for the 32-45 µm particles could be reflecting the apoptosis activation, as has been suggested for multiwall carbon nanotubes (Martínez-Paz et al., 2019). The downregulation of *ATM* may reflect it since it is simultaneous to *Decay* induction. Then, the damage by <32 µm particles would not be enough to trigger apoptosis at 48h, but it would be for 32-45 µm particles. *ATM* showed a similar response on *C. riparius* against vinclozolin with upregulation at low concentration and downregulation at higher concentrations (Aquilino et al., 2019). Moreover, carbon nanotubes provoked increased *ATM* mRNA levels at the same time as no effects on *XRCC1*, identical to the effects of the MPs (Martínez-Paz et al., 2019). Therefore, the damage would activate DSB repair more than on SSB, which is related to *XRCC1*.

Modulation of mRNA levels of genes related to the DDR system has not been previously addressed after exposure to microplastics. But all of these results are consistent with the previously described effects of MPs on DNA. One study detected damage from PE exposure in mussels, although this type of MPs did not cause alterations in oysters (Avio et al., 2015; Revel et al., 2020). Furthermore, several works evaluating PS showed a substantial alteration on DNA also through comet assay in earthworms and clams (Jiang et al., 2020; Ribeiro et al., 2017). To sum up, the LDPE affects the DNA and activates the response to repair it. For the bigger particles used, the damage could be so extensive that it activates the apoptosis. As a consequence, the DNA damage could lead to inhibition of synthesis or disruption on the replication process (Rocha et al., 2014).

The concern about the possible alterations from MPs on the aquatic organisms has been increased over the years due to their exacerbated presence in surface waters as oceans, rivers, and beaches. The present study represents one of the first evaluating the effects of MPs at the cellular level employing multiple biomarkers on the insect *C. riparius*. Diverse mechanisms and pathways were modified by these MPs. On the one hand, the energy reserves showed an altered pattern with increased lipid and sugar content. Besides seems that ROS liberation was derived by the LDPE exposure confirmed by the increased CAT, GST, and TGSH. At the transcriptional level, substantial effects on DNA were developed in line with this oxidative damage, generating even apoptosis induction spite the short exposure time. Surprisingly, these microplastics developed similar actions that typical EDCs, provoking possible defects in the molting or development of the organisms. Finally, the immune response was activated; hence, these compounds can be identified as strange molecules or generated the damage by ROS similarly to the other systems.

In general, LDPEs appear to be toxic for the *C. riparius* mainly through the ROS liberation developing oxidative stress events. One crucial point is the fact that the microplastics can generate direct damage by the physical interaction or indirect action itself by releasing the additives from which it is composed.

So, in many cases, it is difficult to know if the generated effect is due only to the MP alone. Future studies should assess the effect of both the MPs and additives, to understand the real damage of MPs in the aquatic environments. In summary, the LDPEs are able to disrupt different metabolic and cellular processes essential for the correct life on *C. riparius* despite the short exposure time employed. Comparing both particle sizes in general, the 32-45 µm particles can be more toxic to this organism. According to these results, the future of the population could be compromised by delayed development or inadequate immune response or DNA repair mechanisms. Moreover, the excessive energy consumption employed to alleviate the effects of oxidative stress can cause less energy available for other vital processes at the cellular level.

Concluding remarks

8. Concluding remarks

The current context of global change joined to the increased population has generated new environmental conditions by modifying parameters such as temperature or salinity and by incorporating multiple xenobiotics to the environment, mainly derived to the anthropogenic activity (Bindoff et al., 2019; Landis et al., 2013; Noyes et al., 2009). Then, the organisms are now in a scenario where they are submitted to multi-stress, often in a short period, which challenges their ability to survive. As a result, the changing environment alters both the chemical and the organism's behavior, also affecting its distribution (Kimberly & Salice, 2014; Nadal et al., 2015). In this context, the organisms living in aquatic ecosystems are a target of these changes because the water resources and sediments act as sinks for diverse chemical compounds, provoking a continuous flux of compounds and increasing the risk of irreversible alterations that could unbalance their delicate balances.

Nowadays, there is a lack of information available on the toxicity and the effects derived to multi-stress and mixtures, and it is mainly focused on ecological parameters (Godoy et al., 2019; Nieto et al., 2016; Park et al., 2017; Yu et al., 2017). However, some studies have employed molecular analysis by evaluating a limited number of genes or enzymes (Almeida et al., 2018; Kim et al., 2018; Ozáez et al., 2016 a,b,c).

As stated in the introduction, the molecular methods are gaining presence in the ecotoxicological analysis. In this work, it has been used the molecular analysis to improve the knowledge of the processes affecting the organism, centering the study on several cellular processes that have relevance in the cell and organism physiology. Besides provides new information about the effect of the compounds used, the results also help to understand the physiology of *Chironomids* and to identify new putative biomarkers that could be incorporated in standard toxicity tests.

The analysis carried out was focused on the study of fourth instar larvae response to different emergent contaminants. Gene expression and enzymatic activities have provided a picture of the putative mechanisms affected by the toxicant presence, helping to understand the effects at higher levels of complexity and offering clues about the future of the animals in the context of continuous global change. It is worthy to note that the present thesis is one of the first studies on *Chironomus riparius* that tries to get a picture of the complex response that arises in multi-stress scenarios at the molecular level. Furthermore, the study has also involved different times or temperatures, increasing the number of variables used and the complexity of the analysis

The first step in the toxicological analysis is, usually, the analysis of survival. It is employed as an ecological parameter for the toxicity evaluation, and, secondly, to define the concentrations to be used in the later analysis that require no mortality. For most of the conditions performed here, there was previous information concerning survival, allowing to select the experimental parameters (Ozáez et al., 2013; Planelló et al., 2008; Faria et al., 2007; You et al., 2004; Silva et al., 2019). However, others, such as ibuprofen or the combination of benzophenone-3 and temperature, had no previous information. For both cases, these new conditions demonstrated decremental alterations on survival.

One hand, for ibuprofen, even in low concentrations, can alter the survival of *C. riparius*. Therefore, environmentally relevant concentrations would be able to modify the habitat of this species. On the other hand, derived from BP-3 and T results, the mild temperature increased mimic to the climate change could influence the toxicity of UVF BP-3, increasing it on *C. riparius*. However, the impact is less than expected because of the fast capacity of the *Chironomids* to adapt to new temperature conditions.

Concluding remarks

Physiological changes are consequences of molecular events that impact in the cell and, subsequently, at higher levels of complexity. To understand the damage that a pollutant produces, it is essential then to analyze the different levels of complexity. Proteins are one of the main characters in the cell metabolism, and they are coded by genes, which are transcribed in mRNA for a later translation to proteins. Knowing the alteration in the transcriptional activity could provide an early signal of cell damage, allowing us to understand the processes that are in progress during the compound exposure and how the cell manages the situation to maintain the homeostasis. Gene expression profiles are a useful tool to elucidate what happens in the cell during the exposure. The approach in this work was to evaluate different genes involved in multiple pathways to know how they were modulated by exposure to different xenobiotics in single and or combined exposure on fourth instar larvae. Several compounds of different chemical nature were studied as representatives of UVFs, pharmaceutical compounds, and pesticides. Furthermore, it was analyzed the transcriptional profile and biochemical biomarkers in response to microplastic exposure, as it is a class of emerging contaminant with a growing impact on the environment.

The present work is an effort to elucidate the cellular and molecular mechanisms involved in response to toxicants. Furthermore, the approach selected tries to cover different issues that are arising in the analysis of toxicity such as multi-stress and the influence of physical factors on the response to pollution. The task is, sometimes, challenging because the increase of variables adds complexity to the analysis. As a result, some of the data obtained in the present thesis would require additional experiments to be put in value since they could be considered as inconclusive. However, the present thesis is a step forward by opening the analysis to new pathways and offer a more integrative perspective of the cellular response.

On the other hand, the present thesis also provides one array to analyze different conditions and toxicants. It is essential to keep in mind that extensive analysis by transcriptomics often shows a significant lack of deep, so it is necessary to validate the results by other methods such as Real-Time PCR. By selecting specific genes that are relevant in a pathway, an array covering many cell processes can be designed and facilitates the study of other compounds. Furthermore, specific profiles might be obtained as a hallmark of any type of compound. It would help to improve the development of Adverse Outcome Pathways for the AOP wiki. This tool is in progress to fulfill the different demands of the scientific community and policymakers.

As a summary of the thesis, it can be said that the different compounds used here have shown a variable impact on the physiology of *Chironomus riparius*. The damage is produced in several ways, and it can have different consequences in the population future. The mixtures, as expected, are more harmful than single compounds. It confirms the importance of include mixtures in the analysis because some pathways can be altered by mixtures but not by single compounds. One example is the mixture of UVFs, which is more dangerous for the larva than OD-PABA or OC. The damage is even more severe with BPA, suggesting that it is urgent to analyze the interaction between compounds with different chemical nature.

Besides, the study with ibuprofen and microplastics shows that it is required more analysis centered on those contaminants. The growing presence of both pollutants in the environment demands an in-depth analysis to quantify the damage that they can produce and to determine if the damage is reversible.

Moreover, the pesticide endosulfan, even at concentrations similar to that found in the environment, was capable of generating an intense disruption in multiple metabolic pathways in *C. riparius*. It is notable that despite being banned from use in various countries due to its persistence in the

environment, it can be released from the ground to waters many years later. Also, due to its low cost and effectiveness, it continues to be used in multiple underdeveloped countries.

The physical factors are also relevant in the analysis of toxicity. The data obtained with BP-3 at different temperatures confirm the influence, and, especially by the present climate change, it should be considered more often when the studies are designed. The present progress of the climate will change the habitat conditions, and it is necessary to know the ability of the organisms to manage such stress combined with the growing pollution of the ecosystems.

Finally, one of the main achievements is to increase the number of pathways that can be analyzed at the molecular level in *Chironomus riparius*. It would allow us to get a better picture of the processes involved in the toxicity and the response to toxicants. Although it is still limited to around forty genes, it is opening new possibilities to combine the transcriptional activity analysis with other parameters like enzyme activities and comet assay. The power of these tools could add new ways to take advantage of the toxicity test providing the molecular key events that cause one defined physiological response.

CONCLUSIONS

9. CONCLUSIONS

I. It has been designed and validated a specific array for *C. riparius* with forty genes covering five essential metabolic pathways in invertebrates: endocrine system, detoxification mechanisms, stress response, DNA repair, and immune system.

II. The array has proven a useful tool to analyze multi-stress showing specific transcriptional profiles depending on the experimental conditions.

III. Six pollutants with different chemical natures have been evaluated, three of them (ultraviolet filters mixtures, endosulfan, and microplastics) using the array.

IV. Multi-stress analysis on UVFs, using mixtures and simultaneous temperature variation, confirms the need to incorporate these approaches to ecotoxicological assessment.

V. UVFs mixtures are more toxic than single compounds, and the addition of a different nature compound (BPA) increases the toxicity. Mimicking field conditions will provide a closer picture of the actual environmental situation.

VI. Temperature modulates the toxicity of BP-3 on *C. riparius* at survival and gene expression level, offering additional evidence of the influence that climate change could have in the future of aquatic invertebrate populations.

VII. Endosulfan, a pesticide, shows substantial toxicity at the cellular level in *C. riparius.* At environmental concentrations, acts as EDC and, apparently, genotoxic. Mechanisms of action could involve oxidative damage due to the effects on immunity, stress, and detoxification responses.

VIII. Ibuprofen, a pharmaceutical, was toxic at environmental concentrations on the survival of *C. riparius*. Furthermore, it is an endocrine disruptor and causes alterations in stress response, immune system, and GST activity.

IX. The oxidative stress appears to be the primary mechanism of action for LDPE microplastics on *C. riparius*, altering catalase and glutathione-S- transferase activities. The damage disrupts the immune system, and the DNA repairing, probably linked to the ROS effects.

X. Besides, similar to the other xenobiotics LDPE MPs, alters different endocrine-related genes, so it is a possibility that they could act as EDCs.

XI. The designed array has proved the relevance of the simultaneous analysis of different pathways. The growing interest of the scientific community in the Adverse Outcome Pathway database supports the design of new arrays.

XII. The specific molecular response depending on the exposure time analyzed has demonstrated the need to use different times when studying molecular effects.

XIII. The present thesis highlights the need to improve the toxicity tests by including the cellular and molecular endpoints and diversity of test conditions, such as multi-stress. Mimic the environmental condition adds complexity to the analysis but also arises putative interactions and properties that are not observed in single exposures.

10. PERSPECTIVES

- ✓ Design and validate new arrays in *Chironomus riparius* and other invertebrates.
- ✓ Improve the knowledge of the transcriptomics in other chironomids.
- ✓ Evaluate the effect of LDPE as pollution vectors for organic compounds such as UVFs.
- ✓ Study the effects of different types of MPs on gene expression on invertebrates.
- ✓ Analyze the effects of commercial sunscreens on life cycle parameters as survival, growth, or emergence taxes.

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ANNEXES

Annex I: Effects of a single exposure, binary, and ternary mixtures of UV filters OD-PABA and OC, and the plasticizer BPA (Figures)

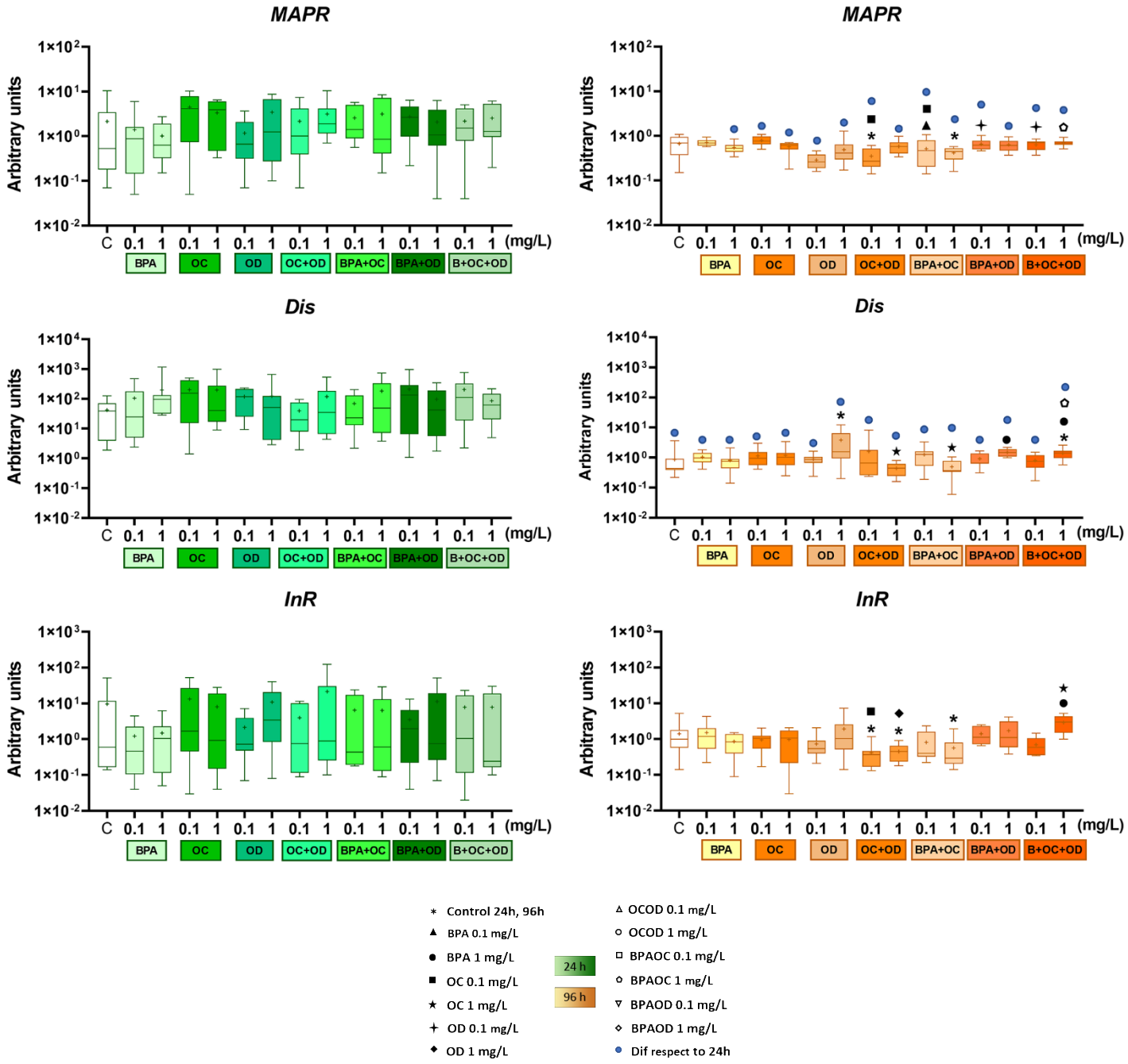

Figure 1. Expression of *MAPR, Dis, and InR* related to the endocrine system in fourth instar *C. riparius* larvae after exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. mRNA levels were normalized using *GAPDH*, *rpL11*, *rpL13*, *RNApol*, *Phfk*, and *TBP* as reference genes. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th and 75th percentiles, while the whiskers denote the highest and lowest results. The mean is indicated by the plus sign inside the box. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

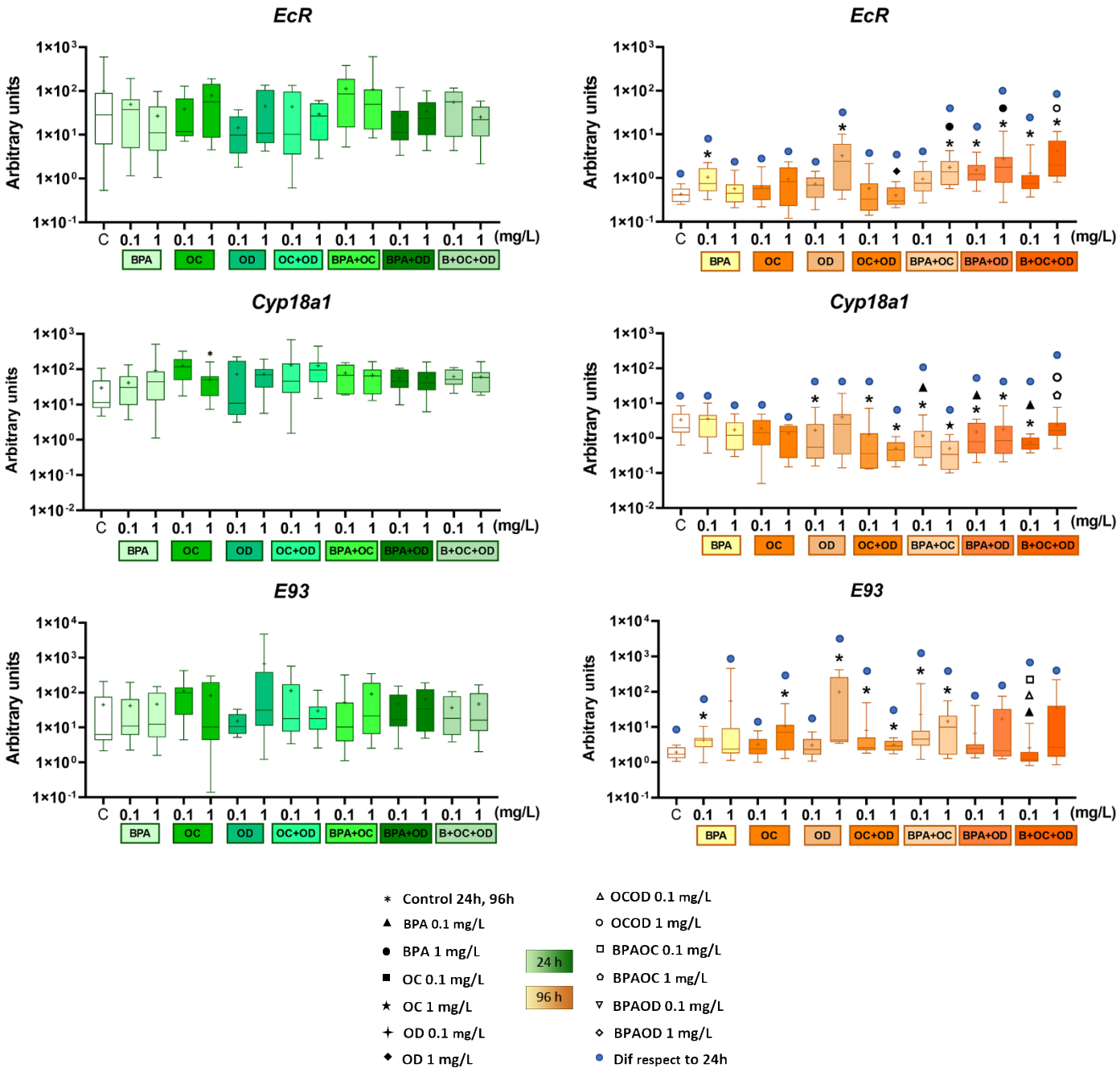

Figure 2. Expression of *EcR*, *Cyp18a1*, and *E93* related to the endocrine system in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

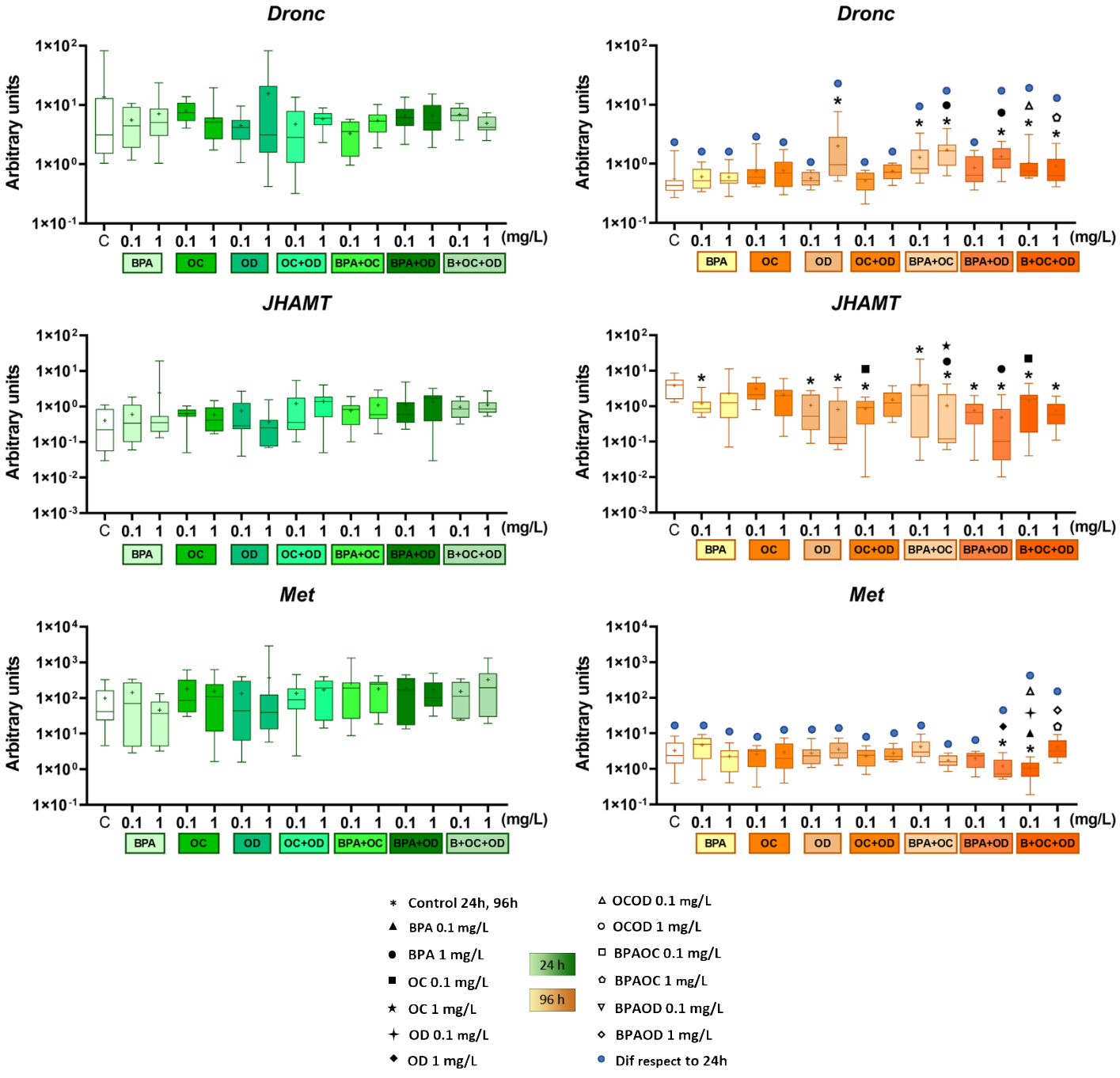

Figure 3. Expression of *Dronc*, *JHAMT*, and *Met* related to the endocrine system in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

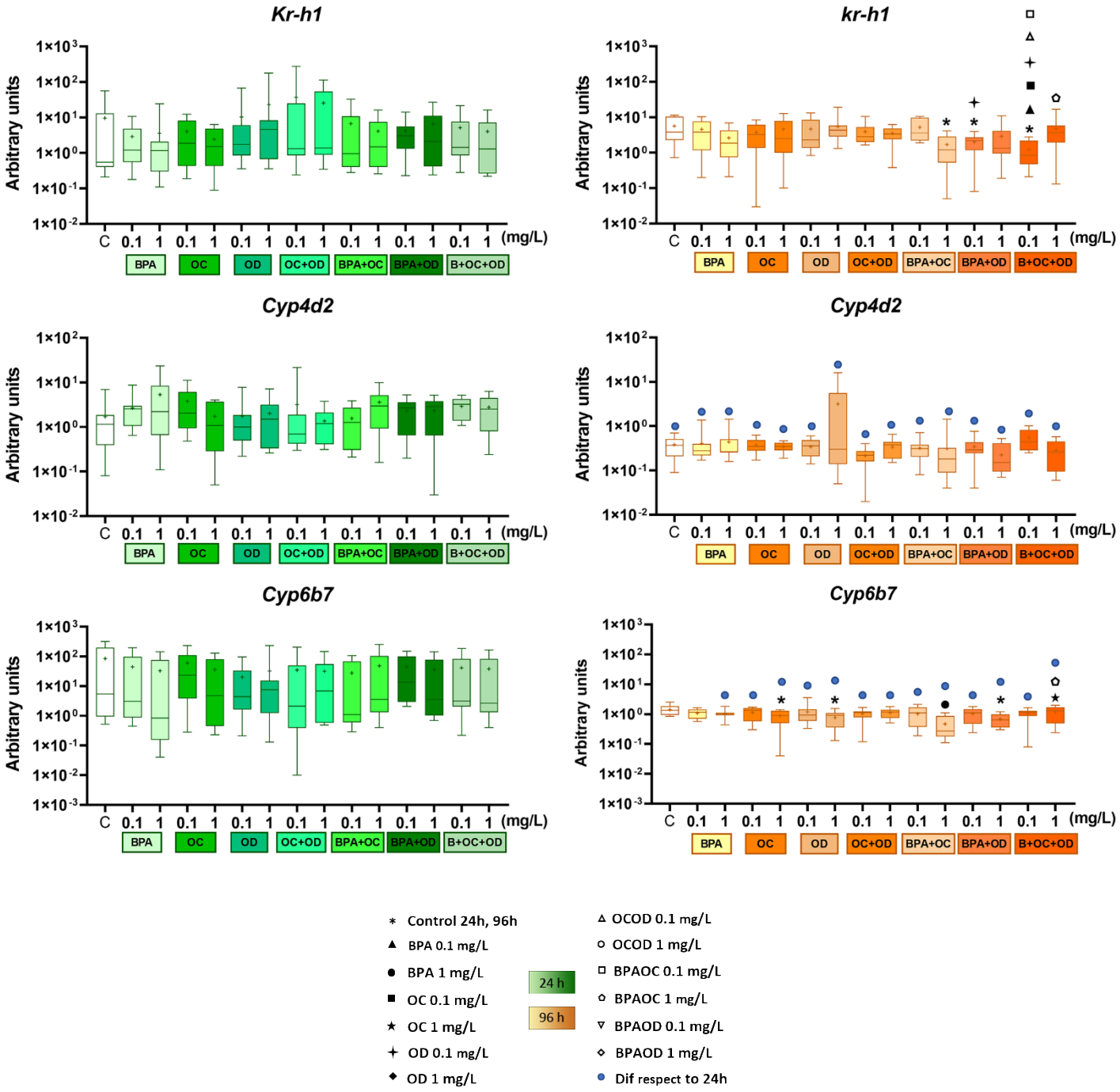

Figure 4. Expression of *Kr-h1* related to the endocrine system; *Cyp4d2*, and *Cyp6b7* related to detoxification response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

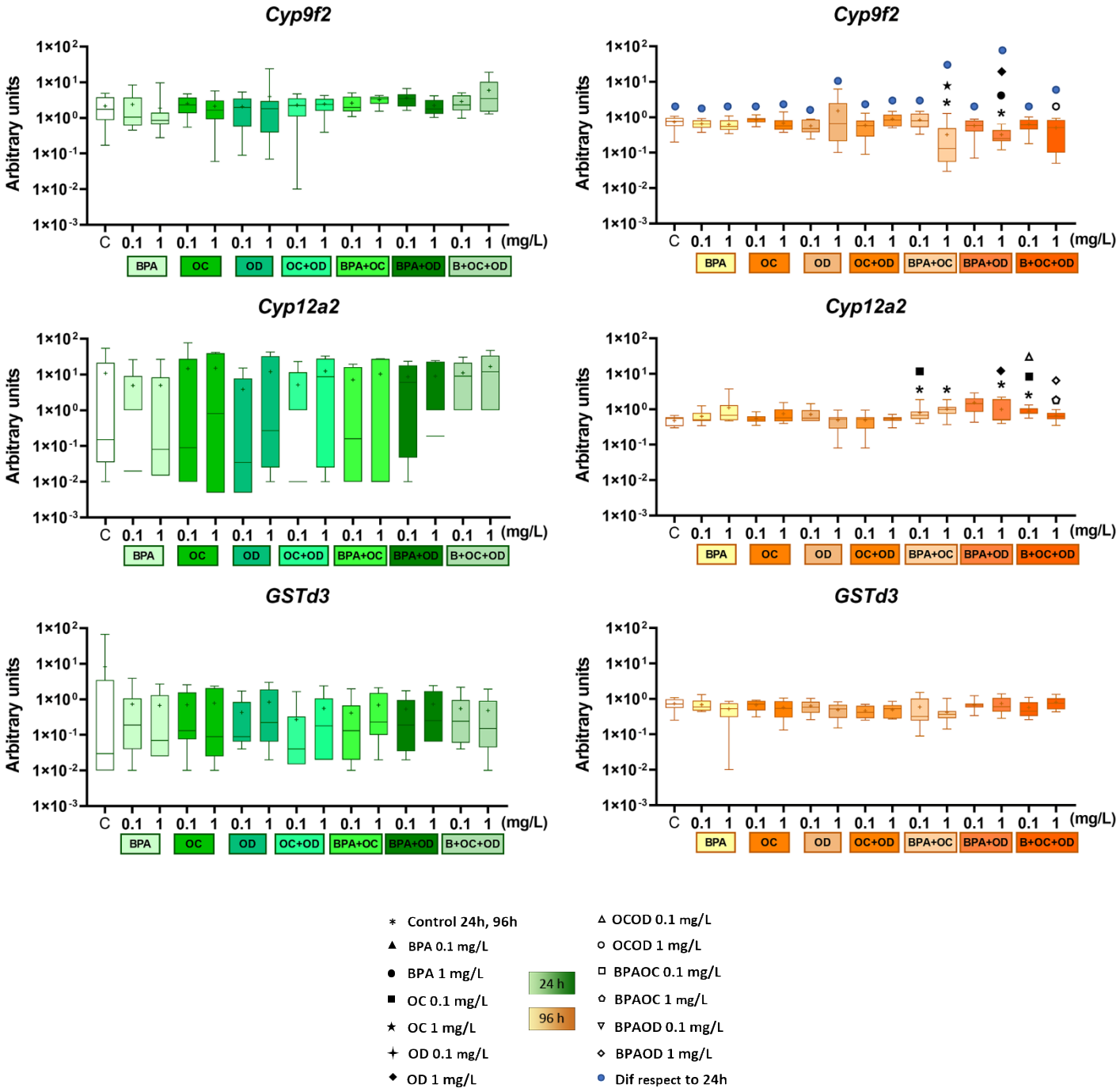

Figure 5. Expression of *Cyp9f2*, *Cyp12a2, and GSTd3* related to detoxification response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

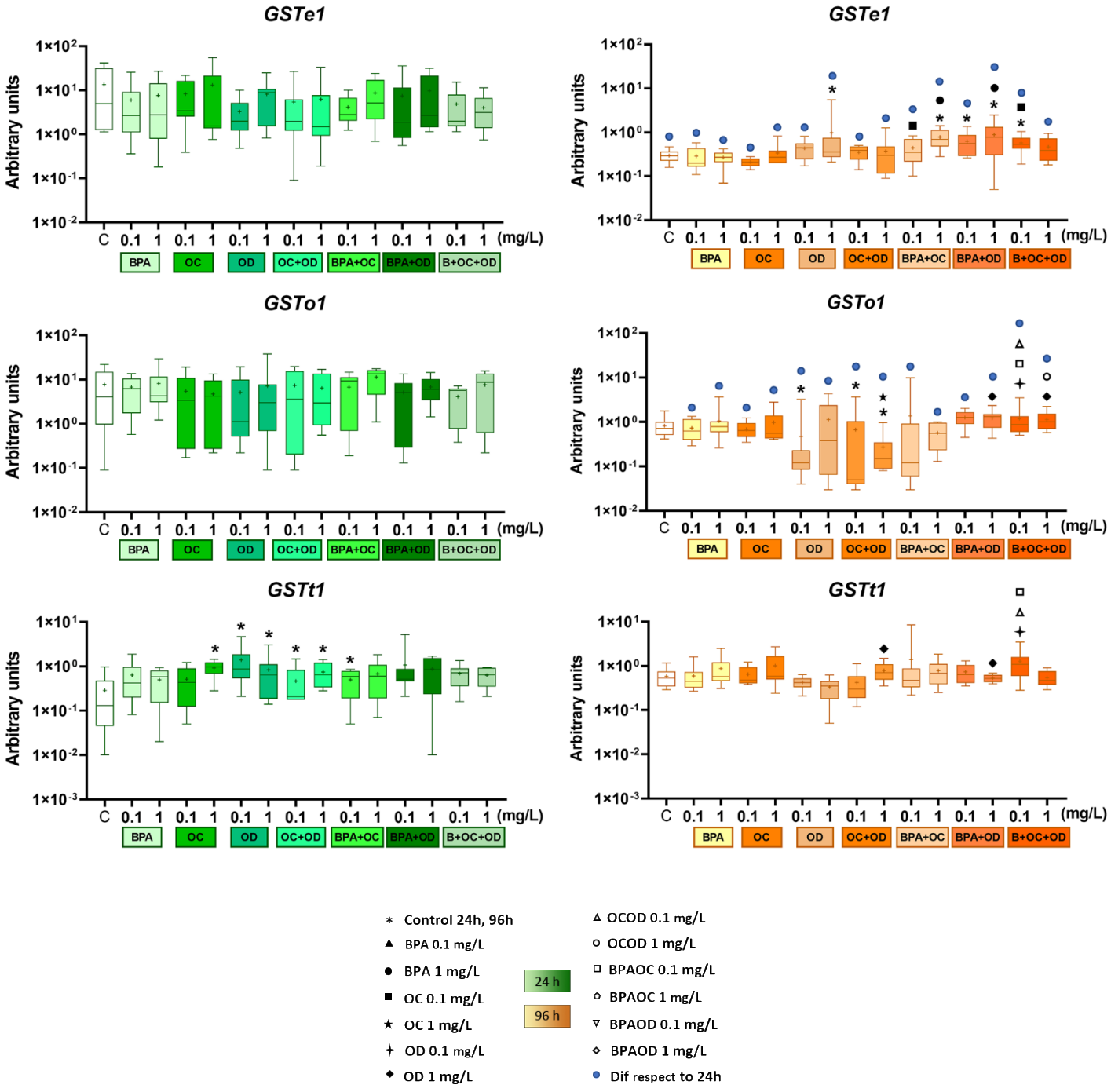

Figure 6. Expression of *GSTe1, GSTo1, and GSTt1* related to detoxification response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

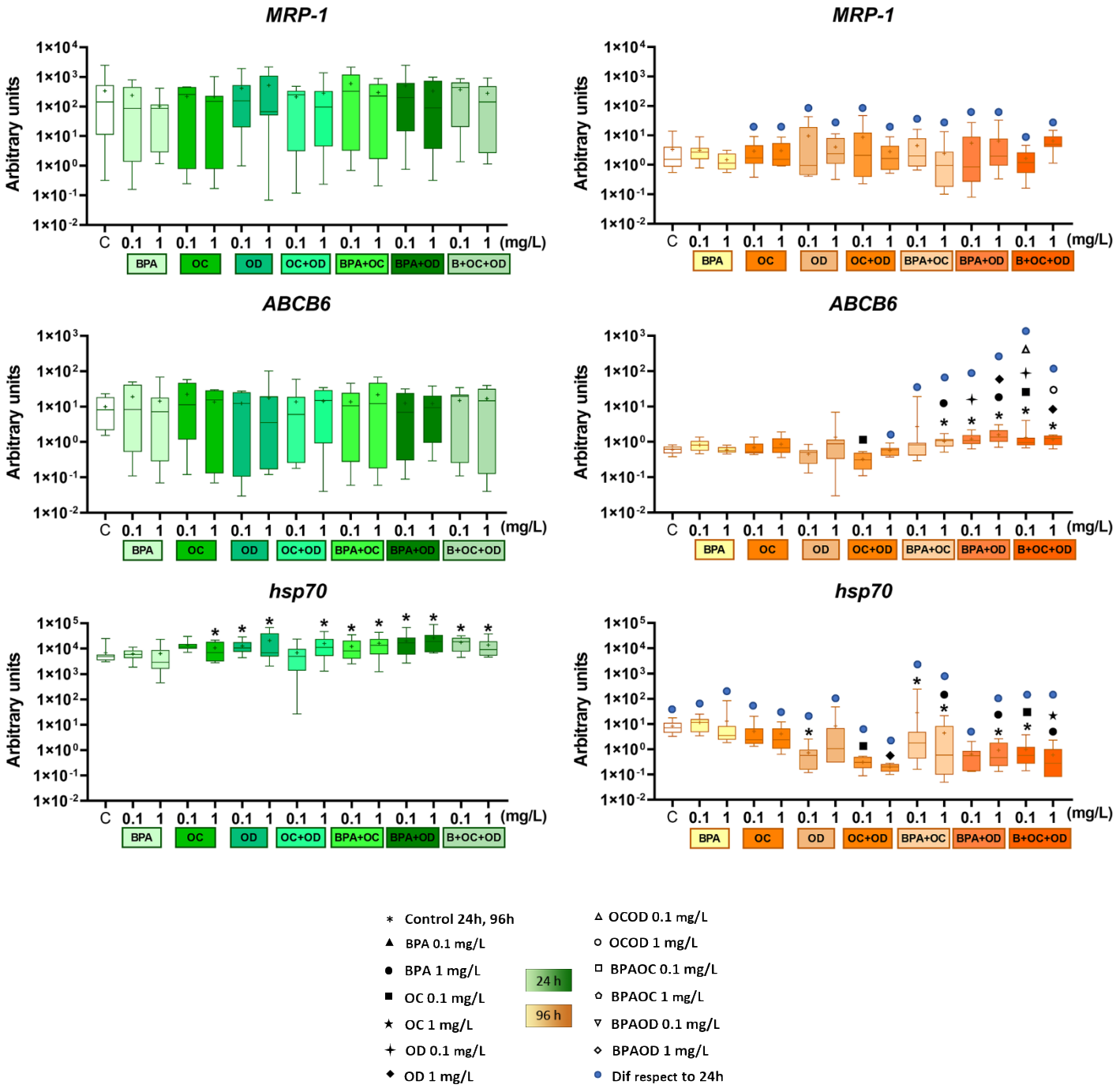

Figure 7. Expression of *MRP-1, and ABCB6* related to detoxification response; *hsp70* from stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

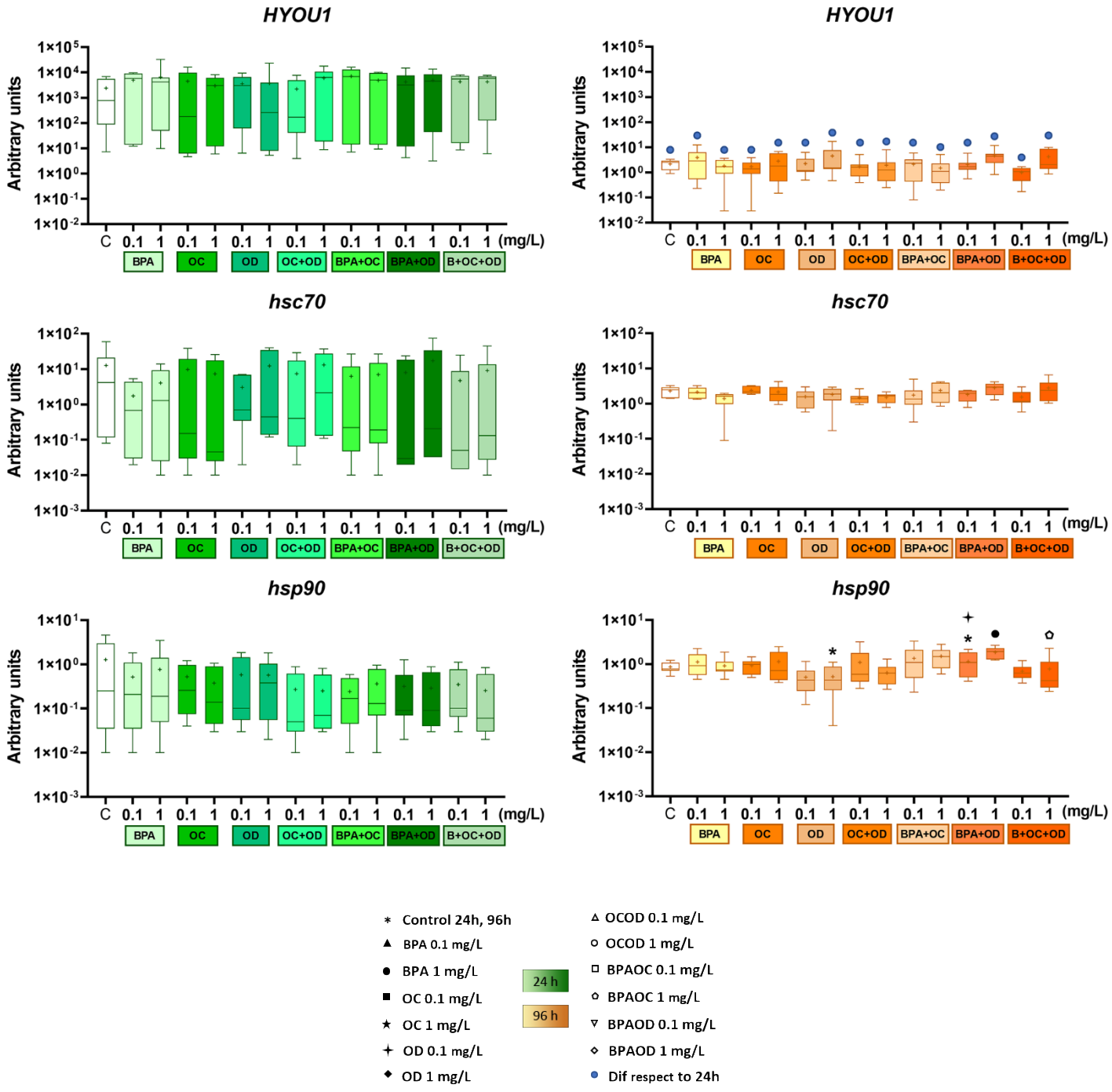

Figure 8. Expression of *HYOU1, hsc70,* and *hsp90* from stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

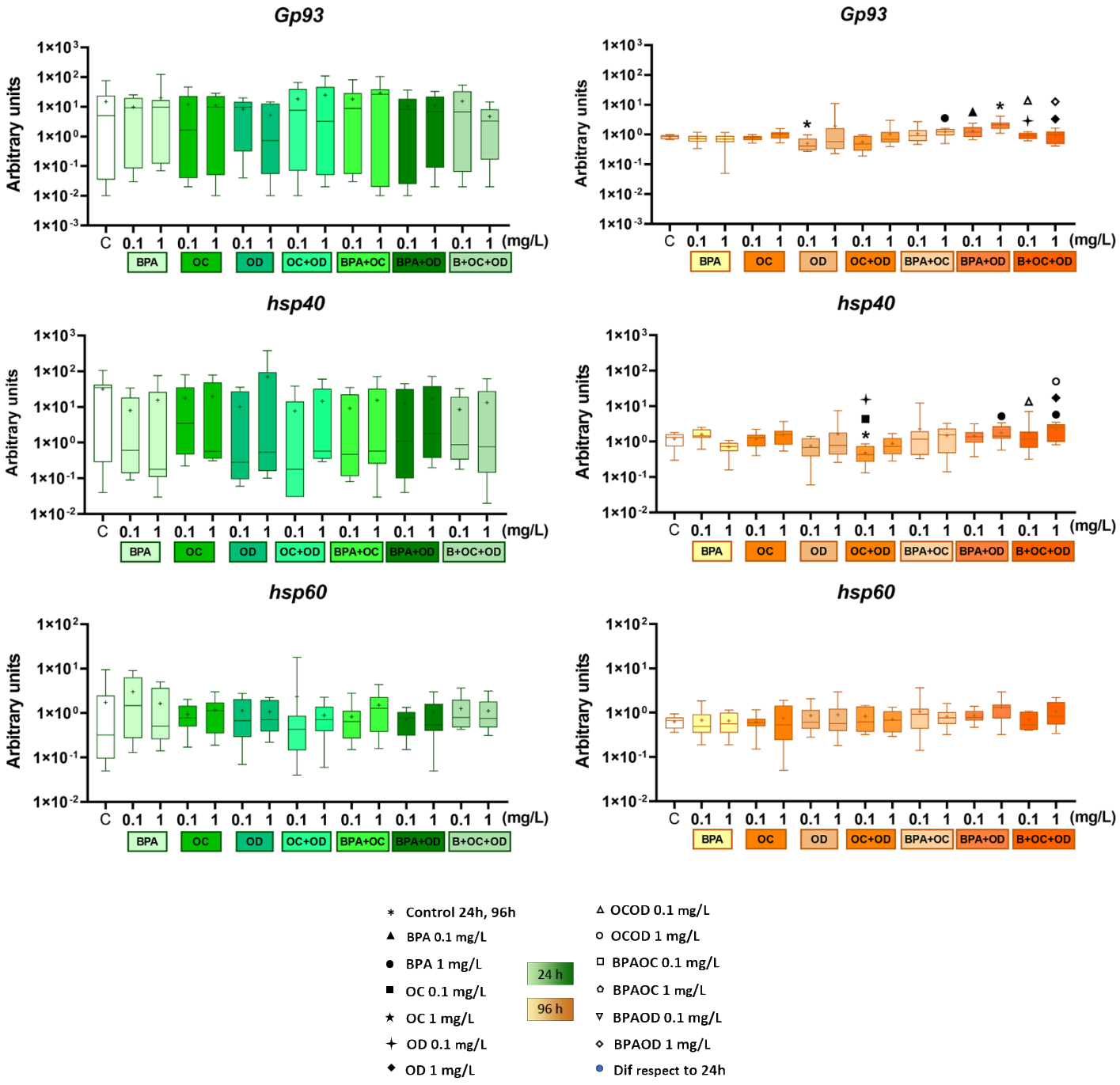

Figure 9. Expression of *Gp93, hsp40* and *hsp60* from stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

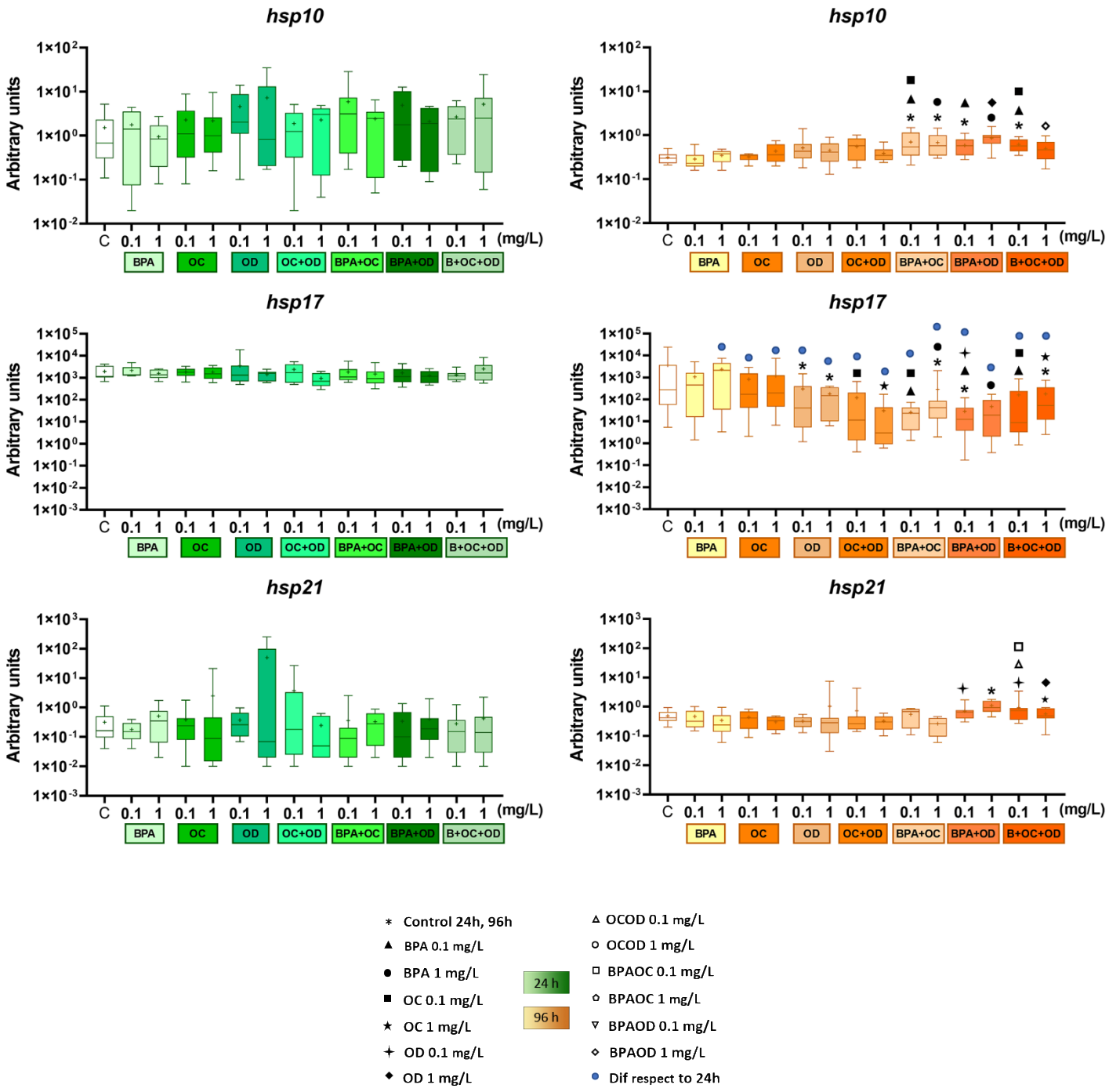

Figure 10. Expression of *hsp10, hsp17* and *hsp21* from stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

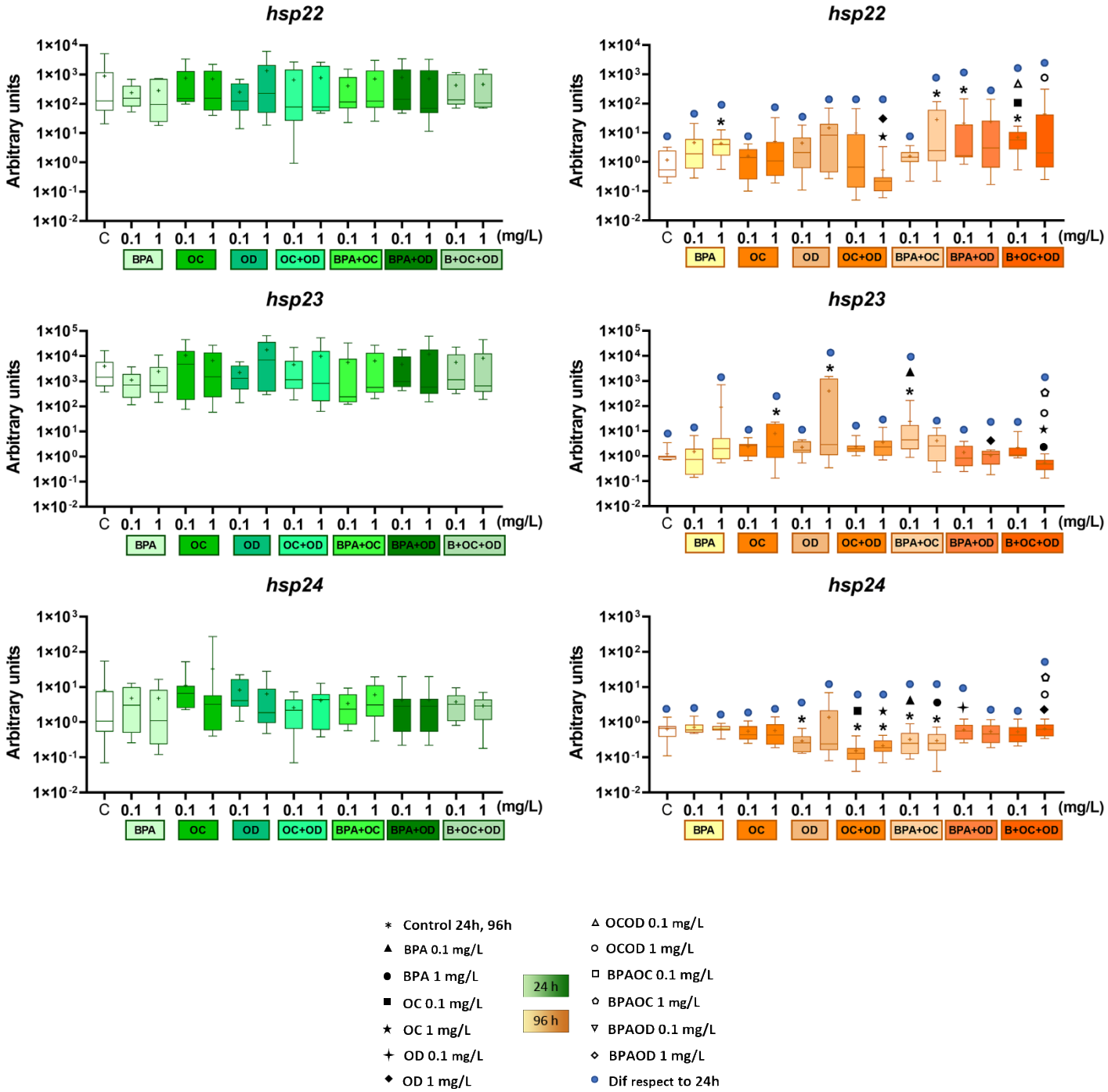

Figure 11. Expression of *hsp22, hsp23* and *hsp24 from* stress response in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

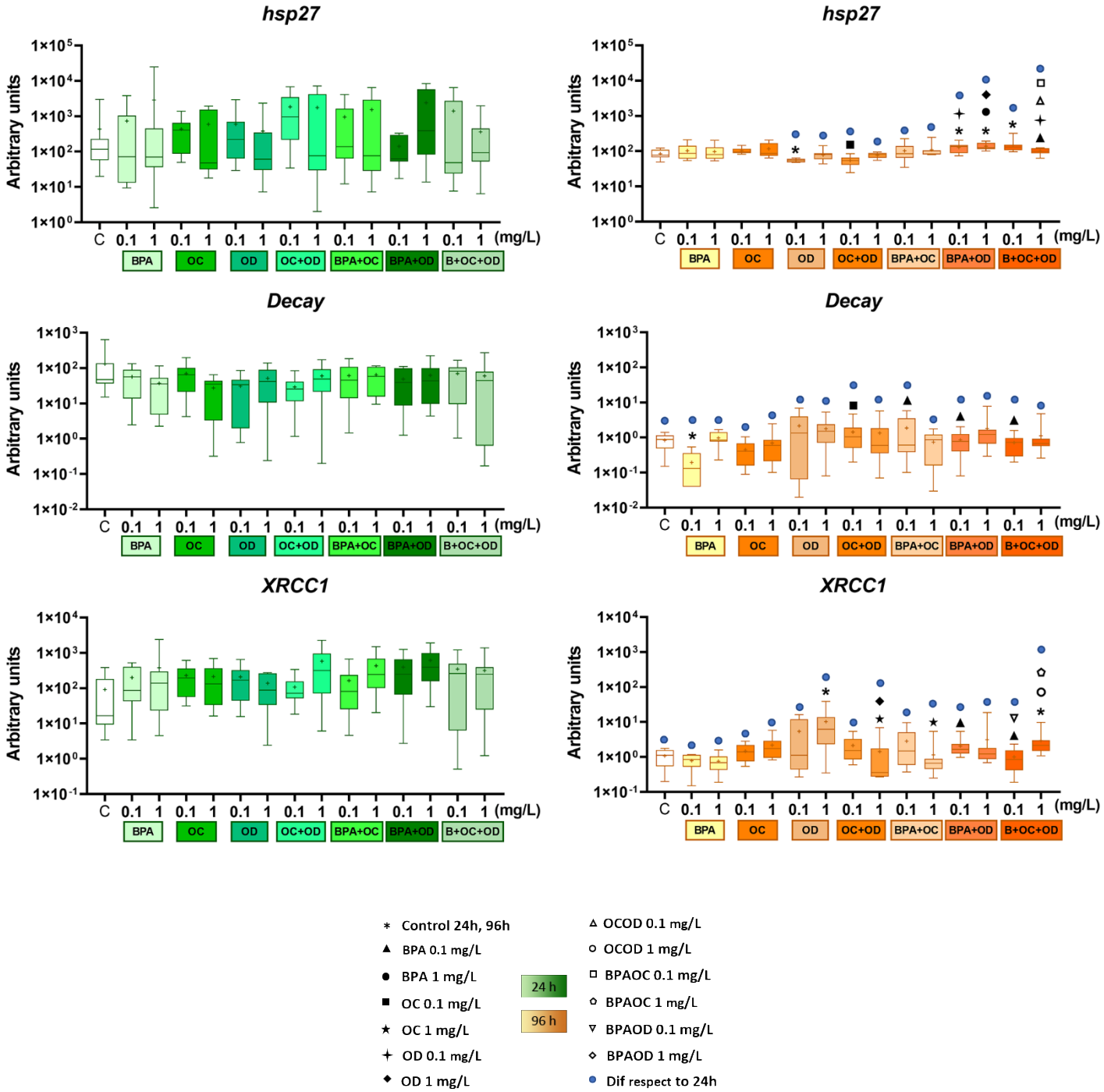

Figure 12. Expression of *hsp27* from stress response; *Decay*, and *XRCC1* related to DNA repair in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

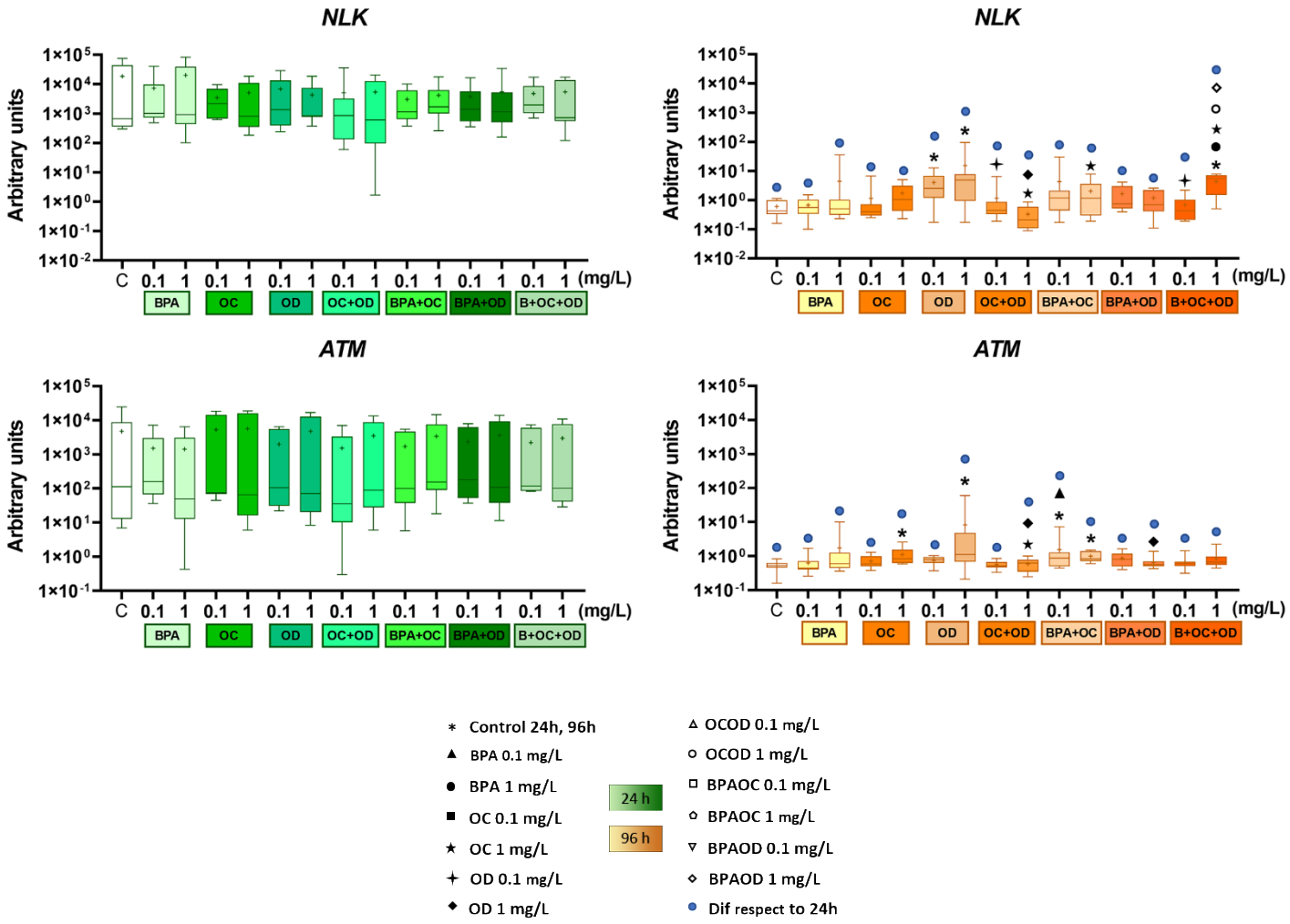

Figure 13. Expression of *NLK*, and *ATM* related to DNA repair in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

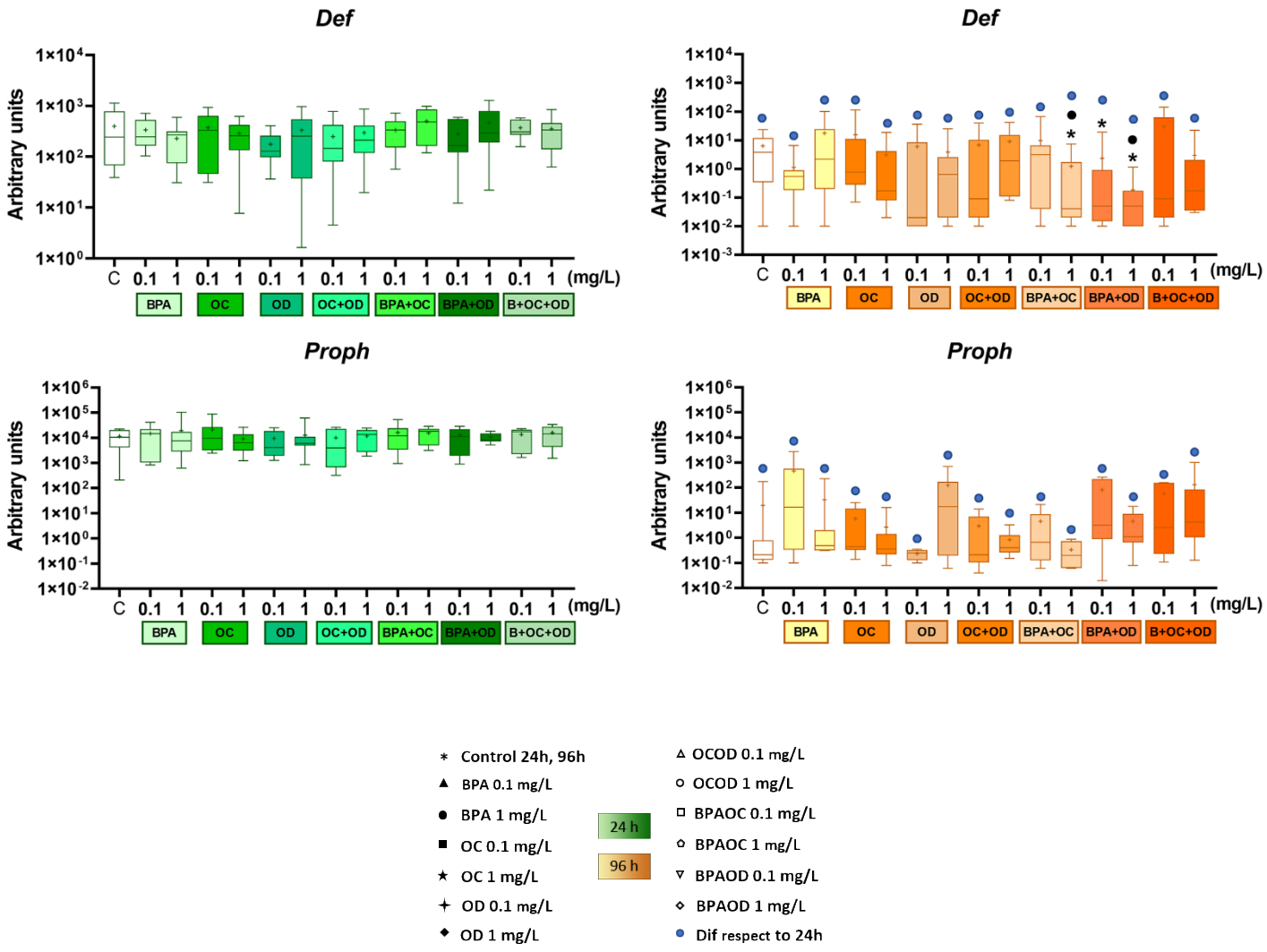

Figure 14. Expression of *Def*, and *Proph* related to immune system in fourth instar *C. riparius* larvae after in vivo exposure to 0.1 or 1 mg/L BPA, OC, OD-PABA, OC+OD, BPA+OC, BPA+OD-PABA, BPA+OC+OD-PABA for 24 hours. All significant differences in respect to the control and the comparisons between treatments are defined in the legend in all the cases ($p < 0.05$).

Annex II: Sequences used for designing of primers

PARP

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ABCB6

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TBP

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HYOU1

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Proph

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L(2)efl

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Def

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JHAMT

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TTGTTTCATCTTTCTACTGTCTTCATTGGGTTCAAGACTTGCCATTAGCATTTGTAAAAATTAAGAATTTTTTGA
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AGTACTCACCTTATATGAACAATTGGAGAGGTAATTTCAACTACATATGCTACCTTGAAAATGCTGACGAAATAA
TAAAGAAAATGCTACGGGAAAGTGGAATTGAAATAATCAAGTTTATTGATGAAAAGAACGACATGTTTAATTTTG
GCACCAAAGAATTCTTTACAGCATCATTGGAAGCTGTCAATCCTTGCTTGAAGCTAATGCCTGAACAGCTTAAAA
ATGAATTCCTTCATGATCAACTCGATGTGCTCTACAACCTACAAAAAGGAA

Annex III: Articles published or submitted and directly related to the present thesis.

Published:

- **Muñiz-González, A.B** & Martinez-Guitarte, J.L. (2018). Effects of single exposure and binary mixtures of ultraviolet filters octocrylene and 2-ethylhexyl 4- (dimethylamino) benzoate on gene expression in the freshwater insect *Chironomus riparius. Environmental Science and Pollution Research*, 25, 35501–35514. https://doi.org/10.1007/s11356-018-3516-7
- **Muñiz-González, A.B** & Martínez-Guitarte, J.L. (2020). Combined effects of benzophenone-3 and temperature on gene expression and enzymatic activity in the aquatic larvae *Chironomus riparius*. *Science of the Total Environment*, 698, 134292. https://doi.org/10.1016/j.scitotenv.2019.134292

Submitted:

- **Muñiz-González, A.B.** Ibuprofen as an emerging pollutant on non-target aquatic invertebrates: effects on *Chironomus riparius.* Environmental Toxicology and Pharmacology**. ETAP-D-20-00629**
- **Muñiz-González, A.B** & MartÍnez-Guitarte, J.L. Unveiling complex responses at the molecular level: transcriptional alterations by mixtures of bisphenol A, octocrylene, and 2'-Ethylhexyl 4- (Dimethylamino)Benzoate on *Chironomus riparius. Ecotoxicology and Environmental Safety.* **YEESA-S-20-03330 (Under review)**
- **Muñiz-González, A.B**, Novo, M., Martínez-Guitarte, J.L. Persistent pesticides: Effects of Endosulfan on gene expression and enzymatic activity of the aquatic invertebrate *Chironomus riparius*. Environmental Science and Pollution Research: **ESPR-D-20-05731(Under review)**

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RESEARCH ARTICLE

Effects of single exposure and binary mixtures of ultraviolet filters octocrylene and 2-ethylhexyl 4-(dimethylamino) benzoate on gene expression in the freshwater insect *Chironomus riparius*

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Abstract
Ultraviolet filters are used extensively in the production of many personal care and industrial products. These products can inadvertently pollute the environment through recreational activities. They have been associated with endocrine disruption in vertebrates but their effects in invertebrates are poorly understood. *Chironomus riparius* is a species of the dipteran order, with aquatic larvae that are frequently used in toxicity tests. Previously, we showed that octocrylene (OC) and 2-ethylhexyl 4-(dimethylamino) benzoate (OD-PABA) differentially affected the mRNA levels of the ecdysone receptor and Hsp70 genes. For a better understanding of their mode of action, transcriptional activity by real-time PCR was analyzed in fourth instar larvae exposed to OC, OD-PABA, or a binary mixture of both. We studied 16 genes related to the endocrine system, stress, the immune system, and biotransformation mechanisms to elucidate the putative interactions between these compounds. No response was observed for the genes involved in biotransformation, suggesting that enzymes other than cytochromes P450 and glutathione-S-transferases (GSTs) could get involved in transformation of these compounds. Similarly, no response was observed for endocrine-related genes while the stress gene *HYOU1* was inhibited by OD-PABA, suggesting an effect in response to hypoxia. In addition, no significant interactions were observed following exposure to a binary mixture of these compounds. Overall, the results suggest a weak, acute response in different metabolic pathways and a lack of interaction between the compounds. Finally, new genes are identified in this organism, opening the possibility to analyze new cellular pathways as targets of toxicants.

Keywords UV filters · Binary mixture · Endocrine system · Stress · Biotransformation · Invertebrate · Endocrine disruptors · *Chironomus riparius*

Introduction

Ultraviolet (UV) filters are compounds used to prevent damage by UV radiation. They are present in several industrial products and are important components of personal care products (PCPs), mainly sunscreens, being either organic (absorbing UV radiation) or inorganic (reflecting UV radiation). They are used as mixtures and there are three to eight UV filters that typically enter the environment through waste water or recreational activities. The amounts of UV filters can be as high as 119 g for 2-ethylhexyl-4-trimethoxycinnamate (EHMC), 49 g for 4-methyl-benzilidine-camphor (4MBC), 69 g for benzophenone-3 (BP3), and 28 g for octocrylene (OC) per 10,000 people per day (Balmer et al. 2005). Analyses of samples from beach water have shown that levels of BP3 reached up to 3316.7 ng/L (Sánchez Rodríguez et al. 2015). In farmed fish, wild mussels, and some other wild organisms, ethylhexyl methoxycinnamate (EHMC) and 2-ethylhexyl 4-(dimethylamino) benzoate (OD-PABA) can accumulate up to 51.3 and 24.1 ng/g (dry weight), respectively (Sang and Leung 2016). Furthermore, UV filters have been found in concentrations from nanogram to microgram per liter in wastewater

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Effects of binary mixtures of ultraviolet filters octocrylene and 2-ethylhexyl 4-(dimethylamino) benzoate on gene expression in the freshwater insect *Chironomus riparius*

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Abstract

Ultraviolet filters are used extensively in the production of many personal care and industrial products. Inadvertently, these products can pollute the environment through recreational activities. They have been associated with endocrine disruption in vertebrates but their effects in invertebrates are poorly understood. *Chironomus riparius* is a species of the Diptera order, with aquatic larvae that are frequently used in toxicity tests. Previously we showed that octocrylene (OC) and 2-ethylhexyl 4-(dimethylamino) benzoate (OD-PABA) differentially affected the mRNA levels of the ecdysone receptor and Hsp70 genes. To improve the knowledge about their mode of action and the putative interactions between these compounds we analyze by Real-Time PCR the transcriptional activity of 16 genes related to the endocrine system, stress, the immune system, and biotransformation mechanisms, on fourth instar larvae exposed to OC, OD-PABA, or a binary mixture of both. No response was observed for the genes involved in biotransformation suggesting that other enzymes than Cyp450s and GSTs could get involved in transformation of these compounds. Similarly, no response was observed for endocrine related genes while the stress gene *HYOU1* was inhibited by OD-PABA suggesting an effect in response to hypoxia. In addition, no significant interactions were observed following exposure to a binary mixture of these compounds. Overall, the results suggest a weak, acute response in different metabolic pathways and a lack of interaction between the compounds. Finally, the number of genes ready to be use with this organism is increased opening the possibility of studying new pathways affected by pollutants.

Keywords: UV filters, binary mixture, endocrine system, stress, biotransformation, invertebrate

1. Introduction

Ultraviolet (UV) filters are compounds used to prevent damage by UV radiation. They are present in several industrial products and are important components of personal care products (PCPs), mainly sunscreens, being either organic (absorbing UV radiation) or inorganic (reflecting UV radiation). They are used as mixtures and typically there are three to eight PCPs that enter in the environment through either effluent in waste water or recreational activities. It has been estimated that the amounts of four different UV filters can be as high as 119 g 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), 49 g 4-methyl-benzilidine-camphor (4MBC), 69 g benzophenone-3 (BP-3), and 28 g octocrylene (OC) per 10,000 people per day (Balmer et al. 2005). Analysis of samples from beach water have shown that levels of BP-3 reached up to 3,316.7 ng / L (Sánchez Rodríguez et al. 2015). Ethylhexyl methoxycinnamate (EHMC) and 2-ethylhexyl 4-(dimethylamino) benzoate (OD-PABA) can accumulate up to 51.3 and 24.1 ng/g (dry weight) in farmed fish, wild mussels and some other wild organisms (Sang and Leung 2016). Furthermore, UV filters, have been found in ng/L to µg/L in wastewater treatment effluent (Kasprzyk-Hordern et al. 2009; Montes-Grajales et al. 2017). Growing evidence of the presence of these compounds in water and animals has raised concerns about the risks that they pose to the wildlife and human health.

UV filters are known endocrine disruptors and can also produce other effects at molecular and physiological levels (Schlumpf et al. 2001, 2008; Schreurs et al. 2005). In fact, OC can cause allergies by contact and molecular effects have been observed in invertebrates. (de Groot and Roberts 2014; Ozáez et al. 2016b) Alterations to hormonal systems can lead to indirect modifications in other pathways and cell processes. Despite the presence of UV filters as a mixture in PCPs and industrial products, they have principally been analyzed individually and there are few studies aimed at determining the effects of mixing. They can also produce synergistic and antagonist effects in estrogenic and androgenic response pathways in vertebrates(Heneweer et al. 2005; Kunz et al. 2006; Kunz and Fent 2009) and complex effects have been observed in invertebrates (Ozáez et al. 2016c). Endocrine disruptors compounds (EDCs) alter the hormonal system by affecting the hormone pathway response and/or the metabolism of the hormone, by either synthesis or degradation (Swedenborg et al. 2009). Insect endocrine systems have been studied for many years and two main hormones, among others, have been linked to molting and development. Ecdysone is responsible for the molting between larval stages. It is a steroid hormone, and its active form is 20-hydroxyecdysone. It is synthesized in several steps by

the Halloween group of proteins (Gilbert 2004). Most of them are cytochrome P450 mono oxygenase enzymes that produce the active form of ecdysone. Degradation of the active form is carried out by another cytochrome P450 enzyme named Cyp18a1 (Guittard et al. 2011). Nevertheless, the regulation of molting and physiological decision to enter into the stage of pupation depends on the presence of the juvenile hormone (JH), an acyclic sesquiterpenoid, secreted by a pair of endocrine glands behind the brain called the *corpora allata*. The biosynthesis of JH is similar to that of cholesterol in animals and the final step is dependent on the JH acid O-methyltransferase (Niwa et al. 2008). Other targets for EDCs are the hormonal response pathways and in insects the best known is the ecdysone response pathway. Ecdysone binds to the ecdysone receptor, a heterodimer formed by the ecdysone receptor (EcR) and ultraspiracle (USP), which activates a cascade of several transcription factors that amplify the signal to the effector genes (Yamanaka et al. 2013). Recently, the protein methoprene-tolerant has been described as the JH receptor and additional information has been obtained regarding the JH response pathway (Jindra et al. 2012). The transcription factor Kr-h1 may be a connecting point between the JH and ecdysone response pathways, since it is downstream of Methoprene-tolerant and upstream of Broad, a protein also related to the ecdysone response pathway (Minakuchi et al. 2008, 2009). Apart from the hormones described above, few data exist about the endocrine system. However, we recently identified an orphan receptor, the membrane associated progesterone receptor (MAPR), which can be induced differentially by two UV filters, BP-3 and 4-MBC, in *Chironomus riparius* embryos. (Ozáez et al. 2016a) This suggests that it could be a promising biomarker to analyze the endocrine disruption of UV filters.

Toxicants can activate different cellular mechanisms such as detoxification systems and stress responses that are involved in homeostasis maintenance. Detoxification involves biotransformation and is usually divided into two phases. In phase I, the compound is transformed by enzymatic reactions that add or expose polar groups. This step is frequently carried out by cytochrome P450 enzymes, a large family of proteins involved in a number of cellular processes (Nelson et al. 1996; Bergé et al. 1998). Phase II biotransformation reactions, involve conjugation with glutathione, glucuronic acid, and other molecules. This serves as a detoxifying step in drug metabolism. The enzyme involved is dependent on the molecule that it is conjugated. In the case of glutathione the reaction is catalyzed by a family of proteins named glutathione-S-transferases (GSTs) (Nebert and Vasiliou 2004). An enzymatic assay to analyze the activity of GST is commonly used in ecotoxicology but it is unable to discriminate between

the activities of different members from this family. The stress response is also frequently activated in response to toxicants (Gupta et al. 2010). It is often analyzed by studying heat shock proteins, but there is a lack of information related to other proteins implicated in this response. However, there is still poor knowledge among the heat shock proteins, for example the Hypoxia up-regulated protein 1 (HYOU1), an HSP70 domain-containing protein that plays an important role in hypoxia/ischemia and angiogenesis. It has been analyzed in humans related with cancer (Stojadinovic et al. 2007) and in the sperm surface (Naaby-Hansen and Herr 2010). To our knowledge, there are no data in the literature about this protein in invertebrates, but it is related to stress, so could be affected by chemicals. On the other hand, lethal 2 essential for life [l(2)efl] is a protein related to development that has been studied in *Drosophila melanogaster* (Kurzik-Dumke and Lohmann 1995; Wójtowicz et al. 2015) and can be induced by several stressors. (Landis et al. 2012)

Toxicants can have physiological effects that cannot be analyzed by the standard toxicity test that focuses on endpoints such as reproduction or development, although they may be important to the survival of the species. One of them is the effect on the immune system that can alter the ability of the organism to fight infection. Insect immune systems are one of the most studied in invertebrates. There are different mechanisms of response to infection, including antimicrobial peptides like the defensins, a family of peptides active against bacteria, fungi, and certain viruses (Bulet and Stöcklin 2005). They are present in invertebrates and vertebrates. Phenoloxidase enzymes are involved in localized melanization and coagulation at wound sites and around intruding microorganisms in the hemolymph (Eleftherianos and Revenis 2011). Analysis of the effects of toxicants on genes related to the immune system, can help to understand the putative effects that occur in the immune system of an individual and how sensitive the individual is to infection during toxic exposure.

The aim of this study was to analyze the transcriptional activity of genes related to the endocrine system, stress response, detoxification mechanisms, and the immune system to elucidate the molecular effects that two UV filters have as single and binary mixtures, on fourth instar *C. riparius* larvae. Filters are commonly found in the environment as mixtures so it is important to know the alterations they can make at cellular and molecular levels when organisms are exposed. These data will help to better understand the effects of these compounds at a molecular level in invertebrates and will aid new studies focused on different aspects of cell metabolism.

2. Material and Methods

2.1 Chemicals

The UV filters OC (CAS No. 6197-30-4; purity ≥ 97%) and OD-PABA (CAS No. 21245-02-3; purity ≥ 98%) were purchased from Sigma-Aldrich (Germany). Stock solutions were made in absolute ethanol and stored in the dark at 4 °C. The stock solution concentrations were 50 mg/mL and 100 mg/mL for single and binary mixtures, respectively.

2.2 Animals

Experimental animals were fourth instar *C. riparius* larvae from stock cultures maintained in the laboratory of Biology and Environmental Toxicology (UNED). They were established from natural populations of midge larvae originally collected from a non-polluted area of Valencia (Spain). They were reared under standard laboratory conditions according to OECD guidelines (OECD 2001). Larvae were grown from egg masses in an aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4) supplemented with nettle leaves, commercial fish food (TetraMint), and cellulose tissue in polyethylene tanks. Cultures were maintained under constant aeration at 20°C and with standard periods of light and dark (16L: 8D).

2.3 Treatment

Nine larvae were exposed to single chemicals or mixtures diluted in 30 mL culture medium for 24 h in polytetrafluoroethylene vessels (100 mL) to prevent adsorption of the compounds to the walls. Nominal concentrations of 0.1, 1 and 10 mg/L of both the UV filters, were used according to previous data from our laboratory (Ozáez et al. 2013, 2016b). Single UV filters, diluted in ethanol, were directly added to the aqueous culture medium. A stock mixture was prepared of both compounds in equal concentration. The mixture was then added to the culture medium. No sediment, cellulose tissue or food were added and exposure was carried out in the absence of light to minimize photodegradation. Non-treated control larvae were exposed to the same concentration of solvent (0.02% ethanol). Three independent experiments were performed for each condition using samples from different egg masses. Three larvae from each experiment were frozen and stored at −80 °C for RNA extraction (therefore the total number of larvae analyzed for each condition was n=9).

2.4 Sequence identification

Several of the genes used in this study have been previously characterized, while others are the first to be described in this species. See results for a summary of the references for those previously described

and the accession number for the new genes. New genes were obtained from a transcriptome project. A file including all assembled sequences from the SRX147945 project (Marinković et al. 2012) was downloaded from the Transcriptome Shotgun Assembly Sequence Database and used to run a systematic search in the GenBank database using the Blast2go tool (Conesa et al. 2005). The conditions of the search were tblastx program, no redundant database and Blast ExpectValue of 1.0E-3. Sequences identified as proteins of interest were selected and an open reading frame (ORF) of each sequence was obtained using SnapGene software (from GSL Biotech; available at snapgene.com). The search in the Blast database confirmed homology of the ORFs for the proteins of interest. Those identified as heat shock proteins were used to design the primers for Real-Time PCR. All newly identified complete cDNA and protein sequences are provided in the supplementary material.

2.5 RNA isolation

Total RNA was extracted from the control and experimental fourth instar larvae using RNAzolRT (MRC) according to the manufacturer's protocol. After completion, the RNA extract was treated with RNase-free DNase (Roche) for 45 min at 37 °C. Later a phenol:chloroform:isoamil alcohol (Fluka) extraction using Phase Lock Light tubes (5prime) was carried out. Next, RNA was precipitated using isopropyl alcohol (0.5 v/v), washed with 75% ethanol, and re-suspended in 30µl diethyl pyrocarbonate (DEPC) treated water. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf). Purified RNA was stored at -80 °C.

2.6 cDNA synthesis and Real Time-PCR

Aliquots of 1 µg isolated RNA were reverse-transcribed using 100 units of M-MLV enzyme (Invitrogen) with 0.5 µg oligonucleotide dT20 primer (Sigma) and 0.5 mM dNTPs (Biotools) at 37ºC for 50 min in a final volume of 100 µL. The cDNA obtained was used as template for Real-time PCR (RT-PCR) to evaluate the mRNA levels of each gene in each condition tested. RT-PCR was performed with a CFX96 thermocycler (BioRad) using 0.5 units of DNA polymerase (Biotools), 0.4 mM dNTPS (Biotools), and Eva Green (Biotium). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13 (rpL13) genes were employed as endogenous references controls. RT-PCR efficiency was carried out with a standard curve. A template diluted 1:2 in five steps was used in the same PCR conditions ($R^2 >$ 0.98 for all primers). The RT-PCR primers and their efficiencies are listed in table 1. The primers were

designed with primer-Blast tool (Ye et al. 2012) and tested using regular PCR to ensure that a unique band was obtained. The RT-PCR was run in the following cycles: initial denaturation at 95°C for 30 seconds followed by 39 cycles of denaturation at 95°C for 5 s, 58°C annealing for 10 s, and 65°C elongation for 5 s. To verify the accuracy of each amplicon, a melting curve analysis was performed after amplification. BioRad CFX Manager 3.1 software was used to determine the total mRNA levels of normalized gene expression ($2^{-\Delta Cq}$). Each sample was run in duplicate wells and at least two independent replicates were performed in each experiment.

2.7 Statistical analysis

Statistical analyses were performed using SPSS 24 (IBM). Normal distribution of data and homogeneity of variance were assessed with Shapiro-Wilk and Levene tests, respectively. Logarithmic transformation of the transcriptional activities to stabilize their variances was necessary in order to fulfill the criteria for analysis of variance (ANOVA) test. Within each ANOVA, two-sided Dunnett's test was used to asses which treatment groups showed transcriptional activity significantly different from the control group at the $\alpha = 0.05$ level. To compare all the samples were used Tukey and Games-Howell as post-hoc test with a significance level $p \leq 0.05$.

3. Results

3.1 Gene sequence identification and characterization

Most of the genes analyzed in this study were previously identified. However, five sequences correspond to new genes not previously described in *C. riparius*. A systematic analysis of a transcriptome project uploaded to the Transcriptome Shotgun Assembly database rendered five sequences with ORFs homologous to Hypoxia up-regulated protein 1 (HYOU1, also known as 150 k Da oxygen-regulated protein, ORP150), prophenoloxidase (proPO), lethal 2 essential for life [l(2)efl], defensin (DEF), and juvenile hormone methyl transferase (JHAMT) proteins, respectively. The accession numbers of the sequences and the highest homologies with insect protein sequences are shown in table 2. The different domains identified in the proteins coded by the cDNA sequences are shown in figure 1. The complete cDNA and protein sequences can be found in the supplementary material. The *C. riparius HYOU1* cDNA (3403 bp) coded for a protein of 940 aa. The protein had the characteristic domain of HSP70, since it is associated with this family of proteins. Furthermore, it showed an HYOU1-like nucleotide binding domain that allowed classification as related to HYOU1, confirmed when comparison to the database was

performed. A cDNA sequence of 2125 bp with an ORF of 683 aa was identified as Phenoloxidase (PO1). This protein shows three domains related to hemocyanin, at the N-terminal, in the medium section, and at the C-terminal, and had highest homology with *Bactrocera latifrons* PO2 (though this protein has only been predicted in this species). It can be proposed that this protein is the *C. riparius* PO1 as POs are known to share homology with hemocyanins and belong to the type-3 copper containing proteins (Lu et al. 2014). *l(2)efl* cDNA was 729 bp in length with an ORF of 198 aa. It shared homology with l(2)efl of *Culex quinquefasciatus* and the protein had the characteristic alpha crystalline domain. This protein is related to small heat shock proteins sharing this domain. The *defensin* cDNA was a 363 bp sequence that contained an ORF of only 76 aa. It showed a defensin 2 domain and 54 % identity with the defensin 1 of *Nyssomyia neivai*, another dipteran. A sequence that codes for an incomplete ORF with homology to JHAMT was identified. The ORF was 251 aa in length and showed homology with the JHAMT protein of *Drosophila melanogaster*. Two domains were identified, one that corresponded to the methyltransferase 11 domain, meaning that it is a S-adenosylmethionine dependent methyltransferase, and a ubiquinone/menaquinone biosynthesis C-methylase UbiE domain, that is related to a prokaryotic enzyme involved in quinol/quinone metabolism. Altogether, these data have allowed us to identify the cDNA sequences of five proteins, two of them related to the stress response (HYOU1, l(2)efl), two related to the immune system (defensin, PO1), and one involved in JH synthesis (JHAMT).

3.2 Endocrine related genes

This study is a first attempt to analyze the acute response of *C. riparius* to mixtures of OD and OD-PABA, combining the expression profile of different genes related to several cellular processes. First, we selected four genes related to the endocrine system and analyzed their mRNA levels by RT-PCR in fourth instar larvae exposed for 24h (figure 2). No significant changes were observed in relation to the control in any case and only differences were observed for *Kr-h1* between 0.1 mg/L mixture with OD-PABA 1 mg/L and 1 mg/L mixture treatments. However, these differences have no biological significance since they are not in comparison to either control or the same concentration of single compound.

To ensure that the acute response did not affect the metabolism of ecdysone, the transcriptional activity of *cytochrome p450 18a1* was also analyzed. No alterations in gene expression were detected although a trend to increase was observed for OC. Molting also depends on the juvenile hormone so to test whether JH metabolism was altered, the transcriptional activity of *JHAMT* was studied. The results showed that no alterations are produced by either OC or OD-PABA. Mixtures showed no differences to

either control or single exposures suggesting these compounds do not affect the metabolism of juvenile hormone.

Although the insect hormonal system is one of the most studied in invertebrates, there is a lack of information about endocrine response involving other hormones. Previously, we described an orphan receptor gene (*MAPR*), which is upregulated in the embryo but not in larvae exposed to either 4MBC or BP-3 (Ozáez et al. 2016a). Here, we analyzed the effect that OD-PABA and OC had on the transcriptional activity of this gene to ascertain whether a different response was observed to that described for 4MBC and BP-3 in larvae. No significant differences in mRNA levels were observed.

3.3 Detoxification response

Recently we have described several genes related with phase I and phase II of biotransformation (Martínez-Guitarte 2018), a key process to survive in contaminated environments. The transcriptional activity of four *CypP450* genes and four *GST* genes was analyzed to elucidate the cell response to single and binary exposures of the two UV filters used (figure 3). OC and OD-PABA did not affect expression of these genes in the conditions analyzed. Similarly, no effect was observed when the binary mixtures were analyzed. However, it is important to note that in some cases, although no statistical significance was obtained, higher mRNA levels were observed (e.g. 10 mg/L OC).

3.4 Stress and immune genes

Stress response is one of main mechanisms that allows the cell to maintain homeostasis when environmental conditions are changing. Previously we showed that *Hsp70* mRNA levels were modified in embryos but not in larvae by OD-PABA and OC (Ozáez et al. 2016b). As mentioned above, we identified two new sequences for stress proteins, HYOU1 and l(2)efl, which have been related to different stressors. Furthermore, HYOU1 belongs to the Hsp70 group of stress proteins since it has an Hsp70 domain. To test whether these two UV filters were able to modify transcriptional activity we analyze the mRNA levels in fourth instar larvae exposed for 24 h (figure 4). OD-PABA downregulated *HYOU1* mRNA levels at 10 mg/L after 24h of exposure. However, the binary mixture did not show any statistically significant differences so a recovery due to the presence of OC suggest a weak antagonism in relation to this gene, as suggested by the increasing trend of 10 mg/L of OC. On the other hand, *l(2)efl* did not show any change in their mRNA levels in comparison with control levels but there was statistical significance between 0.1 mg/L OD-PABA and 1 and 10 mg/L mixture treatments. Finally, the two

sequences coding for proteins involved in the immune system were analyzed. Neither *defensin* nor *phenoloxidase* mRNA levels were affected in comparison to the control. Taking all the results together, it can be proposed that the UV filter did not affect the transcriptional activity of either gene, although longer exposures would determine whether effective downregulation occurs.

4. Discussion

Chironomus riparius is a widely used organism in environmental toxicology which is poorly know at molecular level. The identification of the sequences of five new genes related with juvenile hormone, immunity, and stress offers new possibilities to analyze additional pathways in response to toxicants in this species. *JHAMT* is a key enzyme in the juvenile hormone synthesis so combination with enzymes involved in ecdysone metabolism, such as *Cyp18a1*, can provide a good picture of the effects that a compounds have in these two important hormones for insect development. *Defensin* and *PPO1* are related with immune system and, to our knowledge, they are the two first genes identified in this species which are involved in immunity. Both can be useful to estimate the ability of the individual to respond infection in presence of a pollutant. Identification of HYOU1, a protein involved in the response to hypoxia (Ikeda et al. 1997) and with a role in the prevention of apoptosis and senescence (Krętowski et al. 2013; Montesi et al. 2016), will help to a better understanding of how the toxicants alter the availability of oxygen in the cell. On the other hand, l(2)efl is involved in development and the insulin response pathway (Flatt et al. 2008; Wójtowicz et al. 2015) offering a putative biomarker to monitor how pollutants affect these processes. A combination of the different genes described and the results obtained from standard test will allow the elucidation of the mode of action of the chemicals at a molecular level. This will improve understanding of how the damage is caused and the information used in environmental risk assessments.

The EcR binds ecdysone, initiating the ecdysone response pathway, and previous data suggest that OC does not upregulate EcR in larvae at 24 h, unlike OD-PABA (Ozáez et al. 2013, 2016b). Evaluating the transcriptional activity of *Kr-h1*, a modulator of several ecdysone-regulated genes (Beck et al. 2004), we analyzed the downstream ecdysone response pathway. The absence of response for this gene was striking since *EcR* upregulation by OD-PABA suggested that a downstream activation of the pathway could happen. It is possible that the activated response does not involve this gene or that more exposure time is needed for induction. In this sense is interesting to note that Vinclozolin can increase the mRNA levels at 48 hours while EcR transcription is observed at 24h (Aquilino et al. 2016). Other mechanism

of endocrine disruption is alteration of the ecdysone metabolism, modifying hormone concentration. However, the lack of response for *cytochrome p450 18a1*, the gene that code for the degrading enzyme of ecdysone, suggest that these compounds do not alter the ecdysone levels. There have been no previous studies on the effect of UV filters have on this gene although it can be altered by other toxicants such as Vinclozolin, 2-tridecanone, and 4-vinylcyclohexene 1,2-monoepoxide and 4-vinylcyclohexene diepoxide (Abolaji et al. 2015; Aquilino et al. 2016; Zhang et al. 2016). According with the results, the catabolism of ecdysone was not affected, therefore the putative effect of OD-PABA seems to depend on the ecdysone pathway and more studies are required to elucidate it. However, other hormone also is involved in molting, the juvenile hormone. It is possible that OD-PABA affects JH altering endocrine balance, and secondarily ecdysone response. The analysis of *JHAMT* transcriptional activity showed no changes in any case so these compounds are not altering JH synthesis. To our knowledge, there are no previous studies that have analyzed the effects of UV filters on the metabolism of ecdysone and JH. The results indicate that OC and OD-PABA do not affect metabolism of these hormones and differ in their ability as endocrine disruptors since OD-PABA seems to alter the ecdysone pathway by affecting the EcR (Ozáez et al. 2013), while OC do not show any effect in conditions and genes tested. As ecdysone is involved in development and molting, OD-PABA poses a risk to the population. Although the negative result, JH analogs are used as pesticides (El-Sheikh et al. 2016) and it has been previously described that tea proanthocyanidins can repress *JHAMT* in *Anopheles gambiae* (Muema et al. 2017), showing that JH is also a target for toxicants and requires some attention in future studies of endocrine disruption.

Although no response has been observed for OC and OD-PABA in larvae, similarly to Vinclozolin (Aquilino et al. 2016), *MAPR* is a promising biomarker. It could connect the steroid hormones with cytochrome P450s as the cytochrome b5 domain (CYB5) is in its structure and has been involved in a number of cell processes (Kimura et al. 2012; Ryu et al. 2017). It is known that CYB5 is a modulator of the enzymatic activity of Cyp450s (Henderson et al. 2015) so it is possible that MAPR, using the CYB5 domain, may participate in the detoxification mechanisms or modulate them by interacting with Cyp450s. Longer exposures are needed to analyze this possibility and to evaluate the putative relevance of this orphan receptor in the cell metabolism of insects. Furthermore, its upregulation in the embryo but not in larvae exposed to either 4MBC or BP-3 (Ozáez et al. 2016a) suggest a different sensitivity depending on the development stage.

One of the most important responses to a toxicant is the activation of biotransformation mechanisms that allow the cell to manage the chemical compounds by reducing their toxicity and expelling them from the cell. During this process, different enzymes reduce the toxicity of the compound (phase I) and increase the solubility (phase II). CypP450 enzymes are characteristic of phase I while glutathione-S-transferases are phase II enzymes. All the *CypP450* and *GST* genes showed no effect in the conditions tested. A number of studies have showed that toxicants increase the mRNA levels of Cyp450 genes in invertebrates (Dai et al. 2016; Guo et al. 2017; Sun et al. 2017) and others have described the inhibition of these phase I enzymes (Wang et al. 2016) but a recent work of our laboratory with 4MBC and BP-3 showed that *Cyp4d2*, *Cyp6b7*, *Cyp9f2*, and *Cyp12a2* were inhibited by 4MBC while no response was observed with BP-3 at 24h of exposure (Martínez-Guitarte 2018). The results obtained with OC and OD-PABA were similar to those observed with BP-3, therefore it is possible that some UV filters are processed by other phase I enzymes, such as carboxylesterase (Hatfield et al. 2016), since OD-PABA, OC, and BP-3 all have carboxylester residues that are absent in 4MBC. Further research will elucidate this possibility. On the other hand, the absence of a response showed by genes coding for GST family members, suggests that other phase II enzymes are also involved in the processing of these compounds. This is surprising because GST is a typical phase II enzyme, analyzed by enzyme assay, to study the response of the cell to toxicants. There are several GSTs and some of them have been previously described in *C. riparius* (Nair et al. 2011; Martínez-Guitarte 2018). The activity of GST has been studied in *C*. *riparius* with different compounds showing diverse responses (Park et al. 2010; Morales et al. 2014; Herrero et al. 2015; Saraiva et al. 2017). Messenger RNA levels of members of the GST family have been analyzed with cadmium, silver nanoparticles, and two UV filters, BP-3 and 4MBC (Nair et al. 2011; Martínez-Guitarte 2018). Differential expression of the genes was observed with BP-3 showing upregulation for *GSTd3* and *GSTe1*, while 4MBC inhibited *GSTo1*. The absence of effect in these genes by OD-PABA, OC, or the mixture of both, indicates that another pathway is working. However, it cannot be dismissed that another member of GST family, not analyzed in this work, would be involved in detoxification of OD-PABA and/or OC. Considering these results with those observed with *Cyp450s*, it can be proposed that binary mixtures of the two UV filters did not alter the detoxification mechanisms and there was no interaction in relation to these genes. Furthermore, the differences observed here and those observed in a previous study with 4MBC and BP-3 indicate a specific response to each UV filter. Detoxification of each UV filter follows a different biotransformation, some with low or

no participation of Cyp450s and some conjugated with glutathione in phase II. Further research with other members of these families, and other phase I and phase II enzymes, will help to determine how the cell manage the detoxification of UV filters.

Stress response is a central process for survival of the cell in a changing environment. As mentioned, HYOU1 is a protein related to hypoxia responses (Ikeda et al. 1997) and has also been shown as a component of a complex of several HSP70 family members, such as GRP78, present in the endoplasmic reticulum (ER) and involved in the correct folding of proteins (Ni and Lee 2007). This gene has been studied in mammals with respect to hypoxia and diseases like cancer (Ozawa et al. 1999; Arnouk et al. 2010). However, there are reports on toxicants such as arsenic and diclofenac in zebra fish that showed increased mRNA levels (Lam et al. 2006; De Felice et al. 2012). To our knowledge, no information about the response of this gene in invertebrates, or that shows downregulation, has been published. It is unlikely that the presence of UV filters activated any response related to hypoxia, so downregulation may be related to the chaperone function in the ER reflecting some kind of damage to the folding process. Nevertheless, the downregulation observed suggests that the UV filter may affect the ability of the larvae to respond to hypoxia, putting them at risk if a lack of oxygen occurs simultaneously. The UV filters may then affect the sensitivity of the larvae to other stressors. Results obtained with the mixtures indicate that the presence of both UV filters counterbalance the downregulation. This remain unclear, but suggests that these compounds have a different mode of action in the cell.

On the other hand, *l(2)efl* did not show any change in their mRNA levels. l(2)efl protein is related to the small heat shock proteins (sHSPs) due to its characteristic alpha crystalline domain. The sHSPs have been related to stress and also other cell processes such as apoptosis, the cell cycle and division, development, and differentiation (Morrow et al. 2015; Bakthisaran et al. 2015). In comparison to other sHSPs, *l(2)efl* is poorly studied but is also associated with stress and development. (Landis et al. 2012; Wójtowicz et al. 2015) No data were found in the literature regarding the response to any toxicant, but it is upregulated by ionizing radiation in Drosophila (Landis et al. 2012). Therefore, this is the first study into the *l(2)efl* response to chemicals in invertebrates. The results suggest that there is no significant response in larvae, so l(2)efl probably has a constitutive role similar to other sHSPs, as previously described in *C. riparius* (Martín-Folgar et al. 2015; Martín-Folgar and Martínez-Guitarte 2017). However, it would be interesting to investigate this gene in the embryo since it has been linked to development. (Wójtowicz et al. 2015)

Finally, proteins related to the immune system provide an insight into the ability of the individual to respond to infection. In fact, phenoloxidase activity was analyzed in response to tributyltin in *C. riparius* (Lilley et al. 2012) showing no response in the first generation exposed to the toxicant. There are no data regarding the toxicants effect on defensin in chironomids and only one report describing defensin in *Chironomus plumosus* has been published (Lauth et al. 1998). A similar trend is seen in other insects, although a previous study conducted in honey bees showed that a combination of two acaricides can increase the *defensin-1* mRNA levels in adults (Cizelj et al. 2016). To our knowledge, no previous studies involving UV filters have analyzed this kind of protein. From the results, it can be concluded that under these conditions, the UV filters tested had no effect on this gene. Considering these results obtained with *PO1* and *defensin,* there was no effect detected on immune response from UV filters, and therefore the ability of the larvae to respond to infection was not compromised.

In summary, the acute response to OC and OD-PABA showed either no effects or weak global effects on the endocrine system, the biotransformation mechanisms related to Cyp450s and GSTs, the stress response, and the immune system of *C. riparius* larvae. The downregulation of *HYOU1* mRNA levels compromises the stress response to hypoxia, so the survival of the larvae in certain conditions could be in risk. The mixture of both UV filters did not show interaction effects in the conditions tested but the recovery of normal levels for *HYOU1* in mixtures suggests some kind of antagonism and requires further study. The description of five genes that code proteins involved in hormone biosynthesis, hypoxia response, development, and immune response offers new possibilities for ecotoxicology studies at a molecular level in this model species.

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634 **CAPTIONS**

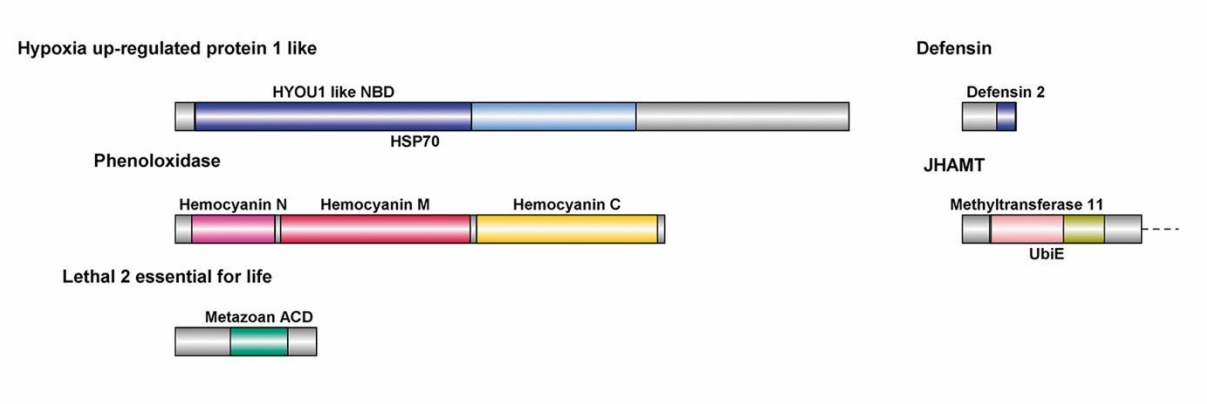

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Figure 2

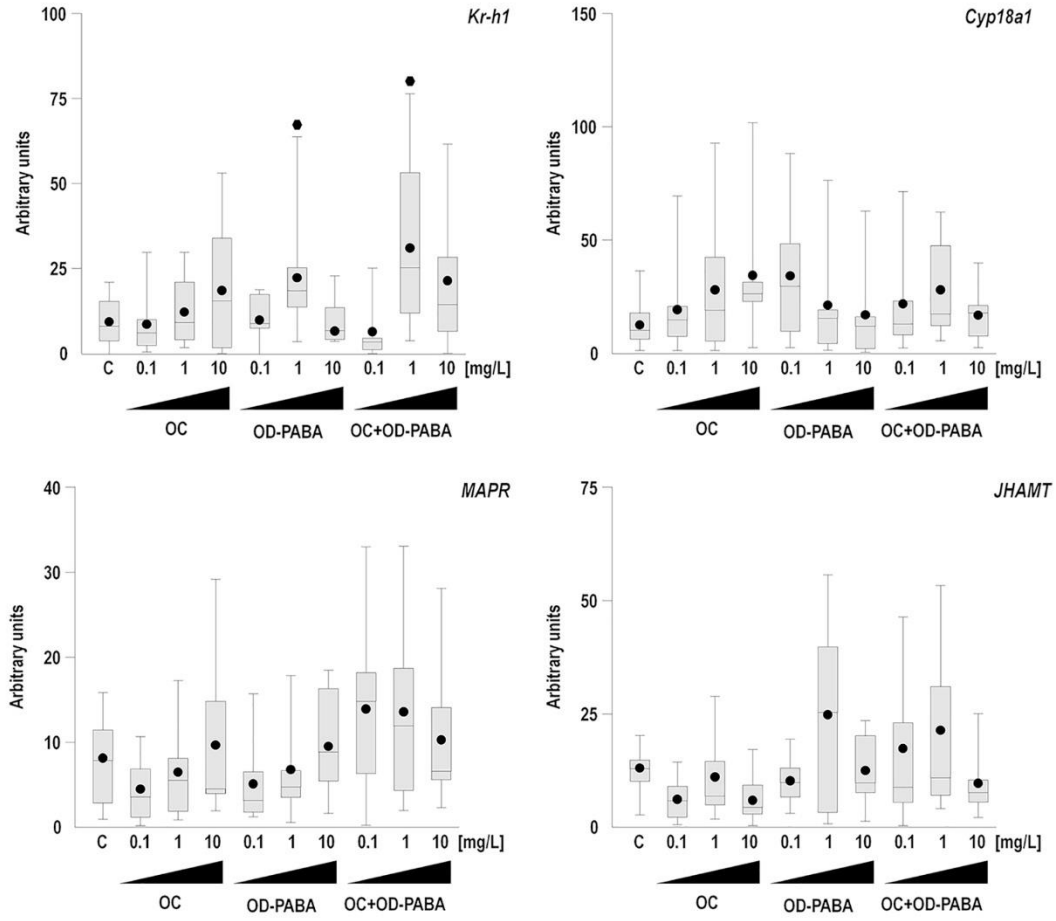

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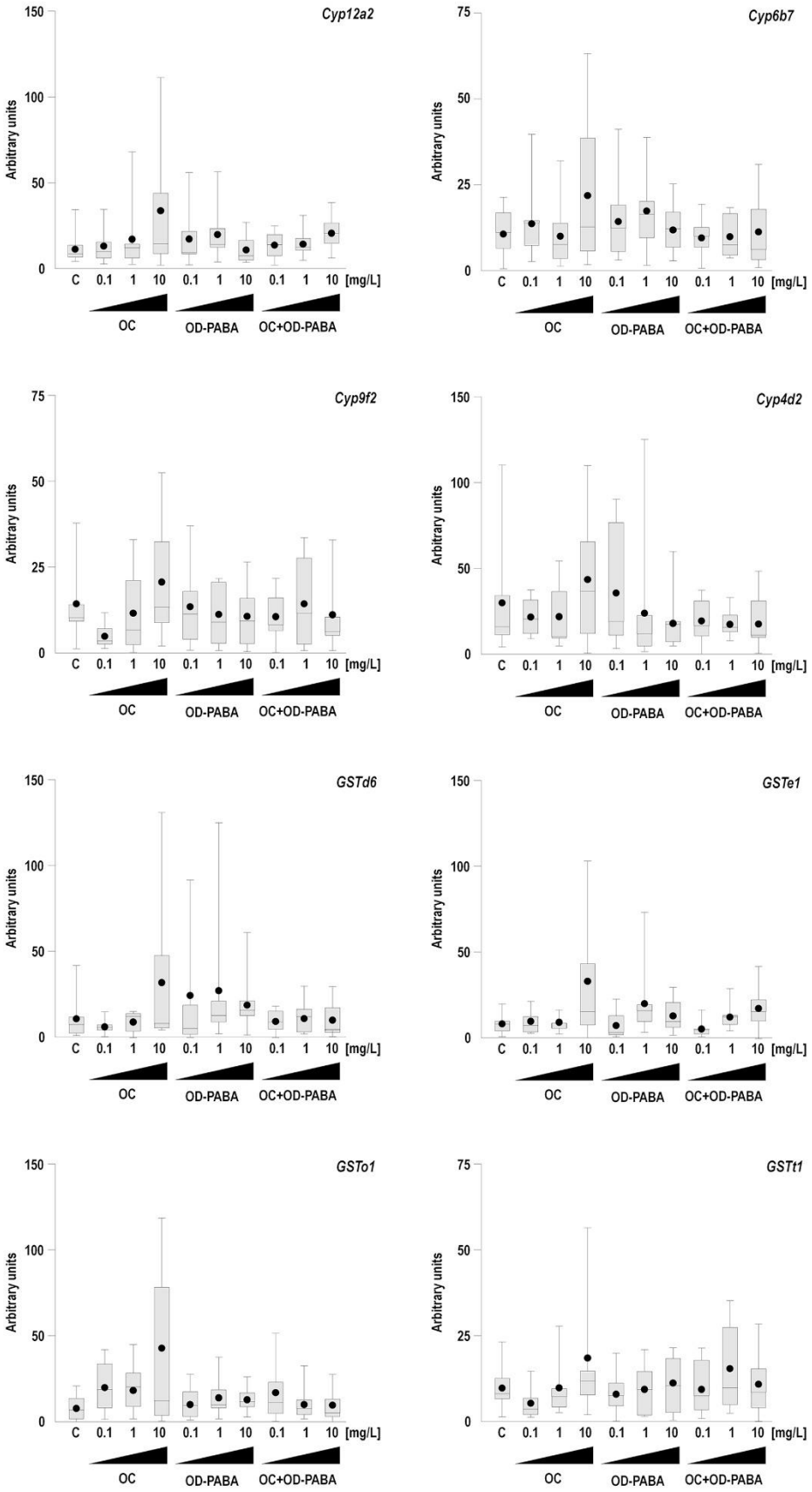
Figure 3


Cyp12a2
Cyp6b7
Cyp9f2
Cyp4d2
GSTd6
GSTe1
GSTo1
GSTt1
Arbitrary units
C 0.1 1 10 0.1 1 10 0.1 1 10 [mg/L]
OC
OD-PABA
OC+OD-PABA

639

Figure 4

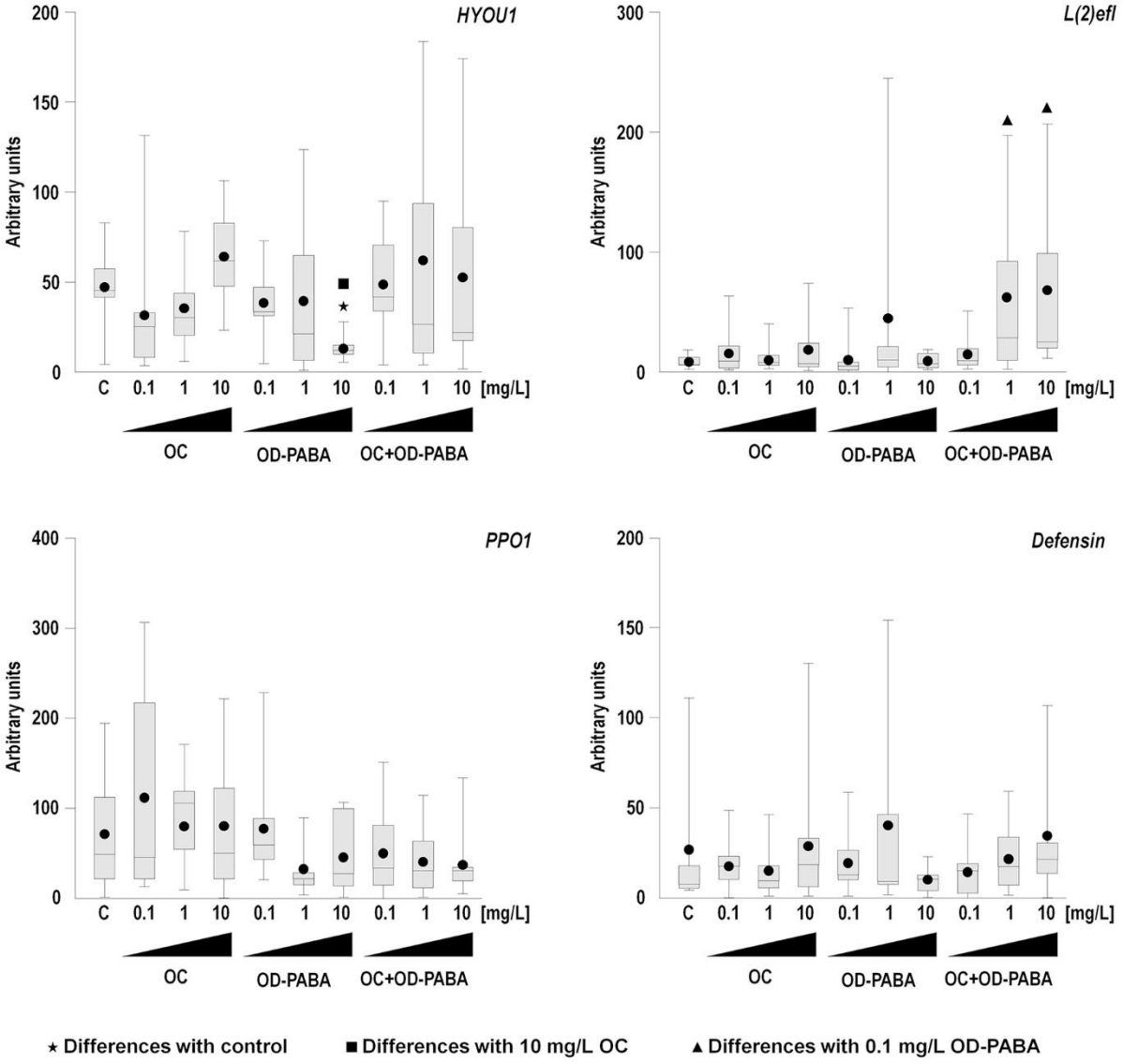


Fig. 1 Diagram of the proteins of *Chironomus riparius* identified as putative mRNAs, and their conserved domains. The different proteins are shown with the characteristic motifs that allow identification. Hypoxia up-regulated protein 1 (HYOU1) showed two characteristic domains, named HYOU1-like NBD and HSP70. For phenoloxidase, three domains were defined. All of them are known as hemocyanin domains with N, M, and C indicating the N-terminal region, the medium region, and the C-terminal region. Lethal 2 essential for life protein showed an alpha-crystallin domain (ACD), characteristic of the small heat shock protein family. Defensin shows a short domain at the C-terminal which is characteristic of these proteins. Finally, juvenile hormone methyl-transferase was incomplete but showed a methyltransferase 11 domain and a ubiquinone/menaquinone biosynthesis C-methylase (UbiE) domain. Diagram was designed using IBS software (Liu et al. 2015)

Fig. 2 mRNA levels of endocrine system related genes *Kr-h1*, *Cyp18a1*, *JHAMT*, and *MAPR* in fourth instar larvae treated with 0.1 mg/L, 1 mg/L, and 10 mg/L octocrylene (OC), Octyl-dimethyl-p-aminobenzoic acid (OD-PABA) or a binary mixture of both (OC+OD-PABA) for 24 h. Analysis was performed by RT-PCR and the reference genes used were *rpL13* and *GAPDH*. Whisker boxes are shown. The number of larvae for each box is nine (n = 9). The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small circle inside the box. ANOVA analysis was performed and the hexagon represents a significant difference from the 0.1 mg/L OC+OD-PABA treatment ($p \leq 0.05$)

Fig. 3 Transcription levels of cytochrome P450 and gluthatione-S-transferase genes in fourth instar larvae treated with 0.1 mg/L, 1 mg/L, and 10 mg/L of octocrylene (OC), Octyl-dimethyl-p-aminobenzoic acid (OD-PABA) or a binary mixture of both (OC+OD-PABA) for 24 h. Transcription levels were quantified by RT-PCR using *rpL13* and *GAPDH* as the reference genes. Whisker boxes are shown. The number of larvae for each box is nine (n = 9). The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small circle inside the box. ANOVA analysis was carried out. No statistical significant differences were observed

Fig. 4 Transcript levels of stress related and immune genes in fourth instar larvae treated with 0.1 mg/L, 1 mg/L, and 10 mg/L of octocrylene (OC), Octyl-dimethyl-p-aminobenzoic acid (OD-PABA) or a binary mixture of both (OC+OD-PABA) for 24 h. The reference genes used were *rpL13* and *GAPDH*. Whisker boxes are shown. The number of larvae for each box is nine (n = 9). The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small circle inside the box. The data were analyzed using ANOVA. The asterisk represents a significant difference from the control ($p \leq 0.05$), the square represents a significant difference from the 10 mg/L OC treatment ($p \leq 0.05$), and the triangle represents a significant difference from the 0.1 mg/L OD-PABA treatment ($p \leq 0.05$)

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Combined effects of benzophenone-3 and temperature on gene expression and enzymatic activity in the aquatic larvae *Chironomus riparius*

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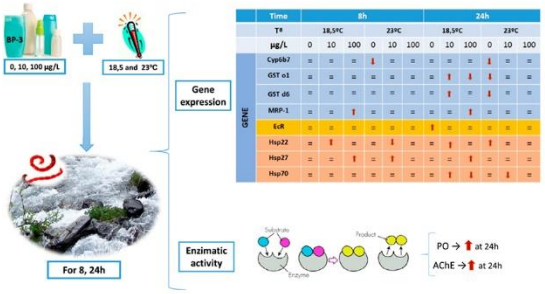
HIGHLIGHTS

- Mild increase of temperature affects survival in presence of BP3.
- Multi-stress alters expression of detoxification genes and *EcR*.
- Cellular ability to eliminate BP3 is affected by the mild increase of temperature.
- *Hsp22*, *Hsp27* and *Hsp70* alteration shows fast stress response at these conditions.
- PO and AchE responses suggest that immunity and nervous system are compromised by BP-3 and temperature.

GRAPHICAL ABSTRACT



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ABSTRACT

Climate change and pollution are two of the main environmental problems living organisms currently face. Temperature can modify a toxicant's effects and the organism's response to it. Global warming is expected to increase the temperature of freshwater ecosystems. In this work, we analyzed the effect of a mild temperature increase on the acute response of the aquatic larvae *Chironomus riparius* to the ultraviolet filter benzophenone-3 (BP3). This substance is commonly used in sunscreens and other commercial products and can reach the environment in different ways. We exposed larvae to BP3 at 18.5 or 23 °C for 8 or 24 h and analyzed the acute response at the molecular level. By quantitative real-time polymerase chain reaction (q-PCR), we studied altered messenger RNA (mRNA) levels of genes related to the endocrine system (*EcR*, *InR* and *Met*), detoxification mechanisms (*Cyp4d2*, *Cyp6b7*, *GST d6*, *GST o1* and *MRP-1*) and stress response (*Hsp22*, *Hsp27*, *Hsp70*, *HYOU* and *Gp93*). Moreover, enzyme activity was evaluated, with a focus on glutathione-S-transferase (GST), phenoloxidase (PO) and acetylcholinesterase (AChE). Results showed that temperature affected the acute response of this organism by modifying the expression of *EcR*, *Cyp6b7*, *GST d6*, *GST o1*, *MRP-1*, *Hsp22*, *Hsp27* and *Hsp70 genes*. These results suggest that even mild temperature change can affect the response of this organism to BP3 influencing short-term progress of the population. Although longer exposures are required to determine the ability of *C. riparius* to manage the pollutants in this novel environmental conditions, in order to know the possible mechanisms of detoxification or adaptation that may develop. This research represents a first step in the analysis of multi-stress

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Short running head: Effects of BP-3 and temperature on *Chironomus riparius* larvae

Keywords: UV-filter, climate change, acute toxicity, Chironomids

Abstract

Climate change and pollution are two of the main environmental problems living organisms currently face. Temperature can modify a toxicant's effects and the organism's response to it. Global warming is expected to increase the temperature of freshwater ecosystems. In this work, we analyzed the effect of a mild temperature increase on the acute response of the aquatic larvae *Chironomus riparius* to the ultraviolet filter benzophenone-3 (BP-3). This substance is commonly used in sunscreens and other commercial products and can reach the environment in different ways. We exposed larvae to BP-3 at 18.5 or 23ºC for 8 or 24 h and analyzed the acute response at the molecular level. By quantitative real-time polymerase chain reaction (q-PCR), we studied altered messenger RNA (mRNA) levels of genes related to the endocrine system (*EcR*, *InR* and *Met*), detoxification mechanisms (*Cyp4d2*, *Cyp6b7*, *GST d6*, *GST o1* and *MRP-1*) and stress response (*Hsp22*,

Hsp27, *Hsp70*, *HYOU* and *Gp93*). Moreover, enzyme activity was evaluated, with a focus on glutathione-S-transferase (GST), phenoloxidase (PO) and acetylcholinesterase (AChE). Results showed that temperature affected the acute response of this organism by modifying the expression of *EcR*, *Cyp6b7*, *GST d6*, *GST o1*, *MRP-1*, *Hsp22*, *Hsp27* and *Hsp70 genes*. These results suggest that even mild temperature change can affect the response of this organism to BP-3 influencing short-term progress of the population. Although longer exposures are required to determine the ability of *C*. *riparius* to manage the pollutants in this novel environmental conditions, in order to know the possible mechanisms of detoxification or adaptation that may develop. This research represents a first step in the analysis of multi-stress response in this animal, and opens new possibilities in the toxicity evaluation of this organism in line with the real scenario that organisms face today.

1. Introduction

Currently, global change is a prominent environmental problem. Global change is understood as any large-scale transformation that affects the functioning of the planet. It involves different processes, including increasing contamination by chemical compounds and the climate change that results from emissions into the atmosphere. The last report of the Intergovernmental Panel on Climate Change showed that there is an increase in the mean temperature in most areas of the planet (IPCC, 2013). Temperature is one physical factor that can alter the distribution of toxicants and an organism's response(s) to them (Gordon et al., 2014; Leon, 2008; Marchand and Haddad, 2017; Nadal et al., 2015). It is especially important in poikilothermic animals because their metabolism is strongly affected by environmental temperature. In this sense, multi-stress has been neglected in toxicity analysis (Holmstrup et al., 2010; Laskowski et al., 2010), and we have limited knowledge about the effects that the increasing temperatures resulting from climate change have on the responses of organisms (Kimberly and Salice, 2014; Landis et al., 2013; Noyes et al., 2009; Park and Kwak, 2014). Although new data has very recently become available (S.-N. Guo et al., 2018; Smeti et al., 2019), there is a gap in information at the molecular level in invertebrates.

Ultraviolet (UV) filters are organic and inorganic compounds used to prevent damage by UV radiation (Serpone et al., 2007). They are found in sunscreens and industrial products and have an increasing presence in the environment. Indeed, they have been found both in freshwater and marine ecosystems (Balmer et al., 2005; Ekpeghere et al., 2016; Sánchez Rodríguez et al., 2015) and organisms (Gago-Ferrero et al., 2013; Molins-Delgado et al., 2018; Zhang et al., 2013). They have several physiological effects, including disrupting endocrine activity and affecting

development (Gao et al., 2013; Schlumpf et al., 2008; Schreurs et al., 2002). One of the most commonly used UV filters is benzophenone-3 (BP-3). It has been detected in different environmental samples and wastewater treatment plants (Archer et al., 2017; Kim and Choi, 2014; Mandaric et al., 2017; Molins-Delgado et al., 2017), ranging from non-detectable levels to amounts such as 2086 ± 1027 ng/L in influents and 153 ± 121 ng/L in effluents (Liu et al., 2012) due to the absence of specific treatments for its complete elimination. It is important to consider that UV filter concentrations can vary during the year; they are seasonally dependent since they are associated with recreational activities (Ekpeghere et al., 2016). BP-3 has been identified as an endocrine disruptor and has environmental relevance because of its lipophilic, photostable and bioaccumulative properties; it causes a variety of toxic reactions in coral and fish that range from reef bleaching to mortality (DiNardo and Downs, 2018; Kim and Choi, 2014). In *Chironomus riparius* BP-3 effects were evaluated at developmental level. BP-3 generated a decrease in the larval growth, delayed development of the females and significant decrease in the average weight of males at 0.75 and 3.41 mg/Kg of BP-3, respectively (Campos et al., 2017).

For several years, we have analyzed the molecular effects of UV filters in *Chironomus riparius*. This animal is a model organism, and both aquatic larvae and terrestrial adults are used for toxicology studies. It plays an important role in aquatic ecosystems as a basic link in the food web (Rieradevall et al., 1995). We found that BP-3 affects embryo development and alters the ecdysone response pathway, stress response and detoxification mechanisms (Martínez-Guitarte, 2018; Martín-Folgar et al., 2018; Ozáez et al., 2013, 2014, 2016a). Further, we studied the effect that BP-3 has on mixtures with other UV filters on the expression of the stress response and

ecdysone receptor (Ozáez et al., 2016b). Together, these data suggest that BP-3 is a representative UV filter with extensive use and effects on invertebrates.

Enzymatic activities are a frequent tool used to analyze toxicity, and they open new paths in the evaluation of xenobiotic effects on invertebrates (Jemec et al., 2010). Glutathione-S-transferase (GST) acts in antioxidant defense by conjugating a compound with glutathione (GSH) to decrease its toxicity. In *C*. *riparius*, GST activity has been evaluated for different compounds (Campos et al., 2016; Park and Kwak, 2018; Saraiva et al., 2017); these studies showed variability in the response. Phenoloxidase (PO) activity, which is related to the immune response (Cerenius and Söderhäll, 2004), is also of interest. In response to microbial infection, the animal induces proteolytic cascades that lead to localized melanization and coagulation. Melanization requires PO to form melanin at wound sites and around intruding microorganisms in the hemolymph (Eleftherianos and Revenis, 2011). PO has been analyzed in *C*. *riparius* in relation to tributyltin effects (Lilley et al., 2012). To evaluate damage to the nervous system, acetylcholinesterase (AChE) activity was examined. It is an esterase that hydrolyzes acetylcholine, a neurotransmitter, and it is a common target for insecticides (Thapa et al., 2017).

The aim of this work was to analyze the influence of temperature, as one factor altered by global change, in the response to BP-3, one of the most common UV filters. We analyzed the early response of *C*. *riparius* larvae by exposing them to BP-3 for 8 or 24 h. We used two concentrations of BP-3, close to those observed in the wild, and two temperatures, one used for a laboratory culture of this species and a second that simulates a mild increase in temperature by climate change. As endpoints, we analyzed alterations in the expression profile of genes related to the endocrine system, stress response and detoxification mechanisms. Previous results

from our laboratory have shown that these processes can be affected by BP-3 having influence in the progress of the population since affect the development, the ability to maintain the cell homeostasis, and the deactivation of the toxicant. Additionally, we analyzed modulation of enzymatic activities related to detoxification (GST), the immune system (PO), and the nervous system (AChE) as indicators of the toxicant biotransformation mechanisms, the vulnerability of the individual to infections, and alterations on behavior.

2. Materials and methods

2.1. Chemicals

The UV filter 2-hydroxy-4methoxybenzophenone (BP-3; CAS No. 131-57-7, purity 98%) was purchased from Sigma-Aldrich (Germany). A stock solution (50 mg/mL) was prepared in absolute ethanol and stored in the dark at 4^{o}C until use.

2.2. Animals

The test organisms were fourth instar *C*. *riparius* larvae. The animals came from a natural population in Valencia (Spain), and they were reared under standard laboratory conditions for several generations according to toxicity testing guidelines (OCDE, 2010). The larvae were grown from egg masses in an aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$ and 0.025 mM KH_2PO_4) in six-liters aquariums supplemented with nettle leaves, commercial fish food and cellulose tissue. Cultures were maintained under constant aeration at 18 ± 2^{o}C and standard periods of light and dark (16 h light: 8 h dark).

2.3. Treatments

For experimental treatments, larvae were exposed to the chemical diluted in 30 mL of culture medium and the appropriate temperature (18.5 or 23ºC). The water was previously tempered and checked before start the experimental set-up using a laboratory thermometer for both conditions. Besides, to control the temperature conditions, the room temperature was maintained at each Tª condition and monitored by atmospheric thermometer. According on the comments of the reviewer we have clarified it in the material and methods section. To generate survival curves, larvae were exposed to 10, 100, 1,000 or 10,000 µg/L of diluted BP-3 for 96 h; mortality was noted every 24 h. The medium was changed and the larvae were fed at 48 h. For gene expression and enzymatic activity, larvae were treated for 8 or 24 h with 10 or 100 µg/L BP-3 (nominal concentrations). In all cases, non-treated larvae (control) were exposed to the same concentration of solvent (0.02% absolute ethanol). Three independent experiments were performed for each set of conditions using samples from different egg masses. The sample size was n = 30 for each survival experiment and n = 9 and n = 12 for gene expression and enzyme activity, respectively. Larvae were frozen and stored at -80ºC for RNA and protein extraction. Experiments were performed in polytetrafluoroethylene vessels to avoid adsorption of the chemical to vessel walls.

2.4. RNA isolation and complementary DNA (cDNA) synthesis

Total RNA was extracted using TRIzol Reagent (Life Technologies, USA) according to the manufacturer's protocol. Next, the samples were treated with RNase-free DNase (Roche, Germany) for 45 minutes at 37ºC. Phenol:chloroform:isoamyl alcohol (Fluka, Germany) organic extraction was carried out using Phase Lock Light tubes (Quantabio, USA). Later, the RNA was precipitated by isopropyl alcohol (0.5 v/v), washed with 75% ethanol and finally resuspended in 25 µL diethyl pyrocarbonate

(DEPC)-treated water. The quality and quantity of purified RNA were evaluated by agarose gel electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf). The total RNA was stored at -80ºC. Reverse transcription was performed using 1 µg of RNA and 100 units of M-MLV enzyme (Invitrogen, Germany) in the presence of 0.5 µg oligo dT20 primer (Sigma, Germany) and 0.5 mM dNTPs (Biotools, Spain) at 37ºC for 50 min in a final volume of 40 µL. The cDNA was stored at -20ºC.

2.5. Quantitative real-time polymerase chain reaction (q-PCR)

q-PCR was used to evaluate messenger RNA (mRNA) levels of the proposed genes. The template was 25 ng of cDNA, and the reaction was carried out with 0.5 units of DNA polymerase (Biotools, Spain), 0.4 mM dNTPs (Biotools, Spain) and 0.5x Eva Green (Biotium, USA). q-PCR was performed with a CFX96 thermocycler (Bio-Rad, USA). The cycling conditions were previously reported (Martínez-Guitarte, 2018). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and two ribosomal protein (*rpL13* and *rpL11*) genes were employed as endogenous references. Efficiency was determined as previously described (Ozáez et al., 2016a). Each sample was run in duplicate wells and two independent replicates were used in each experiment. Primers and efficiencies are listed in Table 1. To analyze and determine total mRNA levels of normalized gene expression ($2^{-\Delta Cq}$), the Bio-Rad CFX Manager 3.1 software was used.

2.6. Protein extraction for enzymatic assays

Protein extraction followed a protocol described for *Drosophila* (Zhong et al., 1996) adapted for *C*. *riparius*. Briefly, each frozen larvae was homogenized in an Eppendorf tube with extraction buffer (10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EGTA pH

8, 0.5 mM DTT, 5% glycerol and 0.5 mM PMSF), frozen on dry ice, thawed and frozen again on dry ice. Later, the samples were maintained for 15 min on ice and centrifuged at 10,000 g for 10 min. Finally, the supernatant was recovered, aliquoted and maintained at -80ºC until use.

Protein extract concentration was quantified using the Bradford method (Bradford, 1976) in a microplate reader (Multiskan Go, Thermo USA) with Bio-Rad Bradford solution following the manufacturer's indications.

2.7. Enzymatic activities

Three enzymes were analyzed: GST, AChE and PO. GST activity was assessed by measuring 1-chloro-2,4-dinitrobenzene (CDNB) and GSH conjugate (Habig et al., 1974). The assay cocktail was prepared with PBS pH 6.5, 100 mM CDNB and 200 mM GSH. The blank consisted of this cocktail without protein. The reaction was developed by mixing 196 µL of the cocktail with 4 µL of protein. Absorbance was measured in a plate reader (Multiskan, Thermo USA) at 340 nm for 5 min, and the activity was calculated according to:

$$(\Delta A340)/\text{min} = \frac{\text{A340 (final read)} - \text{A340 (initial read)}}{\text{reaction time (min.)}}$$

$$\frac{(\Delta A340)/\text{min} \times V\ (\text{ml}) \times \text{dil}}{\varepsilon_{mM} \times V_{enz}\ (\text{ml})} = \mu\text{mol min}^{-1}\ \text{g}^{-1}\ ,$$

where: dil is the dilution factor of the original sample, ε_{mM} (mM^{-1} cm^{-1}) is the extinction coefficient for the CDNB conjugate at 340 nm [5.3 mM^{-1}], V is the reaction volume and V_{enz} is the volume of the sample tested.

PO activity was detected with dihydroxyphenylalanine (L-DOPA) as the substrate. The method was modified from Lilley et al., 2012. Ten µL of protein extract were mixed with 200 µL of 10 mM L-DOPA diluted in PBS pH 7.5 and the reaction was measured at 495 nm for 30 min at 20 °C at 1 min intervals. The blank was the L-DOPA solution without a sample. PO activity is expressed as the total change in optical density per mg of protein (ΔOD/mg protein).

To evaluate AChE activity, a previously described method (Freitas et al., 2014) was used with modifications. It is based on the reaction between thiocholine, which results from AChE activity from the artificial substrate acetylthiocholine iodide (ATCI), with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) used to produce a yellow-colored product. The assay was performed by mixing 100 µL of 8 mM DTNB diluted in PBS pH 8.0 supplemented with 0.75 mg/mL $NaHCO_3$, 50 µL of protein extract and 50 µL of 16 mM acetylthiocholine iodide (ATCI) diluted in PBS pH 8.0. The blank was 50 µL of PBS pH 8.0 with 0.1% Triton-X-100. The reaction was measured at 405 nm for 10 min with intermittent shaking every 30 sec. The activity of the enzyme in µmol min^{-1} g^{-1} protein was by the following equation:

$$\text{Activity} = \frac{(\Delta OD/min)}{(MEC \times C)}$$

where ΔOD/min is the variation of optical density in the time, MEC is the molar extinction coefficient of the colored product at 405 nm, 8160 L/mol.cm , and C is the protein extract concentration in the assay (g/L).

2.8. Statistical analysis

The differences between treatments were tested using SPSS 24 software (IBM, USA). Normal distribution and variance homogeneity were assessed with the

Shapiro-Wilk and Levene tests, respectively. The majority of the data were not normally distributed, and therefore the non-parametric Kruskal-Wallis test was used, ($\alpha = 0.05$).

3. Results and discussion

3.1 Larval survival analysis

Global change is modifying climatic patterns and generating changes in the ecosystems, with aquatic systems being one of the most affected. For a more realistic approach to environmental conditions, we evaluated the combined action of two factors, temperature and chemical stress, by exposing larvae to BP-3 at culture temperature (18.5°C) and a higher temperature (23°C) that mimics mild climate change. In the absence of previous data on the effects of BP-3 combined with temperature, we performed survival analysis with four concentrations of the UV filter at the two test temperatures for 24, 48, 72 or 96 h. The results (Table 2) showed significant differences for BP-3 exposure with respect to the control at 18.5°C for 10 µg/L for 72 h, 100 µg/L for 96 h and 1,000 and 10,000 µg/L for 72 and 96 h. At 23ºC, the differences from control occurred for 10 µg/L BP-3 for 24, 72 and 96 h, 100 µg/L BP-3 for 24 and 72 h and 10,000 µg/L BP-3 for 72 h. According to these data, 10 and 100 µg/L BP-3 concentrations show many significant differences with respect to the control, and between temperatures, in various of the timepoints used. Therefore, these time points were selected for subsequent studies of gene expression and enzymatic activity at 8 and 24 h. Comparison between the same concentrations at different temperatures revealed significant differences for 10 µg/L at 96 h, 100 µg/L at 24 and 72 h and 10,000 µg/L at 72 h. These data revealed the putative effect of temperature on the response of these organisms to BP-3. Survival analysis demonstrated variations due to temperature increase at all concentrations tested.

Temperature can influence organisms at different levels, both in response to the presence of toxins, namely absorption and rate of elimination (Lydy et al., 1999), as well as development in terms of their physiological state (Heugens et al., 2003) or toxicokinetics (Seeland et al., 2012). Previously, we reported that BP-3 was lethal at different concentrations and times (Ozáez et al., 2013, 2016b) at 18.5ºC. Here, the increased temperature appeared to diminish larval survival at different concentrations, a finding that suggests, in some way, a mild increase in the temperature associated with climate change could interfere with population viability. These results are consistent with other reports on *C*. *riparius* that showed the strong effect of temperature change on aquatic organisms (Park and Kwak, 2014). It is worth noting that the lowest survival was observed at 1,000 µg/L BP-3 exposure at 23°C, in contrast to a previous report, where the lowest survival was at 10,000 µg/L (Ozáez et al., 2013). This difference could reflect a putative effect of temperature but it cannot be discarded that the use of polytetrafluoroethylene vessels for the experiments had some influence on the availability of the compound.

3.2 Detoxification mechanisms

Detoxification mechanisms are the main defense of cells against toxicants. In *C*. *riparius*, these mechanisms are activated by compounds such as triclosan, chlorpyrifos and atrazine (Martínez-Paz, 2018; Park and Kwak, 2018; Tang et al., 2018), but previous analysis of several genes related to these processes showed no expression changes induced by BP-3 at 18.5ºC, except for *MRP-1* at 24 h (Martínez-Guitarte, 2018). In order to evaluate the effects of BP-3 and temperature on phase I detoxification, mRNA levels of two cytochrome P450 (cyp450) genes, *cyp4d2* and *cyp6b7*, were analyzed (Figure 1). While there were no significant differences for *cyp4d2*, there were several statistically significant differences for *cyp6b7*. However,

some of them had no biological significance, for example, the differences between 10 µg/L BP-3 at 18.5ºC for 24 h and control at 18.5ºC for 8 h. In our opinion, only the differences observed between controls at 18.5ºC and 23ºC for 24 h were biologically significant. The two phase I genes analyzed followed a similar pattern, with no change at either 8 or 24 h (independent of temperature), but there was a notable *cyp6b7* decrease observed at 24 h between the controls at both temperatures. This result suggests temperature could affect transcription of this gene. In the whitefly (*Bemisia tabaci*), *cyp6cm1* mRNA levels were modified depending on the temperature, with increased expression at 31ºC and decreased expression at 35ºC (L. Guo et al., 2018). However, another report indicated temperature changes increased mRNA levels in sea urchin (Vergara-Amado et al., 2017). Therefore, it is important to consider that even within the same cytochrome family there exists a diversity of responses depending on the temperature. On the other hand, the rest of the significant results were not considered biologically relevant since they were between different concentrations. It cannot be ruled out that other genes involved in phase I metabolism could be activated, such as esterases or other cyp450 family members.

To analyze phase II metabolism, we selected two GST genes because the polymorphic expression of GSTs suggests various biological roles. The selected genes (*GST d6* and *GST o1*) were from different families (Figure 1). While there were statistically significant changes in the expression of both genes (Kruskal-Wallis test), similar to the cyp450 genes, most of the differences had no biological relevance. Statistical analysis indicated that *GST d6* expression differed between controls (8 and 24 h incubations) at 18.5ºC, a change that may be related to an initial mRNA level difference. Expression differences between both time points with 10 µg/L BP-3

exposure at 18.5ºC could be related with the same difference observed for controls and may not represent a real difference. Generally, at both temperatures, the mean gene expression for all the samples was similar, although mRNA levels were slightly higher for 24 compared to 8 h incubation. The results for *GST o1* were similar to *GST d6*, although the mRNA levels after 8 h exposure were usually higher compared to 24 h. However, statistical analysis showed biologically significant differences for 100 µg/L BP-3 exposure at 18.5ºC for 24 h compared to control at the same time and temperature. This difference was not observed at 23ºC. Similarly, we detected differences between both controls, at 18.5 and 23ºC, after 24 h exposure; this finding suggests that a mild increase in temperature can affect *GST o1* transcription. The *GST d6* differences between 8 and 24 h at 18.5ºC suggest its mRNA level could change during the day. Additionally, there was a difference between 8 and 24 h exposure times for 10 µg/L BP-3 at 23ºC. This change could possibly be related to the circadian rhythm since there are previous reports about daily GST variations (Bagheri et al., 2016; Inoue et al., 1999; Maurice et al., 1991). In this sense, *GST o1* mRNA levels in control were similar at different times and temperatures, so variations related to the circadian rhythm could be specific for each GST gene. These data are similar to those obtained previously, although they were not analyzed with regards to temporal changes (Martínez-Guitarte, 2018). More research is needed to clarify the influence of the circadian rhythm on GST genes in this species because the other GST genes analyzed suggest a specific differential expression during the day for each gene (Martinez-Guitarte, 2018). Analysis of *GST o1* differences suggests an influence of BP-3 concentration and exposure time, with downregulation at 24 h exposure of 100 µg/L at 18.5ºC. These results are not in agreement with those obtained previously (Martínez-Guitarte, 2018), but the differences could be related to

the use of glass vessels to carry out the experiments, a protocol which could affect the amount of BP-3 available for absorption by the animals. On the other hand, temperature affected *GST o1* expression by downregulating its transcription at 100 µg/L BP-3 for 24 h compared to control. The fact that there was no difference at 8 h suggests that BP-3 requires time to exert its effect(s). Thus, we propose that BP-3 downregulates *GST o1* transcription; temperature can also further affect its expression, but both stressors appear to work independently. In *B*. *tabaci*, temperature and the pesticide thiamethoxam mildly affected GST gene expression (Guo,L et al., 2018). However, in this species the effect was a weak activation of GST gene transcription. Further, the genes are named differently, a fact that makes it difficult to know equivalence. In any case, GST gene expression showed complex patterns that depended on the conditions and requires more attention.

The multidrug-resistance protein 1 (*MRP1*) gene was also analyzed (Figure 1). This gene codes for an ABC transporter involved in removing toxicants from cells (Kang et al., 2016). Statistical analysis showed differences between control and 100 µg/L BP-3 at 18.5ºC for 8 h while the remaining differences, in our opinion, cannot be considered biologically relevant. Specifically, BP-3 increased *MRP1* expression after 8 h at 18.5ºC. An increase in mRNA levels by BP-3 was previously described after 24 h at higher concentrations than those used here (Martínez-Guitarte, 2018). Temperature effects were also observed at 100 µg/L BP-3 for 24 h, a result that suggests some influence through downregulated transcription compared to the earlier time and lower temperature. No previous data about detoxification mechanisms in relation to simultaneous induction by chemical and heat stress have been found in the literature for invertebrates. Our results suggest a complex picture where a mild increase in temperature can affect cyp450, GST and MRP-1 genes.

Taken together, these expression changes could compromise the ability of the population to manage toxicants in different climatic conditions. Additional research, including other cyp450 and GST family members and extending the exposure time, will help to elucidate whether these alterations are permanent, involve some synergy between the stressors and/or whether there are compensatory mechanisms that allow the cell to respond.

3.3 Endocrine receptors

Since BP-3, as a UV filter, is an endocrine disruptor, mRNA levels of three genes that code for endocrine receptors were studied. Ecdysone receptor (*EcR*), insulin receptor (*InR*) and methoprene-tolerant (*Met*) genes were analyzed to evaluate the BP-3 and temperature effect(s) on ecdysone, insulin and juvenile hormone signaling pathways, respectively (Figure 2). While the difference observed for *EcR* between controls incubated for 24 h at 18.5 or 23ºC could be biologically significant, the remaining differences appear to not be relevant. Previous studies in *C*. *riparius* demonstrated that BP-3 exerts a weak effect on fourth instar larvae at 24 h, but it affects the transcription of different genes than those related to hormonal receptors (Ozáez et al., 2013, 2014, 2016a). No other data have been reported for insects. The results agree with these previous data at culture temperature, with no changes in any of the genes at 8 or 24 h. However, at 23ºC BP-3 appeared to decrease *EcR* expression after 8 h exposure. Additionally, some changes were observed between controls at 24 h that could reflect some influence of the temperature on metabolism. Higher temperatures increase metabolism in poikilotherm animals and climate alterations can be managed in very different ways (Robinet and Roques, 2010). The endocrine system regulates insect development so it is possible that the change observed is due to activation of the ecdysone pathway, a phenomenon that could accelerate

entering the next stage of development. Regardless, a lack of change in *Met* expression suggests that juvenile hormone was not activated. Thus, any effect on development would require more time to manifest. Overall, the larvae endocrine system appears to be refractory to BP-3, although temperature modifies *EcR* expression. Longer exposure times could help to confirm this finding and evaluate whether the alteration in *EcR* has any influence on other endocrine pathways.

3.4 Stress response

The stress response is usually affected by toxicant exposure. Therefore, we analyzed the expression profile of five genes involved in the stress response (Figure 3). Two of them code for the small heat shock proteins (sHSPs) hsp22 and hsp27; these genes were recently described in *C*. *riparius* (Martín-Folgar et al. 2015; Martínez-Paz et al. 2014). They are affected by temperature, heat induced stress was evaluated on *C. riparius*, showing effects on hsp22 and hsp27 gene expression (Martinez-Paz et al 2014; Martín-Folgar et al 2015), or chemicals, including BP-3 (Martín-Folgar et al., 2018; Martín-Folgar and Martínez-Guitarte, 2017). Indeed, *hsp22* and *hsp27* levels increased with culture temperature at 100 µg/L BP-3 according on the results obtained in Martín-Folgar et al., 2018. Additionally, we observed a significant *hsp22* upregulation with 10 µg/L BP-3 exposure compared to control at 18.5ºC for 8 and 24 h. Further, there was a difference at 18.5ºC for 100 µg/L BP-3 compared to control at 24 h, but it is difficult to say whether this was a true difference since the median was higher for 100 µg/L but the mean was higher for control. Furthermore, for controls, *hsp22* was upregulated at 23ºC for 24 h compared to 18.5ºC. Finally, *hsp22* was significantly downregulated after 10 µg/L BP-3 exposure for 8 h at 23ºC compared to the same concentration at 18.5ºC. The rest of the statistical differences lacked, in our opinion, any biological significance. *hsp27* expression, on the other hand, exhibited

some statistical differences between treatments. It was upregulated after 100 µg/L BP-3 exposure at 18.5ºC at both time points. Similarly, 10 µg/L BP-3 upregulated *hsp27* expression at 23ºC for 8 but not 24 h. Additionally, for controls *hsp27* was increased after 23ºC incubation for 24 h compared to 18.5ºC, although the difference was not statistically significant. The remaining significant differences were not biologically relevant.

The expression increase at 8 h for both sHSP genes can be considered a fast response to the presence of BP-3. Further, the temperature effect on *hsp22* suggests that it is a sensitive marker for temperature. While *hsp27* showed a trend for an increase at 23ºC, it could not be considered significant. The differences compared to control were not maintained at 23ºC for *hsp22* or *hsp27*, a finding that suggests temperature in some way affects the response. The fact that some differences were observed between controls, either with 8 h treatment or with the other temperature, could indicate that temperature change masks the response to BP-3 at this temperature. Longer times are needed to know the dynamic of the response to determine what is happening.

Previous reports described that high BP-3 concentrations induced *hsp70* after 24 h exposure (Ozáez et al., 2016b). We obtained a similar result, but with some differences between 8 and 24 h exposure to 100 µg/L BP-3. Indeed, mRNA levels were different between 8 and 24 h for 10 µg/L and 100 µg/L BP-3 at 18.5ºC, results that suggest an alteration with an increase at the shorter time and followed by later recovery to normal levels. For 24 h, 100 µg/L BP-3 downregulated *hsp70* compared to control. No significant difference were observed for 23ºC for the different times, and therefore temperature appears to modify the larval response. Moreover, there was a difference for 10 µg/L BP-3 at 24 h between temperatures, a finding which also

points to the influence of temperature. The results suggest an induction of the *hsp70* after short exposures, a phenomenon that was observed in salivary gland (Ozáez et al., 2014). Subsequently, the levels returned to normal. The differences between 10 µg/L BP-3 at 18.5ºC at 24 and 8 h suggest a later induction with a lower toxicant amount, a result that indicates a certain threshold is necessary to trigger *hsp70* transcription. It is striking that only a difference at 24 h was observed between 18.5 and 23ºC, and it was also related to the 10 µg/L BP-3 concentration. Indeed, there was no temperature effect detected, and so the effects observed were due to the presence of BP-3 rather than the temperature increase.

Taking together the HSP gene expression results, BP-3 induced *hsp22* and *hsp27* expression early. However, the effect of temperature is unclear because there seems to be an induction (as observed in controls), but it is not reflected in the BP-3-exposed larvae. Temperature alone may induce gene expression to such a level that the addition of BP-3 would have no additional effect. The early response of HSP genes to BP-3 was also confirmed by *hsp70* induction, although its levels returned to normal after 24 h.

Glycoprotein 93 (*GP93*) codes for a protein involved in Toll-like receptor folding in the endoplasmic reticulum, but it was not altered by any of the tested conditions (Figure 3). It is related to *Hsp90* and is the ortholog of *Gp96* (Morales et al., 2009). It has been related to stress but also to the immune system, where it can act as a chaperone for cell-surface Toll-like receptors (TLRs) (Anas et al., 2016; Pockley and Henderson, 2018). The lack of a response suggests that this part of the immune response was not affected during short BP-3 exposure, but the results obtained with PO1 activity (Figure 4b) point to some effects of BP-3 in immunity. Finally, we evaluated hypoxia up-regulated 1 (*HYOU1*), but it was not altered by either stressor

alone or in combination (Figure 3). HYOU1 is a protein related to hypoxia responses (Ikeda et al., 1997), and it is a component of a complex of several HSP70 family members, such as GRP78, present in the endoplasmic reticulum and involved in the correct folding of proteins (Ni and Lee, 2007). We previously observed that it was downregulated by the UV filter 2-ethylhexyl 4-(dimethylamino) benzoate (OD-PABA) (Muñiz-González and Martínez-Guitarte, 2018), so it could be differentially expressed depending on the UV filter.

3.5 Enzymatic activity

Enzymatic activity serves as a mechanism to evaluate organism damage at sub organismal level. Thus, we analyzed three enzymatic activities, and the results are shown in Figure 4. Firstly, there were no significant differences in GST activity, but there was a trend for decreased activity for 10 and 100 µg/L BP-3 compared to control for 24 h treatment. Additionally, at 23ºC for 24 h, there was a trend for a median increase. These results point to altered GST activity at later time points, findings that suggests the BP-3 levels were not sufficient to activate this phase. Enzymatic activities are usually analyzed to better understand the cellular responses to certain compounds. GST has been evaluated to study multiple compounds in Chironomids (Chen et al., 2015; Morales et al., 2014; Saraiva et al., 2017). Although there is a lack of data for UV filters in *C*. *riparius*, 3-(4-methylbenzylidene) camphor (4-MBC) activated this enzyme (Campos et al., 2017). Similar results were described for OD-PABA in crucian carp (Ma et al., 2018) and 4-MBC in frog (Martins et al., 2017). In the present study, the global GST activity did not change, but expression of the two GST genes analyzed was altered. It is possible that longer exposures are necessary to detect differences in GST activity. However, it cannot be discarded that the two GST genes analyzed code for enzymes involved in specific processes that

476 are masked by other GSTs. It is worth reminding that, similar to cyp450s, there is
477 great diversity for GSTs with regards to cellular roles (Board and Menon, 2013).
478 Combining specific inhibitors and gene expression analysis could help to elucidate
479 what is happening and discriminate between GSTs.

480 Despite the apparent simplicity of the invertebrate immune system, insects have
481 developed a complete system composed not only of physical barriers but also
482 specific cellular and humoral responses (Kumar et al., 2018). PO1 is part of the
483 innate response. PO activity analysis revealed statistically significant differences.
484 However, the biologically relevant differences suggest that PO activity increased with
485 100 µg/L BP-3 at 18.5ºC for 24 h with respect to the control and 10 µg/L BP-3 at the
486 same temperature. This increased activity was also different with respect to the same
487 exposure at 8 h. Moreover, significant differences were detected at 23ºC for controls
488 incubated for 8 or 24 h. Finally, exposure to 10 µg/L BP-3 at 23ºC for 24 h increased
489 PO activity compared to the same condition at 8 h and at 18.5ºC for 24h. These
490 results suggest higher PO activity at 24 h. Our results indicate that BP-3 and
491 temperature can increase PO activity, and thus it appears that the innate immune
492 response may be modified by both stressors. We showed that *Gp93*, a gene related
493 to Toll-like receptors, was not altered under the conditions tested in this work (Figure
494 3). Toll-like receptors intervene in the cellular response, so taking together the gene
495 expression and PO activity results, the stress from the conditions used can induce
496 innate activity but appears to have no effect on the specific response, at least at the
497 times tested. It would be interesting to explore more about the effect of UV filters on
498 immunity since it can compromise the ability of the population to resist infection. To
499 our knowledge, there are no previous data in insects that analyze the effects of UV
500 filters on immunity.

Finally, we evaluated AChE activity (Figure 4c). This enzyme is associated with the nervous system and is a target for insecticides and drugs (Thapa et al., 2017). One-hundred µg/L BP-3 exposure at 18.5ºC for 24h upregulated AChE activity compared to the same concentration and temperature for 8 h and for 100 µg/L BP-3 at 18.5ºC for 24h. Additionally, AChE activity was increased with 10 µg/L BP-3 at 23ºC for 24 h with respect to the same conditions at 8 h. The rest of the significant differences were not considered biologically relevant. Previously, it was reported that BP-3 did not affect this enzyme in *C. riparius* (Campos et al., 2017). However, the observed differences between 8 and 24 h at different temperatures suggest a trend for an increase that at later times could be significant compared to control. Different experimental conditions could potentially justify this supposition but, in any case, the putative BP-3 effect on AChE activity could compromise larval survival by altering the nervous system.

In summary, analysis of enzymatic activities suggested that BP-3 altered PO and AChE activities with some influence from temperature and time. With regards to GST, it is possibly activated later, but longer exposures are required to confirm this hypothesis.

4. Conclusions

Analysis of two stressors on *C. riparius* fourth instar larvae showed that a mild increase in temperature can affect the response of the organism to BP-3. Detoxification mechanisms were altered, denoted by modified expression of genes involved in phase I, phase II and phase III metabolism. These changes could compromise the ability of the cell to manage the toxicant. BP-3 didn't seem alter the endocrine system, SPs gene expression was altered in response to the toxicant, showing their application as biomarkers. Finally, BP-3 did not change GST activity,

but it is possible that longer treatment times are necessary to manifest alterations in gene transcription. PO and AChE activities were altered, changes that suggest damage to the immune response and nervous system, respectively. Taken together, our results suggest that even a mild increase in the temperature due to global change can affect this insect's toxicant response. Our study represents one of the first analysis related to multi-stress in this model organism that combines different molecular biomarkers. Further research that analyzes additional genes related to other pathways, other enzyme activities and longer exposure times will help to elucidate the mechanism involved in the response to BP-3, the ability of the organism to manage multiple stressors and the putative effect of global change on the survival of populations.

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CAPTIONS

Figure 1. Expression levels of phase I (*cyp4d2* and *cyp6b7*), phase II (*GST d6* and *GST o1*) and phase III (*MRP-1*) metabolism genes in *C*. *riparius* larvae after *in vivo* exposure to BP-3 for 8 or 24 h at 18.5 or 23ºC. mRNA levels were quantified by q-PCR and normalized with *rpL11*, *rpL13* and *GAPDH* as reference genes. Values were compared with controls (solvent-exposed) and among treatments. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small diamond inside the box. All significant differences are indicated (see legend), and those not considered biologically relevant appear in the dashed rectangle ($p < 0.05$).

Figure 2. Expression of endocrine-related genes *EcR*, *InR* and *Met* in *C*. *riparius* larvae after *in vivo* exposure to BP-3 at 18.5 or 23ºC for 8 or 24 h. mRNA levels were

normalized using *rpL11*, *rpL13* and *GAPDH* as reference genes. Values were compared with controls (solvent-exposed) and among treatments. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small diamond inside the box. All significant differences are indicated (see legend), and those not considered biologically relevant appear in the dashed rectangle ($p < 0.05$).

Figure 3. Expression of *hsp22*, *hsp27*, *hsp70*, *Gp93*, and *HYOU-1* genes in fourth instar *C*. *riparius* larvae after *in vivo* exposure to BP-3 at 18.5 or 23ºC for 8 or 24 h. mRNA levels were normalized using *rpL11*, *rpL13* and *GAPDH* as reference genes. Values were compared with controls (solvent-exposed) and among treatments. Whisker boxes are shown. The number of larvae for each box is nine. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small diamond inside the box. All significant differences are indicated (see legend), and those not considered biologically relevant appear in the dashed rectangle ($p < 0.05$).

Figure 4. Analysis of enzyme activities in *C*. *riparius* larvae after *in vivo* exposure to BP-3 at 18.5 or 23ºC for 8 or 24 h. Values were compared with controls (solvent-exposed) and among treatments. Whisker boxes are shown. The number of larvae for each box is twelve. The horizontal line within the box indicates the median. The boundaries of the box indicate the 25th- and 75th-percentiles, and the whiskers indicate the highest and lowest results. The mean is indicated by the small diamond

inside the box. All significant differences are indicated (see legend), and those not considered biologically relevant appear in the dashed rectangle ($p < 0.05$).

Table 1. Primer sequences and efficiencies used for q-PCR.

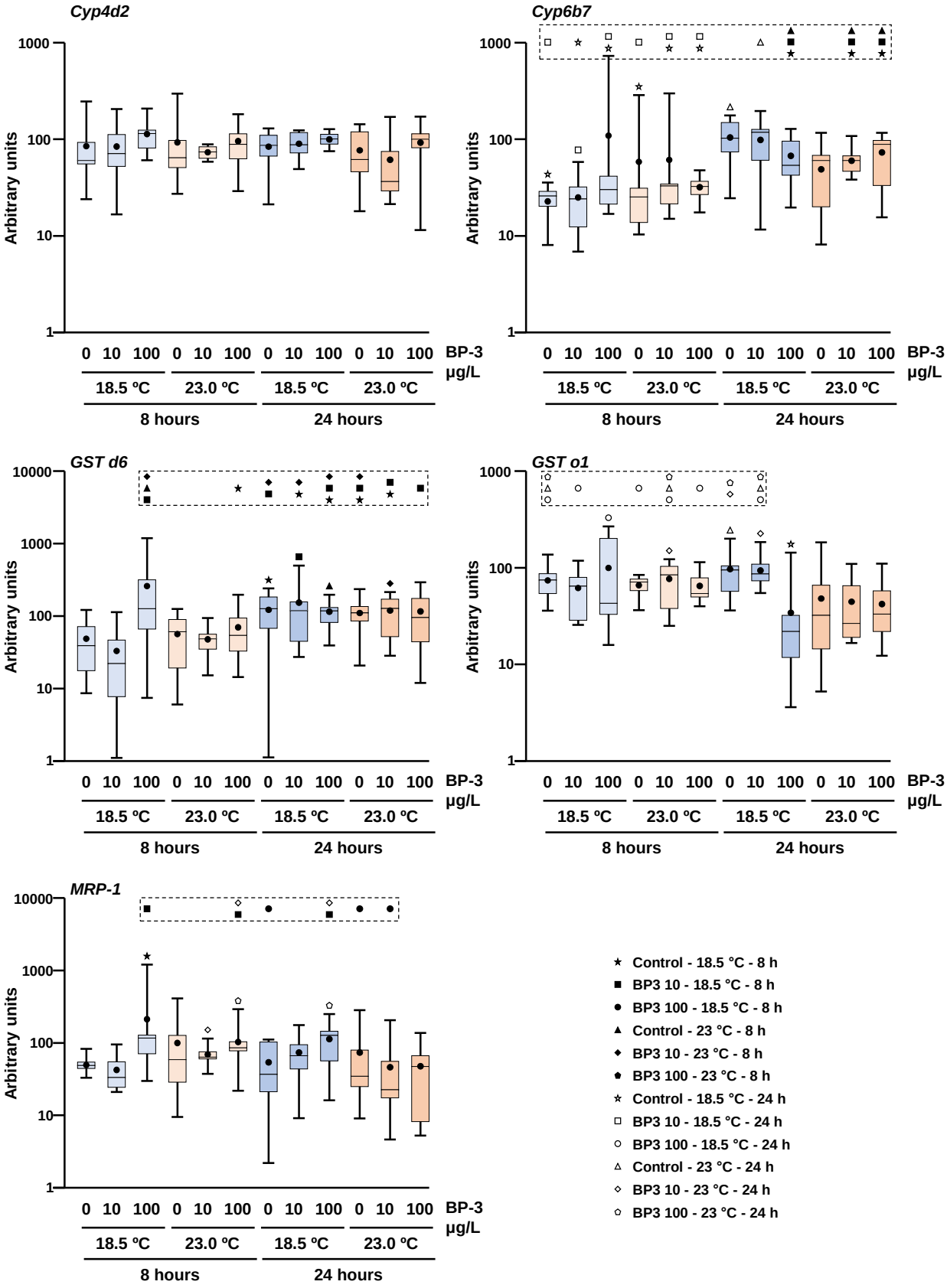
Gene	Forward	Reverse	Efficiency (%)
GAPDH	GGTATTTCATTGAATGATCACTTTG	TAATCCTTGGATTGCATGTACTTG	94.1
rpL13	ACCAGCTAGAAAGCACCGTC	ATGGGCATCTGACGACGATTGGG	97.3
rpL11	TGGCGAAAGCTACCGTCGTA	´TCACCAGATTCTCCGACGCA	106.8
Cyp4d2	AGCACCGATCATCGCAAGAC	ACAAACTCGTCTGGCTTGTCA	108.2
Cyp6b7	AGCGTGAGCAGGAAAAGTCA	TGCTGCTGGTTTCATAGCCT	97.3
Gst d6	GCCTGGAGGATGTGGATTGT	GCTTGAAGAATGCTGCTGACT	100.4
Gst o1	TGAGCTTGATAAGGTGCGCT	TAATCAGGTGTGCCTGCGAG	96.8
MRP1	TGGCCACAAAGTGGAATTGT	CTTTCCAGCTCCTGTTCGTC	102.2
EcR	TCTTCTCACGGCCATCGTCA	GCTGCATCTTGTTTCGCCAC	102.9
InR	CGGAAGTTTCGGTATGGTTT	GACAAATATTGGCTGGCCTT	108.4
Met	GAGTTCAGTACTTAAGCGCACTC	GTGGAGGCTGTTGTGGCACTC	97.3
Hsp22	CCACCACCAGCAATCTCTG	GACAGCAGGTGATTTTCC	99.8
Hsp27	TCAACACACAGGACCG	ATCCTTTATTGGTGATTAATTATG	94.4
Hsp70	ACTTGAACCAGTTGAGCGT	TTGCCACAGAAGAAATCTTG	102.8
HYOU1	CGCTACTATGGGCGCAGTTT	TGGTGCCACTTTCACGTTCA	110.3
Gp93	ACCCCATGTGTACTCGTTGC	CGTGGATTAAATTCGAGAGC	100.9

Table 2. *C. riparius* larvae survival percentages at different temperatures and BP-3 concentrations.

		BP-3 concentration (µg/L)				
T (°C)	**Time (h)**	**0**	**10**	**100**	**1000**	**10000**
18.5	**24**	98.89 ± 1.57	93.33 ± 4.71	90.44 ± 4.43 *	85.55 ± 4.16	96.66 ± 2.72
	48	96.66 ± 0.00	90 ± 2.72	87.77 ± 4.16	83.32 ± 2.72	90 ± 2.72
	72	94.44 ± 1.57	86.66 ± 1.93 c	83.33 ± 4.71 *	76.88 ± 3 c	84.44 ± 4.16 c *
	96	92.22 ± 1.57	85.55 ± 3.14 *	77.78 ± 3.14 c	74.44 ± 4.16 c	82.22 ± 4.16 c
23	**24**	96.66 ± 0.00	92.22 ± 1.57 c	90 ± 1.57 c *	94.44 ± 4.16	93.33 ± 0.00
	48	91.11 ± 3.14	88.22 ± 1.37	85.55 ± 1.57	90 ± 4.71	86.66 ± 2.72
	72	90 ± 2.72	81.11 ± 3.14 c	77.55 ± 1.57 c *	86.67 ± 2.72	79.99 ± 4.71 c *
	96	85.55 ± 3.14	75.55 ± 1.57 c *	75.55 ± 4.16	73.33 ± 2.72	75.55 ± 3.66

C: significantly different from control (0 µg/L) *: significantly different between same concentrations for each temperature

860 **Figure 1**



861

862 **Figure 2**

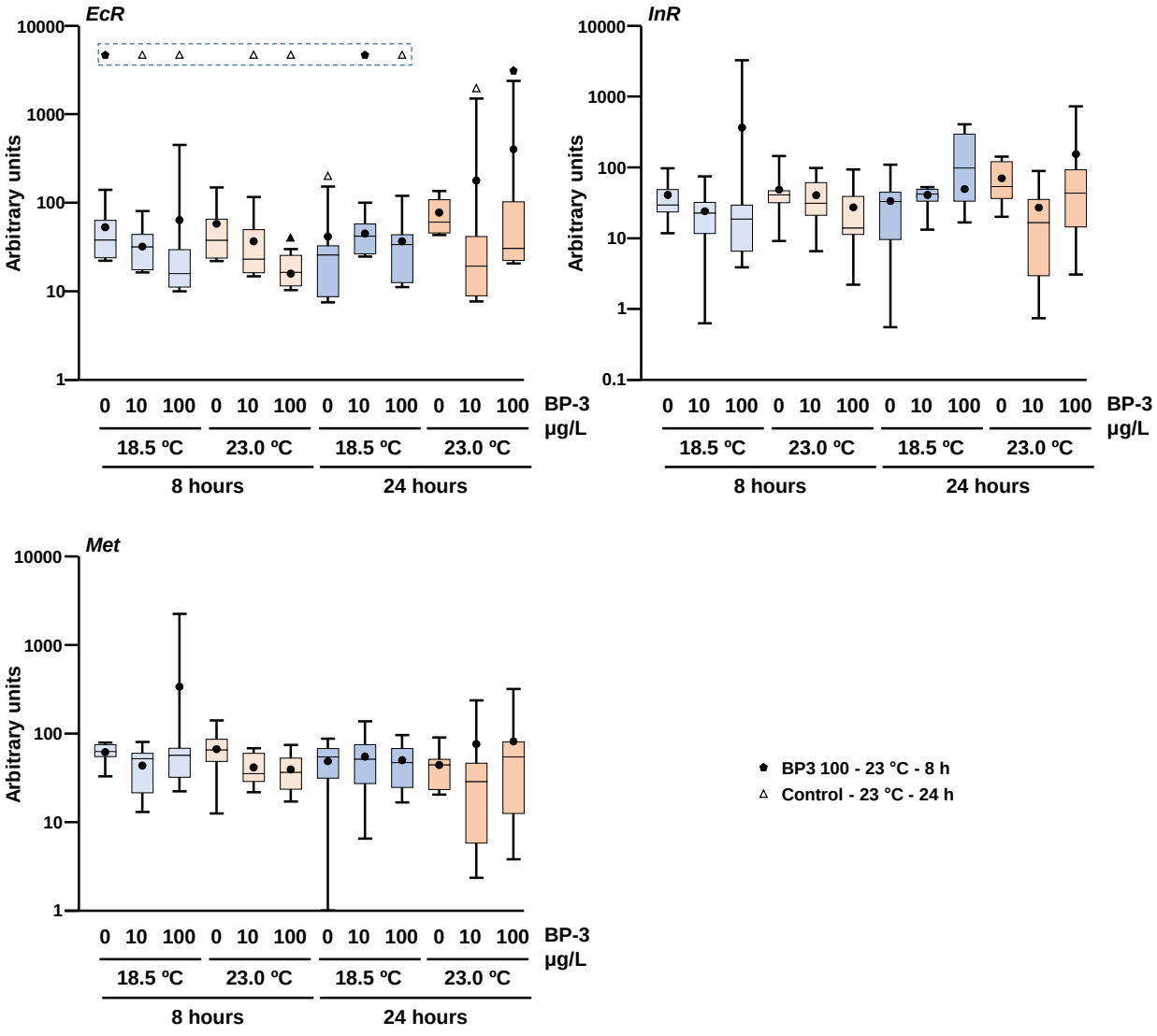


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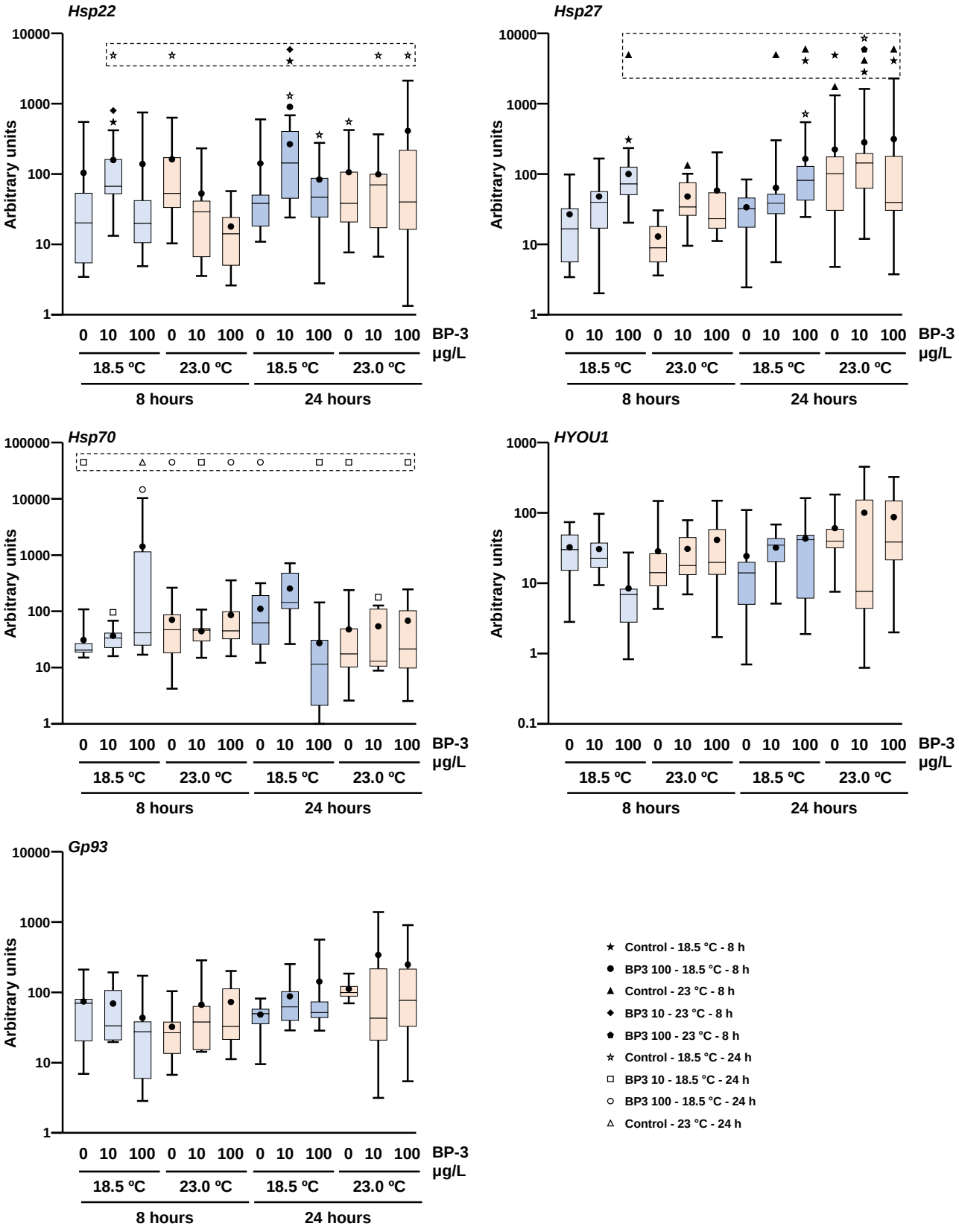
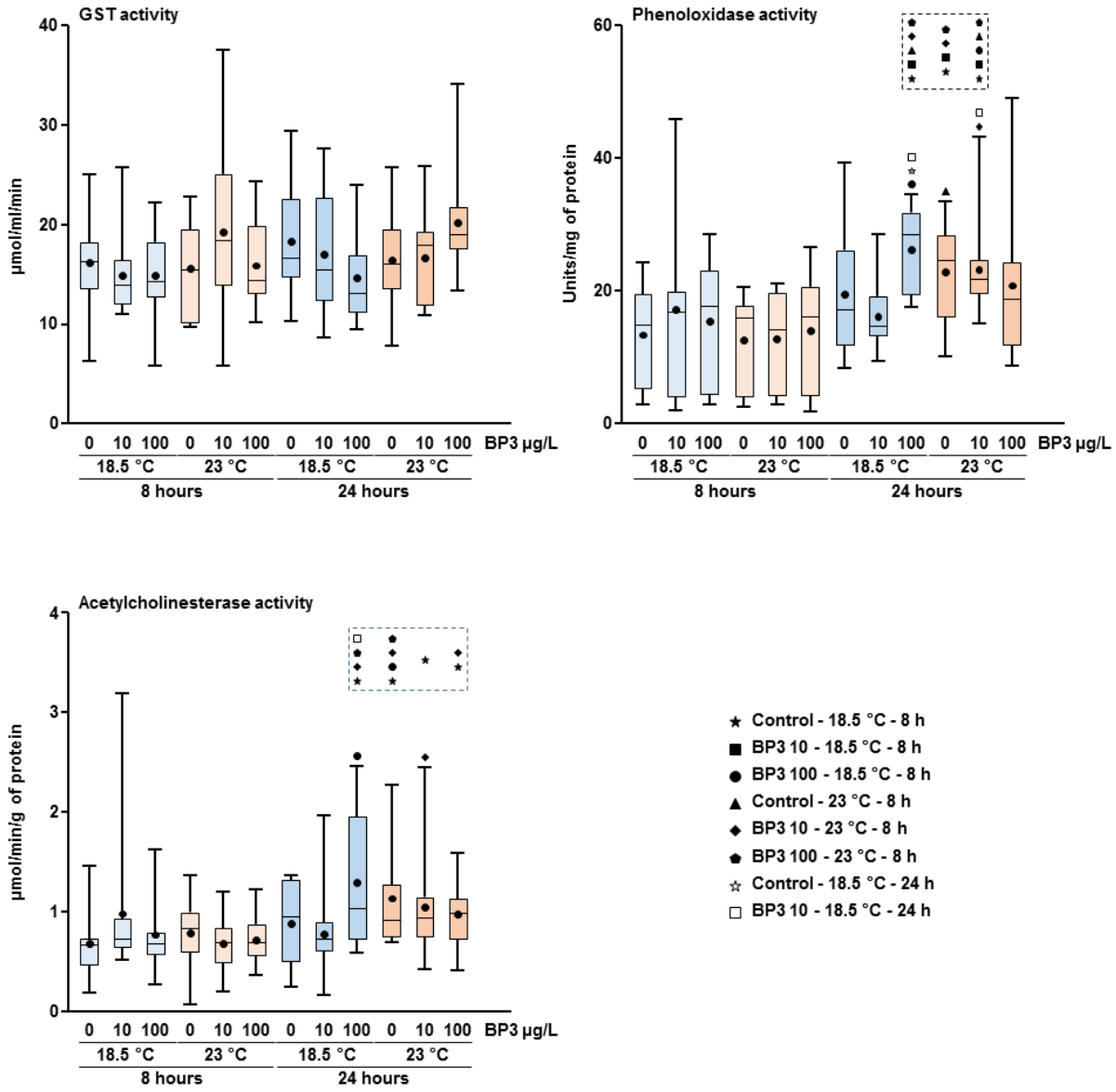
Figure 3


Hsp22
Hsp27
Hsp70
HYOU1
Gp93
Arbitrary units
BP-3 µg/L
0 10 100
18.5 °C
23.0 °C
8 hours
24 hours
Control - 18.5 °C - 8 h
BP3 100 - 18.5 °C - 8 h
Control - 23 °C - 8 h
BP3 10 - 23 °C - 8 h
BP3 100 - 23 °C - 8 h
Control - 18.5 °C - 24 h
BP3 10 - 18.5 °C - 24 h
BP3 100 - 18.5 °C - 24 h
Control - 23 °C - 24 h

866 **Figure 4**



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Annex IV: Other articles published and not directly related to the present thesis

- Novo, M**., Muñiz-González, A. B.,** Trigo, D., Casquero, S., & Martínez Guitarte, J. L. (2019). Applying sunscreens on earthworms: Molecular response of *Eisenia fetida* after direct contact with an organic UV filter. *Science of the Total Environment*, 676, 97–104. https://doi.org/10.1016/j.scitotenv.2019.04.238
- Di Nica, V., **González, A. B. M.,** Lencioni, V., & Villa, S. (2019). Behavioural and biochemical alterations by chlorpyrifos in aquatic insects: an emerging environmental concern for pristine Alpine habitats. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-019-06467-2
- Alonso-Trujillo, M., **Muñiz-González, A. B.,** & Martínez-Guitarte, J. L. (2020). endosulfan exposure alters transcription of genes involved in the detoxification and stress responses in *Physella acuta*. *Scientific Reports*, 10(1), 1–9. https://doi.org/10.1038/s41598-020-64554-8

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Applying sunscreens on earthworms: Molecular response of *Eisenia fetida* after direct contact with an organic UV filter

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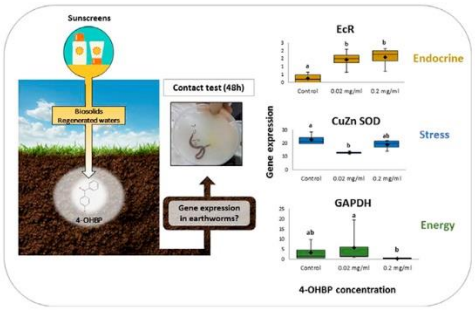
HIGHLIGHTS

- Gene expression changes were studied in *Eisenia fetida* after exposure to a UV filter.
- Whole-body tissue was analyzed after acute (48 h) exposure to 4-OHBP.
- Gene expression of *EcR* increased, indicating endocrine disruption.
- The filter altered *CuZn SOD* expression, related to oxidative stress response.
- Gene expression of *GAPDH*, involved in energy metabolism, decreased.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of organic Ultraviolet (UV) filters has increased in the last years, either in sunscreens, other cosmetics, or even food packaging. These filters may end up in soil and water since the Wastewater Treatment Plants may not successfully remove them. Among them, benzophenones are known to act as endocrine disruptors. However, most of the studies are directed towards vertebrates and aquatic invertebrates, while there is a lack of information on the molecular mechanisms affected by these compounds on soil dwelling invertebrates. Here, we study the impact of direct acute (48 h) contact of 4-hydroxybenzophenone (4-OHBP) at two sublethal concentrations (0.02 and 0.2 mg/mL) on gene expression of the earthworm *Eisenia fetida*. Investigated genes were involved in endocrine pathways, stress response, detoxification mechanisms, genotoxicity, energy metabolism and epigenetics. Three of them were identified for the first time in earthworms. Our results suggest that exposure to 4-OHBP affected endocrine pathways, causing an increase in the Ecdysone receptor gene (*EcR*) expression. Moreover, the UV filter induced changes in the CuZn superoxide dismutase gene (*CuZn SOD*), indicating an effect in the stress response. Finally, significant changes were detected for glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) expression, indicating that energy metabolism is influenced by the 4-OHBP and highlighting the risks of using *GAPDH* as an internal reference for Real Time PCR.

1. Introduction

The emergence of new chemical compounds in the last years has been accompanied with the concern of their effects on humans and environment. Known as xenobiotics, they are rare or nonexistent in the

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CHALLENGES IN EMERGING ENVIRONMENTAL CONTAMINANTS

Behavioural and biochemical alterations by chlorpyrifos in aquatic insects: an emerging environmental concern for pristine Alpine habitats

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Abstract
This study aimed to assess how different concentrations of the insecticide chlorpyrifos (1.1, 5.24, 11, 52.4, 110, 262, 524 and 1100 ng L^{-1}) affect the swimming behaviour of *Diamesa zernyi* larvae following exposure. A video tracking system was employed to analyse two swimming traits (total distance moved and average speed) of the larvae simultaneously after 3 days of exposure to the pesticide at 2 °C. The behavioural results were also interpreted according to biochemical responses to oxidative stress (OS) induced by chlorpyrifos, based on malondialdehyde (MDA) and protein carbonyl (PCC) content. Both distance and speed significantly decreased after 72 h of exposure to chlorpyrifos concentrations of ≥ 110 ng L^{-1}, under which significant OS was detected as lipid peroxidation (level of MDA) and protein carbonylation (level of carbonyl). Analysis of altered swimming behaviour, along with MDA and carbonyl content, indicated that ≥ 110 ng L^{-1} contamination levels of the insecticide cause the organism to reallocate energy normally used for locomotor activity to repair cell damage, which might explain the strong impairment to locomotor performance. Locomotor performance is an ecologically relevant trait for elucidating the population dynamics of key species, with disturbance to this trait having long-term negative impacts on population and community structure. Therefore, chlorpyrifos insecticides represent a serious ecological risk for mountain aquatic species based on the detrimental effects observed in the current study, as the tested concentrations were those at which the insecticide is found in many Alpine rivers of Italy.

Keywords Pesticides · Chironomids · *Diamesa zernyi* · Locomotion ability · Oxidative stress response · Alpine glacier–fed streams

Introduction

Fauna living in pristine areas, such as glacial streams at high altitude in the Alps, is exposed to seasonal contamination by chlorpyrifos (CPF) and other currently used pesticides transported by the atmosphere at median-long distances (Santolaria et al. 2015; Ferrario et al. 2017; Guida et al. 2018; Rizzi et al. 2019). As pesticides are designed to affect biota, there may also be some deleterious effects on non-target organisms living in the aquatic environment where these substances are found (Liu and Schelar 2012; Stehle and Schulz 2015). The presence of pesticides in freshwater ecosystems could impair aquatic fauna, affecting different levels of complexity (including direct acute and chronic effects, bioaccumulation and enrichment in food chains), and might alter the equilibrium of biological communities. Aquatic contamination by pesticides and their effects on wildlife are one of the most studied problems of environmental concern (Darko et al. 2008; Liu et al.

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Endosulfan exposure alters transcription of genes involved in the detoxification and stress responses in *Physella acuta*

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Endosulfan is a persistent pesticide that has been in use for more than five decades. During this time, it has contaminated soil, air, and water reservoirs worldwide. It is extremely toxic and harmful to beneficial non-target invertebrates, aquatic life, and even humans upon consumption, which is one of the many dangers of this pesticide since it biomagnifies in the food chain. The effects of three endosulfan concentrations (1, 10, and 100 µg/L) on the freshwater snail *Physella acuta*, an invasive cosmopolitan species, were examined over a week-long exposure period. Alterations in the expression of ten genes related to stress and xenobiotic detoxification were measured against the endogenous controls *rpL10* and *GAPDH* by Real-Time polymerase chain reaction. Four genes are described here for the first time in this species, namely *Hsp60*, *Grp78*, *GSTk1*, and *GSTm1*. The rest of genes were *Hsp90*, *sHsp16.6*, *cyp2u1*, *cyp3a7*, *cyp4f22*, and *MRP1*. *cyp2u1*, *sHsp16.6*, and *Grp78* expression were all altered by endosulfan. These results suggest a low pesticide concentration activates the acute response in *P. acuta* by affecting detoxification and stress responses and alter endoplasmic reticulum function and lipid metabolism. Furthermore, the newly identified genes extend the number of processes and cellular locations that can be analyzed in this organism.

Endosulfan, also known as Thiodan or Thionex, is a polychlorinated compound used as a pesticide or acaricide. It has been in use since the 1950s in China, the European Union, Australia, Mexico, the United States, and India[1] for crops including maize, soybeans, tomatoes, and cotton[2]. It is particularly effective in removing aphids, fruit worms, beetles, leafhoppers, moth larvae, and whiteflies[3]. However, its highly toxic properties can potentially harm humans and wildlife[4,5]. Further, due to its semi-volatility, endosulfan is often found thousands of kilometers away from its intended area of action in soil, water reservoirs, sediments, and even Arctic and Antarctic lakes. These findings prove its persistent character; indeed, it is listed as a persistent organic pollutant in the atmosphere, soil, and water by the Stockholm Convention[6]. For this reason, it has been banned in over 80 nations. However, one of the main problems with pesticides as endosulfan is the fact that they are persistent and can be dispersed from air to water bodies. The environmental presence of endosulfan has been evaluated by Mohamed *et al.*, 2019[7] showing values from 0.036 µg/L to 62.3 µg/L in water bodies. Indeed, it is an extremely toxic and harmful compound to beneficial non-target invertebrates living in aquatic media[8–10].

Endosulfan is commercially available as a mixture of its isomers, α and β, but higher environmental concentrations correspond to α-endosulfan, since β-endosulfan is naturally converted into α-endosulfan in a slow way due to its higher stability[3]. Although they share similar chemical and physical properties, under aerobic conditions the β-endosulfan half-life is approximately 28 days, whereas α-endosulfan lasts up to 157 days[11]. Certain organisms or chemicals can metabolize endosulfan through oxidation to endosulfan sulfate, which is more toxic and stable[6]. Endosulfan is very toxic to aquatic organisms, especially fish and invertebrates[12–14]. It irreversibly affects fish gills, hindering oxygen intake[15], and interferes with the endocrine system[16]. It is also neurotoxic, since it affects mouse brain development[17]. Although the effects of endosulfan have been extensively analyzed in several species, knowledge about how it affects certain animal groups remains modest.

Mollusks are common model organisms in toxicology, but usually bivalves such as the blue mussel (*Mytilus edulis*) or the Manila clam (*Ruditapes philippinarum*) are used to study pollution in seawater. On the other hand,

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